UCL Division of Infection and Immunity

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[redacted]

Title:

The role of hospital water in the transmission of *Pseudomonas aeruginosa* infection in immunosuppressed patients: Impact of interventions on healthcare water systems, epidemiological characterisation of risk factors for nosocomial bacteraemia and its relationship to reservoirs in the hospital environment using longitudinal sampling, case control studies and whole-genome-sequencing.

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Declaration

I, Özge Yetiş confirm that the work presented in my thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis."

Signed

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that can colonise environmental surfaces, posing a risk of infection to immunocompromised patients. Healthcare waters are a common reservoir. Although neutropenic patients may acquire infection during their course of stay in the hospital, transmission routes are little understood. Molecular identification (PFGE, VNR, MLST, WGS) can determine the relatedness of strains between patients and hospital environment. Although outbreak investigations show indistinguishable strains between environment and patients, the mode and direction of transfer is challenging. In 2018, UCH had the highest number of hospital-onset *P. aeruginosa* bacteraemia cases when compared to other acute trusts in the UK. Consequently, a multi-pronged approach to describe the reservoirs, routes of transmission and epidemiological links between clinical and environmental *P. aeruginosa* in this healthcare setting was proposed. An enhanced monitoring programme was implemented to patient showers in augmented and non-augmented care wards by sampling waters and drains weekly/biweekly and complemented with bespoke remediation including shower interventions and enhanced disinfection to reduce levels of water contamination. Hollow-fibre point-of-use shower filters used to mechanically sequester bacteria from shower waters were evaluated to determine in-use efficacy. In parallel, the epidemiological links between environmental and clinical *P. aeruginosa* strains were compared using Whole-Genome-Sequencing. The importance of patient characteristics were denoted through conditional logistic-regression matched-case-control study to determine factors affecting risk of acquiring *P. aeruginosa*. Biofilm-formation in shower-hoses were replicated in-vitro using microtiter-well assays. Finally, short-read Illumina-sequencing (WGS) of a subset of 190 environmental and clinical isolates determined their relatedness through SNP-distance-matrices and phylogenetic trees and AMR/ biofilm genes characterised. A number of novel findings are presented that challenge national guidelines for testing healthcare waters, highlight risk-factors for risk management of bacteraemia and redefine criteria for the epidemiological characterisation of spatial-temporal distribution of *P. aeruginosa* between clinical and environmental reservoirs.

Impact Statement

This thesis brings together the outcomes of a variety of studies relating the clinical importance of environmental *P. aeruginosa* in healthcare settings. Although this is a relatively well studied field, it also has many unanswered questions especially in terms of transmission links. The results of this thesis benefit both academic and non-academic fields by providing detailed molecular and statistical analysis for further study and evidence concerning the efficacy of methods of control of environmental which will help Hospital Water Safety Groups in reducing the risks to patients.

Enhanced monitoring of shower waters and drains during frequent environmental sampling (every 1-2 weeks) demonstrated that six monthly water sampling as suggested in the current guidelines (HTM04-01) was not frequent enough for timely identification of colonisation of healthcare waters in augmented care wards. Non-augmented care wards were found to be an underreported reservoir of *P. aeruginosa*.

Analysis of different shower head types used in the hospital suggested regardless of the shower head type, remediation and disinfection works should be carried out carefully if contamination is present. Silver impregnated showers may not have sufficient silver concentration to provide antibacterial activity. Research on hollow-fibre shower filter usage in the hospital with the hope of providing safe water to patients showed that patients could still be exposed to high counts of *P. aeruginosa* (32%) even when the filters are in place, although source not being clear. Mechanical filtrations devices at the point of use of shower outlets should be complimented with enhanced monitoring to assure patient safety,

In biofilm studies, a novel laboratory-based method is suggested to extract biofilm from clinical shower heads and hoses. In the conventional crystal-violet assay, many previous studies reported variable results for replicates. However, a revised method is suggested which improved the variance of replicates by excluding edge-effect of spectrophotometric measurements. Analysis of molecular and biofilm studies showed the most prevalent strain isolated from the hospital environment was also a high biofilm producer. However, it did not show close relatedness with clinical isolates, suggesting environmental strains could be using different adaptation strategies to the environment when compared to virulence mechanisms of clinical isolates.

A case-control study showed presence of *P. aeruginosa* in urine as a risk factor for *P. aeruginosa* bacteraemia, suggesting urine culture tests should be done on admission for

immune suppressed patients. Whole Genome Sequencing studies suggested 6 single nucleotide polymorphisms as a relevant cut-off for relatedness analysis for the dataset analysed in this thesis. Phylogenetic trees of environmental and clinical isolates from the same hospital revealed distinct groups, showing that the transmission sources of *P. aeruginosa* could be wider than simply patient to environment or vice versa. Other sources could also introduce the organisms to hospital and promote spread, for example, cleaning practices.

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This thesis is dedicated to Mustafa Kemal Atatürk, founder of Republic of Turkiye who took science as the most reliable guide in life and launched reforms to give Turkish women equal rights, hence shed light on my career.

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List of Abbreviations

AK: Amikacin

ALB: Albumin

AMR: Antimicrobial Resistance

AST: Antibiotic susceptibility test

AZT: Aztreonam

CAZ: Ceftazidime

CBA: Columbia Blood Agar

CEF: Cefepime

CF: Cystic Fibrosis patients

CFU: Colony Forming Unit

CIP: Ciprofloxacin

CLSI: Clinical and Laboratory Standards Institute

CN: Gentamicin

CRE: Creatinine

CRP: C-Reactive Protein

eDNA: Extracellular DNA

EPDM: Ethylene-propylene-diene-monomer

EPS: Extracellular polymeric substance

ESBLs: Extended spectrum beta lactamases

EUCAST: European Committee on Antimicrobial Susceptibility Testing

GFR: Glomerular Filtration Rate

HTM: Health Technical Memoranda

ICU: Intensive Care Unit

IMI: Imipenem

IV line: Intravenous line

LPS: Lipopolysaccharide

MALDI-TOF: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

MBLs: Metallo- beta lactamases

MCA: Milk Cetrimide Agar

MEM: Meropenem

MHA: Muller-Hinton Agar

MLST: Multi locus sequence typing

NA: Nutrient Agar

NEU: Neutrophils

OD: Optical Density

OR: Odds Ratio

PBS: Phosphate-buffered Saline

PCNA: Pseudomonas C-N Agar

PFGE: Pulsed field gel electrophoresis

POU: Point of Use

PRL: Piperacillin

PTZ: Piperacillin – tazobactam

PVC: Polyvinyl Chloride

SIR: Single Isolation Room

SNP: Single Nucleotide Polymorphism

QS: Quorum sensing

TCC: Ticarcillin – clavulanic acid

TOB: Tobramycin

TMV: Thermostatic mixing Valve

UCH: University College Hospital

UKHSA: UK Health Security Agency

UTI: Urinary Tract Infection

WCC: White Cell Count

WGS: Whole Genome Sequencing

VNTR: Variable number tandem repeats

XPS: X-ray photoelectron spectroscopy

Special Notes

Anonymised 1: Shower type 1, manufacturer details available upon request

Anonymised 2: Shower type 2, manufacturer details available upon request

Anonymised 3: Shower type 3, manufacturer details available upon request

Anonymised 4: Shower type 4, manufacturer details available upon request

Chapter 1. Introduction

1.1. Hospital-acquired Infections

Hospital-acquired infections (HAIs), also known as nosocomial infections are defined as infections developed by patients more than 48 hours after admission to a hospital or a healthcare setting. Any infection presenting or incubating at the day of hospital visit are excluded whereas infections developed after discharge are included in this group [1]. Some hospitals like UCH use 72 hours after hospital admission to better define HAI and differentiate from community-acquired infections. There are many types of HAIs such as surgical site, urinary tract, respiratory infections and bacteraemia, pneumonia and hospital-associated diarrhoea [1,2]. Patients staying in intensive care unit (ICU), particularly cancer patients are more vulnerable to these infections in which mortality rates can be high [3].

According to Point Prevalence Surveys of European hospitals on HAIs, 5.2% of patients admitted in hospitals are affected by at least one HAI. This percentage rises to 19.5% in ICU patients. The mortality rate is expected to be two-three times higher in low and middle income countries but lack of data restricts the estimation [4,5].

Hospital pathogens are the microorganisms infecting patients in a hospital or a healthcare setting. Contaminated environmental surfaces play an important role in transmission events. Many pathogens can survive on dry surfaces for long periods of time, reaching up to several months [6]. Transmission via the hands of patient and staff and air are reported in previous studies. Enhanced cleaning and intervention strategies have been tried to clean the environment [7]. Bacteria are the primary cause of nosocomial infections. Gram-positive bacteria include methicillin-resistant *Staphylococcus aureus* (MRSA), *Clostridium difficile*; Gram-negative bacteria include Enterobacteriaceae, *Acinetobacter* spp., *Klebsiella* spp. and *Pseudomonas* spp. Among fungal nosocomial pathogens, *Candida* species cause most infections. Norovirus, Adenovirus, Rotavirus and Hepatitis B virus can also cause HAI [8]. The most common microorganisms causing HAIs are *Escherichia coli* (15.9%), *S. aureus* (12.3%), *Enterococcus* spp. (9.6%) followed by *Pseudomonas aeruginosa* (8.9%) [4]. *P. aeruginosa*, its presence in the hospital environment and clinical importance is studied in detail in this thesis. Its characteristics, antibiotic resistance and biofilm forming properties are explained in below sections. Hospital water systems as *P. aeruginosa* reservoir and water regulations are detailed in introduction as they form the basis of research undertaken in the

following chapters. Finally, typing methods by putting the focus on whole genome sequencing, are explained as background studies.

1.1.1. *Pseudomonas aeruginosa*

P. aeruginosa is a ubiquitous, rod-shaped, monoflagellated, non-spore forming Gram - negative bacterium which grows optimally at 25ºC to 37ºC but can also grow at temperatures up to 42ºC [9]. It can cause disease in plants and animals and is an opportunistic human pathogen causing acute and chronic infections, especially in immunocompromised patients [9,10]. The cell dimensions are 1.5 – 3 µm by 0.5 – 0.8 µm and its genome is relatively large: 5-7 Mbp [10,11]. *P. aeruginosa* may express the pigments: Pyoverdin (yellow-green), pyocyanin (blue), pyorubin (red) and pyomelanin (black). Depending on the growth medium, *P. aeruginosa* colony morphology can be diverse but typically it is usually flat with a metallic sheen and mucoid appearance. On blood media it is beta-haemolytic. It is an aerobic bacteria but can grow anaerobically by using nitrate as a last electron acceptor [12]. Cytochrome c protein is part of its respiratory chain [13]. Microorganisms having cytochrome produce oxidase enzyme which can be tested by oxidase test. The oxidase test uses a reagent, N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride which acts as an artificial electron acceptor and when it is oxidized by the oxidase enzyme, it changes its colour to blue [14].

P. aeruginosa produces many polymers and secondary metabolites as a result of its broad metabolic capacity. Its regulatory genes give it the survival advantage to adapt different environments. Different disinfection methods are used in healthcare facilities to combat with this bacteria but they can regrow and recolonise rapidly posing high risk for vulnerable patients [10]. Presence of efflux pumps and absence of open channels gives the outer membrane a very selective nature thus making it difficult for antimicrobial agents to enter and accumulate within the cell [11].

1.1.1.1. Antibiotic resistance mechanisms

1.1.1.1.1. Beta-lactams

Antibiotics possessing a beta-lactam ring in their structure are known as beta-lactam antibiotics. Penicillins (piperacillin, tazobactam, ticarcillin/clavulanic acid, etc.) and their derivatives, cephalosporins (ceftazidime, cefepime etc.), monobactams (e.g. aztreonam) and carbapenems (meropenem, imipenem, ertapenem etc.) fall into this group [15]. Their

structures are given in Figure 1.1 [16]. Their mode of action targets peptidoglycan biogenesis of bacterial cell wall by inactivating penicillin binding proteins (PBPs). Inhibition of high and low molecular weight PBPs results in loss of wall integrity followed by cell lysis [17].

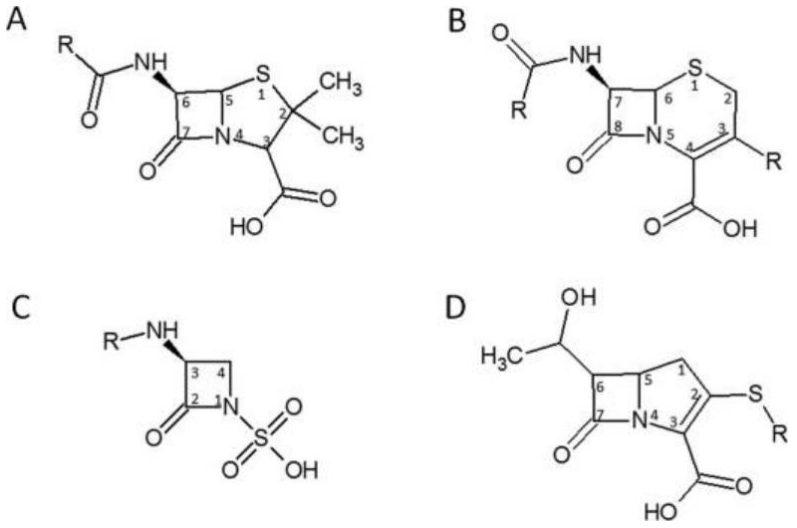


Figure 1.1. Beta-lactam ring structures of different antibiotic groups. A. Penicillin B. Cephalosporin C. Monobactam D. Carbapenem [16]

Beta-lactamases are enzymes which break down the beta-lactam ring of the antibiotic making it unavailable to bind to PBPs. *P. aeruginosa* produces over a hundred beta-lactamases, the most important ones being AmpC, extended spectrum beta lactamases (ESBLs), and metallo-beta lactamases (MBLs) [18].

AmpC which is encoded by the gene *ampc*, is a cephalosporinase giving *P. aeruginosa* intrinsic resistance to beta lactam antibiotics (excluding carbapenems) when expressed in high levels. Resistance depends on the level of expression of AmpC which can be induced by penicillin G and narrow spectrum cephalosporins such as cefazolin [9]. Weak inducers of AmpC expression such as ceftazidime, cefepime and piperacillin can be used to treat pseudomonal infections, yet their prolonged use selects mutants which hyper-produce AmpC resulting in failure of treatment [18].

ESBLs hydrolyse penicillin, cephalosporin and aztreonam (from monobactam) but can be inhibited by clavulanic acid, tazobactam or sulbactam which are beta-lactamase inhibitors [18,19]. This enzyme group is mainly produced by plasmids or encoded in integrons [20]. Among many types of ESBLs described in *P. aeruginosa*; TEM, SHV and CTX-M belonging to Class A serine Enterobacteriaceae types are gained by horizontal gene transfer. OXA-type

enzymes (except OXA-50 which is naturally produced) follows horizontal transfer of mobile genetic elements such as integrons, cassettes and plasmids [21].

MBLs show action against all beta-lactam antibiotics (including carbapenems) and are resistant to beta-lactamase inhibitors. An exception is aztreonam where MBLs are unable to hydrolyse its structure. They are not inhibited by tazobactam or clavulanic acid but may be suppressed by chelators of bivalent ions [20,21].

1.1.1.1.2. Aminoglycosides

This antibiotic group shows bactericidal effect by binding to 16S rRNA of bacterial ribosome which affects proofreading process in the rRNA. Once embedded in the cell membrane, this leads to synthesis of defective proteins which further affect the membrane permeability [22]. Amikacin, gentamicin and tobramycin fall into this group [15].

There are many aminoglycoside resistance mechanisms predominantly arising from enzyme modification, reduced outer membrane permeability and active efflux. Some studies showed target modification takes place on rare occasions. Once a phosphate, adenyl or acetyl radical is attached to the antibiotic molecule, its binding affinity to cell wall is decreased. Target modification is done by 16S rRNA methylases which are encoded in transposons in transferrable plasmids and might be transferred horizontally [20].

1.1.1.1.3. Fluoroquinolones

Fluoroquinolones (e.g. ciprofloxacin) affect DNA replication and transcription by targeting DNA gyrase and topoisomerase [15,23].

Fluoroquinolone resistance is accomplished by two mechanisms: Modification in target enzyme and active efflux. Change in target enzyme is produced by point mutations in genes encoding DNA gyrase which lowers the binding affinity to quinolones. Fluoroquinolones are substrates for MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM which are efflux systems identified in *P. aeruginosa* [20].

1.1.1.2. Pathogenesis

P. aeruginosa controls its virulence factors via regulatory networks to enable bacterial survival and infect host cells. Adhesins (flagella and type 4 pili), secreted enzymes, type III secretion system, proteases, exotoxins, endotoxins (lipopolysaccharides), iron acquisition, alginate overproduction and biofilm forming properties take part in pathogenesis [9,12].

1.1.1.2.1. Flagella

A single polar flagellum gives *P. aeruginosa* swimming motility as well as taking part in adhesion and biofilm formation. Once adhered to the mucins on the cell surface, it initiates NFκB-mediated inflammatory response [9,24].

1.1.1.2.2. Type-4 pili

Long filament-like appendages produced by many Gram-negative bacteria are known as Type-4 pili and are encoded by more than 50 genes *P. aeruginosa* [25]. The main role of Type-4 pili is to enable bacteria to adhere host epithelial cells but it also enables twitching motility and contributes to biofilm formation. They can also protect bacteria from the host immune system by forming microcolonies (concentrating bacteria in a certain location) in tissues [9,24].

1.1.1,2.3. Secreted Toxins

Secretion systems in *P. aeruginosa* are listed as type I, type II, type III, type V and type VI. Type I secretions system (T1SS) constitutes of Apr system (secretes alkaline protease AprA) and iron utilization (HasAp protein binds to haemoglobin). Type II secretion system (T2SS) secretes exotoxin A, phospholipase C, LasA and LasB. Type V secretion system (T5SS) secretes EstA, LepA and LepB. Type VI secretion system (T6SS) takes part in competition with other species in the environment [9].

1.1.1.2.4. Type III Secretion System

P. aeruginosa has at least four secretion systems taking part in virulence (Type I, II, III and IV)[26]. Type III Secretion System (T3SS) has appendages in the form of needles which form pores in the host cell membrane and subsequently injecting bacterial effector proteins through these pores [24]. There are four T3SS effector molecules in *P. aeruginosa*: ExoU, ExoS, ExoT and ExoY. Generally, ExoU and ExoS do not present in the same strain whereas ExoT and ExoY present in almost all strains. Strains producing ExoU and ExoT cause rapid host cell death, on contrary strains producing ExoS and ExoT lead to slower host cell death. Both of these groups are found in acute human infections but the expression of T3SS is downregulated in cystic fibrosis (CF) patients [27].

1.1.1.2.5. Proteases

Extracellular proteases induce host tissue damage and supress immune responses, therefore facilitate bacterial colonisation. Alkaline protease (e.g. AprA) which is a zinc metalloprotease degrades host fibronectin, complement proteins and flagellin monomers so that *P. aeruginosa*

can escape immune recognition. Other proteases identified in *P. aeruginosa* are elastases (LasA and LasB) and protease IV [24,28].

1.1.1.2.6. Bacteriocins

Bacteriocins (pyocins) are produced to compete with other bacteria. There are three types of pyocins: R, F and S. R-type pyocins cause cell death by arresting the synthesis of macromolecules and lyse target cells by depolarizing their cytoplasmic membrane upon attachment to the cell surface [29,30]. Together with F-type pyocins, they are protease and nuclease resistant. S-type pyocins are soluble and they inhibit phospholipid synthesis [29].

Production of pyocins confers *P. aeruginosa* survival advantage to compete with other bacteria in environments by making them the predominant strain in a bacterial niche [31]. Pyocin producing strains isolated from drains are predicted to eliminate susceptible profiles [32].

1.1.1.2.7. Exotoxins

Exotoxin A, the major cellular toxin inactivates elongation factor-2 and thus inhibits protein synthesis by ADP-ribosylating. It is also highly toxic for cells and takes part in local and systemic pathology [12].

1.1.1.2.8. Endotoxins (Lipopolysaccharides)

Lipopolysaccharides (LPSs) are composed of three units: the lipid A, core oligosaccharide and O-chain polysaccharide. Prevention of lysis is enabled by expression of O-chains. Structure of this side chains affects antimicrobial resistance. Some chronic strains of *P. aeruginosa* (e.g. in CF) have altered O-chain which alters host inflammatory responses [12,33]. Studies showed that LPS serves as a protective barrier and is a receptor for R-pyocins [29]. Innate immune system recognizes LPS inducing bacterial infection inflammation [34]. Secreted toxins together with adhesins and proteases take part in pathogenesis [26]. Plasticity of *P. aeruginosa* genome (inactivation of genes, recombination events, etc.) can alter the virulence of LPS, hence giving *P. aeruginosa* the survival advantage to adjust itself according to stress in the environment, transiting to a chronic infection as in CF patients [35].

1.1.1.2.9. Iron Acquisition

Iron acquisition is mainly enabled by two siderophores (iron chelating molecules) produced by *P. aeruginosa* which are the pigments pyoverdin and pyochelin; their receptors are the

outer membrane proteins FpvA and FptA respectively. A repressor protein (Fur) acts as a master regulator of the system, regulating the transcription of genes according to the iron concentration. Fur also controls the expression of small regulatory RNAs which control quorum sensor production. Iron is an essential requirement for *P. aeruginosa* and its acquisition takes part in regulation of genes affecting survival in oxidative stress and virulence genes. Production of exotoxin A is also is also regulated by iron concentration [12,36].

Iron acquisition is essential for both host and environmental survival. Iron acquisition systems of *P. aeruginosa* gives it the survival advantage to compete with other microorganisms [37]. Studies showed that iron-rich environments enhances production of Psl which takes essential part in biofilm production, serving as a signal for *P. aeruginosa* to produce more biofilm [38,39].

1.1.1.3. Biofilm

Extracellular polymeric substance (EPS) which constitutes of exopolysaccharides, polypeptides, extracellular DNA (eDNA) and biomolecules is the essential component of biofilm. Stages of biofilm formation are given in Figure 1.2. Initially, planktonic bacterial cells attach to the surface reversibly (a) and then they start producing EPS and adhesins which enables them to attach to the surface irreversibly (b). After the formation of microcolonies (c) biofilm matures and covers noncolonized surfaces (d). As a last step, bacteria start dispersing from the sessile biofilm in planktonic freely floating forms and travels to other surfaces€ [40,41].

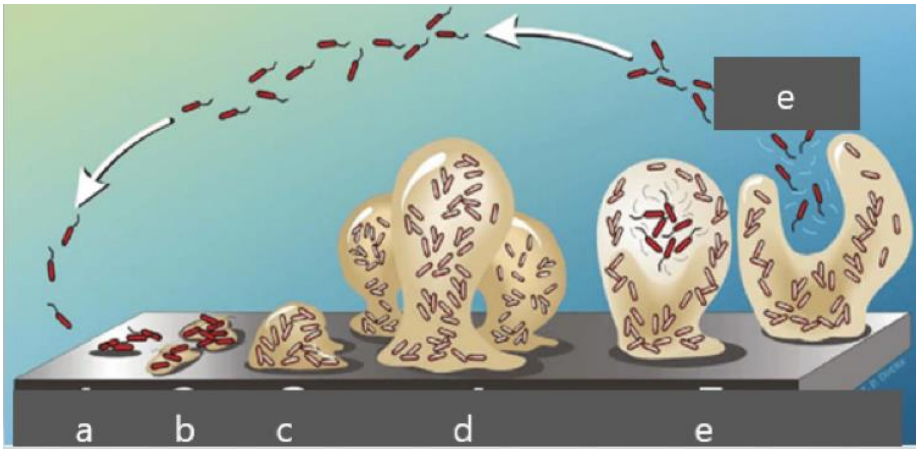


Figure 1.2. Phases of biofilm formation. A. Reversible attachment to the surface b. Irreversible attachment to the surface c. Microcolony formation d. Biofilm maturation e. Dispersion of planktonic bacteria from the biofilm [41].

Stability of the biofilm structure depends on three polysaccharides: Alginate, Pel and Psl. Alginate is an anionic linear polymer which gives structural stability to biofilms. Mucoid strains overproduce alginate (e.g. strains isolated from lungs of CF patients). Pel and Psl is mainly seen in non-mucoid strains. Pel is a glucose rich cationic polysaccharide and the *pel* (polysaccharide encoding locus) has seven genes. Psl (neutral) has repeating pentasaccharides and the *psl* (polysaccharide synthesis locus) has twelve genes. Pel and Psl together form the primary structure scaffold in biofilm development [40,42].

PAO1 and PA14 are the two most commonly studied non-mucoid laboratory strains; the former needs Psl while the latter requires Pel production for mature biofilm growth [42].

In addition to polysaccharides, matrix proteins (e.g. adhesins, amyloids, nucleoid-associated proteins) contribute to structural support of biofilm [43]. Biofilm mode of growth enables bacteria to be tolerant to antibiotics and biocides as well as increasing the chance of horizontal gene transfer with the help of close proximity [44].

For the purposes of this thesis, biofilms already formed in the hospital shower heads and hoses were studied and ways to eradicate them were investigated. Clinical and environmental studies to remove biofilms done by different groups are detailed below:

1.1.1.3.1. Removal of biofilms

1.1.1.3.1.1. Clinical

Treatment of biofilms using therapeutic enzymes aims to degrade the biofilm matrix. Studies on PAO1 showed that nitric oxide, antibiofilm peptide 1080 and cis-2-decenoic acid prevent and disrupt biofilm formation when applied for more than 24 hours. However, those treatments are not strain-specific and they might harm the microbiota. The only enzyme used in clinical practices to disrupt biofilms is dornase Alpha I (Deoxyribonuclease I) which hydrolyses eDNA [45].

Biofilms formed by the strains overproducing alginate (mucoid strains) were shown to be up to 1,000 times more resistant to tobramycin when compared to non-mucoid strains whereas resistance of planktonic cells were the same [46]. A further study used seaweed alginate beads as a model for EPS of *P. aeruginosa* biofilm in which diffusion of tobramycin to the biofilm was shown to be retarded as a consequence of positively charged tobramycin molecules binding well to the anionic surface of the alginate matrix, therefore slowing down the bulk of tobramycin to diffuse to the biofilm interior [47]. When treated with alginate

lyase, antibiotic diffusion through the EPS is enhanced, suggesting that alginate forms a physical barrier. Early biofilm formations can be dispersed by DNAase (targeting eDNA) [48].

Many studies have attempted disrupting biofilms by using biosurfactants (e.g. rhamnolipids), chemical surfactants (e.g. caprylic acid) or antimicrobial agents (e.g. norspermidine), thermal disinfection (including self-cleaning drain systems) and pressured steam [49–53]. This laboratory-based studies are aiming to have use in disrupting biofilms in clinical instruments (endotracheal tubes, IV lines) or clinical environments (e.g. pipework of hospitals) [51,54] . For this purpose, surface properties such as hydrophobicity and electrical charge are well studied to produce biofilm-repellent clinical devices [55].

1.1.1.3.1.2. Environmental

Among biocides, chlorine is commonly used in water systems to control biofilms. Chlorine leads to cell death by disrupting the cell membrane. Monochloramine (as a secondary disinfectant) and chlorine dioxide (strong oxidant) are also used. Thermal disinfection (raising the water temperature to greater than 60ºC), dismantling the water outlets (for descaling and disinfection), and ultraviolet (UV) irradiation are other ways to control biofilms. However, those methods often fail to eradicate already formed biofilms [56].

Strain and surface properties are the two main factors affecting the biofilm forming properties of *P. aeruginosa* as given below.

1.1.1.3.2. Strain properties

In order to understand the cellular factors involved in biofilm forming properties of *P. aeruginosa*, its LPS mutants have been studied. Studies suggest that hydrophobicity and motility are the two most important factors promoting biofilm formation. Zeta potential (cell charge) and chemical composition of the surface are ranked after those two variables. Aggregation, production of outer membrane vesicles (OMVs) and exopolysaccharides have negative effect on biofilm formation [57]. Further studies showed that near neutral zeta potential, hydrophobic surface and motility of the strain helps producing more biofilm on both hydrophobic and hydrophilic surfaces [58]. Biofilm microbial community composition is also important for surface charge by different bacterial communities altering the surface charge [59].

1.1.1.3.3. Surface properties

Studies with PAO1 showed that negatively charged surfaces reduce motility, adhesion and biofilm formation [60]. A more comprehensive study on five strains (including PAO1 and PA14) demonstrated that either positive or negative charge modification of surfaces decrease adherence to surface when compared to unmodified surface. In vivo studies on rats where *P. aeruginosa* were added on titanium screws on skulls support in vitro findings: *P. aeruginosa* grew on unmodified implants leading to persistent infection whereas no persistent infection was observed on charge-modified implants (both positively and negatively charged), decreasing colonisation of implants [61].

In addition to strain and surface properties, quorum sensing has an important role in communication between the cells, coordinating communication and sensing the already formed biofilms around the cells. This system and signalling molecules are detailed below:

1.1.1.3.4. Quorum sensing

Quorum sensing (QS) is the regulation of gene expression to be able to adapt the environment and response to other bacterial cells. Activation of transcription factors is enabled by production of secondary metabolites, coordinating QS target genes. QS mechanisms include regulations in virulence, pathogenicity, motility and biofilm formation. Bacterial communities secrete QS signalling metabolites which further regulate the construction and behaviour of neighbouring biofilms [62].

1.1.1.3.4.1. Quorum sensing systems

Three major QS systems regulating virulence factors are LasR, RhlR and MvfR (PqsR), with LasR (located at the top of QS cascade) and RhlR (shown to function in the absence of its canonical autoinducer *N*-butanoyl-l-homoserine lactone (C4-HSL)) playing the most important roles and MvfR contributing to the induction of both [63,64].

Three major groups of signalling molecules are acylhomoserine lactones (AHLs), oligopeptides and the LuxS/autoinducer 2. LasR's cognate signal is N-(3-oxododecanoyl)-L-homoserine and PqsR's signalling molecule is Pseudomonas Quinolone Signal (PQS, 2-heptyl-3-hydroxy-4(1H)-quinolone) [44]. Pyocyanin (nitrogen containing compound from phenazine family) serves as an intercellular signalling molecule in biofilm formation and its expression is controlled by QS mechanisms [65].

1.1.1.3.4.2. Rhamnolipids

Rhamnolipids (amphipathic glycolipids serving as biosurfactants) are under QS control and have important roles in biofilm maintenance. There are open channels in between microcolonies enabling nutrient and oxygen access and waste product removal. Rhamnolipids are essential in keeping those channels open. They also take part in detachment of cells from the biofilm. They are also involved in mushroom cap formation of the biofilm structure [44].

1.1.1.3.4.3. Environmental conditions

Studies showed that iron limiting conditions enhance QS gene expression via upregulation of *pqs* genes and high release of eDNA. Low shear wet environments induce *psl* operon resulting in Psl production which is one of the main components of EPS. Rhamnolipid production was found to increase in those environments [44]. In hospital water systems, high shear is tried to be established by flushing to disrupt and deform biofilms [66].

Hospital water systems, regulations to manage safe water and their relation to outbreaks are now reviewed:

1.2. Hospital Water Systems

1.2.1. Safety Management

Water quality in hospitals and healthcare settings are under regulation of guidelines set by local or joint groups in European countries and USA (US Centers for Disease Control and Prevention) [67]. World Health Organisation (WHO) issued guidelines for drinking water quality by taking into account not only hospitalised patients but also public health [68]. Health Technical Memoranda (HTMs) created by Department of Health, England set up best practice engineering standards and give advice for design, installation and commissioning of safe water in healthcare premises in the UK [69]. HTM 04-01 C focuses on *P. aeruginosa* in augmented care units and gives advice to National Health Service Managers. As advised by these guidelines, many National Health Service (NHS) institutions set up Water Safety Groups and have implemented water safety plans [70,71].

1.2.2. Water Quality and *P. aeruginosa* colonisation

Contaminated water outlets in healthcare settings possess high risk to the following group of patients: ICU, neonates, cystic fibrosis, renal dialysis, transplantation, haematology, cancer therapy and burn treatment patients. Generally, immunocompromised patients are at higher risk of infection when compared to immune competent patients [72]. There are various sources of contaminated outlets such as hospital water, plumbing equipment, sinks and

faucets, showers and shower heads, ice machines, humidifiers, sink traps, flow straighteners, tap water aerators/diffusers, and hydrotherapy tools [72,73].

In a water distribution system, as the water travels from large diameter supply pipes to downstream pipes with smaller diameter, bacteria start to form biofilms by taking advantage of the varying water flow rate and stagnation. As *P. aeruginosa* survives prefentially in distal parts of water system such as sinks, taps and showers to get easy access to oxygen (as it is an aerobic bacteria and can only grow to some extent by using nitrate in anaerobic conditions); they can easily form biofilms near the point of use and disperse planktonic bacteria to the water system consequently passing to the patients and staff, medical devices and other instruments and surfaces contacting water outlet. It is difficult to eradicate biofilms from the hospital water system especially in the presence of dead-legs where water is stagnant and biocides do not diffuse [12,73,74]. When outlets were found to be contaminated, sampling back several points towards the water supply system are tried. Contamination was found to be on distal parts of the system (e.g., reservoir of sensor taps), showing the importance of parameters such as pipe corrosion and shear stress [67]. In rare cases, water supply was also found to be contaminated which led to decommissioning all sinks [75].

1.2.3. Significant Infective Dose

Significant infective dose depends on many factors, hence there is no certain criteria given in literature or suggested in this thesis [76]. Dose response is not only dependent on the quantity of bacteria but also to exposure duration and frequency, predisposing factors of the individuals, etc. There are dose response studies done for dermal dose of *P. aeruginosa* where an earlier study reported 1000 CFU/mL as folliculitis minimum infective dose [77,78].

Although many studies focused on quantifying *P. aeruginosa* from whirlpools and hydrotherapy pool water sampling, aerosol production during showering and its risk to *P. aeruginosa* infection through inhalation is also important. Aerosol production depends on shower specifications such as design of the shower head, size of the nozzle as well as shower stall configuration and inlet water flowrate and temperature. Previous studies characterised aerosol concentrations through test chambers with estimated bacteria concentration, exposure time, breathing rate, etc [79]. In a real life example from a hospital setting where average bacterial count was 2.2 x 10^7 CFU/L in shower water, there was 3.4 x10^3 CFU/m^3 aerosol production [80]. Infective dose estimates for *P. aeruginosa* for humans are not available but mice studies suggested this dose should be greater than 10^7 CFU [76].

1.2.4. Risk of Infection

Although it has been known that *P. aeruginosa* is one of the main inhabitants of hospital water outlets, it was not suspected as the causative agent of for hospital-acquired bacteraemia until 2009. After the use of DNA –based typing techniques, the link of these infections to hospital water pathogens started being investigated by many researchers [67].

A one year prospective epidemiological investigation held by Blanc *et. al* in a 870-bed tertiary care hospital showed that 56/132 *P. aeruginosa* case strains in ICU were not distinguishable from the ones found in faucet swabs (taken by dismantling faucets, plated on blood and cetrimide agars and incubated at 35°C) which were found to be belonging to nine different genotypes by PFGE. Another important aspect of this study was that the *P. aeruginosa* positive faucet swabs were taken from hot-cold water mixing chambers but the presence of *P. aeruginosa* was not found in any of the water outlets demonstrating that although the faucets were the reservoir of colonization, cross contamination from the water system is also possible [81] . In another study, Aumeran *et. al*. investigated contaminated water sources following an outbreak where *P. aeruginosa* was recovered from central venous catheter (CVC) blood cultures from children in the oncohaematology paediatric unit. Investigation was done by repetitive intergenic consensus polymerase chain reaction which showed that the *P. aeruginosa* strains found in the heavily contaminated showers frequently used by children with CVC were indistinguishable from clinical strains [82].

In the UK, following *P. aeruginosa* outbreaks in neonatal units in Northern Ireland in 2011 and 2012, examination of environmental sites showed their importance as sources of infection. In one of the hospitals where three pre-term babies were affected by the outbreak; two of them were indistinguishable from the strain present in the tap in ICU room. The third strain was found in the swab from a sink in the entrance of the ICU room. In another hospital where five babies were infected and ten babies were colonised with the outbreak strain; water samples from the five out of six taps in neonatal ICU and one tap from Special Care Baby Unit showed the same outbreak strain. The babies might have been infected by the contaminated taps through washing during nappy changes or by the usage of tap water when defrosting breast milk. In two other hospitals, although *P. aeruginosa* was found in sink and tap swabs and water samples, no direct association was found with the outbreak strain [83].

Following these outbreaks, further assessments were made to understand the risk of environmental contamination. Flow straighteners and associated components were found to

be highly contaminated by *P. aeruginosa* with VNTR profiles similar to the infected neonates and tap water samples. Higher counts of *P. aeruginosa* were found in flow straighteners in sensor taps when compared to the ones in non-sensor taps, the emphasis of complex flow straighteners harbouring more bacterial counts than their simpler versions by providing greater surface area for biofilm to form, using more EPDM for tap solenoids, etc. In addition, thermostatic mixer valves (TMVs) provide favourable water temperature for *P. aeruginosa* to proliferate [71]. As a result, HTM 04-01 advises the removal of flow straighteners and presenting non-TMV mixing taps, checking the less frequently used outlets and flexible hoses (found to have three times more *P. aeruginosa* counts when compared to copper pipes) if there is *P. aeruginosa* contamination in the water system. EPDM is a common material used in shower hoses on which *P. aeruginosa* biofilm found to persist, therefore guidance suggests avoiding EPDM [74,84,85] . However, one study investigating silicone and nitrile rubber as alternative materials to EPDM on automatic taps. did not show significant difference in *P. aeruginosa* counts to suggest replacement of EPDM [86].

This literature review showed the clinical importance of environmental *P. aeruginosa* and given the continuing shower water contamination and *P. aeruginosa* bacteraemia in UCH which is a multistorey building teaching hospital where this research is undertaken, this PhD thesis is planned by focusing on many aspects of the problems in following chapters.

Relatedness analysis of strains collected from shower waters and *P. aeruginosa* bacteraemia patients were determined by molecular typing methods. Below is a summary of typing methods. VNTR and WGS are used in this thesis.

1.3. Molecular Typing Methods

Molecular and genomic typing methods are being used to find out the relatedness of bacterial strains. Traditional methods such as bacteriophage typing and serotyping left their places to modern molecular methods like ribotyping, PCR based methods, applications based on electrophoresis, etc. [87]. Below are some of the most common methods employed for genomic characterisation:

1.3.1. PFGE

Pulsed field gel electrophoresis (PFGE) relies on chromosomal DNA restriction patterns. Tenover *et al.* [87] set the methodology of this electrophoresis by using restriction

endonucleases and then comparing discrete bands to analyse the relatedness; however no standardized criteria was used, hence making different studies difficult to compare . DNA fragments produced by restriction endonucleases (*SpeI* and *XbaI* for *P. aeruginosa*) show discrete bands when run on agarose gel which are compared between each other to search for their relatedness. However, point mutations, insertions and deletions of DNA can change the PFGE patterns which might be misleading when investigating outbreaks [87]. Another limitation of PFGE is that the procedure is long taking days and requires expertise and experience in the area [88].

1.3.2. MLST

Another widely used method, multilocus sequence typing (MLST) is based on PCR and sequences fragments in housekeeping genes. Relatedness of isolates is shown by comparing differences between allelic patterns. Although it looks for a small proportion of the genome, it is more discriminatory when compared to PFGE [88].

1.3.3. MLVA (VNTR)

Multilocus variable number tandem repeat (VNTR) analysis (MLVA) which is another PCR-based method provides more rapid (hours) robust results when compared to MLST and PFGE. The number of repeats in the selected VNTR regions constitute a code which can be used to compare between other isolates. 19 VNTR loci were described for *P. aeruginosa* and the VNTR characteristics of the 15 loci (ms77, ms127, ms142, ms172, ms207, ms209, ms211, ms212, ms213, ms214, ms215, ms216, ms217, ms222 and ms223) are given in Table 1.1. Characteristics of the remaining four loci are not given in the table since ms10 and ms61 are highly variable, ms173 has a large allele size range and ms194 has small repeat unit when compared to its allele size. Details of locus names, primer sequences, repeat unit sizes and Hunter-Gaston Discriminatory Indexes (HGDI) are listed with the examples of reference strains PAO1 and PA14. HGDI gives the probability of two unrelated strains to be classified as the same strain and it is advised to be greater than 0.90 for higher confidence level of interpretation [89,90].

Table 1.1.VNTR characteristics, loci names and primer sequence details [89]

Locus name	Position (kb) in PAO1	Primer name	Primer sequence	Repeat unit size (bp)	Product size bp (repeat copy no.)		No. of observed alleles (alleles)[a]	HGDI index[b]
					PAO1	PA14		
ms77	2263	ms77L ms77R	GCGTCATGGTCTGCATGTC TATACCCTCTTCGCCCAGTC	39	442 (4)	364 (2)	7 (2–6 + 1.5, 2.5)[c]	0.51
ms127	3496	ms127L ms127R	CTCGGAGTCTCTGCCAACTC GGCAGGACAGGATCTCGAC	15	210 (8)	225 (9)	2 (8–9)	0.33
ms142	3876	ms142L ms142R	AGCAGTGCCAGTTGATGTTG GTGGGGCGAAGGAGTGAG	115	890 (7)	201 (1)	9 (1–7 + 2.5, 3.5)	0.81
ms172	5084	ms172L ms172R	GGATTCTCTCGCACGAGGT TACGTGACCTGACGTTGGTG	54	789 (12)	789 (12)	6 (8, 10–13 + 8.5)	0.73
ms211	264	ms211L ms211R	ACAAGCGCCAGCCGAACCTGT CTTCGAACAGGTGCTGACCGC	101	663 (5)	360 (2)	8 (2–8 + 1.5)	0.76
ms212	521	ms212L ms212R	TGCTGGTCGACTACTTCGGCAA ACTACGAGAACGACCCGGTGTT	40	522 (9)	324 (4)	10 (3–9, 11–12, 14)	0.75
ms213	2568	ms213L ms213R	CTGGGCAAGTGTTGGTGGATC TGGCGTACTCCGAGCTGATG	103	640 (5)	221 (1)	7 (3–7, 9 + 4.5)	0.85
ms214	2705	ms214L ms214R	AAACGCTGTTCGCCAACCTCTA CCATCATCCTCCTACTGGGTT	115	426 (3)	655 (5)	4 (3–6)	0.81
ms215	4376	ms215L ms215R	GACGAAACCCGTCGCGAACA CTGTACAACGCCGAGCCGTA	129	765 (4)	507 (2)	7 (1–6 + 2.5)	0.80
ms216	4528	ms216L ms216R	ACTACTACGTCGAACACGCCA GATCGAAGACAAGAACCTCG	113	543 (3)	315 (1)	4 (1–4)	0.64
ms217	6187	ms217L ms217R	TTCTGGCTGTCGCGACTGAT GAACAGCGTCTTTTCCTCGC	109	606 (2)	933 (5)	7 (1–6 + 1.5)	0.79
ms222	5361	ms222L ms222R	AGAGGTGCTTAACGACGGAT TGCAGTTCTGCGAGGAAGGCG	101	390 (2)	391 (2)	7 (1–6 + 1.5)	0.76
ms223	5455	ms223L ms223R	TTGGCAATATGCCGGTTCGC TGAGCTGATCGCCTACTGG	106	454 (4)	453 (4)	7 (2–7 + 1.5)	0.77
ms207	2735	ms207L ms207R	ACGGCGAACAGCACCAGCA CTCTTGAGCCTCGGTCACT	6	146 (7)	134 (5)	12 (4–14, 17)	0.89
ms209	4541	ms209L ms209R	CAGCCAGGAACTGCGGAGT CTTCTCGCAACTGAGCTGGT	6	148 (6)	148 (6)	7 (4–10)	0.77

1.3.4. FTIR approach for clustering bacteria

In addition to previously mentioned DNA-based methods, surface cell polysaccharides were found to be useful when identifying and clustering Gram negative bacteria in outbreak investigations[91,92]. For this purpose, Fourier Transform Infrared Spectroscopy (FTIR) was used to group bacteria involved in outbreaks caused by Gram negative bacilli (*P. aeruginosa, Klebsiella pneumonia, Enterobacter cloacae, Acinetobacter baumannii*) which can give results in three hours. Comparison of the clustering done by FTIR with PFGE and MLST showed that all *P. aeruginosa* isolates (n=20) were grouped correctly except one which was shown to have undergone a 131-kb chromosomal deletion, a potential reason for misclassification. As the rapid turnaround of FTIR is promising, it was thought to be useful in prospective study, hence the investigators simulated an ongoing outbreak by running eighty

samples over eight days. Isolates of seven out of thirteen outbreaks were clustered correctly whereas the others had one or two misclassifications [92].

1.3.5. WGS

Whole Genome Sequencing (WGS) is used to determine the entire nucleotide sequence of an organism. Generally, short sequences are read by shotgun sequencing which are further assembled *de novo* or by mapping onto a reference genome. WGS data can be used for antimicrobial resistance (AMR) profiling, strain identification, tracking sources of infection in outbreaks. WGS takes longer (24 hours) to complete than other methods and requires bioinformatics analysis. Resolution depends on sequencing depth and coverage. Despite this, WGS is superior as it gives higher resolution in strain identification when compared to PFGE, MLST and VNTR [93–96]. Feasibility of WGS has increased with recent developments which made it fast and affordable [97]. It is widely used in outbreak analysis and previous studies showed its advantage in early detection of outbreaks as well as detection of MDR pathogens [98]. Despite its advantages, WGS has some limitations. Most of the WGS analyses rely on SNP differences by comparing against a reference genome, hence making the analyses highly dependent on the quality and selection of reference genome. Having the detailed genomic information does not give information about gene expression. Genomic data should be analysed alongside with phenotypic screening methods [99].

1.3.5.1. Interpreting WGS data - *P. aeruginosa* genome analysis

1.3.5.1.1. Genetic similarities

Although earlier DNA sequencing studies suggested that *P. aeruginosa* can be represented by two main groups indicated by PAO1 (a chronic wound infection strain isolated in 1950s) and PA14 (a plant infection pathogen); a recent genomic study analysing 1,311 *P. aeruginosa* genomes suggested the presence of at least five major clades [100,101]. Even more detailed structure was suggested in a phylogeny study done by Belkum *et al.* who focused on sequencing and further analysis of genomic diversity of 672 strains isolated from Europe and the United States, five of which originating from nonhospital settings and the rest from clinical isolates which were mainly isolated from non-cystic fibrosis infections. Phylogenetic tree given in Figure 1.3 shows the high genetic similarity between clinical and non-clinical isolates [102,103].

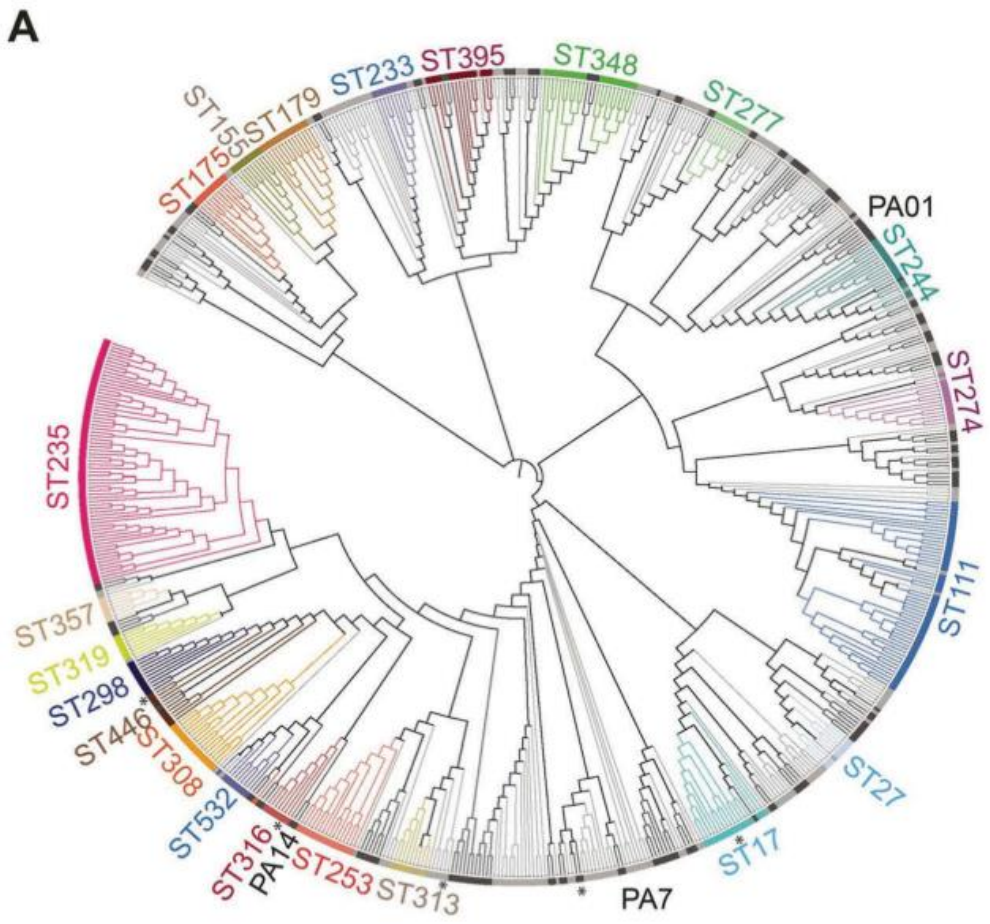


Figure 1.3. Phylogenetic tree constructed by sequencing 672 *P. aeruginosa* isolates, representing their genomic diversity. PAO1, PA7 and PA14 are the common laboratory strains. Black asterisks represent the four nonclinical isolates took part in the study [102].

1.3.5.1.2. Core Genome

P. aeruginosa has a large accessory genome (with a small core genome and a large pan-genome) which gives it the ability to grow under a wide variety of places (e.g. soil and water) under different environmental conditions, causing disease in plants and animals [101]. A study investigating 1,311 genomes showed that core genome constitutes 1% of the pan-genome [100]. In another study focusing on analysis of nine strains from blood, wound, urine, respiratory and ocular infection types out of 2,560 *P. aeruginosa* genomes, 321 core essential genes have been defined, accounting for 6.6% of the genome [104]. Mobile elements such as insertion sequences, transposons and plasmids take part in antibiotic resistance and they constitute a high proportion of the pan-genome. Analysis of the core

genome showed that recombination events through horizontal gene transfer occur frequently [105].

1.3.5.1.3. Epidemiological Analysis

Epidemiological interpretation of WGS data is conventionally done through SNPs (single nucleotide polymorphisms) and cgMLST (core-genome MLST) analysis. As SNP and cgMLST analyses will be restricted to the core genome, a further more sensitive epidemiological analysis could be completed by typing the accessory genome [106].

PubMLST is an open access database which stores information of more than 100 microbial species and genera [107]. In this thesis, genome assemblies were annotated using PathogenWatch which is a global platform using schemes from PubMLST, Pasteur, and Enterobase [108].

SNPs are identified via building a core genome alignment or using a reference genome and mapping sequenced reads. When undertaking the phylogeny analysis through SNPs, diversity in sequences resulting from recombination events (e.g. highly variable regions) should be excluded [106,109]. When performing epidemiological relatedness studies, there is no certain SNP cut-off used by all groups; relevant cut-offs are chosen according to metadata and statistical index calculations [110–112]

cgMLST is also used in epidemiological studies, by comparing the whole genome sequences (assembled or unassembled) with the information available in PubMLST website [107]. In this approach, gene by gene comparisons will be made and recombinant regions will be collapsed into allelic changes [109]. Newly sequenced isolates can be compared phylogenetically and phylogeographically with the isolates present in the database and the identification of high risk clones can be made [101]. Antibiotic resistance genes, especially carbapenemase genes are frequently found in high-risk clones. Metallo-beta lactamases (*bla_{VIM}, bla_{IMP}, bla_{SPM}, and bla_{NDM}*) are the globally prevalent carbapenem resistance genes taking part in multidrug-resistance [101,113]. Antimicrobial resistance should also be analysed by plasmid epidemiology, where short read sequences will be challenging in assembly, therefore plasmid isolation before sequencing or long read sequencing is needed [106,114].

1.3.5.1.4. Global clones of *P. aeruginosa* and MDR interpretation from WGS data

Worldwide distribution of *P. aeruginosa* follows a non-clonal epidemic population structure but the distribution of some sequence types (ST111, ST175, ST235, ST244 and ST395) are associated with outbreaks from different locations [115]. Epidemiological studies making use of the *P. aeruginosa* stains typed by MLST showed that ST235 is the global predominant clone showing MDR properties, potentially explaining its evolutionary success [116]. A study involving genome analysis of 79 clinical isolates from a 27-year period showed that the emergence of ST235 sublineage shows Europe, dating back to 1984. This study revealed fourteen distinct clades of ST235, with a global dispersion and countrywide spread [115].

1.3.5.1.5. Emergence of fluoroquinolone resistance

Treepong *et al.* demonstrated appearance of the most recent common ancestor of ST235 approximating to 1984 coincides with the usage of fluoroquinolones to treat pseudomonal infections. Earliest strains involved in the study showed full susceptible fluoroquinolone genotype whereas the latter ones had mutations in quinolone-resistance-determining regions (QRDR). This supports the finding that the start of usage of this group antibiotics caused fluoroquinolone resistance in strains and those having QRDR mutations were selected. This selection especially favoured the spread of strains having *exoU* gene which was found in all ST235 strains typed [115]. ExoU, which is a part of T3SS affects the severity of the disease and previous studies reported its association with multidrug resistance, including ciprofloxacin [117,118].

1.3.5.1.6. WGS and Phenotypic AST comparison

Antibiotic resistome defines intrinsic and acquired genes in the genome. Environmental resistome consists of pathogens found in natural and built environments [119]. Analysing resistome (set of primary antibiotic resistance genes in the pathogen's genome) of *P. aeruginosa* through WGS data helped scientists to understand antibiotic resistance pathways and their evolutionary perspectives. Correlation of antibiotic resistant genotype and phenotype was thought to be important in designing therapeutic strategies [120]. A schematic representation of WGS in relation with its AST applications is given in Figure 1.4 [121]. However, studies analysing WGS data for genotypic-phenotypic concordance of antibiotic susceptibility patterns of bacteria showed that there is insufficient data to use WGS results as a guide to decide on antibiotic resistance for clinical purposes. In order for an AMR gene to be identified from a sequenced strain, the region should have full-length identical genes to the previously defined AMR genes. Because of this, genetic variations and emergence of new

resistance mechanisms resulting from altered expression of resistance genes might be missed when interpreting WGS data. Furthermore, RNA sequencing or microarray expression is needed as WGS alone is not able to provide data on levels of gene expression [122].

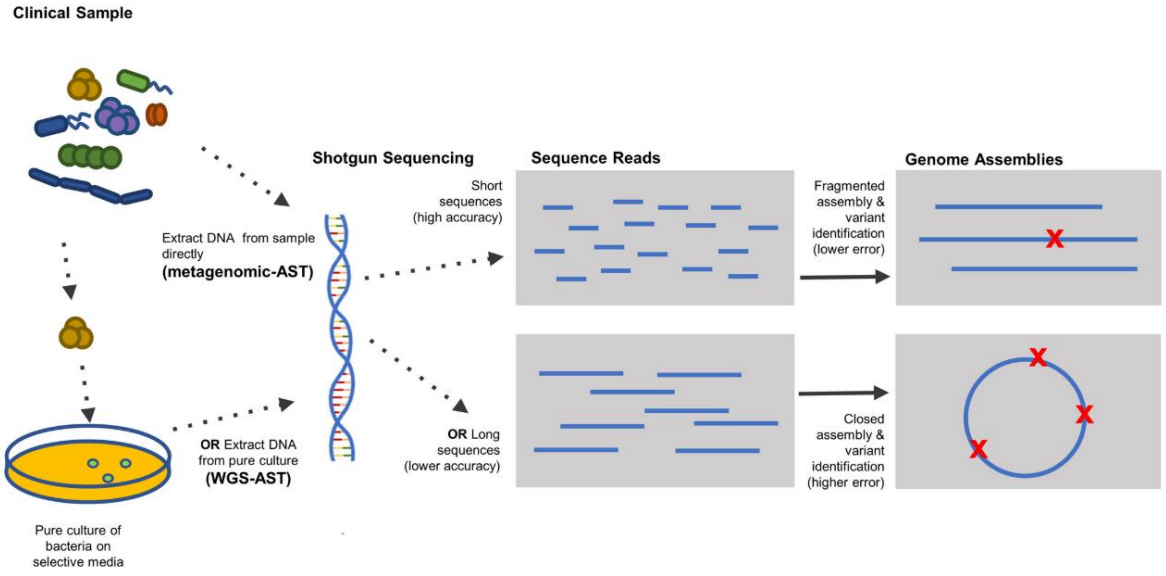


Figure 1.4. Schematic representation of WGS and its application on AST profiles. Shotgun sequencing of short or long sequences of DNA regions and the illustration of their assembly [121].

Transfer of mobile genetic elements, genome-wide homologous recombinations, insertion/deletion events and modification of endogenous genes give *P. aeruginosa* a high genomic diversity [121]. A previous comparative study on WGS data and MIC results showed that 63 of 105 (60%) of isolates which were non-susceptible to amikacin after MIC tests actually contained a genomic element that could be used to predict resistance whereas 42 of them did not show any elements related to amikacin resistance in WGS data. Furthermore, 283 isolates were concluded to be sensitive to amikacin after MIC tests, 30 of which were shown to be carrying amikacin-resistance genes further emphasizing the importance of gene expression. Analysis of meropenem and levofloxacin resistance in the same study revealed 91% and 94% sensitivity and specificity for genomic inference of resistance genes where *gyrA* and *gyrB* mutations were observed [103,122]. Given that the presence of a resistance gene does not necessarily mean it will be expressed; phenotypic AST results are included in this thesis in addition to the genotypic WGS AMR gene absence/presence study.

1.3.5.1.7. Outbreak investigation from AST and WGS data

Investigation of outbreaks in two adjacent hospitals were initially thought to be monoclonal as the strains had similar antibiotic susceptibility patterns and all carrying carbapenemase bla_{VIM} gene. However, WGS analysis proved them to be polyclonal, grouping the strains into two main clusters. Comparison of the strains collected through 4 years showed the bacteria's adaptation to environment mainly due to mobile genetic elements [123]. Another study investigating outbreaks from two hospitals from the UK involved MDR *P. aeruginosa* strains only sensitive to colistin and also sensitive to disinfectants [124,125]. Although VNTR analysis concluded that the outbreak strain did not match the isolates on the national database, WGS revealed the strain to be belonging to ST111, one of the most common epidemic clones in Europe, showing WGS had superior discriminatory power when compared to VNTR. Genome analysis of ST111 showed key genetic mutations resulting in XDR phenotypes [125]. WGS investigation of an outbreak in Germany demonstrated outbreak-specific SNPs forming four dominant clades which enabled researchers to predict the date of the outbreak as a year prior to the diagnosis of the index case [126].

1.3.5.1.8. Recombination

Recombination is the transfer of genetic material in between bacterial cells. Gene transfer is enabled by three mechanisms: Conjugation (transfer of plasmid from one cell to another), transduction (injection of DNA to the cell by phage), and transformation (incorporation of DNA from the environment into the bacterial cell) [127]. Recombination occurs more often between the same species when compared to the different species. Homologous recombination is the import of genes and gene fragments replacing the homologous genetic material in their genome (recA-mediated recombination). The rate of recombination differs in different species and within linages of the same species. There are software packages available which use developed algorithms analysing nucleotide sequencing data and relative rates of recombination (e.g. ClonalFrame, BratNextGen, Gubbits, etc.) [128,129].

BEAST framework which is based on Bayesian paradigm is widely used and it is later optimized based on maximum likelihood through Treetime tool which used Phyton-based framework. This new methodology enables researchers to estimate evolutionary rates, account the effect of recombination and finally construct dated phylogenies [130,131].

1.3.5.1.8.1. Effect of Recombination Rate on Outbreak Analysis

Some species have lower recombination rates. *Staphylococcus aureus* has a lower rate than other nasopharyngeal pathogens (relative recombination rate = 0.7). For *Escherichia coli*, there are different recombination rates reported, but higher rates of recombination are found in virulent lineages [128]. In some cases, high recombination rates are observed during adaptation, but lower following adaptation. An important example of this is *Salmonella enterica* serovar *typhi* which exchanged 1/4th of its genome with *S. paratyphi* A [132]. High rates of recombination are shown in *Helicobacter pylori*, *Streptococcus pneumoniae*, *Flavobacterium psychrophilus*, etc [133,134].

P. aeruginosa has genome plasticity consisting of conserved core and variable accessory genome [135]. Diversity in *P. aeruginosa* clones suggest frequent recombination but not analysed in detail by MLST data [117]. Although in vitro recombination studies have been done in vitro, they do not tell us exactly what happens in nature [127]. For the purposes of this thesis, focus was put on shower and handwash drain environment as they were thought to be the long-term reservoirs of MDR bacteria, hence resistance genes [136]. Horizontal gene transfer in natural environments was shown to happen frequently where there is high cell population, e.g., biofilms. Sink drains were good examples having these properties and were reported to be the cause of outbreaks [137,138]. Following *P. aeruginosa* bacteraemia outbreak in 2017 in UCH, relatedness analysis according to SNP differences were determined as given in Chapter 5; however, it should be noted that high recombination rate will result in more genomic differences, hence less strict criteria should be used when determining the likely matches.

Aims and Hypothesis

This PhD study investigates the relation between outbreaks of infection with *P. aeruginosa* in the hospital and contamination of the environment with a particular focus on shower waters and drains.

Three hypotheses supporting this statement of intent are given as follows:

Hypothesis 1:

The increase in *P. aeruginosa* bacteraemia in the hospital in 2017/8 was caused by spread of contamination of showers.

Aims:

To determine whether there is a correlation between environmental *P. aeruginosa* isolated from hospital showers and blood isolates from patients using these showers.

To determine the time for recolonization of a new shower hose by *P. aeruginosa*.

Hypothesis 2:

The type of shower, arrangement of hose, shower head holder and shower can minimize the formation of *P. aeruginosa* biofilm in the hose and reduce the spread of microorganisms from the drain.

Aims:

To determine which arrangement of shower head, shower hose and pipeline minimises *P. aeruginosa* colonization in hospital water systems.

To develop new configurations such as shortening hose length, reducing water stasis through the hose and proposing alternative disinfection methods to prevent recontamination.

Hypothesis 3:

The risk of *P. aeruginosa* infection is dependent on patient characteristics and the proximity of the patient bed to contaminated sites such as showers and drains in the hospital.

Aims:

To correlate *P. aeruginosa* bacteraemia with colonization of showers patients are using through a case control study.

Chapter 2. Materials and Methods

2.1. Materials

2.1.1. Solid Media (90mm agar plates; 4mm deep-filled):

Pseudomonas C-N Agar (PCNA), Oxoid, Thermo Fisher Scientific, US

Columbia Blood Agar (CBA), Oxoid, Thermo Fisher Scientific, US

Nutrient Agar (NA), Oxoid, Thermo Fisher Scientific, US

Milk Cetrimide Agar (MCA), Oxoid, Thermo Fisher Scientific, US

Muller-Hinton Agar (MHA), 8mm deep-filled - Oxoid, Thermo Fisher Scientific, US

2.1.2. Liquid Culture Media and Diluent

Phosphate Buffered Saline (9mL, pre-sterilised) - Oxoid, Thermo Fisher, US

Nutrient Broth (10mL, pre-sterilised) - Oxoid, Thermo Fisher, US

Neutraliser solutions

Neutraliser A: 3% (w/v) Tween 80, 0.3% (w/v) Lecithin, 0.1 % (w/v) Sodium thiosulfate, (Sigma-Aldrich, UK) and prepared in Phosphate-buffered saline (PBS) solution (Oxoid, Thermo Fisher, US).

Neutraliser B: 3% (w/v) Tween 80, 0.3% (w/v) Lecithin, 1.0 % (w/v) Sodium thiosulfate, 1.5% (w/v) K2HPO4, KH2PO4 0.05% (w/v), 1% (w/v) Poly-[sodium-4-styrenesulfonate], 0.1% (v/v) Triton® X100 (Sigma-Aldrich, UK) and prepared in Phosphate-buffered saline (PBS) solution (Oxoid, Thermo Fisher, US).

Solutions were sterilised by autoclaving (121°C for 15minutes) and refrigerated (2-5°C) until required.

2.1.3. General Laboratory Equipment

MALDI-TOF mass spectrometry, MALDI biotyper system Bruker, US

MALDI-TOF tile, Bruker, US

Incubators (37ºC and 35ºC), Thermofisher, US

Cooled Incubator, Memmert, Germany

Hotplate (55ºC), Grant-bio, UK

UV – fluorescence lamp with viewing cabinet (long-wave; 365nm wavelength), Analytik Jena, UK

Vortex (high speed range: 700-1100g), Scientific Industries, US

Centrifuge (Thermo Scientific Heraus megafuge 8; capable of 1100xg), US

Micro centrifuge (Thermo Scientific Heraus megafuge 8; capable of 5000g), US

Vacuum filtration Manifold – Millipore, Germany

Vacuum pump (Max 65kPa), Millipore, Germany

Freezers (-20°C and -80°C), Liebherr, Germany

Pressure Gauge (0-4 bar range, RS Components, UK)

Autoclave, LTE Scientific, UK

Microplate photometer UV/Vis microplate reader, Multiskan FC, Thermo Scientific, US

2.1.3. Water Processing Materials and Equipment

Water sample collection containers (100mL capacity; dosed with 18mg sodium thiosulfate), IDEXX Laboratories Inc, US

Filtrration Funnels (100mL capacity; code: M1HAWG100) Millipore, Germany

Manual Filter membrane holders (47mm diameter capacity; Advantec, Toyo Kaisha Ltd. Japan) - Cole-Parmer, US

Filtration membrane (47mm diameter, 0.45µm pore-size, black-gridded; Advantec, Toyo Kaisha Ltd. Japan) - Cole-Parmer, US

Bunsen burner (Campingaz; propane/butane; portable) – VWR US

Stainless steel tweezer (blunt-nose; flat-tipped) – VWR US

Pipettes (100µL, 500µL, 1000µL, 10000µL operational capacity), VWR, US

Multichannel pipettes (100µL, 500µL, 1000µL operational capacity), Finnpipette Systems Thermo Fisher Scientific, US

2.1.4. Antibiotic Susceptibility Testing (AST) Materials and Reagents

Antibiotic-impregnated discs:

Ticarcillin – clavulanic acid (TCC), Biorad, US, 75/10µg

Piperacillin (PRL), Oxoid, Thermo Fisher Scientific, US, 30µg

Piperacillin – tazobactam (PTZ), Oxoid, Thermo Fisher Scientific, US, 36µg

Amikacin (AK), Oxoid, Thermo Fisher Scientific, US, 30µg

Ceftazidime (CAZ), Oxoid, Thermo Fisher Scientific, US, 10µg

Cefepime (CEF), Oxoid, Thermo Fisher Scientific, US, 30µg

Ciprofloxacin (CIP), Oxoid, Thermo Fisher Scientific, US, 5µg

Tobramycin (TOB), Oxoid, Thermo Fisher Scientific, US, 10µg

Aztreonam (AZT), Oxoid, Thermo Fisher Scientific, US, 30µg

Imipenem (IMI), Oxoid, Thermo Fisher Scientific, US, 10µg

Meropenem (MEM), Oxoid, Thermo Fisher Scientific, US, 10µg

Gentamicin (CN), Oxoid, Thermo Fisher Scientific, US, 10µg

McFarland Standards, Pro-Lab Diagnostics, US

Six-cartridge Antibiotic disc dispenser, Oxoid, Thermo Fisher Scientific, US

Vernier Calliper, France

Graduated Scientific ruler, SR1 Shatter Resistant, UK

Curved-nose and flat-ended stainless-steel tweezers, VWR, US

2.1.5. Molecular ID by MALDI-TOF Mass Spectrometry

Bacterial Test Standard (BTS)

IVD Matrix HCCA (α-cyano-4-hydroxycinnamic acid) portioned

Microscout plate (MSP) 96 polished steel target plate

Maldi-TOF Biotyper® IVD system (Bruker Daltronics)

Wooden toothpicks

2.1.6. DNA Extraction Reagents

DNA extraction kit (Zymo Research Quick-DNA Miniprep Plus Kit, Catalogue no: D4068)

DNA away solution, MBP DNA Away Surface Decontaminant, VWR, US

2.1.7. General Consumables

L-shaped spreaders

Inoculation loops (1µl and 10µl)

Universal tubes (15mL and 30mL capacity)

Microcentrifuge tubes (~1.5mL)

Oxidase test strips

Deep-well plates (2.2mL volume per well)

Glass beads (3-4mm diameter)

47mm diameter filter membranes; nitrocellulose; 0.45 µm pore-size; gridded

96 well plate, Corning, US

2.1.8. Storage of Bacterial Isolates:

Nutrient agar slopes (3mL; pre-sterilised), Oxoid, Thermo Fisher Scientific, US

CryoBeads in vials with cryopreservant (Microbank™, UK)

2.1.9. Software

Microsoft Office (Excel, Word, Powerpoint) – Microsoft Inc. US

SPSS - IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp

R - R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

2.2. Methods

2.2.1. Shower Water Sample Collection

1. 1 ml Neutraliser A (composition: 1g/L Sodium Thiosulphate, 30 mL/L Tween 80 and 3 g/L Lecithin in PBS) was dispensed into sterile sample collection bottles (Capacity 120 ml).
2. Shower head was disinfected by wiping the outlet surfaces with a sterile alcohol wipe (70% ethyl-alcohol).
3. A sterile water collection bag (VWR International) was secured to the shower head, the bottom corner cut using sterile scissors before turning the shower on. A minimum of 100mL shower water sample was collected using the water collection bag to direct the water-flow into the sample container.
4. Shower water was aseptically transferred to the sample collection bottle containing 1 mL Neutraliser A (Making the final neutraliser concentration 1% (v/v).
5. Water samples were either processed straight after the water collection or put in 4ºC fridge up to 24 hours.
6. A minimum contact time of 30 minutes was permitted for all samples prior to processing. Minimum contact time was used to allow the neutraliser to quench the activity of residual biocide within the sample. This was done to prevent likelihood of false negative results.

2.2.2. Water Sample Processing

Sample-concentration by filtration

One of the following two methods were used to process water samples. Decision was based on number of samples. At the beginning of the study, where there were 20 water samples each week, manual filtering was used. When more samples need to be processed after adding the targeted water sampling, Manifold filtering which is a faster method was used.

Manually filtering water samples

1. 0.45 µm pore sized filter membranes were placed into sterile filter membrane holders using flame-sterilised tweezers.
2. Water samples were passed through the filter into the 0.45 µm pore sized membranes and manually filtrated by using sterile 50 mL syringes (minimum of 100 mL water sample was filtered).
3. Filter membranes were transferred from the filter holders and placed onto the surface of a Pseudomonas C-N (PCN) Agar using sterile tweezers and incubated at 37ºC for 48 hours (+/- 4hrs).

Filtering water samples through water manifold

1. Autoclaved stainless steel filtration heads (Manifod, Sigma-Aldrich) (123°C; 30 minutes) were placed on water manifold.
2. Filtration heads were flame sterilized with 70% ethyl alcohol (VWR International).
3. 0.45 µm pore sized filter membranes were placed onto the manifold heads.
4. 100 mL filter funnels were placed on membranes.
5. Water samples were thoroughly mixed (by inverting 3-5 times) and pouring into filter funnels. The samples were concentrated onto membranes by evacuating the water via the membrane into a waste carboy using a vacuum pump Millipore pump (max 65kPa).
6. Funnels were discarded and membranes were plated onto PCN Agar with the help of sterile tweezers and incubated at 37ºC for 48 hours.
7. In between each processing step, filtration heads were flame sterilised with 70% ethyl alcohol.

2.2.3. Drain Sample Collection

1. Sterile cotton swabs were soaked into Neutraliser-A solution.
2. Drain exteriors and interiors were swabbed with the pre-moisturised cotton swabs.
3. Cotton swabs were streaked on PCN Agar plates by making sure to cover all plate surface.
4. Plates were incubated at 37ºC for 24 hours.

2.2.4. Confirmation of *P. aeruginosa*

1. After incubation periods were completed, presumptive *P. aeruginosa* colonies were determined by colour and morphology (usually green/yellow coloured colonies).
2. CFU (colony forming unit) counts were noted down with any other observations.

3. Presumptive *P. aeruginosa* colonies were streaked on Columbia Blood Agar (CBA) plates and incubated at 37ºC for 24 hours.
4. Single colonies from the streaks were re-streaked in parallel onto Nutrient Agar (NA) and Milk Cetrimite Agar (MCA) plates.
5. An oxidase test was performed on the streaks by using oxidase test strips[14].
6. MCA plates were checked for casein hydrolysis.
7. Fluorescence properties of the strains were checked under UV.
8. Isolates giving positive oxidase reaction (observation of colour change to blue on strips) and hydrolysing casein on MCA plates were saved on nutrient agar slopes and/or beads.
9. Single colonies are streaked on nutrient agar slopes, lids were loosened and incubated aerobically at 37ºC for overnight growth, then the lids were tightened and stored at room temperature.
10. 10 µl loop full of streaks were inoculated in the bead tubes. Tubes were inverted for 10 times and incubated at room temperature for 2 minutes. Cryopreservative liquid is discarded and bead tubes were stored at -20ºC.
11. Maldi-TOF identification were done as last step of confirmation.

2.2.5. Sample Preparation for MALDI-TOF Identification

1. Strains going to be identified by MALDI-TOF were streaked on appropriate media (CBA or NA) and incubated at 37ºC for overnight growth. Direct transfer sample preparation procedure was applied for MALDI-TOF identification:
2. BTS (Bacterial Test Standard: *E.coli*) was removed from the freezer and allowed to wait at room temperature for 5 minutes and mixed by vortexing.
3. 1 µl BTS was inoculated onto A1 and A2 spots of the MSP 96 polished steel target plate.
4. Small amount of biological matter (Sufficient amount to create a thin layer of the bacterial culture onto the tip of the toothpick) from the agar plates were taken using a wooden toothpick (pathology laboratory-grade) and smeared in the appropriate spot of the target plate. The duplicate sample spot was smeared by using the same toothpick without revisiting the agar plate.
5. IVD Matrix HCCA was removed from the fridge, warmed in room temperature and vortexed.

6. Each sample spot (including BTS) was covered with 1 µl IVD Matrix HCCA and allowed to incubate at room temperature until the spots are visibly dry. Care was taken to overlay BTS with IVD Matrix HCCA within 30 minutes as otherwise results would not be valid.
7. Once the spots are visibly dry, the target plate was transferred to the vacuum chamber of the MALDI Biotyper® for mass-spectrometry. The Protein signatures were measured by Maldi-TOF and compared to the library of the Biotyper. Identification scores (Log-scores) were recorded. Acceptance values: Scores of >1.7 to <2.0 identifies satisfactorily at the genus level; score>2.0 identifies satisfactorily to the the species level)

2.2.6. Antibiotic Susceptibility Tests

1. *P. aeruginosa* isolates were revived on CBA or NA media.
2. Sterile cotton swabs or 1 µl inoculation loops were used to inoculate bacteria into 3 mL PBS containing tubes, shaken by vortex and the turbidity was set equal to 0.5 McFarland standard.
3. With a sterile cotton swab, the bacterial solution was loaded on MHA plates by surface smearing across the entire plate.
4. Twelve antibiotic discs (TCC, PTZ, AK, CAZ, FEP, PRL, CIP, TOB, ATM, IPM, CN and MEM) were dispensed on MHA plates pre-loaned with bacteria (six antibiotic discs per plate).
5. MHA plates were incubated at 35ºC for 18 hours.
6. Inhibition zones were measured with the help of a graduated scientific ruler or a Vernier-calliper. Sensitivity patterns were determined by comparing with EUCAST breakpoints[15].

2.2.7. Biofilm Quantification in Shower Hoses

1. Shower hoses were collected from hospital wards during three-monthly shower change. These shower changes were done by the hospital as per the shower company guidelines.
2. Hoses were put in sealed plastic bags containing sterile PBS to keep the environment moist and stored in 4ºC until the study.
3. Outer surface of the shower hoses was disinfected with 70% ethanol and allowed to air dry.

4. 1 cm^2 cross sections from proximal to shower head, middle and distant parts of the hose were cut by using a sterilised tin-snips (hand-tool).
5. 1 cm^2 cross sections were cut in half to expose the inner surface.
6. Both halves of the cross sections were transferred to universal tubes (If the testing was to be done later, 5 ml sterile PBS was added to the universal tubes and stored in 4ºC until the study).
7. Storage PBS was discarded from the tubes and 9 mL sterile PBS was added.
8. To remove the planktonic cells, universal tubes were inverted 10 times.
9. To check the planktonic growth, 0.1 mL aliquot from step 8 (first wash) was plated on PCN agar.
10. PBS from step 9 was discarded. Steps 7 and 8 (wash stage) was repeated for three times in total by discarding the PBS after each step.
11. Hose cross-sections were transferred to clean universal tubes containing 10 mL sterile PBS and 4-6 glass beads.
12. Universal tubes were vigorously shaken by vortexing at high speed for 30 seconds.
13. 1 mL from the tube from step 12 was added to 9 mL sterile PBS to obtain 10^{-1} dilution. mL aliquot from 10^{-1} dilution was plated onto PCN agar plate.
14. 10 µl from the 10^{-1} dilution was plated onto PCN agar plate to obtain approximately 10^{-2} dilution.
15. Plates were incubated at 37ºC for 48 hours.
16. CFU counts were noted down for first wash and biofilm dilutions.

2.2.8. Microplate Assay for Biofilm Measurement

Microplate assay methods were adapted by O'Toole [139].

1. Loopful of *P. aeruginosa* strains were inoculated into 10 mL nutrient broth, lid of the tube is loosened, placed on a shaking platform and incubated for 18 hours at 37°C.
2. 100 µl nutrient broth was dispensed into the wells of microtiter well plate, 20 µl from overnight grown bacterial suspension added (~$2x10^6$ CFU)
3. Microtiter well plate was incubated for 24 hours at 37°C.
4. Wells were rinsed thoroughly with sterile PBS.
5. 130 µl crystal violet (1%) was added to each well, incubated at room temperature for 15 mins.

6. Crystal violet was rinsed several times with sterile PBS (until the excess dye washes off)
7. Remaining crystal violet is resolubilized in 200 µl acetic acid (30%), incubated at room temperature for 15 mins.
8. OD measures of 570nm and 595nm were performed with a spectrophotometer.

2.2.9. Pressure Measurement of Showers

Shower heads were dismantled from the hose and pressure gauge fitted directly at the end of shower hose.

1. Shower head was dismantled and the pressure gauge (RS Components) was placed at the end of the shower hose.
2. Water flow was put on highest power and the resulting pressure measures were recorded in Pascal units.
3. The pressure gauge (RS Components) was dismantled from the shower hose and its end was disinfected by immersion into absolute ethanol for 2-3 seconds and then wiping the excess with an alcohol wipe.
4. The shower head was put back on the hose end and disinfected by wiping with a sterile alcohol wipe (70% ethyl-alcohol).

2.2.10. Quantification of *P. aeruginosa* in Shower Water

2.2.10.1. Dilutions by Direct Inoculation into Manifold Funnel

1. Approximately 110 mL shower water samples were collected in 1 mL Neutraliser-A containing collection bottles as described in shower water sample collection section.
2. 10 mL from the neat shower water sample was added to 90 mL PBS to obtain 10^{-1} dilution.
3. 1 mL from the neat shower water sample was added to 99 mL PBS to obtain 10^{-2} dilution.
4. 1 mL from 10^{-1} dilution was added to 99 mL PBS to obtain 10^{-3}dilution.
5. Dilutions and the neat shower water samples were processed through Manifold method as explained in water sample processing section
6. After incubation at 37ºC for 48 hours, CFU counts were noted down.

2.2.10.2. Dilutions by Spread Plating

1. Up to 100 mL shower water samples were collected as described in shower water sample collection section.
2. 0.9 mL sterile PBS was dispensed in well of each deep-well plate. 0.1 mL from neat water samples were inoculated in the first column of deep-well plate (creating 10^{-1} dilution). 0.1 mL samples from the first column were transferred to the second column (creating 10^{-2} dilution) after mixing well with multichannel pipette.
3. Dilutions were carried on in the same manner described in Step 4 up to 10^{-6} dilution. 0.1 mL aliquot from each dilution (10^{-1} to 10^{-6}) were plated on CBA plates.
4. Following incubation at 37ºC for 24 hours, CFU counts were noted down.
5. Confirmation tests of presumptive *P. aeruginosa* colonies were as described in confirmation section.

2.2.11. DNA Extraction

Zymo Research Quick-DNA Miniprep Plus Kit was used for DNA extraction [140].

2.2.11.1. Preparation of Bacteria

To obtain the bacterial count needed for DNA extraction (1-5 x 10^6 cells in 200µl PBS), a loop full biological matter from fresh bacterial isolates on CBA media was inoculated to 10 mL nutrient broth and left for overnight incubation aerobically at 37ºC.

1. Bacteria grown on nutrient broth media was centrifuged for 10 minutes at 110g.
2. Supernatant was discarded and 10 mL sterile PBS was added and shaken well to dissolve the bacterial precipitate by vortexing.
3. 10^{-1} dilution was created by adding 1 mL aliquot from step 2 to 9 mL sterile PBS, resulting in desired cell count (CFU counts were double checked by plating 10^{-5} and 10^{-6} dilutions.

2.1.11.2. Applying DNA Extraction Procedure

1. Proteinase K solution was prepared by adding 1040 µl Proteinase K Storage Buffer to 20 mg Proteinase K tube to make ~20 mg/ml final concentration.
2. 200 µl aliquot from step 4 (preparation of bacteria) was transferred to microcentrifuge tubes.

3. 200 µl BioFluid & Cell Buffer and 20 µl Proteinase K was added to 200 µl bacterial sample from step 2.
4. Mixture was vortexed for 15 seconds and incubated on hot plate at 55ºC for 10 minutes.
5. 420 µl Genomic Binding Buffer was added to the digested sample and vortexed for 15 seconds.
6. Mixture was transferred to a spin column in a collection tube and centrifuged at 12000 g for 1 minute. Collection tube with the flow through was discarded.
7. Spin column was transferred to a new collection tube and 400 µl DNA Pre-Wash Buffer was added followed by centrifugation at 12000 g for 1 minute. Collection tube was emptied.
8. 700 µl g-DNA Wash Buffer was added to the spin column followed by centrifugation at 12000 g for 1 minute. Collection tube was emptied.
9. 200 µl g-DNA Wash Buffer was added to the spin column followed by centrifugation at 12000 g for 1 minute. Collection tube with the flow through was discarded.
10. Spin column was transferred to a clean micro centrifuge tube and 50 µl DNA Elution Buffer was added followed by incubation at room temperature for 5 minutes.
11. Spin column in the micro centrifuge tube was centrifuged at maximum speed (by pulsing the micro centrifuge) for 1 minute where the DNA was eluted. The eluted DNA was stored in -20ºC.

2.2.12. Randomised Selection of Showers

A random number generator in excel was used to select the showers to be included in the *P. aeruginosa* quantification study and the strains to be included in antibiotic susceptibility tests.

2.2.13. VNTR Methods for Multi-Locus Variable Number Tandem Repeat Analysis typing

MLVA targeted nine loci: ms172, ms211, ms213, ms214, ms217, ms222, ms2017, ms209 and ms61, method as described by Turton et al [90]. 188 *P. aeruginosa* strains (111 patient blood isolates and 77 environmental isolates) retrieved between 2017 - 2019 were typed by nine-loci VNTR by UKHSA.

2.2.14. WGS Methods

WGS methodology and analyses were provided by Lucy van Dorp, UCL. Inferences on relatedness of the strains, investigation of resistance and biofilm genes and incorporating them with the case-control study outcomes were performed by myself.
Whole genome sequencing data (fastq format) downloaded from Illumina BaseSpace in three batches totalling 192 isolates.

FastQ files were concatenated within samples to create joined fastq files (suffix XX.joined.R1.fastq.gz, XX.joined.R2.fastq.gz; available at: /SAN/ballouxlab/Precision_AMR/Pseudomonas/Fastq_generator_files*). All files were inspected using FastQC and no additional trimming/filtering of reads was performed.

2.2.15. Genome Assembly Procedure for Whole Genome Sequence Analysis

De novo genome assembly was performed using UniCycler1 v0.4.8.

Species identity was assessed using PathogenWatch (https://pathogen.watch). One isolate – DNA164 was assigned as *Enterococcus faecalis* – and one isolate assembled into a large number of contigs (DNA77 ~13.5Mb genome) with about ~69% of the data being assigned to Achromobacter species. These two isolates were therefore removed before subsequent analysis.

The resulting 190 *P. aeruginosa* assemblies ranged in length from 6151361-7111628 nucleotides with an average number of contigs of 144 (91-240) of mean lengths 49151 nucleotides (29137-72657 nucleotides; 95% CI). GC content ranged from 65.7-66.6%.

Genome assemblies were automatically annotated for ST status and closest published assemblies using PathogenWatch (https://pathogen.watch. Gene by gene ST annotations are provided in the excel table (PA_mlst-PubMLST.csv).

2.2.15.1 Resistance Gene Characterisation

Genome assemblies were annotated for the presence and absence of antimicrobial resistance (AMR) genes using the Comprehensive Antibiotic Resistance Database (CARD)2 Resistance

Gene Identifier tool. Across the 190 isolates we identify a total of 100 unique AMR genes (mean of 55 per isolate; 48-84 95% CI assuming a gene presence threshold of 98%) spanning multiple drug classes.

2.3. Statistical Analyses

Statistical methods for the study given in Chapter 3 were provided and the analyses done by Paul Bassett, Statsconsultancy Ltd.

2.3.1. Environmental Sample Collection

The design of the study was such that showers and drains from two wards (A and B) were measured through a 29-week period. The outcome variable in all analyses was the occurrence of *P. aeruginosa* at a given location at a given point during the follow-up. This was considered as a binary outcome (present/absent). A feature of the study design was the drains and showers from the same locations were measured throughout the study. To allow for this non-independence of the data, all analysis was performed using logistic multilevel (mixed) regression methods. Two level models were used with individual measurements nested with specific locations. Data from showers and drains were analysed separately. However, data from the two wards were included in the same regression model. An interaction between time and ward was included in the models. A significant interaction would imply that changes over time varied between wards. If an interaction was present, changes over time were quantified separately for each ward.

The final set of measurements at week 29 were not included in the analyses as intended to show long term outcome.

Two approaches to the analysis were considered. The first approach considered time as a continuous variable, and the analyses examined changes in the occurrence of *P. aeruginosa* over the course of the study. The shape of the relationship between time and the outcome was examined. Quadratic and cubic terms for time were considered, but these were omitted from

the model if not found to be statistically significant. A second approach considered the follow-up time in 3 periods:

Period 1: Before the first shower change (weeks 1-3)

Period 2: After the first shower change (weeks 4-13)

Period 3: After the second shower change (weeks 15-21)

The differences in *P. aeruginosa* occurrence between the three time periods were assessed.

a) Approach 1 – Changes over time

Using time as a continuous variable, the data suggested that quadratic and cubic terms for time did not significantly improve the fit of the regression model, and so only a linear term for time was included in the analysis.

The analyses suggested a significant ward by time interaction for both showers and drains. *P. aeruginosa* contamination over time varied for Wards A and B. As a result, the change over time for each of the wards was quantified separately.

b) Approach 2 – Time in categories

The second analysis approach divided the follow-up time into three time periods, and comparisons were made between them.

The regression models showed some evidence of a significant interaction between time and ward for both showers ($p=0.04$) and drains ($p=0.05$). Differences between time periods were present in the two wards and were quantified separately.

2.3.2. Statistical Analysis of Antibiotic Susceptibility Profiles

The Kruskal-Wallis and Mann-Whitney tests were used.

The design of the study was such that showers and drains from two wards (A and B) were measured through a 29-week period. For the purposes of these analyses, data for each ward/location were analysed separately, giving four sets of analyses.

The outcome variables were levels of antibiotic susceptibility for each antibiotic. These were assessed on a three-point ordinal scale; sensitive, intermediate or resistant.

A feature of the study design was the drains and showers from the some of the same locations were measured throughout the study (although sampling was performed randomly). In principle, the analysis would allow for this non-independence of the data. However, the overall sample size in each location was relatively small, and for many antibiotics, the majority of responses were in one of the three outcome categories. Therefore, there was insufficient data to implement more complicated methods that would allow for non-independence of the data. Due to the data limitations, a simpler approach was implemented, assuming independence of the data values.

Measurements were split into occurring in one of the 3 time periods, namely:

Period 1: Before the first intervention (weeks 1-3)
Period 2: After the first intervention (weeks 4-13)
Period 3: After the second intervention (weeks 15-21)

The differences in antibiotic susceptibility between the three time periods were assessed.

Due to the ordinal nature of the outcome, the Kruskal-Wallis was primarily used to compare outcomes between the three time periods. The exception was for Ward A drains. Here only two measurements were made in period 1, an insufficient number for analysis. Thus, for this data, comparisons were made between periods 2 and 3 only, with the Mann-Whitney test used for the data analysis.

Chapter 3. Enhanced monitoring of augmented and non-augmented care ward shower waters by longitudinal environmental sampling of *P. aeruginosa* in hospital waters and drains during remediation works

Most of the studies included in this chapter have been peer-reviewed and published by Journal of Medical Microbiology and revised and published (DOI 10.1099/jmm.0.001698). Published paper is given in Appendix E.1.

Statistical analysis and interpretation were provided by Paul Bassett. XPS measurements, results graphs and conclusions were provided by Sanjayan Sathasivam.

This chapter investigates the effects of remediation works on shower water *P. aeruginosa* contamination through weekly / once in two weeks sampling frequency. In the time of the sampling, showers were changed from standard to Ag+ impregnated units.

3.1. Introduction

P. aeruginosa can colonize and persist in the hospital environment for long periods; previous studies state its environmental survival time from 6 hours to 16 months [141]. It can survive under variety of environmental conditions, such as nutrient-depleted settings [142]. In a hospital setting, water is a well-known source of *P. aeruginosa*. Hot tubs, hydrotherapy pools, sink taps, tap aerators, faucets, showers, plumbing systems are common reservoirs [56,143].

The ability of *P. aeruginosa* to form biofilm allows it to colonize wet areas such as shower hoses, water plumbing systems and sink/shower drains but makes it difficult to eradicate [144]. It has intrinsic resistance or reduced susceptibility to many antibiotics, can acquire resistance from other bacterial species in the environment and biofilm enables it to resist to environmental stresses including some antibiotics [145,146]. Presence in the hospital environment presents a risk for immune suppressed patients. Many *P. aeruginosa* outbreaks are reported in the literature and often are linked to the environmental reservoirs [82,147].

Given the importance of safe water for patients in hospitals, many countries implemented regulation strategies to test the hospital water regularly. In the UK, the HTM guidelines of Department of Health include installation and commissioning of water systems as well as water testing. Guidelines state that water sampling for the presence of *P. aeruginosa* should be performed every 6 months where water has direct contact with patients, where staff wash hands or it contacts with equipment which later will be used by patients [69]. HTM 04-01 C

focuses particularly on the risk of *P. aeruginosa* in augmented care settings and gives best practice advice [70].

In this study, two wards were selected for more frequent testing for *P. aeruginosa* (every 1-2 weeks) for a 7-month period between 21.11.2018 and 06.06.2019. Shower water and drain samples were collected. One ward was an augmented care ward where regular water sampling was advised by HTM guidelines, and the other ward was a non – augmented ward where previous cases of wound infections by *P. aeruginosa* had been reported. Following *P. aeruginosa* outbreak in 2017 in the hospital by the hospital epidemiologist, water was found to be the main reservoir.

Although sampling the drains is not suggested by HTM guidelines, previous studies showed that drains harbour antibiotic resistant *P. aeruginosa* stains, e.g. VIM-2 β-lactamase-producing isolates and their dissemination poses a risk to the patients [145]. Drain contents can be disseminated to the surface around drains by splashing or through aerosols [136,148]. In our hospital setting, there was also the contamination risk through direct contact of the shower heads to the drains: Shower hoses were in 1.2 m length before and through the first three weeks of this study and it was observed that patients do not necessarily hang the shower heads to the shower head hangers and when they left them freely, shower heads were either touching the shower floor or falling directly into the shower drains. Hence, the first remediation was to shorten the shower hoses to 0.8 m so that they would not rest on the shower floor but hang freely. At the same time the Water Safety Group of the Hospital (Estates head, authorised engineer, infection control teams, consultant microbiologist, ward managers and nurse in charge) ordered that the hose and head were changed to silver impregnated units in an attempt to reduce the bioburden.

In our hospital, before and together with changes of the shower hose and head, various cleaning and disinfection strategies were tried to eradicate *P. aeruginosa* including TMV replacement and disinfection but none were effective in the long term. This sampling study corresponded to the period when short-hosed silver impregnated showers were first used in the wards and the effect on presence of *P. aeruginosa* was investigated. A XPS (X-ray photoelectron spectroscopy) analysis was carried out later to see the silver concentration in those showers to comment on antibacterial activity.

3.2. Materials and Methods

3.2.1. Ward Types and Sampling Frequency

Two wards (Ward A and Ward B) on separate floors were selected for enhanced environmental sampling of shower water and drains. Ten patient beds were selected at random from each floor.

Ward A was a non-augmented care unit accommodating surgical patients located in the middle level of the building. Beds in this ward were either for single-occupancy, i.e., within a Single-Isolation Room (SIR), or multiple-occupancy in bays accommodating up to four patients. SIRs provided a dedicated en-suite bathroom while bays areas provided a single bathroom facility shared between patients in that bay. This ward comprised 21 bathrooms in total of which nine were within the SIR en-suite unit and 12 were multiple occupancy access facilities.

Ward B was an augmented care unit caring for haematology patients. This ward was located on the highest floor of the building and comprised of 34 SIRs, each with en-suite bathroom facilities exclusive to the occupying patient.

UCH is a 700-bed multi-storey building teaching hospital in London, UK which was opened in 2005. It has sixteen floors where diverse patient population stay in specialised wards, mainly: Adult/adolescent haematology/oncology, paediatrics, and neurosurgery. Both Ward A and Ward B studied in this chapter are at the same hospital building. Ward A was selected because patients had wound infections caused by *P. aeruginosa*. Ward B was selected as this is a haematology ward housing immune-suppressed patients who were susceptible to *P. aeruginosa* infections. Enhanced monitoring of the effect of shower water contamination was essential in both wards. Both wards had high patient occupancy. These wards had different clinical teams treating the patients, hence there were no shared staff.

The study was conducted over seven months with environmental samples collected a total of 14 time points between 21.11.2018 – 06.06.2019 from both wards (Table 3.1). Monitoring of shower waters and drains surfaces (swabs) for *P. aeruginosa* was undertaken at weeks: 3-4, 13-15 and after week 21 (Ward A) and week 29 (ward B).

The study was divided into three phases. At the outset there was a control period of three weeks, after which Phases 1, 2 and 3 occurred at weeks: 3-4, 13-15 and after week 21 (ward A) / week 29 (ward B).

Existing shower heads comprised of either one of: a) standard non-antimicrobial plastic, b) antimicrobial silver-impregnated plastic or c) point-of-use antimicrobial filtration units; the hose types at the start of the study were: i) ethylene propylene diene monomer (EPDM) or ii) polyvinyl chloride (PVC), all of length 1.2m

At each new phase, the showers were replaced with pristine antimicrobial silver-impregnated shower heads with 0.8m antimicrobial silver-impregnated hoses.

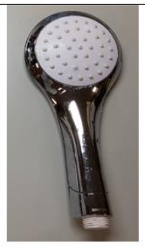
In all shower areas, signage was affixed to walls to instruct users to avoid replacing the shower head onto its mount after use, i.e., to allow the shower head to drain freely under gravity into the shower tray.

Environmental sample collection from hospital waters and drains were performed as described in Chapter 2. Water sample collection was done aseptically followed by filter-concentrating on 0.45 µm pore sized filter membranes, plating on PCN Agar, incubating at 37ºC for 48 hours (+/- 4hrs). Drain sample collection used pre-moisturized cotton swabs and direct plating on PCN Agar, incubating at 37ºC for 24 hours. Oxidase and casein hydrolysis tests were performed on presumptive *P. aeruginosa* colonies followed by MALDI-TOF identification.

3.2.2. Shower Head Types

Shower head types used in this chapter are given in Table 3.1 (non-antimicrobial and antimicrobial shower heads). Details of hollow fibre filter is given Chapter 4.

Table 3.1. Anonymised shower head types described in this thesis.

Shower head Types	Old small head (non-antimicrobial)	Wide flat head (non-antimicrobial)	Small flat head (non-antimicrobial)	POU membrane filter; non- antimicrobial	Antimicrobial shower head; non-filter type)	POU hollow-fibre filter; antimicrobial
						

3.2.3. Pressure Measurements

Pressure measurements were done by a pressure gauge (Bourdon, 0-4 bar range, RS Components, UK) as detailed on Chapter 2 and recorded.

3.2.4. Antibiotic Susceptibility Testing

Antibiotic susceptibility tests were performed by disk diffusion assay against 12 antibiotics as described in Chapter 2. Inhibition zones were measured and susceptibilities were determined by comparing with EUCAST breakpoints [149]. EUCAST is widely used in European countries and in the UK. In this thesis, EUCAST breakpoints are used to cross-reference with other studies [150].

3.2.5. Routine Cleaning Procedure and Protocol

Throughout this study, the existing hospital cleaning practices remained unchanged on both wards. Briefly, the routine daily cleaning of all bathroom surfaces consisted of manual application of a sporicidal disinfectant (peracetic acid; 0.1%, Diff X: MTP Innovations Ltd, UK) with a pre-wetted microfibre cloth. The cleaning technique employed involved folding of the cloth into half twice to create 8-faces and using a new (unused) face of the cloth for each different surface.

The cleaning procedure was performed In the following order in all bathrooms: (i) shower outlet: (ii) sink/tap outlet; (iii) drains; (iv) toilet; and (v) floor surface. A new pair of nitrile gloves were used for each bathroom. A pristine pair of nitrile gloves and a new cloth was used to clean the shower exclusively.

The cleaning of outlets (showers and taps) involved visual inspection for limescale, and where present, dissolved by application of a descaler solution (WTP Showerhead Plus, UK) and removed after a dwell time (1minute) by mechanical scrubbing. The outlet was then opened fully for two minutes, and the water discharged into the corresponding drain. Drains were inspected for hair and debris and removed using a paper towel before discarding into a clinical waste receptacle. Floors were cleaned using microfibre-cloth mops pre-wetted with the same disinfectant solution; a new (unused) mop was used in each bathroom.

3.2.6. Standard Remediation Procedure During the Study

Management of remedial actions was undertaken by the hospital estates department and any testing of the waters (routine and retest samples) performed by the hospital water testing services.

The frequency of sampling was based on a risk assessment by the local Water Safety Group and deemed appropriate for sampling every 6 months at the start of the study. On identifying a contaminated outlet on an augmented care ward, cleaning, flushing, chemical disinfection with peracetic acid disinfectant (0.1%) and retesting were performed as recommended [70]. Colonised showers were withdrawn from use but flushed daily and sampled every two weeks until cleared. Patients and staff were instructed to avoid placing shower handles onto the wall mount but to suspend the shower vertically to allow the excess water to drain under gravity.

3.2.7. TMV Remediation (replacement) Procedure

If outlets remained positive for *P. aeruginosa* following hose change, contamination of the thermostatic mixing valve (TMV) or contiguous plumbing was assumed. Where subsequent remedial actions failed, replacement of the TMV was considered. New TMVs were chlorinated prior to fitting and, when installed, the external surfaces of the housing sprayed with a chlorine disinfectant. The TMV unit was then flushed for 2 minutes. Water samples were taken for microbiological analysis for *P. aeruginosa* a week before and 72 hours after TMV replacement.

3.2.8. Enhanced Remediation Procedures

Where standard remediation measures failed to eradicate *P. aeruginosa* contamination despite several attempts, the Water Safety Group agreed to test enhanced measures in 3 shower-rooms from each ward (i.e., 6 in total). Injection points were installed to allow disinfectant concentrate (50% Hydrogen peroxide and 0.05% silver ions) dosing of the cold and hot water supplies.

To determine whether the *P. aeruginosa* was introduced to the building from an external source, water was sampled and tested from the incoming mains supply and from the basement level of the hospital building. Water samples were also collected for testing from shower rooms adjacent to the test shower rooms.

A control shower room was allocated on a separate floor located in the mid-point between ward A and B. This shower was installed with a pristine TMV and showerhead and hose units and closed off to patient use. The shower was not subjected to daily cleaning but was flushed daily (10-minute cycles with fully-open valve).

3.2.9. Extraordinary Remediation Procedure

Where standard and enhanced remediation of shower outlets contaminated with *P. aeruginosa* failed, extraordinary remediation measures were adopted. In this case, the TMV and all copper pipework supplying the affected room was replaced and fully disinfected (systemic chemical flushing with 0.1% peracetic acid; Peracide; Sky Chemical UK Ltd). These procedures were undertaken after the survey period.

3.2.10. XPS Methodology for Silver Composition Analysis

XPS analysis was performed to determine the silver composition of the antimicrobial shower materials. Methodology and Results are provided by Sanjay Sathasivam, UCL Chemistry.

XPS was carried out on a Thermo Fisher Scientific K-Alpha instrument, using Al Kα radiation (1486.6 eV) with a 400 μm (diameter) spot size. Two spots were analysed for each sample. Survey spectra were taken at a pass energy of 200 eV. High resolution C 1s and Ag 3d were taken at a pass energy of 50 eV. Charge neutralisation was carried out using a dual-beam Ar+ and electron flood gun. Charge correction of spectra was carried out using adventitious C 1s set to a binding energy of 285.0 eV.

3.3. Results

3.3.1. Environmental *P. aeruginosa* in Shower Waters and Drains

Two floors of the hospital tower were selected for environmental sampling of shower waters and drains for *P. aeruginosa* isolation. Water samples were collected from ten showers and drains from Ward A (6^{th} floor; non-augmented care unit) and Ward B (16^{th} floor; augmented care unit) at a frequency of weekly/once in two weeks. Sampling was performed for seven months, 21.11.2018–- 06.06.2019. In total 560 samples were taken, 267 of which were positive for presence of *P. aeruginosa*.

Seven out of ten showers from non-augmented care unit belonged to bay units where the showers were shared between four patients. The other three showers from non-augmented care unit and ten showers from augmented care unit belonged to single isolation rooms where showers were assigned to one patient only.

3.3.1.1. *P. aeruginosa* in Non-augmented Care Ward

3.3.1.1.1. *P. aeruginosa* in Shower Water

As shown in Table 3.2, all showers from Ward A had long hoses (1.2m) except for one with a short hose (0.8m) at the beginning of the study. Of these, four showers were fitted with a point-of-use filtration device (Anonymised 3 Medical filter showers), four were standard plastic-chrome shower heads (Bristan) and two were Anonymised 1 Ag+ antimicrobial showers. Details of shower heads are given in Appendix A. Green highlighted cells of Table 3.2 show the presence of *P. aeruginosa* contamination.

Photographs of filter membranes on PCN plates after processing water samples following incubation at 37ºC for 48 hours are given in Appendix A.1. Phenotypic confirmations were performed for all presumptive *Pseudomonas aeruginosa*. *P. aeruginosa* typically exhibits green/yellow colonies but confirmation tests (oxidase and milk hydrolysis) were performed for all colonies observed on filter papers. MALDI-TOF confirmation details of shower water and drain strains from Ward A are given in Appendix A.3.

Initial sampling results showed that 4/10 shower outlets (40%) were contaminated with high counts (>300CFU/100mL) of *P. aeruginosa* (Appendix A.1.) All samples were collected with the shower heads on (including the filtered shower heads). Water sample collection in the following two weeks showed the same results as the start of the study.

After the third sampling (week 3), all shower heads were changed to Anonymised 1 Ag+ with short hoses. Shower water sample were collected a day after the first remediation. As seen in Table 3.2, outlet of shower#1 started showing *P. aeruginosa* contamination after the first remediation; shower#6 had persistent *P. aeruginosa* contamination whereas shower#3, shower#7 and shower#10 outlets were cleared of *P. aeruginosa*. Appendix A.1 indicates how *P. aeruginosa* colonies started to reappear in shower#1 outlet from the fourth sampling.

In the fifth sampling (corresponding to approximately two weeks after the first shower change, shower#4 started to be colonised with *P. aeruginosa* (Appendix A.1). In the sixth sampling (week 7 in Table 3.2), shower#9 and shower#10 started showing *P. aeruginosa*

contamination making the total number of contaminated showers to five of ten (50%). After the ninth sampling (week 13 in Table 3.2), a second remediation occurred where the shower heads and hoses were changed to new Anonymised 1 Ag+ ones. Later sampling showed that *P. aeruginosa* contamination in shower#3 reappeared after two months of clear results. In the following sampling (week 17 in Table 3.2), *P. aeruginosa* contamination in shower#5 reappeared, raising the total number of contaminated showers to seven out of ten. The same pattern continued in the following two samples covering a month. After the thirteenth sampling (week 21 in Table 3.2), the showers were changed to new Anonymised 1 Ag+ ones. In the following sampling, shower#3 and shower#5 were cleared from *P. aeruginosa* contamination whereas the other five showers continued to give high counts as can be seen in Appendix A.1.

3.3.1.1.2. *P. aeruginosa* in Shower Drains

Confirmed *P. aeruginosa* contamination results from drains of Ward A showers are given in Table 3.2. No specific pattern of contaminated drains was observed through the weeks of sampling but the number contaminated increased after sixth sampling (week 9 in Table 3.2) where four out of ten drains (40%) were found to have *P. aeruginosa*. In the last sampling, this number increased to seven out of ten drains.

3.3.1.2. *P. aeruginosa* in Augmented Care Ward

3.3.1.2.1. *P. aeruginosa* in Shower Waters

At the start of the sampling, all ten showers had Anonymised 1 Ag+ shower heads with long hoses. The initial sampling results revealed that eight out of ten shower waters (80%) had *P. aeruginosa* contamination. PCN plate pictures are provided in Appendix A.2. The second sampling of shower#3 did not show presence of *P. aeruginosa*, but contamination reappeared in the following week sampling. Remediation happened after the third week of sampling where all showers were replaced with new Anonymised 1 Ag+ shower heads with short hoses. At the fourth sampling, only shower#10 found to be cleared from contamination but it reappeared in the following week. Shower#9 water outlet was found to be contaminated at the sixth sampling (week 7 in table 3.2) (Appendix A.2) but this contamination was cleared in the following week. Starting from the seventh sampling, the same seven shower waters were found to have *P. aeruginosa* contamination regardless of shower replacement with new Anonymised 1 Ag+ shower heads and short hoses. In the last sampling, contamination in

shower#4 and shower#10 was found to have *P. aeruginosa* contamination making the total number of contaminated showers nine out of ten (90%).

MALDI-TOF identifications for confirmation of shower water and drain strains from Ward B are given in Appendix A.4.

3.3.1.2.2. *P. aeruginosa* in Shower Drains

Drain swab results of Ward B showers for presence of *P. aeruginosa* are given in Table 3.2. As in Ward A drain swab results, no specific pattern was observed. Shower#12, shower#15 and shower#16 drains were frequently contaminated with *P. aeruginosa* (average 64%).

Table 3.2. Presence and persistence of *P. aeruginosa* in shower waters and drain surfaces before and after change of showerhead-hose units with 3-monthly replacement programme and during corrective actions.

Ward A *(non-augmented care)*

Room #	Room type	Presence/Absence of *P. aeruginosa* in Shower waters or drains (per week) during successive showerhead+hose replacement																														
		Control Phase	Shower type	Hose length	Hose Material	Wk 1		Wk 2		Wk 3		Phase 1	Shower type / Hose Length/ Material	Wk 4		Wk 5		Wk 7		Wk 9		Wk 11		Wk 13		Phase 2	Wk 15		Wk 17		New TMV		Wk 19		Wk 21		Phase 3	Wk 29	
1	Bay		POU Filter	Long	EPDM	*		*		*			Ag+ /Short/PVC																								
2	Bay		POU Filter	Long	EPDM	*		*		*			Ag+ /Short/PVC																								
3	Side Room		Standard	Long	EPDM								Ag+ /Short/PVC																								
4	Side Room		POU Filter	Long	EPDM	*		*		*			Ag+ /Short/PVC																								
5	Bay		POU Filter	Long	EPDM	*		*		*			Ag+ /Short/PVC																								
6	Bay		Ag+	Short	PVC								Ag+ /Short/PVC																								
7	Bay		Ag+	Long	PVC								Ag+ /Short/PVC																								
8	Side Room		Standard	Long	EPDM								Ag+ /Short/PVC																								
9	Bay		Standard	Long	EPDM								Ag+ /Short/PVC																								
10	Bay		Standard	Long	EPDM								Ag+ /Short/PVC																								

Ward B *(augmented care)*

Room #	Room type	Presence/Absence of *P. aeruginosa* in Shower waters or drains (per week) during successive showerhead+hose replacement																														
		Control Phase	Shower type	Hose length	Hose Material	Wk 1		Wk 2		Wk 3		Phase 1	Shower type / Hose Length/ Material	Wk 4		Wk 5		Wk 7		Wk 9		Wk 11		Wk 13		Phase 2	Wk 15		Wk 17		New TMV		Wk 19		Wk 21		No shower head/hose change	Wk 29	
11	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																								
12	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																								
13	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																								
14	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																								
15	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																								
16	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																								
17	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																								
18	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																								
19	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																								
20	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																								

Table Key		
	POU Filter	Point-of-Use membrane filter device
	Standard	Non-antimicrobial plastic body
	Ag+	Antimicrobial Silver-impregnated materials
	Long	>>1.2m length hose
	Short	0.8m length hose
	EPDM	Ethylene Propylene Diene Monomer
	PVC	Poly-Vinyl-Chloride

	PsA in Shower water
	PsA in Drain
	TMV not replaced
	Drain sample not applicable
*	Outlet with POU filter

Control Phase: Pre- showerhead+hose change
Phase 1: First shower head+hose change
Phase 2: Second shower head+hose change
Phase 3: Third shower head+hose change

The effect of introducing new antimicrobial shower head-hose units (remediations) into non-augmented care (Table 3.2.A) and augmented care (Table 3.2.B) wards on the presence of *P. aeruginosa* in showers (green) and corresponding drains (orange) over a 29-week period. Blank cells represent no contamination by *P. aeruginosa*. Quarterly shower hose changes are marked by column.

3.3.1.3. Statistical Analyses

Statistical analyses done by Paul Bassett, Statsconsultancy Ltd.

a) Approach 1 – Changes over time

Analysis results are summarized in Table 3.3. Second column gives the p-values from the ward by time interactions. Odds ratios representing the change in the odds of *P. aeruginosa* for a one-week increase in time are given with corresponding confidence intervals in fourth column showing the strength of association between time and the occurrence of *P. aeruginosa*.

Table 3.3: Changes in of *P. aeruginosa* over time, odds ratios and p-values

Location	Ward x time interaction p-value	Ward	Odds Ratio (*) (95% CI)	Time P-value
Showers	0.004	A	1.19 (1.09, 1.31)	<0.001
		B	0.95 (0.84, 1.07)	0.42
Drains	0.03	A	1.18 (1.09, 1.30)	<0.001
		B	1.04 (0.98, 1.11)	0.23

(*) Odds ratio for a one-week increase in time

Logistic multi-level regression modelling demonstrated a significant association between duration in use of the shower in the clinical setting and occurrence of *P. aeruginosa* in shower waters (p=0.004) and drains (p=0.03), irrespective of hose/head changes (Figures 3.1&3.2). For each week elapsed, the odds for colonisation/contamination with *P. aeruginosa* in the non-augmented care setting increased by 19% in shower water (OR= 1.19; CI= 1.09 – 1.31, P<0.001) and 18% in the drains (OR = 1.18; CI= 1.09 – 1.30, P<0.001). Odds for *P. aeruginosa* colonisation/contamination over time remained unchanged in the augmented care wards for showers (OR= 0.95; CI= 0.84 – 1.07, P=0.42) and drains (OR= 1.04; CI= 0.98 – 1.11, P=0.23).

Graphical illustrations of the fitted change over time are shown for showers in Figure 3.1 and for drains Figure 3.2 together with the correlation equations and the R-squared values.

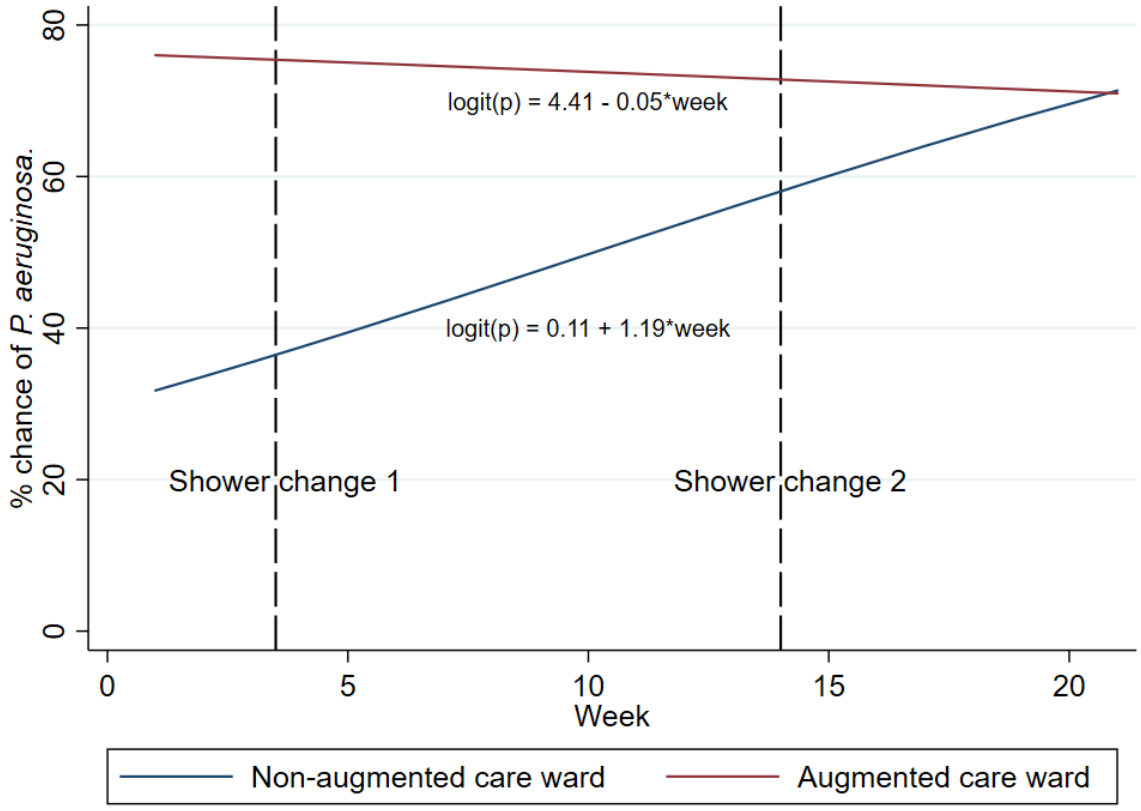


Figure 3.1. *Probability of P. aeruginosa contamination of shower waters with time (weeks) in augmented and non-augmented care settings. Y-axis represents the occurrence ratio as a function of duration of exposure (X-axis). No changes were made to drains. Shower head-hose units were replaced with unused antimicrobial silver-impregnated replacements between weeks 3-4 and 13-15 (shower changes 1 and 2 respectively).*

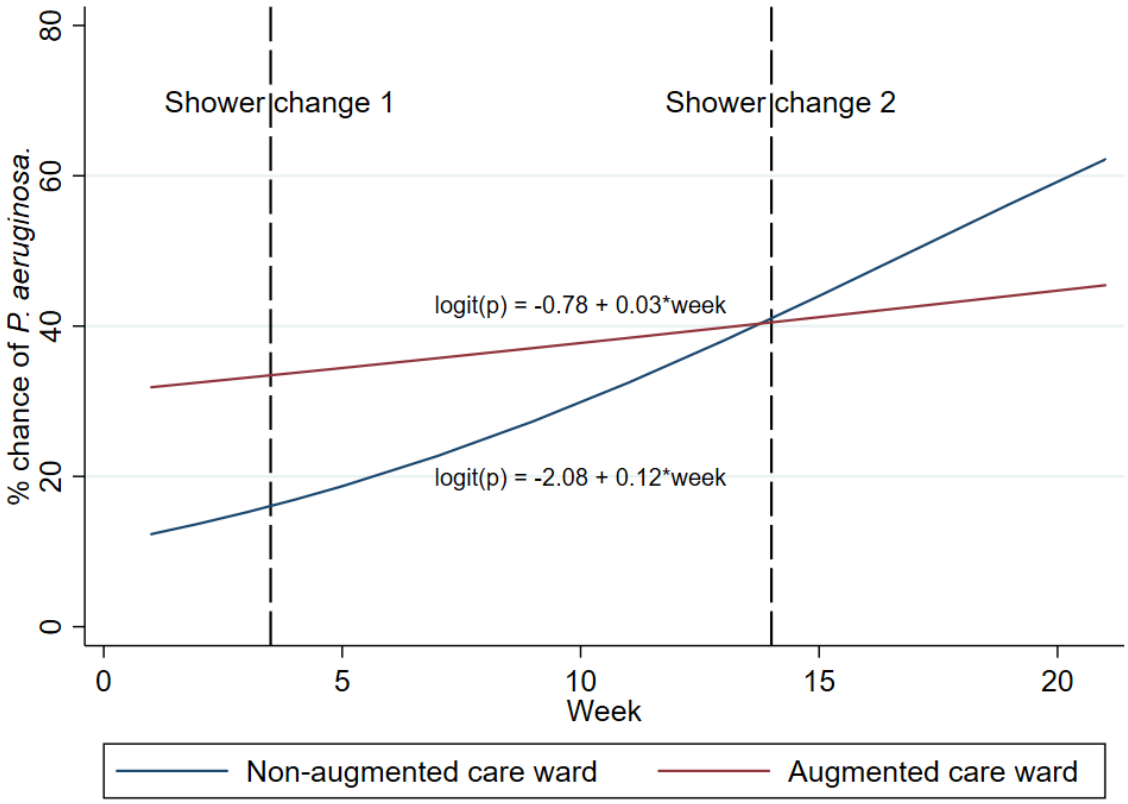


Figure 3.2. *Probability of P. aeruginosa contamination of drain surfaces with time (weeks) in augmented and non-augmented care settings. Y-axis represents the occurrence ratio as a function of duration of exposure (X-axis). Shower head-hose units were replaced with unused antimicrobial silver-impregnated replacements between weeks 3-4 and 13-15 (shower changes 1 and 2 respectively).*

b) Approach 2 – Time in categories

Results of the analyses are summarised in Table 3.4. The third column shows the number and percentage of measurements where *P. aeruginosa* was present for each location/ward/timepoint combined. Following the percentages, difference in *P. aeruginosa* between periods are given as expressed as odds ratios, representing the odds of *P. aeruginosa* in each period relative to the odds for the control period.

Table 3.4: Comparison of *P. aeruginosa* in different study periods, odds ratios and p-values

Location	Ward				Time P-value
		P aeruginosa n/N % Odds ratio (95% CI)			
		Week 1-3 Pre shower change	Week 4-13 First shower change	Week 15-21 shower change	
Showers	Non-augmented care (A)	12/30 (40%) 1	25/60 (42%) 1.14 (0.33, 3.91)	27/40 (68%) 11.4 (2.35, 55.1)	0.003
	Augmented care (B)	23/30 (77%) 1	44/60 (73%) 0.52 (0.08, 3.62)	29/40 (73%) 0.45 (0.05, 3.66)	0.73
Drains	Non-augmented care (A)	4/30 (13%) 1	14/60 (23%) 2.31 (0.61, 8.83)	23/43 (58%) 18.2 (4.22, 78.6)	<0.001
	Augmented care (B)	7/30 (23%) 1	24/60 (40%) 2.84 (0.91, 8.88)	18/40 (45%) 3.79 (1.11, 12.9)	0.09

The frequency of contamination of shower water and drains with *P. aeruginosa* and odds of occurrence between hose changes in augmented and non-augmented care wards is shown in Table 3.3. Relative to the period before the first hose change, the likelihood of shower water and drains in the general setting (ward A) becoming colonised with *P. aeruginosa* increased over time ($P<0.05$), with the highest density of colonised showers/drains after the second head/hose change. Colonisation of showers and drains in augmented care (Ward B) persisted at high frequency regardless of the head/hose change.

3.3.1.4. Antibiotic Susceptibility Profiling of Environmental *P. aeruginosa* Strains

Susceptibility of isolates from Ward A – Ward B sampling were tested against 12 antibiotics amikacin (AK – 30µg), gentamicin (CN – 10µg), tobramycin (TOB – 10µg), aztreonam (AZT – 30µg), meropenem (MEM – 10µg), imipenem (IMI – 10µg), ceftazidime (CAZ – 10µg), cefepime (CEF – 30µg), ciprofloxacin (CIP – 5µg), piperacillin (PRL – 30µg), piperacillin/tazobactam (PTZ – 36µg), and ticarcillin/clavulanic acid (TCC – 75/10µg) from aminoglycoside, monobactam, carbapenem, cephalosporin, fluoroquinolone and penicillin groups by using EUCAST standards [15].

Of 560 samples taken, 274 *P. aeruginosa* strains were isolated. 117 were isolated from Ward A (non-augmented care; 69 shower head water; 48 shower drain) and 157 were isolated from Ward B (augmented care; 105 shower water and 52 shower drain). Antibiotic susceptibility test (AST) was performed on randomly selected half of the isolates (35/69 showers; 25/48 drains from Ward A and 53/105 showers; 26/52 drains from Ward B). Significance of the differences in antibiotic susceptibility results were analyzed by comparing 3 time periods with Kruskal-Wallis test by taking into account remediation times: Period 1 (Before the first remediation (weeks 1-3)); Period 2 (After the first remediation (weeks 4-13)); Period 3 (After the second Remediation (weeks 15-21)).

3.3.1.5. Statistical Analyses of Antibiotic Susceptibility Test Results

Summary of the results are given in Appendix A.5 as Tables A.5.1-4. There were significant reductions in the prevalence of resistance to cefepime in *P. aeruginosa* in showers in ward A and to imipenem in showers in ward B during the study ($p<0.05$). In the drains in ward B, *P. aeruginosa* resistance to ciprofloxacin and aztreonam declined significantly. In shower water

in ward B, 50% of strains at the beginning of the study were resistant to imipenem. Frequency of imipenem-resistance in *P. aeruginosa* declined to 12% and 31% after the first and second remediation respectively ($p<0.05$).

The first set of analyses was done for the measurements taken from showers in Ward A, examining changes over the course of the study as summarized in Appendix Table A.5.1. The columns show the number and percentage of responses in each antibiotic susceptibility category for each time period where the final column shows p-values from the analyses, indicating the significance of the differences between the three periods.

The results showed a significant difference between the three time periods for cefepime only. The highest proportion of resistant measurements was in period 1, where over 40% were resistant. This proportion was lower in the subsequent periods, especially the second time period. There were no significant differences between time periods for the other antibiotics.

The second series of analyses were performed for data from Ward A drains. As there were few measurements from period 1, the formal statistical comparisons were made between periods 2 and 3 only. The analysis results are given in Appendix Table A.5.2. No significant differences between periods 2 and 3 were observed for any of the antibiotics.

Similar analyses were also performed to compare between time periods for the data collected from showers in Ward B. The results are given in Appendix Table A.5.3.

A significant difference between the three time periods was observed for imipenem. The highest percentage of resistant cases was in period 1, where half of measurements were resistant. This fell to only 12% of measurements in period 2 and increased to 31% for period 3. No other significant differences were observed for the other antibiotics.

The fourth and final set of analyses compared between time periods for data from drains in Ward B. The results are summarised in Appendix Table A.5.4.

Statistically significant differences between the three time periods were observed for ciprofloxacin and aztreonam. Resistant measurements were most frequently seen in period 1, with a lesser occurrence in the subsequent time periods for both antibiotics (As detailed in Appendix A.5). 75% of aztreonam measurements were resistant in period 1, which fell to 20% in period 2 and no resistant measurements in period 3. No significant differences were observed for the other antibiotics.

3.3.1.6. Water Pressure Measurements

8 shower water pressure measurements from Ward A averaged 2.71 bar (range: 2 – 3.9).

6 shower water pressure measurements from Ward B averaged 0.83 bar (range: 0.8 – 0.9).

3.3.1.6. Effect of TMV Replacement

Between week 17-19, TMV remediation was applied to a random selection of showers in which the TMV was thought to be contaminated i.e. the TMVs were replaced (3 from each ward), and the pipework disinfected (hydrogen peroxide 50%, silver ions 0.05%) as shown in Table 3.2.

Of the six showers sampled, with one exception, changing the TMV and disinfection of the housing was ineffective in clearing contamination. Neither hydrogen peroxide nor peracetic acid injected into the pipe between TMV and outlet eradicated *P. aeruginosa*. In the rooms adjacent to the ones where TMV was changed, there was no effect on the isolation of *P. aeruginosa* from showers. Sampling of supply water in the basement of the building did not show any growth of *P. aeruginosa*. A single shower was closed to both patient and staff access and was flushed but not cleaned for 3 months. No contamination with *P. aeruginosa* was observed at repeated monthly sampling.

3.3.1.7. *P. aeruginosa* Colonisation Status of Handwash Basin Taps

All handwash basin taps in the study rooms on Ward A and B remained clear of *P. aeruginosa* contamination, in hospital laboratory tests before and after the monitoring period for showers.

3.3.1.8. Silver Composition of Shower Materials by XPS Analysis

XPS analysis demonstrated silver composition of showers was below the detection limit in all but one case. In addition to Anonymised 1 silver showers, other showers used/ had potential use in the hospital were also tested. In total, 18 shower piece samples from three different companies were analysed by XPS for the presence of silver. List of the samples is as follows in Table 3.5:

Table 3.5. Description of shower components (head/hose materials) assigned for XPS Analysis to determine concentration of elemental silver present in the material.

Number	Sample Description
1	Anonymised 2 shower head
2	Anonymised 2 shower head
3	Anonymised 2 shower head
4	Anonymised 2 shower head
5	Anonymised 1 shower head blue
6	Anonymised 1 shower head blue
7	Anonymised 1 shower head blue
8	Anonymised 1 shower head blue
9	Anonymised 1 shower head purple
10	Anonymised 1 shower head purple
11	Anonymised 1 shower head purple
12	Anonymised 1 shower head purple
13	Anonymised 1 blue hose interior
14	Anonymised 1 blue hose exterior
15	Anonymised 1 purple hose interior
16	Anonymised 1 purple hose exterior
17	Anonymised 4 head
18	Anonymised 4 hose

Three manufacturer types promoting antimicrobial silver were analysed. Replicate samples were taken from different batches. Shower pieces are measured in duplicates (e.g., #1 and #2 are duplicates of the same shower. Anonymised 1 shower head blue and purple belong to different batches.

X-ray photoelectron spectroscopy (XPS) is powerful analytical tool that can be used to determine the chemical composition and environment of surfaces (<10 nm). A recent study by Shard *et al.* has shown that detection limits down to 0.01 at.% are possible for heavy elements such as Ag in a relatively light elemental matrix such as carbon [151]. XPS was carried out on all 18 samples with high resolution scans taken in the Ag 3d region (Figure 3.3). No Ag peaks were detected for samples 1-2 and 5-18 however, 3 and 4 showed small

peaks indicating the presence of Ag on the surface. Despite the very low signal to noise ratio of these peaks, fitting was carried out to determine the chemical state and concentration of Ag (Figure 3.4). The signals were best fit with an asymmetric line shape that showed the Ag 3d 5/2 peaks centred at 368.3 eV and matching literature values for metallic Ag at 368.2 eV (±0.1) [152]. The Ag concentration relative to C, the major component polymer matrix was 0.025 and 0.013 at% for sample 3 and 4, respectively.

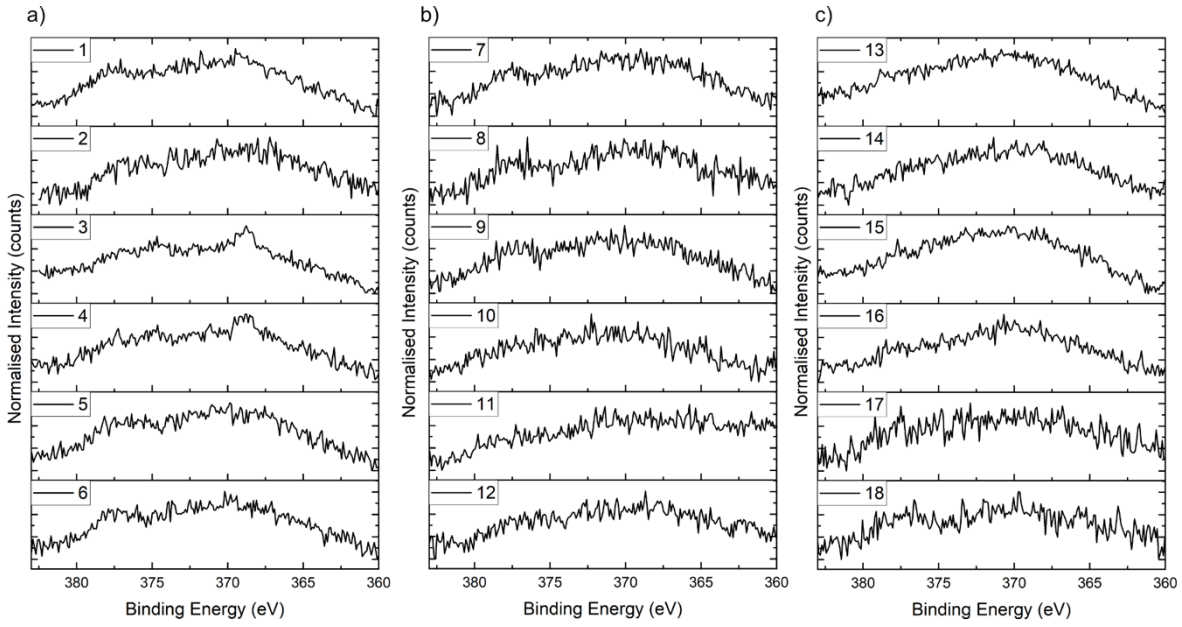


Figure 3.3: Ag 3d high resolution XPS scan data for samples a) 1-6, b) 7-12 and c) 13-18.

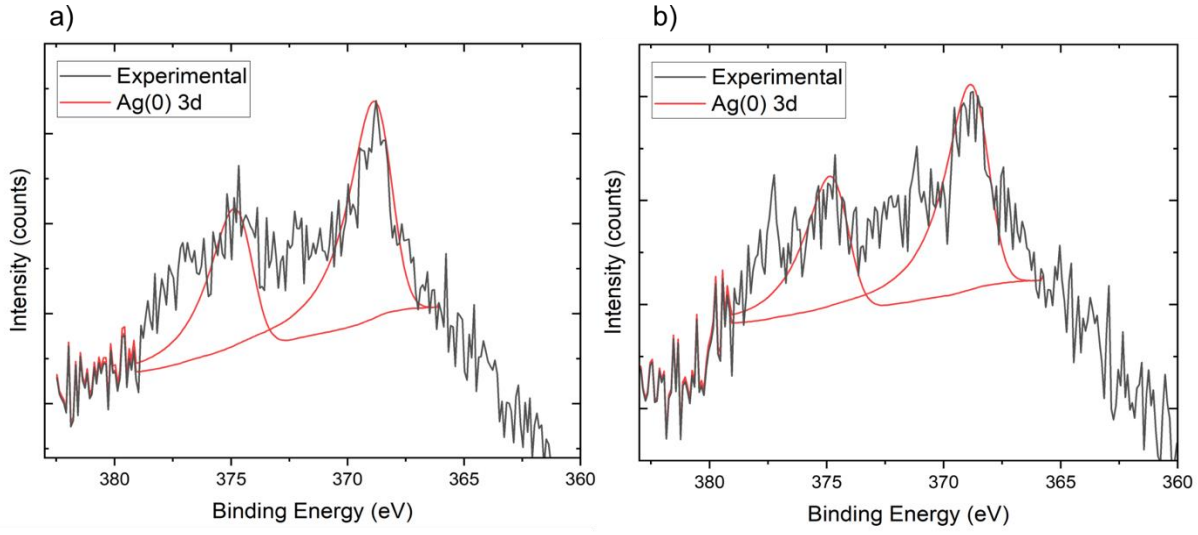


Figure 3.4: Ag 3d high resolution XPS scan data including fitting for samples a) 3 and b) 4. The peak positions of the Ag 3d5/2 were both 368.3 eV that matches to Ag metal.

3.4. Discussion

There were no differences in cleaning frequency or bed occupancy between the wards and both bays and single rooms were almost fully occupied throughout the period of study. Under HTM 04-01 guidelines, routine monitoring for *P. aeruginosa* is not required in non-augmented care wards but showers were shared between patients and transmission was a possibility. Drains were a reservoir of *P. aeruginosa,* and these drains were open. Contamination of water outlets from drains in baths has been demonstrated [153]. Shower heads can be contaminated from drains through contaminated droplets/bioaerosols generated during ablutions and/or handling of the shower head with contaminated hands.

Sampling the same showers over seven months showed that once the shower is colonised with *P. aeruginosa,* it stays colonised for extended periods. More showers in augmented care ward were found to have *P. aeruginosa* in their water outlets than in the non-augmented care ward. At the beginning of the study, 8/10 showers were colonised by *P. aeruginosa* in augmented care ward rising to 9/10 at the end of study whereas showers it was 4/10 for the non-augmented care ward rising to 5/10 in the end.

The initial change in augmented care wards (December 2018) was to shorten the hose length from 1.2m to 0.8m so that shower head did not reach the floor which can be a potential source of contamination. Quarterly change of Anonymised 1 Ag+ showers to unused heads and hose occurred twice during this study. However, this did not have any effect on contaminated water outlets except one shower (shower#10) in which contamination was initially not detected but reappeared eventually (Table 3.2). Other groups seeing drains as a potential contamination source tried different remediations such as introducing copper and dry-membrane shower drains but none was found to prevent colonisation by *P. aeruginosa* [154].

All shower types present in the hospital at the study time are given in Appendix A. The remediation was to change all showers to Anonymised 1 Ag+ short hosed (December 2018). Shower#1 had a Anonymised 3 bacterial filter shower head at the beginning of the study but showed *P. aeruginosa* in the outlet with the Anonymised 1 Ag+ shower head in situ after the first shower change suggesting contamination had been present and the Anonymised 3 shower head was filtering the outlet effectively. As can be seen from Table 3.2, first shower

change helped reduce contamination of outlets at the beginning except one shower (shower#7) which had a Anonymised 1 short hose before the remediation in Ward A. *P. aeruginosa* contamination tended to persist unless the type of shower head was changed. Following a *P. aeruginosa* outbreak in a haematology unit in another hospital, potential contamination sources were investigated, and *P. aeruginosa* contamination was found on 29/85 samples collected from showers, sinks and siphons. All taps and shower heads were fitted with disposable membrane water filters (0.2 μm pore size) with the requirement of a weekly replacement significantly reducing *P. aeruginosa* bacteraemia but adding to costs [155]. A similar approach on replacing the showers with filtered heads is described in detail on Chapter 4.

Anonymised 1 Ag+ product sheet states that their shower units have a silver based antimicrobial additive which reduces bacterial viable counts by 99.9% when compared to controls [156]. However, antimicrobial activity was not observed in majority of the showers. Following collaboration with Prof Ivan Parkin's group (UCL Chemistry Department), Anonymised 1 Ag+ shower pieces analysed by X-ray photoelectron spectroscopy (XPS) but did not detect any Ag apart from the low signals in shower samples #2 and #3 (Table 3.5, Figures 3.3 and 3.4).

Presence of *P. aeruginosa* in the drains did not reflect the shower water contamination (Table 3.2). Drains harbour a variety of different microorganisms other than *P. aeruginosa*, of which some also grew on Pseudomonas selective media (PCNA); therefore it was difficult to distinguish some *P. aeruginosa* species for selection. Species that also grew on PCNA included: *P. alcaligenes, P. fulva, P. guariconensis*, *P. mosselii*, *P. nitroreducens*, and *P. stutzeri* and *Aeromonas hydrophilia, Aeromonas caviae, Citrobakter braaki, Citrobacter freundii, Achromobacter insolitus, Achromobacter denitrificans* and *Enterobacter cloacae.* Other studies focussing on shower drain bacteria found *Enterobacter cloacae* as a source for outbreak as well as other bacteria such as *Leclercia adecarboxylata*, and Pantoea species. [157,158]

The risk of contamination of showers was greater than taps, possibly due to the larger volume of stagnant water in a shower between TMV and outlet. Contaminating organisms in a shower hose have a large available luminal surface area on which biofilm plaques can develop. Biofilms have been reported to attach more firmly to surfaces where there is a high shear stress [66,159] but in this study contamination at the outset was more common in the

ward where water pressure was low. The cleaning protocols would not have removed biofilm from the inside surfaces of the shower and external decontamination relied on mechanical scrubbing to remove biofilm. Application of disinfectant alone would not be adequate to eradicate *P aeruginosa* within biofilm [160]. Although shower hoses and heads were replaced periodically, biofilm in the plumbing and TMV was not affected. Hence a more radical approach to biofilm removal was needed, such as local thermal disinfection [52].

TMV and plumbing were progressively colonised during the investigation, likely by patients cross-contaminating the hose during use, rendering hose/head replacement ineffective. Thermostatic mixer valves were behind wall panels and not easily accessible for ad-hoc disinfection, and the housing of the TMV could not be readily replaced. In this hospital, replacement of the TMV was ineffective likely due to colonisation of inaccessible pipework and TMV housing. Water supplies in the hospital had between 80 – 365 milligrams per litre calcium carbonate (Thames Water) [161]. Biofilm formation was likely on surfaces exposed to hard water as limescale deposits were common.

Showers and taps represent a significant reservoir of *P. aeruginosa* for patients vulnerable to developing bacteraemia. The presence of indistinguishable genotypes of *P. aeruginosa* in water outlets and patients has been described, although the direction of transmission is often unclear [82,146,162]. Patients with mucositis, intravascular catheters or foot wounds may be susceptible to potentially invasive contamination from the environment. In a study of outlets in 23 augmented care units over 16 weeks [95], between 0.9% and 16% of outlets demonstrated colonisation. Whole genome sequencing suggested a single genotype persisted within an outlet, possibly related to contamination in manufacture. Judging by epidemiological links in time and place, indistinguishable isolates suggested acquisition from the environment in 5% of patients. In another study, taps in 10 ICUs were repeatedly sampled and isolates typed by pulsed field electrophoresis [163]. More infections appeared to be transmitted between patients than from the outlets to patients. A tap water source of organisms detected on patient screening was implicated in 17% of patient acquisitions. Strains persisted in taps a median of five weeks (or longer in electronic taps). However, non-augmented care areas were not sampled in either study. In 141 isolates taken from showers in a burns unit, whole genome sequencing showed clustering of isolates by room and outlet and

three patients had identical genotypes to their environment [96]. A thermostatic mixer valve was shown to be a source of water contamination.

HTM guidelines suggest that materials such as EPDM may be prone to colonisation by *P. aeruginosa* on surfaces such as the inside of flexible lined hoses [69]. However, in this study, changing all hoses from EPDM to PVC materials did not affect the proportion of showers becoming contaminated/colonised [86].

Among the antibiotic susceptibility tests against twelve antibiotics, there were few statistically significant differences over the period of study and some resistance was to antibiotics not used in the wards, such as aztreonam. Resistance to aztreonam has been reported to be persistent, even in the absence of any selective pressure [164]. Since there were few significant differences in antibiograms of strains isolated in this study, it was not possible to deduce any links in transmission of isolates between rooms and time-points. Consequently, epidemiological links would require molecular analyses, such as whole-genome-sequencing.

After the end of the surveillance study, full replacement of pipework (extraordinary remediation), TMV and shower head/hose unit followed by systemic disinfection was undertaken and subsequent sampling demonstrated eradication of *P. aeruginosa* colonisation.

Studies showed multidrug resistant *P. aeruginosa* strains in hospital effluents and waste waters associated with hospital outbreaks due to poor design of toilets, sinks and showers leading to splash backs as well as blockages and leaks. [124] Other studies showed *P. aeruginosa* harbouring virulence factors in hospital waste waters as a potential source for disseminating strains to the environment.[165]

When isolates from patients with bacteraemia and corresponding showers were compared, it was seen that patient isolates showed more antibiotic resistance, probably due to antibiotic therapy and excretion of antimicrobials and their metabolites into the drain. Studies investigating shower, sink and waste water drains demonstrated drain-associated genetic transfer of antibiotic resistance genes. Intra- and interspecies plasmid-based exchange of the resistance genes raised concern that there was horizontal transfer of carbapenemase-encoding plasmids [166,167].

In other studies, timely identification of *P. aeruginosa* contamination required sampling schedules more frequent than those based on local risk assessment [69,70], even when there is no evidence of contamination of the mains water supply. However, the cost of sampling all showers in augmented care every two weeks would be difficult to justify in the long term without proving that transmission from shower to patient was occurring and that methods used to eradicate it were effective. Nonetheless, safeguarding vulnerable patient groups requires monitoring of shower water at intervals sufficient to identify clinical risks and implement effective remedial interventions in a timely fashion.

3.5. Conclusions: Findings of Enhanced Monitoring of Augmented and Non-augmented Care Ward Shower Waters

In conclusion, this study showed that routine 6-monthly sampling of augmented care wards was insufficient to prevent vulnerable patients being exposed to a risk of infection. The limited frequency of mandatory testing as per national guidelines HTM 04-01 means patients could be exposed to *P. aeruginosa* for several months if a shower becomes contaminated. As a result of enhanced monitoring, all contaminated showers were later removed together with adjacent plumbing, and new showers were installed that could be more easily disinfected along with the wall plumbing. There were limitations to this investigation. The distribution of single and shared showers and water pressures within the building may not be generalisable to other hospitals. The time and frequency of shower use and the volume of water used per episode was not known. The presence of a length of pipe in the wall between TMV and outlet and fixed TMV housing was inaccessible for disinfection and compromised control of *P. aeruginosa* and made changing the TMV ineffective. Dead legs (blind-ended or unused pipes) or a very low level of contamination in supply water could not be excluded as a source.

Findings of this chapter also demonstrated that silver impregnated shower units were not an effective solution for eradicating *P. aeruginosa* contamination. Following this outcome, shower units were replaced with hallow-fibre filtered heads as explained in the following chapter.

Chapter 4. Efficacy and limitations of point of use hollow-fibre technology filtration devices used in healthcare showers

Part of this Chapter is published in Journal of Hospital Infection on December 2022, doi: 10.1016/j.jhin.2022.08.007. Published paper is given in Appendix E.2.

4.1. Introduction

P. aeruginosa commonly colonizes hospital water systems and has been associated with outbreaks of infection in vulnerable patients [73]. Installation of filters on water outlets therefore has been recommended when disinfection fails to eradicate the organism.

P. aeruginosa tends to become established in distal parts of water system such as sinks, taps and showers [3]. Showers are liable to develop *P. aeruginosa* biofilm due to the materials used, low flow rates and operating water temperatures of between 25°C and 40°C which favour growth of this pathogen [168]. The aerosol droplets produced can be inhaled by patients or contaminate intravenous line insertion sites and damaged mucous membranes, posing a risk of infection particularly to patients following chemotherapy.

In the UK, remedial actions to mitigate the risks posed by *P. aeruginosa* contamination in water systems are described in the Health Technical Memorandum (HTM) 04-01 guidance [69,70]. Where efforts to reduce the numbers of *P. aeruginosa* using mechanical (shearing by flushing water) and chemical (e.g. chlorine dioxide, silver/copper ion etc.) methods fail, a physical barrier-approach such as a point-of-use (POU) membrane filter unit may be implemented if water pressure is adequate.

The two main types of POU filter units used on faucets and shower outlets in the healthcare setting are membrane filters (disposable or reusable) and hollow fibre filters; depending on the manufacturer, standard membrane filter units comprise of a double layer membrane with 0.1– - 0.2µm pore size that prevents the passage of *P. aeruginosa* and a pre-filtration layer that retains larger particulates and organic matter [168]. Hollow-fibre filter units consist of a sealed chamber into which the incoming water must pass through 0.1µm diameter pores spanning the length of a matrix of hollow fibres before exiting the outlet. Standard membrane and hollow-fibre filter units operate as pass-through water filtration systems and are prone to biofouling and bioscaling with organic debris and inorganic salts (e.g. calcium/magnesium carbonates). The ability of these filters to sequester *P. aeruginosa* effectively depends on the duration and frequency of usage as well as water quality. Efficiency of membrane POU water filtration has been demonstrated but some studies report that *P. aeruginosa*

contamination can occur within the recommended term of usage given by the manufacturer [9,10]. Companies producing shower filters for medical are T-safe, , Challis Aquafree and Pentair X-flow; Anonymised 2 showers are investigated in this study [156,171–174].

Hollow fibre filters started to gain popularity as they allow greater flow of water especially when water pressure is low [175,176]. The advantage of hollow fibre filters against conventional flat membrane filters is to attain high membrane surface within a limited volume as the membrane is in the form of hollow fibre bundles [177]. Polysulfone and polyethylene are the two commonly used materials in hollow fibres with average pore diameter range of 0.25 to 1.5 µm and 0.5 to 2µm respectively [177,178]. Hollow fibres provide structural strength, hence increasing the average membrane life. They increase water permeability due to their hydrophilic properties and can work in lower pressures than membrane filters [175]. To determine whether these POU filters continue to prevent egress of *P. aeruginosa* during the manufacturer usage period, the efficacy of historically used 25 polysulfone hollow-fibre shower filter units (Medical shower filter; 0.1µm pore-size; polysulfone body; antimicrobial silver-impregnated; in-use lifecycle expiry of 92 day) in patient bathrooms in augmented and non-augmented care wards were surveyed [171]. In vitro validation testing was done on 15 hollow fibre filters in total in order to understand the filtering efficacy and potential contamination sources.

Investigation of hollow fibre membrane in Anonymised 2 showers used in this study are given in Figure 4.10. Figure 4.10.1 shows the inner cartridge (hollow-fibre matrix) of the Anonymised 2 shower head. Hollow fibres to the bottom part of the shower head is shown in detail in Figure 4.10.2. Blue colour should not be taken into account since it stems from the colouring of the blades used to cut the material. Hollow fibres can be seen in Figure 4.10.3 at the bottom part of the shower head where water inlet enters. Detailed close-up photograph of hollow fibre threads is given in Figure 10.4. Photograph as given in Figure 4.10.5 was taken from bottom part of the tube (water inlet) showing hollow fibres. Photograph taken from top of the tube (water outlet) showing hollow fibres is given in Figure 4.10.6. Zoom in photograph of hollow fibres is given in Figure 4.10.7.

Figure 4.10.1. Inner tube of Anonymised 2 shower head.

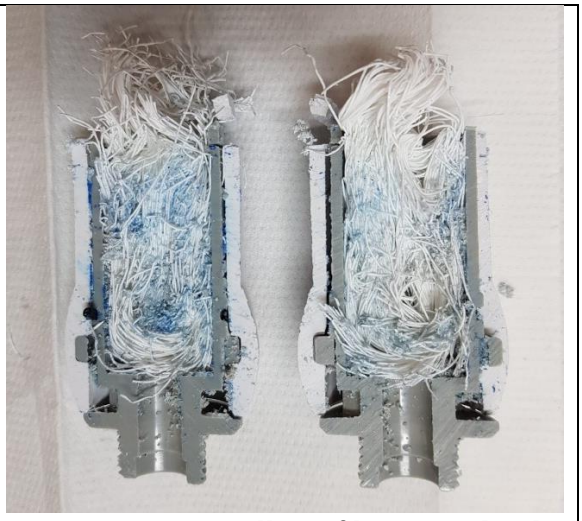


Figure 4.10.2. Hollow fibres inside the tube

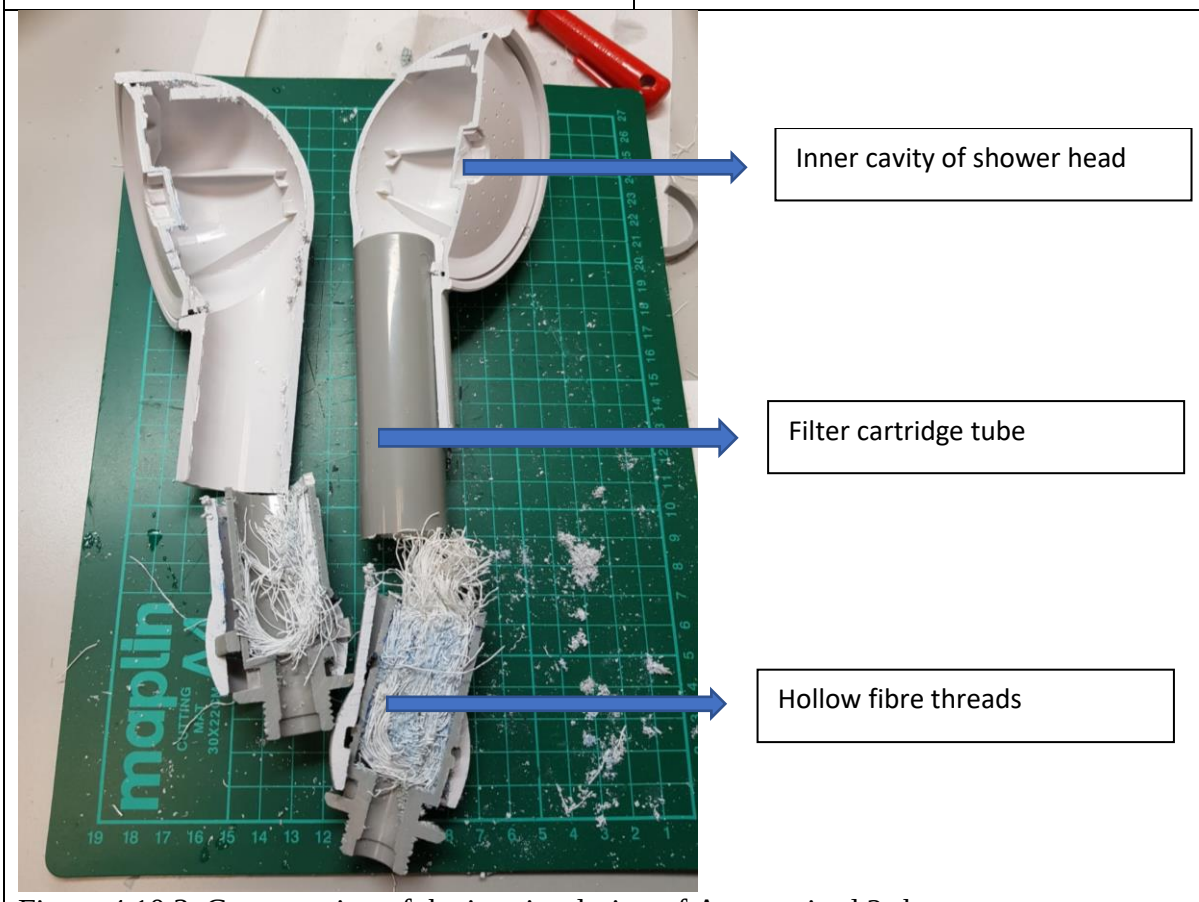


Figure 4.10.3. Cross section of the interior design of Anonymised 2 shower

Figure 4.10.4. Hollow fibre threads from a transverse section of the filter cartridge

Figure 4.10.5. Water inlet part of the inner tube

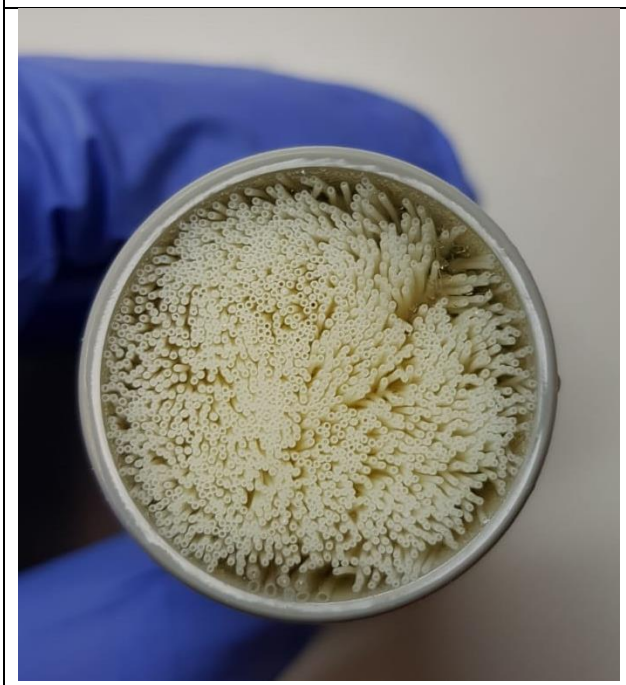


Figure 4.10.6. Hollow fibres at water outlet

Figure 4.10.7. Zoom in photograph of hollow fibres

Figure 4.10. Investigation of hollow fibre membrane in Anonymised 2 showers

4.2. Materials and Methods

4.2.1. Investigation of hollow fibre membrane in Anonymised 2 showers

4.2.1.1. Breaking open the shower heads and filter cartridges for investigation

A pristine Anonymised 2 shower head was dismantled and the shower head was opened to reveal the interior design and to locate the hollow fibre. The shower head cut in half is shown in Figure 4.10 to reveal the cross-section and the dead spaces in the head where water may reside permitting can stagnate and form bacterial biofilm.

Anonymised 2 filters (Anonymised 2 UK Ltd., Northern Ireland, UK) were installed on showers in the upper half of the UCH tower (8^{th} – 16^{th} floor) in July 2019 with the aim to have bacteria free water outlet for safe patient usage. Environmental sampling of showers was performed in the rooms of patients having pseudomonas bacteraemia. In two of these locations, shower water from the filter was found to have high counts of *Pseudomonas aeruginosa* (>300CFU/100mL). Retesting by the manufacturer did not reveal any faults in the filter, suggesting external contamination. However, leakage around the filter following damage to the resin was another possible cause.

4.2.1.2. Clinical setting and selection criteria

Twenty-five patient bathrooms were selected at random from six wards with patients requiring augmented care (haematology, elderly care, adolescent haematology/oncology and infectious diseases) at a 700-bed multi-storey building teaching hospital in London, UK (UCH). Each ward was a single floor of the hospital building. The bathrooms selected were en-suite for single-isolation rooms (SIRs) or those serving shared-occupancy bed bays (room with 4-6 beds). Apart from elderly care, cases of *P. aeruginosa* bacteraemia had occurred in all of the wards in the preceding six months but the source had not been demonstrated as hospital or community.

All the bathrooms had a POU hollow-fibre filter integrated showerhead.

4.2.1.3. Shower water sample collection and assay by membrane-concentration:

Prior to sample collection the showerheads were disinfected by wiping the entire outer surface with a sterile alcohol wipe (70% isopropyl alcohol) and allowed to air dry (~15s).

The opening of a water sample collection bag (sterile-grade) was placed over a showerhead and secured to capture a water sample. An incision was made aseptically to the bottom corner

of the bag to create a second opening via which water could be channelled. The shower valve was opened and an aliquot of at least 100 mL water was collected using the water collection bag into a sample container (pre-dosed with 1mL neutraliser solutions; composition: 1g/L sodium thiosulphate, 30 mL/L Tween 80 and3 g/L Lecithin in PBS). The showerhead was then removed aseptically and placed onto a pre-sterilised tray. A second 100 mL water sample collected in the same manner into a second sample container. These two samples represent "with/without POU filter" sample arrays respectively. The showerhead was then re-attached and the entire surfaces of the showerhead and hose wiped with a sterile alcohol wipe prior to reinstating the shower. This process was repeated for 25 individual showers within the hospital. The number of showers targeted was 25 (21%) of 119 showers on the test wards. The sampling period was 19.08.2019 – 10.01.2020. Follow-up sampling was performed for two of the showers 24 days after the first water collection.

Water samples were transferred to refrigeration (2-8°C) within 2 hours of collection and processed within 24 hours. Shower samples (100±5mL) were concentrated by vacuum filtration (max 65kPa pressure) through a 47mm nitrocellulose membrane of pore size: 0.45µm followed by plating the membrane onto a Pseudomonas C-N agar plate. Plates were incubated aerobically at 37°C for 48 hours prior to counting the colonies. Water sampling and following procedures were in line with HTM guidelines recommended by NHS England [69].

4.2.1.4. Confirmation of *P. aeruginosa* isolates

Confirmation of suspected colonies are done as detailed in Chapter 2 by milk hydrolysis and oxidase tests and final confirmation by Maldi-TOF.

4.2.1.5. Measurement of *P. aeruginosa*

The upper reading/counting-limit of samples analysed using the membrane-concentration assay technique was 300CFU/100mL.

A sub-set of four showers, selected at random, were assayed further by taking a one-millilitre aliquot from the original sample and performing serial 1/10, 1/100 and 1/1000 dilutions before plating 100uL onto Columbia Blood Agar from the neat, 1/10, 1/100 and 1/1000 arrays. Confirmation of *P. aeruginosa* was as previously described.

4.2.1.6. Shower water pressure measurements

Water pressure measurements were performed with a pressure gauge (Bourdon Pressure Gauge 0-4 bar, RS Components, UK) as detailed in Chapter 2 on randomly selected 74 showers from 10 wards.

4.2.1.7. Statistical Analysis

Chi-squared test with Yates' correction was performed for the difference between days of usage of those shower groups (showers effectively filtering the bacterial load and failing to filter). Chi-squared test was used to compare observed results with expected results; Yates' correction was applied to reduce the error in approximation [179].

4.2.2. In vitro validation of Hollow-fibre POU filter devices

4.2.2.1. Pilot Study on 5 Showers

4.2.2.1.1. Determination of retrograde contamination to the internal cavity of the shower head

The hollow fibre cartridges were removed aseptically. The exterior of the shower heads was disinfected using 70% isopropyl alcohol. This disinfection is validated in laboratory but beyond the scope of this study The interior cavity of the shower heads was swabbed (1 cm^2 area) with a pre-moistened swab (with neutraliser solution as previously described). Swabs were streaked onto Pseudomonas C-N agar media and incubated at 37°C for 48 hours and colonies counted.

4.2.2.1.2. Testing hollow-fibre cartridge for retrograde contamination

In order to test the cartridges for retrograde contamination, all shower head parts were disinfected by using the following procedure prior to further tests. All outer surfaces of the hollow fibre cartridges were disinfected with 70% ethanol solution. Up to 100mL of sterile water were passed through the cartridges with 20mL sterile syringes. Effluents were captured in sterile containers (with neutralizer). 0.1mL of the effluents were spread plated onto Pseudomonas C-N agar media, incubated at 37°C for 48 hours and the colonies were counted.

4.2.2.1.3. Testing the efficacy of (cleaned) hollow fibre cartridges to retain *P. aeruginosa*

Hollow fibre cartridges were disinfected by using ~2000 ppm peracetic acid solution. Approximately 250mL of the peracetic acid solution was flushed through manually using a syringe. This was left for a few minutes of contact time after which 10 mL of neutraliser B was flushed through followed by a rinse volume of sterile deionised water (250mL). These were left overnight to dry prior to further experimental use. The next day, sterility was checked by passing 100 mL sterile water through the cartridge and filter. The effluent was concentrated by 0.45 µm pore sized membranes, which were plated onto Pseudomonas C-N agar media incubated at 37°C for 48 hours and the colonies were counted.

Overnight broth tubes of a type strain (NCTC 10662) of *P. aeruginosa* titres (~10^7 CFU/mL) were prepared and 1 ml were inoculated into sterile water. Using a 20 ml syringe, the inoculum plus additional sterile water was passed through previously cleaned filter cartridge until ~100mL is passed through. Effluents were captured in sterile containers (with neutralizer). 0.1mL of the effluent was plated onto Pseudomonas C-N agar. The remaining effluent was filter concentrated on 0.45 µm pore sized membranes which were plated onto Pseudomonas C-N agar media and incubated at 37°C for 48 hours and the colonies were counted.

4.2.2.2. Main Study on 10 Showers

Ten showers were selected for analysis listed in following arrays

Procedure A - The entire surface of the face of the shower head was swabbed with a cotton-tipped swab (pre-moistened with neutraliser). The surface was then disinfected with an alcohol wipe (70% IPA).

Procedure B - 100mL of water was collected on opening the shower outlet (pre-flush sample).

Procedure C - The entire shower head and hose was removed aseptically and a second (pre-flush) 100ml of unfiltered shower water taken from the same shower.

Procedure D - a pristine Anonymised 2 shower head was fitted by estates engineer and 100mL of water was collected on opening the shower outlet (pre-flush sample).

Procedure E - The inner face of the shower head was sampled with a cotton-tipped swab (pre-moistened with neutraliser).

Procedure F - sterile tap water was passed through the filter cartridge until and 100mL captured.

Procedure G - The filter cartridge was disinfected with peracetic acid solution (2000ppm) passed though by syringe and rinsed three times with 1 litre sterile deionised water. And final rinse with 100mL alcohol solution (70%). Allowed to air dry overnight.

Procedure H - A type culture of *Pseudomonas aeruginosa* (NCTC 10662) was inoculated into the shower cartridge and flushed through with 1 litre of sterile tap water. The first 100mL of water post-filter was collected for examination.

P. aeruginosa isolates recovered from this study are sent for WGS.

4.3. Results

4.3.1. Ward Study Results

P. aeruginosa was found in the effluent from 8 (32%) showers, despite the filter being in place (Table 4.1). Six out of those eight showerheads were found to have high bacterial counts (>300CFU/100mL). One filter (shower #16) reduced the *P. aeruginosa* load in effluent from >300 CFU to 8 CFU while another (shower #17) reduced the count to ~100 CFU. These 8 showers had been in use for a mean of 60.87 days (95% CI 15.3 to 106). Shower #16 and shower #17 were sampled on 15th day of usage. At the second sampling (39th day), these two showers showed 100 and >300 CFU/mL *P aeruginosa* in the effluent respectively with the shower filter in place.

The remaining 18 showers effectively filtered out *P. aeruginosa* bioburden despite presence at high numbers (i.e. >300 CFU/100mL). The duration of usage of the POU filters screened averaged 20.65 days (SD=12.57). There was no significant difference in the days of usage between those shower groups (showers effectively filtering the bacterial load and failing to filter) (p=0.075).

Table 4.1. Presence of *P. aeruginosa* of effluent in hospital shower waters fitted with a POU filter unit at various durations of usage. Numbers of *P. aeruginosa* present in shower waters with and without a POU filter unit determined by membrane-concentrations assay.

POU shower filter details					**Effluent Water Quality (presence of *P. aeruginosa*)***	
Shower Ref. number	Ward Ref.	Ward Specialty	Location of corresponding Shower (Bay/ SIR)	Age of filter (Days in use)**	Without POU filter (CFU/100 mL)	With POU filter in place (CFU/100 mL)
1	Ward E	Haematology	SIR	15	>300	>300
2	Ward E	Haematology	SIR	15	>300	0
3	Ward E	Haematology	SIR	15	>300	0
4	Ward E	Haematology	SIR	15	>300	0
5	Ward E	Haematology	SIR	15	>300	0
6	Ward E	Haematology	SIR	15	>300	0
7	Ward E	Haematology	SIR	15	>300	0
8	Ward E	Haematology	SIR	15	>300	0
9	Ward B	Haematology	SIR	15	>300	0
10	Ward B	Haematology	SIR	15	>300	0
11	Ward B	Haematology	SIR	15	>300	0
12	Ward B	Haematology	SIR	15	>300	0
13	Ward B	Haematology	SIR	15	>300	0
14	Ward B	Haematology	SIR	15	>300	0
15	Ward B	Haematology	SIR	15	>300	0
16	Ward B	Haematology	SIR	15	>300	8
17	Ward B	Haematology	SIR	15	>300	100
18	Ward F	Elderly care	Bay	45	>300	>300
19	Ward C	Adolescent Haematology/Oncology	Bay	45	>300	>300
20	Ward C	Adolescent haematology/oncology	Bay	47	>300	0
21	Ward D	Oncology (Adult)	Bay	47	>300	0
22	Ward G	Infectious Diseases	Bay	47	>300	0
23	Ward B	Haematology	SIR	52	>300	>300
24	Ward C	Adolescent haematology/oncology	Bay	150	>300	>300
25	Ward C	Adolescent haematology/oncology	SIR	150	>300	>300

* Counts reported as 0 CFU are below the detection limit (1CFU)

** - expiry date of POU filter units are 92 days from date of installation (manufacturer specifications).

P. aeruginosa was quantified in four out of eight showers that had over 300 CFU/100mL of *P. aeruginosa* with the filtered showerhead in place. There was a geometric mean of $4x10^6$CFU/100mL (6.8x10^4 – 2x10^8) (Table 4.2).

Table 4.2. Quantification of *P. aeruginosa* bioburden to determine water quality of effluent from four showers

Shower description and details			**Effluent Water Quality (presence of *P. aeruginosa*)**
Shower Ref. Number	Ward Reference	Ward Specialty	CFU/100 mL Without POU filter
16	Ward B	Haematology	6.8 x10^4
17	Ward B	Haematology	1.45x10^7
23	Ward B	Haematology	1.6x10^6
25	Ward C	Adolescent Haematology/Oncology Teenage cancer	2.02x10^ 8

A total of 74 shower water pressure measurements were taken from ten floors of the hospital with values averaging 2.94 bar (range 0.3 – 3.9). Pressure measurements of the four wards tested in this study were:

- Ward C: 10 shower water pressure measurements, mean 2.43 bar (range:2.3-2.8)
- Ward D: 8 shower water pressure measurements mean 1.8 bar (range:1.6-2.2)
- Ward E: 8 shower water pressure measurements mean 1.17 bar (range:1.1-1.25)
- Ward B: 6 shower water pressure measurements mean 0.83 bar (range:0.8-0.9)

Correlation analyses on shower pressures and average colony count of *P. aeruginosa* in those 4 wards are shown in Figure 4.2. R2= 0.926, p= 0.073 (Pearson correlation test)

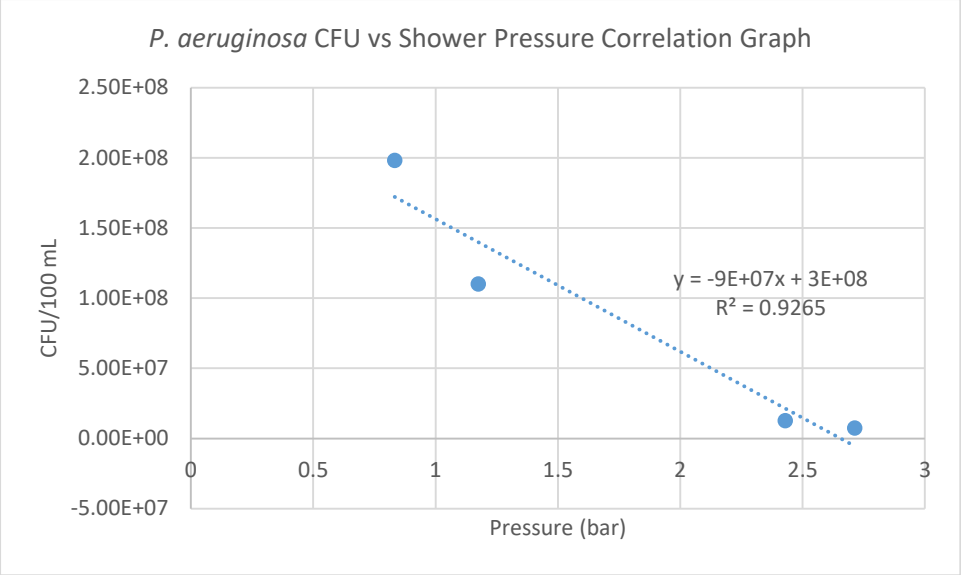


Figure 4.2. Average *P. aeruginosa* – pressure correlation on Wards C, D, E and B.

Correlation analyses was also done with actual *P. aeruginosa* and pressure levels as follows in Figure 4.3 R2=0.499, p=0.01.

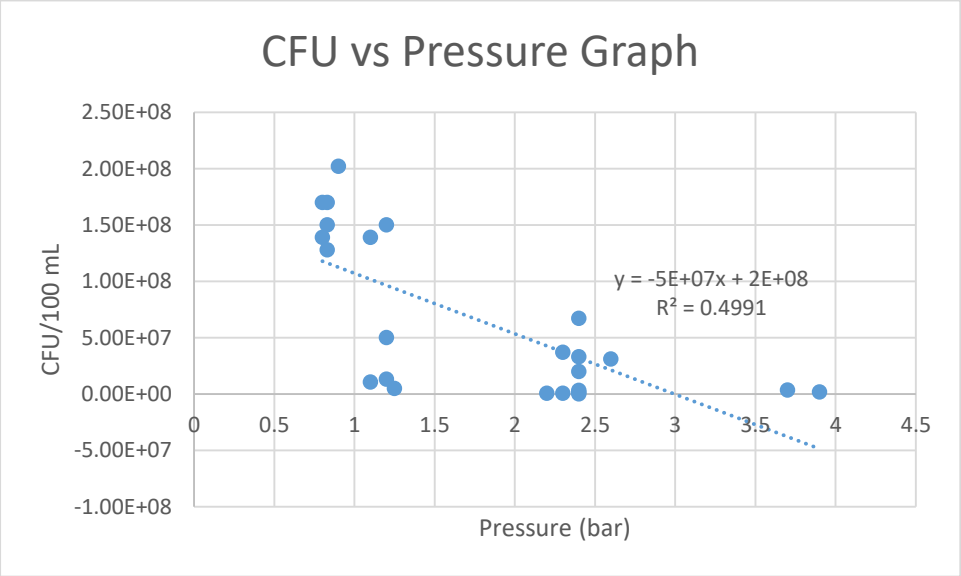


Figure 4.3. *P. aeruginosa* – pressure correlation with actual measures on Wards C, D, E and B.

4.3.2. Laboratory-based Study Results

4.3.2.1. Pilot Study on 5 Showers

Total of 5 showers collected from augmented care wards were used in laboratory-based study, two of which (showers #26 and #27) showed high counts of *P. aeruginosa* with the filtered head in place. Duration of usage of the showers averaged 37.2 days (SD=1.64). One unused shower and filter head was included in the studies as a control.

Three of five (60%) of the used showers (#27, #28, #29) had contamination with *P. aeruginosa* inside the shower head with average count 40 CFU/cm2 (SD=38.59).

Four out of five (80%) showers (#26, #27, #28, #29) had contamination with *P. aeruginosa* within the hollow fibre cartridge, average count 503 CFU/0.1 mL (SD=195.07).

After disinfecting the cartridge with peracetic acid, the efficacy of disinfection was tested by passing sterile water and filter concentrating 100 mL, all of which showed 0 CFU/100 mL.

All the *P. aeruginosa* strains identified were confirmed by Maldi-TOF Mass Spectrometry analysis.

To test the efficacy of used/unused filter showers to retain *P. aeruginosa* in water source, an inoculum of 3.9×10^7 CFU *P. aeruginosa* (NCTC 10662) was passed through the filter (from proximal to distal).

3 out of 5 (60%) of the used showers (#26, #27, #28) failed to prevent water contaminated with *P. aeruginosa* from passing through the outlet, average count 1520.33 CFU/100 mL (SD=2408.32).

4.3.2.2. Main Study on 10 Showers

P. aeruginosa isolates recovered from the main study on 10 showers given in Section 4.2.2.2. are sent for WGS. Analysis of the results are planned as future study.

4.4. Discussion

4.4.1. Investigation of hollow fibre cartridge

In order to understand the water flow pathway during shower usage, water was run through the inner tube of the hollow fibre cartridge. Although the path was not clear, water seemed to go through the edges of the fibres rather than going through the middle, possibly allowing unfiltered water coming out of the water outlet.

Dead space in the shower head harbours water which could eventually promote biofilm growth.

The 0.1um pores along each hollow-fibre thread is effective in sequestering bacteria when water is forced through the lumen of the hollow fibre thread and can egress only via the pores. To prevent water egress outside of this route, the spaces between the fibres at bound at the terminal ends with resin.

4.4.2. Ward Study

Exposure to *P. aeruginosa* colonized shower water is a potential risk for the development of bacteraemia in immune suppressed patients [73,82]. In this study setting, the use of hollow fibre shower filters did not provide assurance of safety for the patient in the shower environment. Although not necessarily due to a failure of the filter itself, external contamination and growth inside the shower head had a similar effect, exposing some patients to high levels of organisms with a risk of serious subsequent infection in immune suppressed individuals. Without repeated monitoring, clinical teams may be unaware of the potential source of *P. aeruginosa* bacteraemia in vulnerable patients.

The hollow-fibre POU filter showerheads were in-situ for three months before the sampling survey commenced; this replaced showers comprising of non-filtration antimicrobial-impregnated showerhead/hose units.

The selection of the hollow-fibre technology was due to the high-capacity filtration via the 0.1um-diameter pores in the filter-matrices and long shelf-life of 92 days (manufacturer communications). The POU filters were subjected to routine surveillance to assure efficacy against *P. aeruginosa* during the period of usage.

Although the POU-filters were effective in removing *P aeruginosa* from the effluent in a majority of cases, the organism was found distal to the filter in a third (8/25) of showers.

While this study did not explore the sources of contamination, the isolation of *P. aeruginosa* from filter-treated waters was likely due to retrograde contamination from external reservoirs or failure of the filter-matrices in sequestering bacteria.

In this study a high bacterial burden (>10^6 CFU/100mL) in the pipework proximal to the filter may have overwhelmed the efficacy of the hollow-fibre filter matrix. However, a study using a 0.1μm porous polyethylene hollow-fibre filter demonstrated >log6 reduction when challenged with *Klebsiella terrigena* [180]. Retrograde contamination of taps, and even proximal piping, from drains despite point of use filters has been reported [146].

Point-of-use filters are an alternative to chemical disinfection using chlorine dioxide, hydrogen peroxide or copper-silver ionisation and are effective when endemic potential pathogens cannot be eliminated [168]. In a surgical ICU, point of use filters were associated with elimination of tap water contamination and reduction of pseudomonas colonization and infection in patients by 95% and 56% respectively [169]. Use of 0.2 μm filters in wards in Japan removed all Gram-negative bacterial contamination in water for up to 2 months [168]. Studies in ICU and bone marrow transplant units found installation of filters reduced nosocomial pseudomonas infections [181,182].

However, external contamination can affect the efficacy of POU filter-devices and represents a potentially indefinite revenue commitment for replacements. In our study, the hollow-fibre filters adopted had a specified lifespan of approximately 3 months. Nevertheless 26% (6/23) of the POU filters became colonized before the expiry-date of the device had elapsed. Two of the filters screened in this study were in-situ beyond the expiry date and were decommissioned from use immediately by the hospital estates and facilities management. Membrane filter devices are an alternative to hollow-fibre filter units but contamination with *P. aeruginosa* has been demonstrated to occur within the recommended duration of use [9]. A study from France reported *P. aeruginosa* contamination at weeks 4 and 5 after installation [170]. Although contamination level may be low initially, *P. aeruginosa* can proliferate quickly, presenting a risk for cross contamination. Polysulfone or polyethylene hollow-fibre filters have practical utility over standard membrane filters in low-pressure water systems where water output would otherwise be severely attenuated [11,12]. However, they are susceptible to the same problems of external contamination within a few weeks of installation. In a laboratory study involving experimental contamination of pristine hollow-fibre filter devices (0.2um pore-size) before placing on uncontaminated faucets and showers

were compared. Membrane and hollow-fibre shower filters were effective in removing *P. aeruginosa* [175]. However, despite a recommended use time of 31 days, faucet hollow fibre filters showed early growth of *P. aeruginosa*, in one case from day 16. There was no back contamination after filters were removed.

The mains water supply of the hospital was screened at the incoming site to the hospital and found to be free of *P. aeruginosa* (data upon request). In our survey, the water proximal to the filters harboured 10^6 CFU/100mL *P. aeruginosa*. In cases where *P. aeruginosa* was isolated post-filtration, it could not be ascertained whether the contamination originated by retrograde contamination (e.g. aerosolised droplets from shower trays/drains), translocation through the filter-matrix by high-pressure water flow or as a consequence of perforation of the POU filter cartridge within the showerhead body. The pressure of water flow in the test building was below the upper tolerance (5 bar; manufacturer product specification) of the POU filter cartridge. Further exploratory and destructive analysis of the filter device, including microbiological and molecular characterisation, is required. Low pressures present another risk because patients may then remove the shower heads and expose themselves to unfiltered shower water colonized by *P. aeruginosa*. Low shower pressures averaged 0.83 bar on Ward B a haematology area where immune-suppressed patients stayed. In some cases, shower heads had already been removed by the patients when showers were inspected, despite warnings by nurses, ward sisters and wall posters not to do so.

An audit conducted after this study screened patients for rectal colonization between 24/01/2020 and 13/05/2020 (110 days). There were 155 patients and 606 samples were collected (groin/rectal swabs). Seven *P. aeruginosa* positive samples were taken belonging to six patients in total. Two patients were *P. aeruginosa* positive when they were first admitted making the prevalence at admission 1.29%. Four patients were *P. aeruginosa* negative in the first sample but acquired *P. aeruginosa* during their stay making the proportion that acquired *P. aeruginosa* 2.6%. Length of stay until acquiring *P. aeruginosa* averaged 33 (SD=25.57) (unpublished data). Patient locations and presence of *P. aeruginosa* shower contamination data were checked. One of the patient locations where *P. aeruginosa* was acquired showed shower water with >10 ^{8}CFU/100mL in a sample near the time of bacteraemia. This was one of the hollow-fibre shower locations, however its efficacy in filtering and whether the patient exposed themselves to unfiltered water by pulling off the filtered shower head is not known. Various devices are marketed on the premise of delaying retrograde biofilm formation but efficacy in use against *Pseudomonas* sp has not been demonstrated in peer reviewed studies,

for example, copper inserts for faucet outlets and silver-impregnated hoses. Although it is important to demonstrate the source of contamination, investigation of all possible routes of transmission is difficult. Hollow-fibre medical filter devices may be useful in preventing exposure of patients to *P. aeruginosa* from colonized shower water for short periods of use. However, application of POU shower filter units should be complemented with regular water testing, daily cleaning, and internal disinfection of filtered water outlets in augmented care wards, especially when growth of *P aeruginosa* persists.

4.4.3. Laboratory-based Study

In-vitro efficacy testing of shower filters showed that external *P. aeruginosa* contamination on outside of the shower may enter the inside cavity of the shower and contaminate the hollow fibre filter cartridge. The external shower surface needs daily cleaning and if internal contamination develops the filtered head needs to be removed and disinfected. Failure of the filters within the 92 days shelf life given by the manufacturer cannot be controlled by external cleaning.

The laboratory study showed contamination can be either retrograde contamination or filter failure. Laboratory-based study could not be conducted immediately after the collection of used showers, but they were kept in moist bags in 4°C fridge which may have resulted in desiccation/death of *P. aeruginosa*.

Although it is important to demonstrate the source of contamination (external contamination or failure of filters), it is challenging and not practical to do further investigation routinely. Therefore despite the use of POU filters, it is prudent to continue regular water testing for *P. aeruginosa* screening, as well as frequent cleaning especially in augmented care wards.

As described by the outcomes of this chapter, *P. aeruginosa* contamination was present in the hollow-fibre filter shower heads. Subsequently, thermal disinfection shower heads were put in place in UCH, however, sample collection for this PhD thesis was completed by that time, therefore results regarding their efficacy were not outlined in this thesis but completed by the Environmental Research Laboratory [52].

Molecular analysis of the environmental and clinical isolates collected within this thesis study is described in the following chapter.

Chapter 5. Molecular analysis of environmental and clinical isolates of *P. aeruginosa* to determine their relatedness, AMR and biofilm properties

VNTR analysis was provided by Jane Turton and Zoe Payne, UKHSA. WGS analysis of the pilot study was provided by the team at Quadram Institute (Justin O Grady, Andrew Page and Leonardo de Oliveira Martins). WGS analysis of the main study is provided by Lucy van Dorp, UCL Genetics Institute.

5.1. Introduction

P. aeruginosa is present in hospital water systems such as tap and shower water, plumbing equipment, flow straighteners and thermostatic mixer valves [56,74]. Presence of multidrug resistant gram negative bacteria in the hospital environment presents a particular risk for vulnerable patients [183]. *P. aeruginosa* is intrinsically resistant to many antibiotics and can easily gain resistance through gene transfer [184]. The biofilm forming capacity of *P. aeruginosa* allows them to form stable structures which are difficult to eradicate, presenting a challenge for environmental cleaning [185].

Although the presence of *P. aeruginosa* in hospital water systems has been known for many years, it is only after the development of molecular methods such as PFGE, VNTR, MLST and more importantly WGS that its epidemiological risk to patients started to be understood [94]. Previous studies showed genetic relatedness between environmental and patient *P. aeruginosa* isolates pointing to plausible human-to-environment-to-human transmission [145]. More detailed (data-driven) investigations are needed to accurately depict and understand the direction of transfer. Metadata, e.g. times (environmental presence or patient acquisition dates) and special locations of sample collection/patient occupancy give good information relating this question which should be studied in conjunction with information from phylogenetic inference.

At UCH, there was an outbreak of *P. aeruginosa* in 2017. Early in-house environmental sampling data from the Environmental Research Laboratory (A commercial laboratory (UKAS Laboratory No. 10147) accredited to undertake water microbiology testing, based within the UCH site and provides testing and monitoring services to UCLH Trust) showed shower water to be the main reservoir of *P. aeruginosa*. To be able to compare environmental and patient strains, antibiotic susceptibility tests were undertaken by disk diffusion assays. It was observed that hospital water strains were almost always fully susceptible to the 12

antibiotics tested (shown in Chapter 3) and drain strains showed resistance to some antibiotics. Extensive and multidrug resistance were mainly observed in patient strains. The first molecular typing and comparison between environmental and patient strains used VNTR, a PCR-based method which characterizes isolates by the number of tandem repeats in their DNA. Nineteen loci have been identified for *P. aeruginosa*; of these, 15 loci are the preferred criteria for epidemiological risk analyses although identification through a nine-locus scheme is also possible, which was the service offered by UKHSA and used in this thesis [90].

Although molecular typing methods like PFGE and VNTR have been used in previous studies, developments in technology suggest WGS as the gold standard. One study comparing these three methods showed that WGS gives the most detailed and accurate results when compared to PFGE and VNTR in an outbreak analysis by considering full genome, not only marker regions [94]. In addition to outbreak analysis, genotypic characterisation of antibiotic resistance genes can be found and further epidemiological investigations can be done through cgMLST data although WGS is far more powerful for outbreak reconstruction [93]. WGS typically considers an aligned genome, achieved either through assembly or reference mapping, and the calling of variants which distinguish the diversity of the species in question [109].

In this thesis, two samples from the total collection of isolates were compared; isolates typed by VNTR and WGS. 188 *P. aeruginosa* strains (111 patient blood isolates and 77 environmental isolates) retrieved between 2017 - 2019 were typed by nine-loci VNTR. Given the limitations of VNTR analysis when compared to WGS, VNTR results were considered as a preliminary approach to highlight important strains of interest for WGS [94]. 190 *P. aeruginosa* isolates (86 patient blood isolates and 104 environmental isolates) retrieved between 2014 - 2020 were typed by WGS. Antibiotic susceptibility tests were performed by disc-diffusion assay against 12 antibiotics and AMR genes were screened for their presence in WGS assemblies as detailed in the methods.

Through a synthesis of epidemiological data, characterization of STs and phylogenetic reconstruction the aim is to recover the relatedness of environmental and clinical strains and identify possible routes of transmission. Analysis of genotypic AMR data in comparison with phenotypic antibiotic profiles were done to investigate the presence of AMR genes and to

demonstrate their expression. Biofilm genes were screened by prioritising biofilm-producing strains as this could be a survival advantage by adhering to host tissues and invading patients [186].

5.1.2. Hypothesis

P. aeruginosa bacteraemia patients contaminate the shower environment, especially shower heads, which further become a source transmitting infection to further patients.

Chaperone-usher pathway (*cup*) genes are important determinants of environmental colonisation as well as contributing to bacteraemia by infecting the host via urinary tract infection and disseminating to the bloodstream.

5.1.3. Aims/objectives

To estimate the relatedness between environmental and clinical isolates

To characterise the AMR and biofilm profiles of the isolates

To identify if environmental and clinical isolates share common traits giving them a survival advantage to colonize the environment and infect the patients.

5.2. Materials and Methods

5.2.1. General metadata acquisition

Environmental samples were collected from shower and handwash basin waters and drains and biofilm material from the shower hoses as described in Chapter 2 - Methods.

Antibiotic resistance profiles were determined by disc diffusion assays against 12 antibiotics of relevance to *P. aeruginosa* treatment (TCC, PTZ, AK, CAZ, FEP, PRL, CIP, TOB, ATM, IPM, CN and MEM) as detailed in Chapter 2.

Isolates for VNTR profiling were selected according to epidemiological links. Clinical and environmental strains were isolated from similar sites and dates, then the selection was widened to e.g. whole ward or neighbouring wards. VNTR typing of nine loci (ms172, ms211, ms213, ms214, ms217, ms222, ms2017, ms209 and ms61) were performed by Jane Turton and Zoe Payne, UKHSA [90].

5.2.1.1. Inclusion/exclusion criteria

Clinical isolates included in this study were isolates from *P. aeruginosa* bacteraemia patients from UCH. Environmental water and drain isolates were selected from the *P. aeruginosa*

isolate collection generated during this PhD and from the existing laboratory collection where historical isolates were selected for wider temporal context. Community acquired *P. aeruginosa* isolates were excluded from WGS analysis (except for one isolate). Isolates from other hospitals (other Trust sites) were excluded from WGS analysis but some were included in the VNTR analysis.

5.2.2. Bacterial Genomic Extraction

DNA extractions were performed using the Zymo Research Quick-DNA Miniprep Plus Kit as detailed in Chapter 2.

Isolates for WGS typing were selected in a similar manner to VNTR isolate selection (perceived epidemiological links) and according to the results of VNTR typing. In total, 190 *P. aeruginosa* isolates from 9/4/2014 - 18/9/2020 period were typed by WGS. 86 were patient bacteraemia isolates and 104 were environmental isolates. 69 of the environmental isolates were from shower water, 13 shower drains, 13 hand wash basin water, 6 from sink drains and 3 were recovered from the biofilm of shower hoses.

5.2.3. Bacterial DNA Quality Control and Quantification

Following DNA extraction, quality and quantity was measured by Nanodrop and Qubit. Nanodrop results were used for quality measurements: Absorbance(260)/Absorbance(280) and Absorbance(260)/Absorbance(230) values. Absorbance(260)/Absorbance(280) ratio close to 1.8 is accepted as a satisfactory result. Absorbance(260)/Absorbance(230) ratio close enough to 2.0 is accepted as satisfactory result, but the interference-effect of the buffer to this measurement is explained later in the discussion. Qubit results were used to determine DNA quantity (ng/µl). A total input of at least 100-200ng DNA was obtained after DNA extractions for WGS.

5.2.4. Illumina Short-read WGS sequencing and analysis

We acknowledge Quadram Institute (Justin O Grady, Andrew Page and Leonardo de Oliveira Martins) for the WGS of pilot study including 24 isolates.

Sequencing Stream at UCL Genomics for WGS analysis of 190 isolates are acknowledged; carrying out the library preparation and sequencing. Result analysis was done in collaboration with Lucy van Dorp from UCL Genetics Institute.

5.2.4.1. Library preparation

Samples were processed using the NEB DNA Ultra II Library Kit (p/n E7645) according to manufacturer's instructions.

Briefly, 200 ng of DNA was sheared using a Covaris E220 focused ultra-sonication system (42 seconds, PIP 75, duty factor 10, cycles per burst 1000). The fragmented DNA was end-repaired then "A-tailed" at the 3' end to prevent self-ligation and adapter dimerisation. Truncated 15µM adaptors, containing a T overhang are ligated to the A-Tailed cDNA followed by an AMPure XP bead clean up without size selection.

Double ligated DNA molecules are then enriched with limited cycle PCR (4 cycles) utilising NEB's Q5 high fidelity polymerase. The primers used in this PCR extend the adaptor to full length and contain sequences that allow each library to be uniquely identified by way of a sample-specific 8bp index sequence.

5.2.4.2. Sequencing

High yield, adaptor-dimer free libraries were confirmed on the Agilent TapeStation 4200 (Agilent DNA 1000 assay) and library quantification was estimated using the Qubit dsDNA HS assay (Life Technologies). Libraries to be multiplexed in the same run are pooled in equimolar quantities, calculated from Qubit and Tape station fragment analysis.

The library pool was denatured and sequenced on the NextSeq 500 instrument (Illumina, San Diego, US) at 1.5pM, using a 150 bp paired-read run with corresponding 8bp unique dual sample indexes.

5.2.4.3. Data Analysis

Run data were demultiplexed and converted to fastq files using Illumina's BCL Convert Software v3.7.5.

5.2.5. Specific methodology for WGS:

Fastq files were concatenated within samples to create joined fastq files (suffix XX.joined.R1.fastq.gz, XX.joined.R2.fastq.gz; available on the UCL Computer Science Cluster at: /SAN/ballouxlab/Precision_AMR/Pseudomonas/Fastq_generator_files*). All files were inspected using FastQC and no additional trimming/filtering of reads was performed.

5.2.6. Genome Assembly

De novo genome assembly was performed using UniCycler1 v0.4.8 and species identity was confirmed using the database of assemblies provided by PathogenWatch (https://pathogen.watch).

The resulting 190 *P. aeruginosa* assemblies ranged in length from 6151361-7111628 nucleotides with an average number of contigs of 144 (91-240) of mean lengths 49151 nucleotides (29137-72657 nucleotides; 95% CI). GC content ranged from 65.7-66.6%.

Genome assemblies were automatically annotated for ST status and to identify the closest RefSeq assemblies using PathogenWatch (https://pathogen.watch).

5.2.7. Resistance Gene Characterisation

Genome assemblies were annotated for the presence and absence of antimicrobial resistance (AMR) genes using the Comprehensive Antibiotic Resistance Database (CARD)2 Resistance Gene Identifier tool.

5.3. Results

5.3.1. VNTR analysis of environmental and clinical isolates of *P. aeruginosa*

In this thesis, 111 *P. aeruginosa* patient blood isolates were typed by VNTR between 04.09.2017 – 01.12.2019. Repeats of nine loci (ms172, ms211, ms213, ms214, ms217, ms222, ms2017, ms209 and ms61) were identified. The number of cases typed per year was: 24 from 2017; 78 from 2018 and 9 from 2019. Total number of cases in UCH was: 76 (51 hospital-acquired) in 2017, 87 (67 hospital-acquired) in 2018 and 73 (47 hospital-acquired) in 2019. A total of 77 environmental *P. aeruginosa* isolates from 24.04.2017 – 19.08.2019 were typed by VNTR analysis, three of which were isolated from the shower drain, one from the hand wash basin and the remainder from shower waters.

Both patient (clinical) and shower water (environmental) isolates had diverse VNTR profiles. The most common VNTR profile seen in both patient and environmental strains was the pattern (12, 5, 3, 2, 6, 1, 6, 4, 13) – on some occasions ms61 position had a different repeat and some strains had one missing repeat. This VNTR profile was observed in twelve patient cases (12/112, ~11%) belonging to nine patients (two patients had multiple bacteraemia cases (one twice, other three times), two of which were community-acquired cases. The same

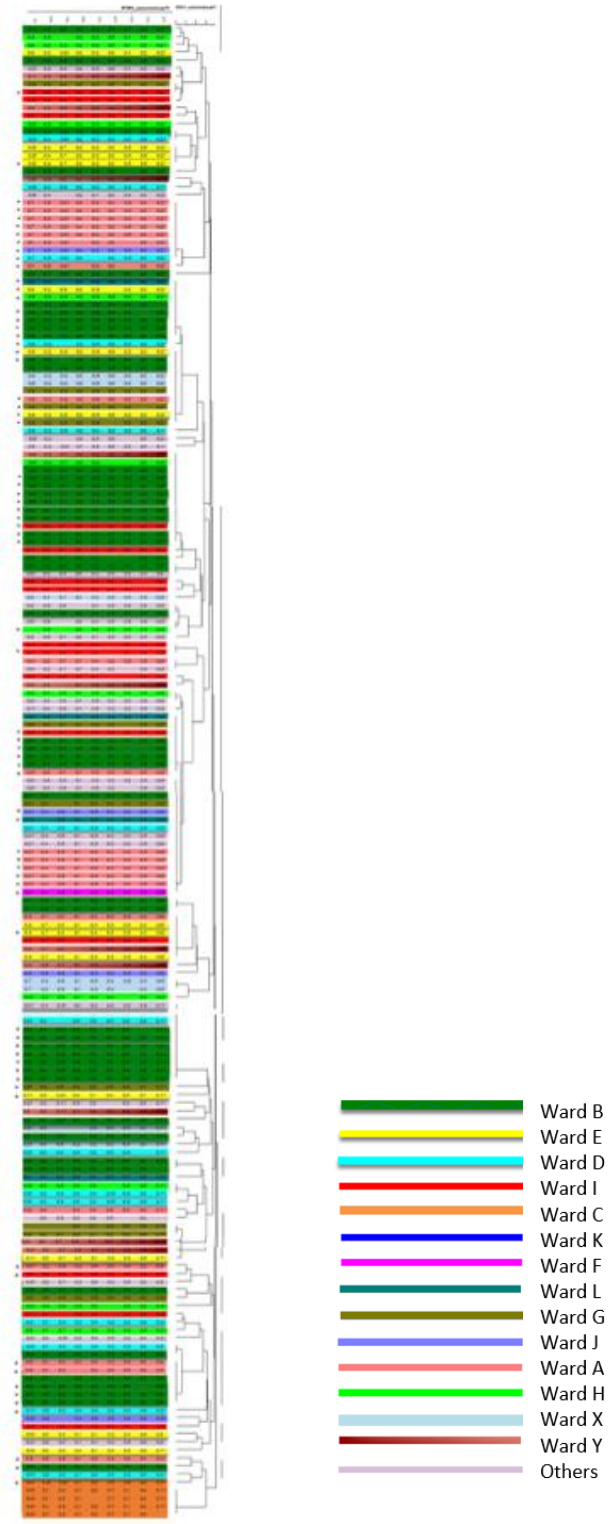
VNTR type was also identified in seven shower water samples from different ward locations and one from a shower drain.

Related VNTR profiles of patient and shower water isolates were observed. Matching by time (chronology) and location; four *P. aeruginosa* isolates showed exact matches between the patient and the shower in their bathroom in which they occupied. On two occasions the shower water strain preceded the patient bacteraemia (VNTR profiles 12, 3, 5, 5, 2, 3, 7, 2, 14 and 11, 4, 1, 7, 3, 1, 3, 1, 10). On two other occasions the patient isolate preceded the shower water strain (VNTR profiles 12, 2, 7, ?, 4, 1, 9, 2, 11 and 9, 2, 5, 2, 4, 3, 3, 1, 9).

Shower isolates from the same ward tended to have the same VNTR profiles. Repeated samplings over time showed that the same *P. aeruginosa* VNTR strain persisted for at least three months.

Phylogenetic trees created by VNTR results are given in detail in supplementary Appendix B. Some of the historical environmental and clinical were already typed by VNTR before the start of this PhD. To better illustrate the potential transmission events, locations of the samples collected/bacteraemia patients stayed are highlighted by different colours. A compressed image of a phylogenetic tree created based on VNTR profiles are given in Figure 5.1 including a total of 185 isolates: 71 environmental, 114 clinical *P. aeruginosa* strains. Detailed version of this table is included in supplementary.

Figure 5.1. Phylogenetic tree constructed by VNTR results of 185 *P. aeruginosa* isolates using methods previously described [90]. Colours represent different wards.



Visual illustration highlighting clusters of *P. aeruginosa* isolates by ward and hospital. Detailed version of this table is included in Appendix B.

VNTR result analysis for most prevalent isolates and clusters

First cluster (E):

Table 5.1. Summary characterisation of first set of isolates having same/similar VNTR profile of 11-8-3-3-2-3-10-4

Ref number	Source	Ward	Bed number	Site	Collection date
19E12	Environmental	Ward B	B3	Shower water	21/11/18
19E19	Environmental	Ward B	B26	Shower water	21/11/18
19E20	Environmental	Ward B	B3	Shower water	11/12/18
19E25	Environmental	Ward B	B26	Shower water	11/12/18
19E26	Environmental	Ward B	B3	Shower water	27/02/19
19E32	Environmental	Ward B	B26	Shower water	27/02/19
19E35	Environmental	Ward B	B3	Shower drain	11/12/18
17V394507	Patient	Ward B	B4	Blood	20/12/17
17V397890	Patient*	Ward D	B37	Blood	12/12/17

*community-acquired bacteraemia

Same VNTR profiles were observed from:

1. Shower head water of B3&B26 from Ward B for 3 months.
2. Shower head water of B3&B26 from Ward B before and after the shower change (Anonymised 1 antimicrobial shower head & hose change happened once in 3 months; sample collection was a day after the shower change).
3. Shower head water and shower drain of B3 from Ward B.

Matches with the previous batches sent for VNTR typing:

1. Blood isolate of a patient who was staying at B4 from Ward B one year before the collection of previously stated environmental samples. B3 & B4 are adjacent rooms and their showers share a wall & plumbing.
2. Blood isolate of a patient who was staying at B37 from Ward D. This bacteraemia is a community-acquired case.

Second and third clusters (J):

Table 5.2.Second set of isolates having same/similar VNTR profile of 12-5-3-2-6-1-?-4-13

Ref number	Source	Ward	Bed number	Site	Collection date
19E13	Environmental	Ward B	B5	Shower water	21/11/18
19E16	Environmental	Ward B	B32	Shower water	21/11/18
19E21	Environmental	Ward B	B5	Shower water	11/12/18
19E29	Environmental	Ward B	B32	Shower water	27/02/19

19E34	Environmental	Ward A	B5-8	Shower drain	19/11/18
307	Environmental	Ward I	B34-37	Shower water	05/09/17
18V056397	Patient	Ward H	B1	Blood	24/01/18
17V366004	Patient	Ward H	B4	Blood	02/01/17

Same VNTR profiles were observed from:

1. Shower head water of B5&B32 from Ward B for 3 months.
2. Shower head water of B5&B32 from Ward B before and after the shower change.

Matches with the previous batches sent for VNTR typing:

1. Shower head water of B34-B37 from Ward I.
2. Blood isolate of a patient who was staying at B1 from Ward H.
3. Blood isolate of a patient who was staying at B4 from Ward H.

Table 5.3.Third set of isolates sharing same/similar VNTR profile of 12-5-3-2-6-1-?-4-13

Ref number	Source	Ward	Bed number	Site	Collection date
19P4	Patient*	Ward D	N/A	Blood	24/05/18
19P10	Patient	WS3	N/A	Blood	02/08/18
19P21	Patient	WS3	N/A	Blood	19/10/18
19E3	Environmental	Ward A	B42-45	Shower water	19/11/18
19E4	Environmental	Ward A	B50-53	Shower water	19/11/18
19E10	Environmental	Ward A	B58-60	Shower water	27/02/19
19E11	Environmental	Ward A	B50-53	Shower water	27/02/19
19E33	Environmental	Ward A	B42-45	Shower drain	19/11/18
19E46	Environmental	Ward F	B5-8	Shower water	10/12/18
393	Environmental	Ward J	B19-22	Shower water	04/07/18
395	Environmental	Ward L	B37-40	Shower water	04/07/18
18V031341	Patient	Ward G	B16	Blood	23/02/18
18V108050	Patient	Ward B	B32	Blood	07/03/18
17V288830	Patient	Ward G	B40	Blood	18/09/17

*community acquired bacteraemia

The same VNTR profiles were observed from:

1. Blood isolate of a patient who was staying at Ward D. This bacteraemia is a community acquired case.
2. Shower head water of B42-45, B50-53, B58-60 from Ward A and B5-8 from Ward F.
3. Shower head water and shower drain of B42-45 from Ward A.
4. Shower head water of B50-53 from Ward A before and after the shower change.

Matches with the previous batches sent for VNTR typing:

1. Shower head water of B19-22 from T07 and B37-40 from T09.
2. Blood isolates of patients who were staying at B16 & B40 from Ward G.
3. Blood isolate of a patient who was staying at B32 from Ward B.

Fourth cluster (H):

Table 5.4. Fourth set of isolates having same/similar VNTR profile of 12-3-6-3-2-4-13-5-7

Ref number	Source	Ward	Bed number	Site	Collection date
19E2	Environmental	Ward A	B9	Shower water	19/11/18
19E5	Environmental	Ward A	B1-4	Shower water	11/12/18
19E8	Environmental	Ward A	B12	Shower water	27/02/19
19E9	Environmental	Ward A	B23-26	Shower water	27/02/19
19E39	Environmental	Ward J	B4	Shower water	15/01/19
19E49	Environmental	Ward D	B30	Shower water	17/04/19
388	Environmental	Ward A	B1-4	Shower water	08/05/18
392	Environmental	Ward A	B19-22	Shower water	08/05/18

Same VNTR profiles were observed from:

1. Shower head water of B1-4, B9, B12 and B23-26 from Ward A.
2. Shower head water of B4 from Ward J and B30 from Ward D.

Matches with the previous batches sent for VNTR typing:

1. Shower head water of B1-4 and B19-22 from Ward A suggesting that the same strain persisted for more than 7 months in B1-4 shower.

Fifth cluster (G):

Table 5.5.Fifth set of isolates having same/similar VNTR profile of 12-3-3-6-6-3-8-2-8

Ref number	Source	Ward	Bed number	Site	Collection date
19P32	Patient	Ward G	N/A	Blood	26/05/18
19E1	Environmental	Ward A	B9	Shower water	19/11/18
19E41	Environmental	Ward G	B22	Toilet s water	14/03/19
19E44	Environmental	Ward E	B15	Shower water	10/12/18
19E50	Environmental	Ward G	Bay 4 (B15)	Shower water	09/04/19
306	Environmental	Ward B	Staff WC	HWB water	24/04/17
318	Environmental	Ward E	B23	Shower water	10/07/17
18V094129	Patient	Ward B	B41	Blood	18/05/18
18V247661	Patient	Ward X	N/A	Blood	29/10/18

Same VNTR profiles were observed from:

1. Blood isolate of a patient who was staying at Ward G and shower head water of B4 from Ward G.
2. Shower head water of B15 from Ward E and B4 from Ward G.

Matches with the previous batches sent for VNTR typing:

1. Hand wash basin water of Ward B N and shower head water of B23 from Ward E.
2. Blood isolate of a patient who was staying at B41 from Ward B.

Sixth cluster (G):

Table 5.6. Sixth set of isolates having same/similar VNTR profile of 12-3-3-6-6-3-8-2-8

Ref number	Source	Ward	Bed number	Site	Collection date
19P27	Patient	Ward B	N/A	Blood	19/11/18
19E17	Environmental	Ward B	B34	Shower water	21/11/18
19E18	Environmental	Ward B	B41	Shower water	21/11/18
19E23	Environmental	Ward B	B34	Shower water	11/12/18
19E30	Environmental	Ward B	B34	Shower water	27/02/19
309	Environmental	Ward D	B1	Shower water	09/05/17
310	Environmental	Ward H	Staff WC	HWB Water	18/05/17
316	Environmental	Ward E	B15	Shower water	10/07/17
394	Environmental	Ward L	B23-26	Shower water	04/07/18

Same VNTR profiles were observed from:

1. Shower head water of B34&B41 from Ward B.
2. Shower head water of B34 from Ward B before and after the shower change.
3. Blood isolate of a patient who was staying at WardBS.

Matches with the previous batches sent for VNTR typing:

1. Shower head water of B1 from Ward D, B15 from Ward E and B23-26 from T09.
2. Hand wash basin water of staff WC from Ward H.

Seventh cluster (Ga):

Table 5.7. Seventh set of isolates having same/similar VNTR profile of 12-3-5-5-2-3-7-2-14

Ref number	Source	Ward	Bed number	Site	Collection date
19P29	Patient	Ward H	N/A	Blood	10/12/18
19P41	Patient	Ward B	B35	Blood	14/12/18
19E14	Environmental	Ward B	B8	Shower water	21/11/18
19E24	Environmental	Ward B	B35	Shower water	11/12/18
19E27	Environmental	Ward B	B8	Shower water	27/02/19
19E31	Environmental	Ward B	B35	Shower water	27/02/19
19E37	Environmental	Ward I	B4	Shower water	17/04/19
19E42	Environmental	Ward B	B35	Shower water	11/12/18
19E43	Environmental	Ward B	B35	Shower water	19/12/18
18V024528	Patient	Ward A	B49	Blood	10/02/2018

Same VNTR profiles were observed from:

1. Shower head water of B8&B35 from Ward B and B4 from Ward I.
2. Blood isolate of a patient who was staying at Ward H.
3. Blood isolate of a patient who was staying at Ward B-B35. The shower head strains isolated from this room 3 days prior to sample collection from the patient shows the same VNTR profile. The same strain isolated from this shower for 3 months.

Eighth cluster (Fa):

Table 5.8. Eighth set of isolates sharing same/similar VNTR profile of 12-2-7-?-4-1-9-2-11

Ref number	Source	Ward	Bed number	Site	Collection date
19P30	Patient	Ward I	B15	Blood	10/12/18
19E45	Environmental	Ward I	B15	Shower water	13/12/18

Same VNTR profiles were observed from:

1. Blood isolate of a patient who was staying at Ward I-B15 and shower head water sample taken from this room 3 days after the sample collection from the patient.

UKHSA reports comment that this is a unique isolate from UCH.

Ninth cluster (B):

Table 5.9. Ninth set of isolates sharing same/similar VNTR profile of 9-2-5-2-4-3-?-1-9

Ref number	Source	Ward	Bed number	Site	Collection date
19E15	Environmental	Ward B	B27	Shower water	21/11/18
19E22	Environmental	Ward B	B27	Shower water	11/12/18
19E28	Environmental	Ward B	B27	Shower water	27/02/18
387	Environmental	Ward A	B13-17	HWB	08/05/18
390	Environmental	Ward A	B5-8	Shower water	27/06/18
18V24701	Patient	Ward B	N/A	Blood	N/A

Same VNTR profiles were observed from:

1. Shower head water of B27 from Ward B before and after the shower change & for 3 months.

Matches with the previous batches sent for VNTR typing:

1. Hand wash basin water of B13-17 from Ward A.
2. Shower head water of B5-8 from Ward A.
3. Blood isolate of a patient who was staying at Ward B.

Tenth cluster (C):

Table 5.10.Tenth set of isolates sharing same/similar VNTR profile of 10-3-5-5-4-1-3-7-8

Ref number	Source	Ward	Bed number	Site	Collection date
19P23*	Patient	Ward I	N/A	Blood	31/10/18
19P26*	Patient	Ward Y	-	Blood	19/11/18
19P37	Patient	Ward E	N/A	Blood	20/10/18
18V056688	Patient	Ward E	N/A	Blood	28/1/2018
18v024345	Patient	Ward A	N/A	Blood	7/2/2018
317	Environment	Ward E	17	Shower water	10/7/2017

*community acquired bacteraemia

Same VNTR profiles were observed from:

1. Blood isolate of patients from Ward I and Ward Y, both were community acquired cases.
2. Blood isolate of a patients from Ward E.

The most common VNTR profiles among patients in the clinical setting investigated UCH were as follows:

171 *P. aeruginosa* patient blood isolates are typed by VNTR covering 11.12.2016 – 09.01.2019. Number of cases typed per year was: 1from 2016; 29 from 2017; 140 from 2018 and 1 case from 2019. VNTR profiles among patients are found to be quite diverse. (12, 5, 3, 2, 6, 1, 6, 4, 13) was observed in nine cases from six different patients. (10, 3, 5, 5, 4, 1, 3, 7,

8) was observed in five cases from four different patients. (12, 3, 5, 5, 2, 3, 7, 2, 14) was observed in three cases, three patients.

A subset of this VNTR results were analysed for potential environmental – clinical links: Nineteen environmental (shower water from six ward: A, E, F, H, I, J) and nine clinical (hospital-acquired bacteraemia from five wards B, E, H, I, J) isolates collected between November 2018 and January 2019 were selected.

One shower water isolate from a single-isolation-room in augmented-care ward (B) was taken three days before the occupying patient (same room) developed bacteraemia. Isolates showed indistinguishable VNTR profiles (12,3,5,5,2,3,7,2,14). This profile was found in another shower water isolate (ward B). A similar VNTR profile (12,3,-,-,2,3,7,2,14) caused bacteraemia on ward H during the same week.

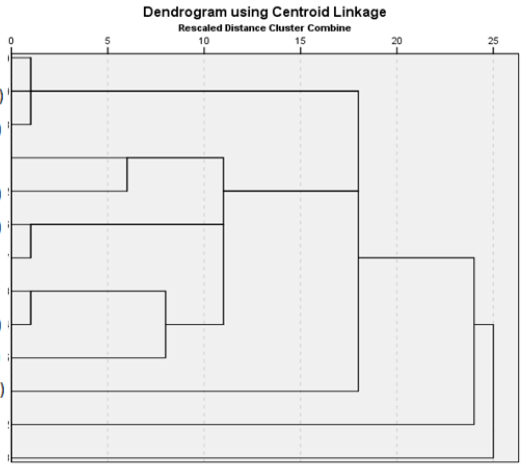
On ward I, the same VNTR profile (12,2,7,-,4,1,9,2,11) was detected in a shower three days after isolation from a patient who probably had used the shower. Two similar VNTR profiles (12,3,3,6,6,3,8,2,9 and 12,-,3,6,6,3,8,2,9) were isolated from two showers (ward B) on the same day with a third related profile (-,3,3,6,6,3,8,2,9) causing bacteraemia in a different room (same ward). Their single-locus-variant (12,3,3,6,6,3,8,2,8) was seen in two different shower water isolates (wards A&E). A further strain (VNTR:12,3,6,3,2,4,13,5,7) was isolated in two different showers (wards A,J). The most prevalent environmental strain (VNTR:12,5,3,2,6,1,6,4,13) was isolated on 3/19 occasions (16%). More than half of patient (5/9) and 21% of environmental (4/19) strains showed unique VNTR profiles within this cohort.

A targeted analysis of VNTR profiles was performed on thirteen isolates collected in December 2018. A dendrogram of these six clinical and seven environmental isolates is given in Figure 5.2. Out of ten different VNTR profiles isolated in the same month, two indistinguishable pairs were observed as deriving from patient and environmental strains. This highlights the importance of the shower environment as a source of transmission in this setting.

Figure 5.2. Dendrogram plot on VNTR results of thirteen *P. aeruginosa* isolates isolated on December 2018 showing relatedness patterns

Pseudomonas_VNTR

172	211	213	214	217	222	207	209	61	
12	3	?	?	2	3	7	2	14	Ward E Room 7 (Patient)
12	3	5	5	2	3	7	2	14	Ward A Room 6 (Shower)
12	3	5	5	2	3	7	2	14	Ward A Room 6 (Patient)
11	6	9	?	3	3	8	2	10	Ward E Room 1 (Patient)
12	5	5	3	2	2	7	4	10	Ward E Room 8 (Shower)
12	3	3	6	6	3	8	2	8	Ward B Room 5 (Shower)
?	3	3	6	6	3	8	2	9	Ward A Room 2 (Patient)
12	2	7	?	4	1	9	2	11	Ward C Room 3 (Patient)
12	2	7	?	4	1	9	2	11	Ward C Room 3 (Shower)
12	3	1	5	2	2	10	2	9	Ward B Room 4 (Patient)
11	8	3	3	2	3	10	4	13	Ward A Room 2 (Shower)
12	5	3	2	6	1	6	4	13	Ward D Bay 1 (Shower)
10	2	6	?	3	6	6	6	13	Ward E Room 9 (Patient)



Blue and yellow highlighted cells shows indistinguishable VNTR pairs

Table 5.11. Metadata (room locations and dates of sample collections) and AST profiles of 24 isolates for 12 antibiotics (AK, GM, TN, PRL, TZP, TCC, ATM, MEM, IMI, CAZ, CIP, CFE)

DNA extractions	Ward/Room Location	DOSC	AK	GM	TN	PRL	TZP	TCC	ATM	MEM	IMI	CAZ	CIP	CFE
DNA isolate 1	Ward B – Room 1	14/12/2018	S	S	S	S	S	S	I	S	S	S	S	R
DNA isolate 2	Ward B– Room 1	11/12/2018	S	S	S	S	S	S	I	S	S	S	S	R
DNA isolate 3	Ward I– Room 1	10/12/2018	S	S	S	S	S	S	I	S	S	S	S	S
DNA isolate 4	Ward I– Room 1	13/12/2018	S	S	S	S	S	S	I	S	S	S	S	R
DNA isolate 5	Ward B– Room 2	12/02/2018	S	S	S	S	S	S	I	S	S	S	S	S
DNA isolate 6	Ward B– Room 2	21/11/2018	S	S	S	S	S	S	I	S	S	S	S	S
DNA isolate 7	Ward E– Room 1	20/10/2018	I	S	S	S	S	S	I	S	S	S	S	R
DNA isolate 8	Ward E– Room 1	28/01/2018	I	S	S	S	S	S	I	S	S	S	S	R
DNA isolate 9	Ward E– Room 2	10/07/2017	S	S	S	S	S	S	S	S	S	S	S	R
DNA isolate 10	Ward A– Room 1	27/02/2019	S	S	S	S	S	S	I	S	S	S	S	S
DNA isolate 11	Ward B– Room 3	11/12/2018	S	S	S	S	S	S	I	S	S	S	S	S
DNA isolate 12	Ward E– Room 1	10/12/2018	S	S	S	S	S	S	I	S	S	S	S	R
DNA isolate 13	Ward B– Room 3	06/12/2018	S	S	S	S	S	S	I	S	S	S	S	R
DNA isolate 14	Ward B– Room 4	06/12/2018	S	S	S	S	S	S	I	S	S	S	S	R
DNA isolate 15	Ward E– Room 1	18/02/2020	S	S	S	S	S	S	I	S	S	S	S	R
DNA isolate 16	Ward C– Room 1	31/10/2019	S	S	S	R	R	R	R	R	S	R	R	R
DNA isolate 17	Ward C– Room 1	30/04/2019	S	S	S	S	S	S	I	S	S	S	S	R
DNA isolate 18	Ward E– Room 3	19/12/2018	S	S	S	S	S	S	I	S	S	S	S	S
DNA isolate 19	Ward E– Room 3	02/07/2018	S	S	S	S	S	S	I	S	S	S	S	R
DNA isolate 20	Ward E– Room 3	04/09/2017	S	S	S	S	S	S	I	S	S	S	S	S
DNA isolate 21	Ward D– Room N/A	24/05/2018	S	S	S	S	S	S	I	S	S	S	S	R
DNA isolate 22	Ward B– Room 3	11/12/2018	S	S	S	S	S	S	I	S	S	S	S	S
DNA isolate 23	Ward B– Room 4	03/12/2018	S	S	S	S	S	S	I	S	S	S	S	S
DNA isolate 24	Ward B– Room 4	18/05/2018	S	S	S	S	S	S	I	S	S	S	S	R

Phylogenetic trees created by VNTR results suggested potential links between environmental samples collected from similar locations (same rooms/wards) and between environmental and patient isolates. However given limitations of the VNTR method, typing 9 loci from the suggested 15 loci and missing repeats in some of the isolates' results as shown in Tables 5.2. – 5.10, decreased the discriminatory power of this method [90,94].

5.3.1.1. Overall conclusions: VNTR sequence typing of *P. aeruginosa*

Conclusions above were made according to matching VNTR profiles. Even though most had the same profiles; some results had some gaps where the VNTR test was not able to obtain amplicons. They were still considered to be in the same family in dendrogram since they did not have different VNTR values. It was observed that shower isolates from the same ward tended to have the same VNTR profiles. Comparison of the same showers through months showed that the same *P. aeruginosa* strain may persist for at least 3 months. But there were examples of different profiles from the same shower through time: Ward E-B15 shower head water had isolates having different VNTR profiles after 17 months. When shower water and patient bacteremia isolates are compared, it was found that in one case, a strain isolated from the shower head water of Ward B-B35 matched with the blood isolate of a patient staying in this room.

5.3.2. WGS analysis of environmental and clinical isolates of *P. aeruginosa*

192 isolates were typed by WGS, details are given below:

• 64 hospital rooms

• Samples from patient bacteraemia (n=87), shower water (n=70), shower drain (n=13), HWB (n=12), toilet sink drain (n=4), biofilm from shower hose (n=3), room sink drain (n=1), bay sink tap water (n=1), bay sink drain (n=1)

• Samples were collected between 9/4/2014 until 18/9/2020.

5.3.2.1. Molecular (WGS) analysis of environmental and clinical isolates of *P. aeruginosa*

5.3.2.1.1. Pilot WGS Study on 24 isolates

In the light of the results obtained by VNTR sequencing, initially 24 *P. aeruginosa* isolates were selected according to the time and location of sample collection and were sequenced by WGS (10 patient bacteraemia and 14 environmental shower isolates). Metadata and AST results against 12 antibiotics can be seen in Table 5.11.

In this thesis, disk diffusion antimicrobial sensitivity is measured by a phenotypic test. Possibly the effect of some resistance genes might not have yet been expressed in phenotype due to phenotypic lag, e.g. if the mutations occurred late in culture growing time [187]. Phenotypic lag or phenotypic delay is the time difference between when the genetic mutation happens and when it is expressed phenotypically [188]. This can be observed for expression

of AMR genes in different culture media and is taken into account when comparing genotypic results with phenotypic tests.

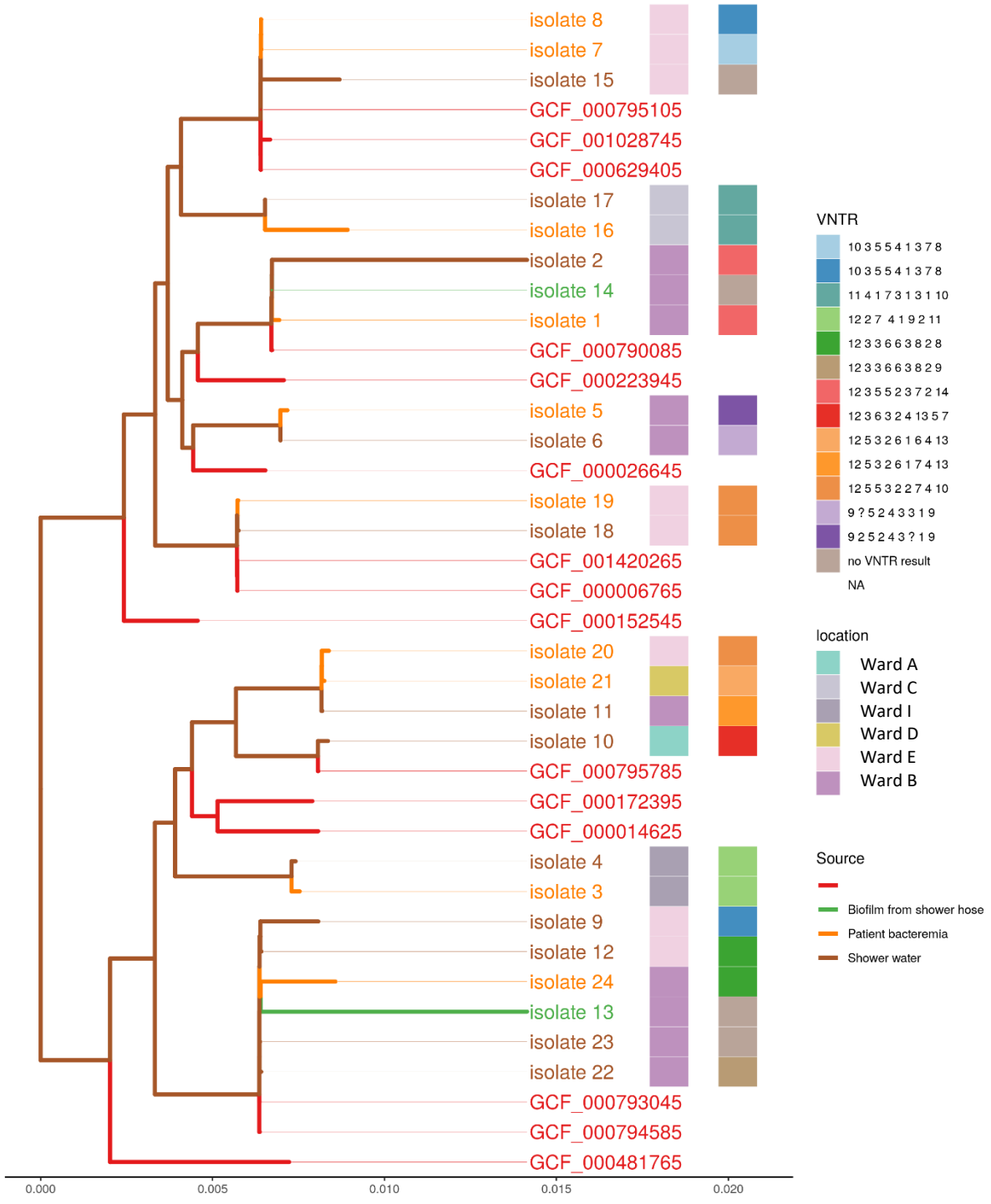


Figure 5.3. Phylogenetic tree of 24 isolates in question as well as publicly available isolates with metadata. Corresponding VNTR profiles, locations and source of isolates are included in the legend.

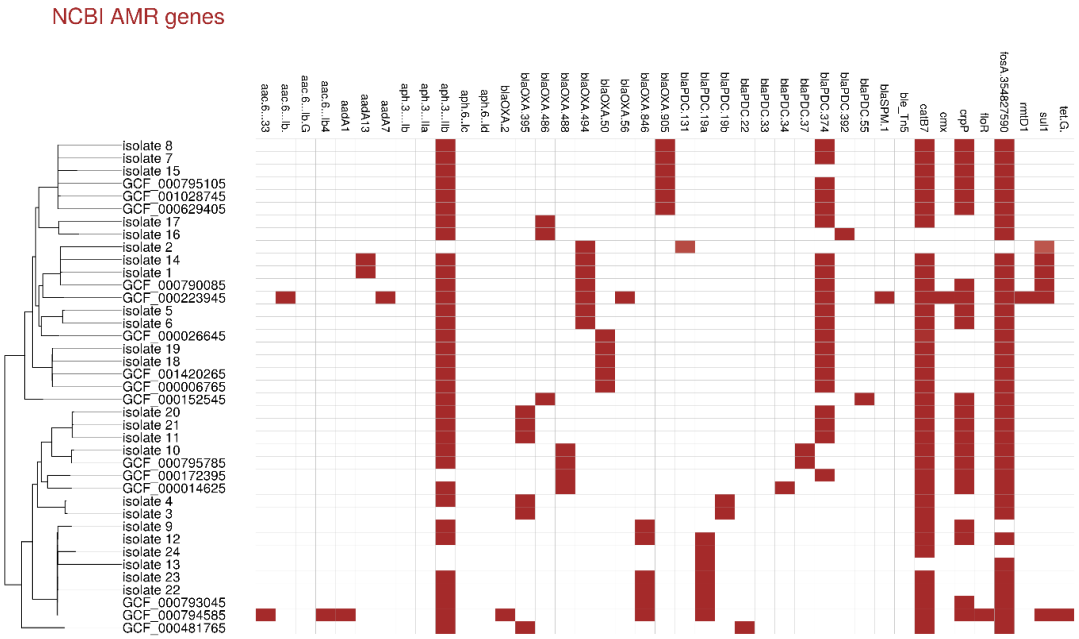


Figure 5.4. NCBI AMR genes of 24 clinical and environmental isolates (from NCBI AMR database, including the most common AMR genes (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA313047) , screened with abricate (https://github.com/tseemann/abricate), and the colour intensity represents the gene coverage (proportion of the gene found in the sample, with the darkest colour representing 100%)

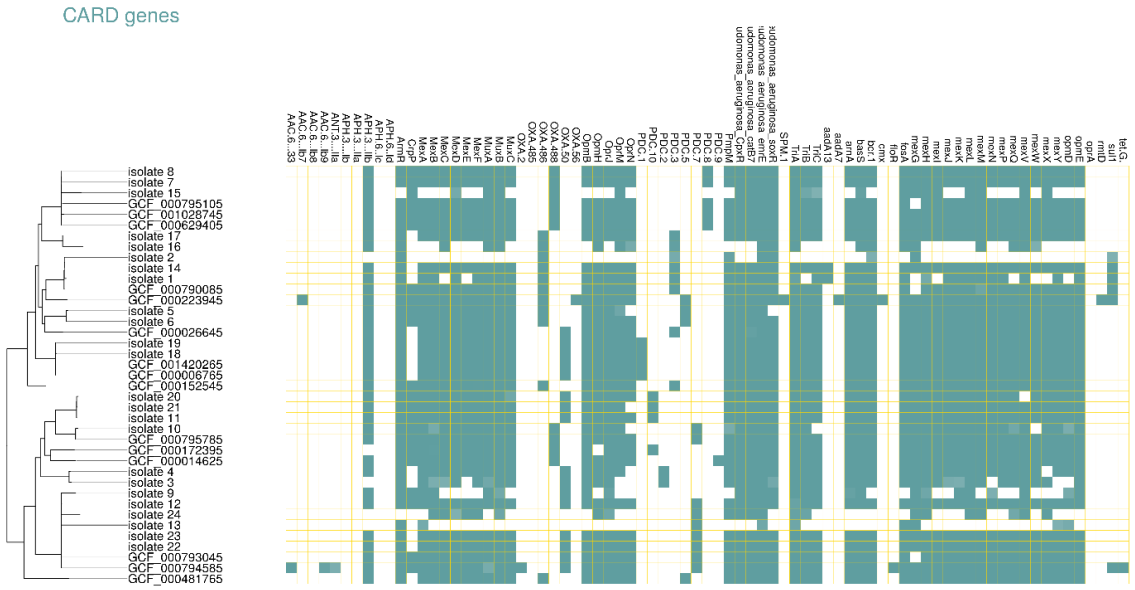


Figure 5.5. CARD AMR genes of 24 clinical and environmental isolates (from CARD database (https://card.mcmaster.ca/)), screened with abricate (https://github.com/tseemann/abricate), and the colour intensity represents the gene coverage (proportion of the gene found in the sample, with the darkest colour representing 100%)

WGS sequencing of 24 isolates were analysed in comparison with the VNTR results. Based on the phylogenetic tree, set of related groups were arbitrarily defined a and evaluated for epidemiological significance and in order to select further relevant samples for later analysis. These are described as below, data referring to Tables 5.1 – 5.10.

First group:

Isolate 7: Patient bacteraemia, Ward E– Room 1, 20/10/2018

Isolate 8: Patient bacteraemia, Ward E– Room 1, 28/01/2018

Isolate 15: Shower water, Ward E– Room 1, 18/02/2020

Two different patients staying in the same room (Ward E– Room 1) nine months apart were found to have the same (indistinguishable) VNTR and WGS results. A shower water isolate obtained two years later fell into the same clade (VNTR profile not known) with more substitutions per site (according to branch lengths but more detailed study was suggested on raw SNP differences).

The close match detected between these patient and shower water isolates suggests plausible transmission. To better understand the direction of transmission, one historical shower water isolate from laboratory database was planned to send for WGS (Shower water isolate from 17/05/2015, Ward E– Room 1 was found). In this clade, there are some strains shown to be closely related from publicly available database, suggesting other sources of transmission from nosocomial may also be important, further analysis for this including sampling sites and times of publicly available isolates are suggested.

Second group:

Isolate 16: Patient bacteraemia, Ward C– Room 1, 31/10/2019

Isolate 17: Shower water, Ward C– Room 1, 30/04/2019

Shower water isolate preceding patient isolate by six months, indistinguishable VNTR and WGS results. Patient bacteraemia isolate has more substitutions per site (according to branch lengths but more detailed study was suggested on raw SNP differences)).

Third group:

Isolate 1: Patient bacteraemia, Ward B – Room 1, 14/12/2018

Isolate 2: Shower water, Ward B – Room 1, 11/12/2018

Isolate 14: Biofilm from shower hose, Ward B– Room 4, 06/12/2018

Shower water isolate preceding patient isolate by three days, indistinguishable VNTR and WGS results. Shower water isolate has more substitutions per site (according to branch lengths but more detailed study was suggested on raw SNP differences)). One biofilm isolate from the same ward but isolated in a different room but in the same clade suggests it can be a prevalent strain or suggesting a transmission on equipment or carriage.

Fourth group:

Isolate 5: Patient bacteraemia, Ward B– Room 2, 12/02/2018

Isolate 6: Shower water, Ward B– Room 2, 21/11/2018

Patient isolate preceding shower water isolate by nine months, indistinguishable VNTR and WGS results.

Historical shower water isolates from Ward F– Room 2 from laboratory library is planned to be sequenced in the next batch to better understand the direction of transmission and to understand how long the isolate persists in showers (two shower water isolates from 10/04/2015 and 09/07/2019 were found).

Fifth group:

Isolate 18: Shower water, Ward E– Room 3, 19/12/2018

Isolate 19: Patient bacteraemia, Ward E– Room 3, 02/07/2018

Patient isolate preceding shower water isolate by five months, indistinguishable VNTR and WGS results.

Historical shower water isolates from Ward E– Room 3 from laboratory library can be sequenced to better understand the direction of transmission (a shower water isolate from 17/04/2015 was found).

Sixth group:

Isolate 11: Shower water, Ward B– Room 3, 11/12/2018

Isolate 20: Patient bacteraemia, Ward E– Room 3, 04/09/2017

Isolate 21: Patient bacteraemia, Ward D– Room N/A, 24/05/2018

Patient bacteraemia and shower water isolates from different locations falling into the same clade after WGS. They have different VNTR results.

Seventh group:

Isolate 10: Shower water, Ward A– Room 1, 27/02/2019

No other isolate fell into this clade from this isolate set.

Eighth group:

Isolate 3: Patient bacteraemia, Ward I– Room 1, 10/12/2018

Isolate 4: Shower water, Ward I– Room 1, 13/12/2018

The patient isolate preceded the shower water isolate by three days. Results were indistinguishable by VNTR and WGS.

Historical shower water isolated from Ward C obtained from the laboratory library is planned (in light of these results) to be sequenced to better understand the direction of transmission (Historical isolate from 02/07/2014 was found).

Ninth group:

Isolate 9: Shower water, Ward E– Room 2, 10/07/2017

Isolate 12: Shower water, Ward E– Room 1, 10/12/2018

Isolate 13: Biofilm from shower hose, Ward B– Room 3, 06/12/2018

Isolate 22: Shower water, Ward B– Room 3, 11/12/2018

Isolate 23: Shower water, Ward B– Room 4, 03/12/2018

Isolate 24: Patient bacteraemia, Ward B– Room 4, 18/05/2018

The patient isolate we identified preceded the shower water isolate by seven months, with indistinguishable WGS results (Ward B– Room 4). The patient isolate had more substitutions per site (according to branch length).

Shower water and hose biofilm isolates listed above were in the same group, indicating this could be a prevalent strain in this hospital.

Shower biofilm isolate of Ward B– Room 4 (isolate 14) showed that it is in a different group (third group). This suggested that shower hose biofilm isolates and shower water isolates may be different.

Similar to the first group, there are some strains shown to be closely related from publicly available database, suggesting other sources of transmission from nosocomial may also be important, further analysis for this including sampling sites and times of publicly available isolates are suggested.

5.3.2.1.2. WGS analysis of 190 environmental/clinical *P. aeruginosa* isolates

Following pilot WGS study on 24 isolates, a wider study on 190 isolates were carried out by including further environmental and clinical isolates isolated from similar locations and close in time.

5.3.2.1.2.1. AMR Gene Carriage

Across the 190 isolates a total of 100 unique AMR genes (mean of 55 per isolate; 48-84 95% CI assuming a gene presence threshold of 98%) were identified spanning multiple drug classes. We detect no significant difference in the total AMR gene carriage in isolates obtained from different sources (Figure 5.6). However, we do observe large outliers e.g. DNA114 (109 AMR genes), DNA183 (176 AMR genes), DNA192 (184 AMR genes), DNA195 (187 AMR genes). DNA163, DNA183 and DNA192 were all sampled on the same

location (Ward D-36 in Summer 2019) and include two shower and one patient bacteraemia isolate.

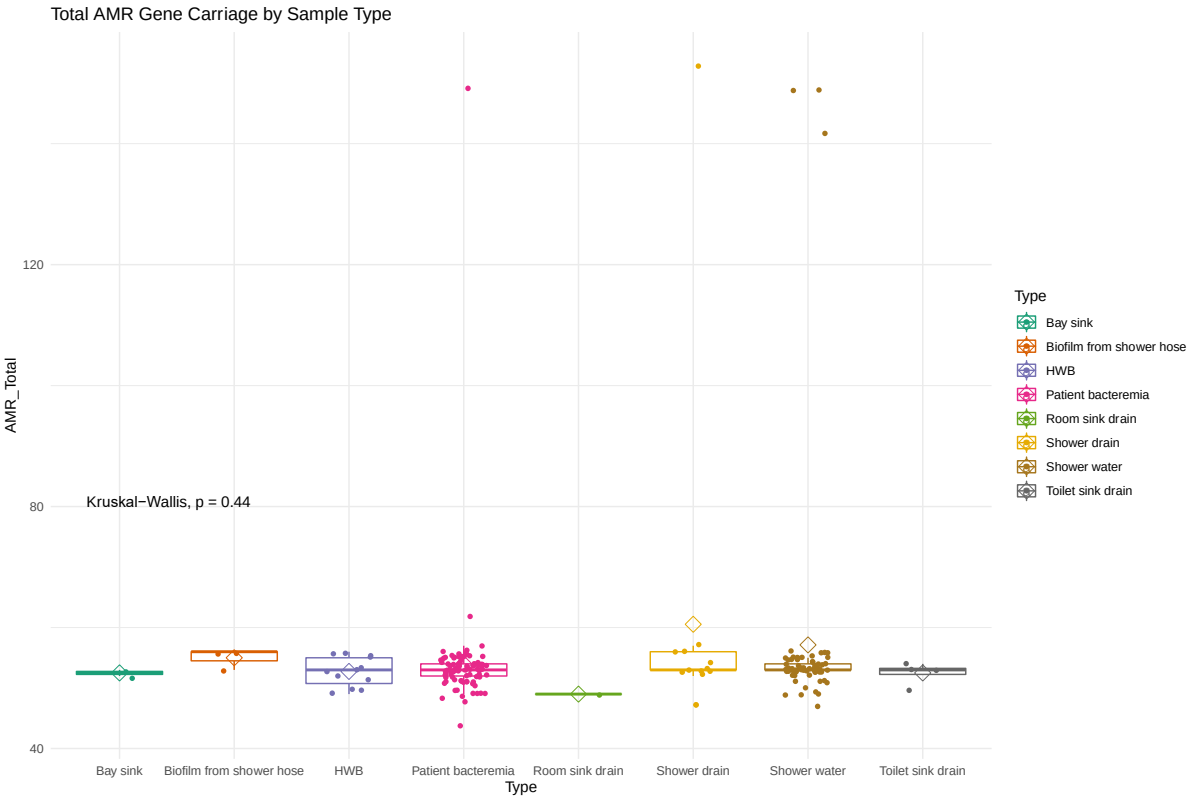


Figure 5.6: *Total AMR gene carriage (y-axis) by isolate sampling type (x-axis). No pairwise comparison was assessed as significantly different based on Wilcoxin rank test. Note here resistance genes were considered present if exhibiting >99% nucleotide identity to the resistance gene in question. Hits with nucleotide identity <99% were not considered in the total count.*

5.3.2.1.2.2. Biofilm Gene Characterisation

A suite of genes putatively implicated in biofilm formation in *P. aeruginosa* were screened (**Table 5.12**) using a *blastn* identity threshold of 98% identity and 98% gene coverage. Isolates on average harboured 18.8 of these genes (15.7-22). All carried algD, pelA-pelD, pelF-pelG and at least some of the *cup* gene cluster (98% carrying cupA, with less carrying other *cup* genes for instance cupC1 and cupC2 were carried by only 6% of isolates) (**Figure 5.7**). Again, as with AMR gene carriage, no combinations of categories were significantly different between isolates obtained from different sources (clincal, environmental etc).

Table 5.12: Gene sequences queried, with NCBI accession used, as obtained from the *Pseudomonas* Genome DB (https://www.pseudomonas.com).

Gene	Name	Accession Used
algD	alginate synthesis	NC_002516.2:3962825-3964135
pelA	Pellicle operon	NC_002516.2:c3433891-3431045
pelB	Pellicle operon	NC_002516.2:c3431067-3427486
pelC	Pellicle operon	NC_002516.2:c3427446-3426928
pelD	Pellicle operon	NC_002516.2:c3426922-3425555
pelE	Pellicle operon	NC_002516.2:c3425577-3424588
pelF	Pellicle operon	NC_002516.2:c3424591-3423068
pelG	Pellicle operon	NC_002516.2:c3423066-3421696
psLD	polysaccharide synthesis locus	NC_002516.2:2457510-2458280
cupA1	Chaperone-usher pathway	NC_002516.2:2342493-2343044
cupA2	Chaperone-usher pathway	NC_002516.2:2343132-2343878
cupA3	Chaperone-usher pathway	NC_002516.2:2343862-2346480
cupA4	Chaperone-usher pathway	NC_002516.2:2346477-2347838
cupA5	Chaperone-usher pathway	NC_002516.2:2347828-2348541
cupB1	Chaperone-usher pathway	NC_002516.2:c4569623-4569054
cupB2	Chaperone-usher pathway	NC_002516.2:c4569023-4568277
cupB3	Chaperone-usher pathway	NC_002516.2:c4567955-4565421
cupB4	Chaperone-usher pathway	NC_002516.2:c4565424-4564684
cupB5	Chaperone-usher pathway	NC_002516.2:c4564596-4561540
cupC1	Chaperone-usher pathway	NC_002516.2:1073285-1073902
cupC2	Chaperone-usher pathway	NC_002516.2:1073960-1074673
cupC3	Chaperone-usher pathway	NC_002516.2:1074784-1077303

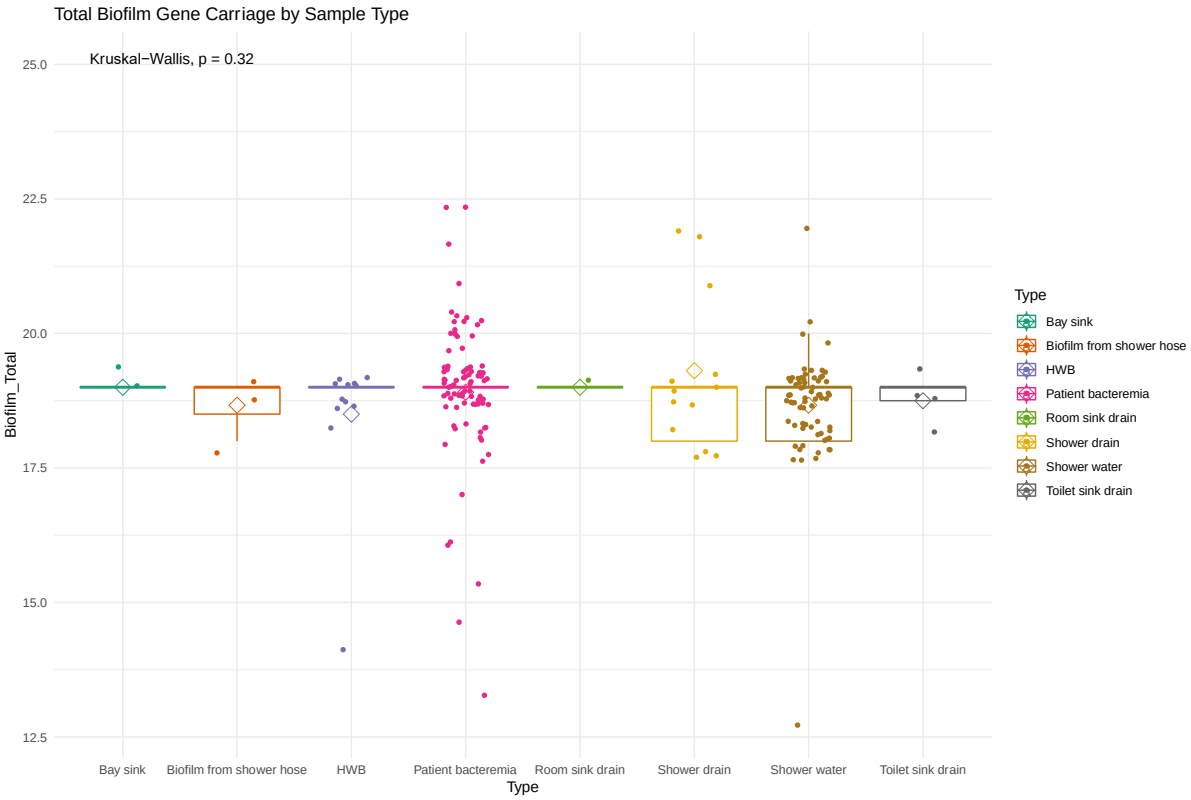


Figure 5.7: *Total biofilm gene carriage (y-axis) by isolate sampling type (x-axis). No pairwise comparison was assessed as significantly different based on Wilcoxon rank test. Note here biofilm formation genes were considered present if exhibiting >99% nucleotide identity to the gene in question, obtained from a curated list. Hits with nucleotide identity <99% were not considered in the total count.*

5.3.2.1.2.3. Pan-genome analysis

Genome assemblies were annotated for gene presence using Prokka v1.14.6 [189]. Included in the analysis was a *P. composti* genome (GCF_900115475.1) to be used as a phylogenetic out-group to orientate the phylogeny. *P. composti* was selected as a suitable out-group species based on Gomila *et al* 2015 [190]. Core, accessory and pan-genomes were obtained across the 190 annotated isolates (with and without outgroup) using Roary v3.13.0[191] (**Table 5.13, Figure 5.8**).

Table 5.13: *Roary identified pan-genome statistics for two core genome alignments of P. aeruginosa.*

Isolates considered	Core genes (99% <= strains <= 100%)	Soft core genes (95% <= strains < 99%	Shell genes (15% <= strains < 95%)	Cloud genes (0% <= strains < 15%)	Total Genes (99% <= strains <= 100%)
190 *P. aeruginosa* (	3172 (16%)	1124	2864	12680	19840
190 *P. aeruginosa* + 1 *P. composti*	2204 (11%)	2053	2900	12699	19856

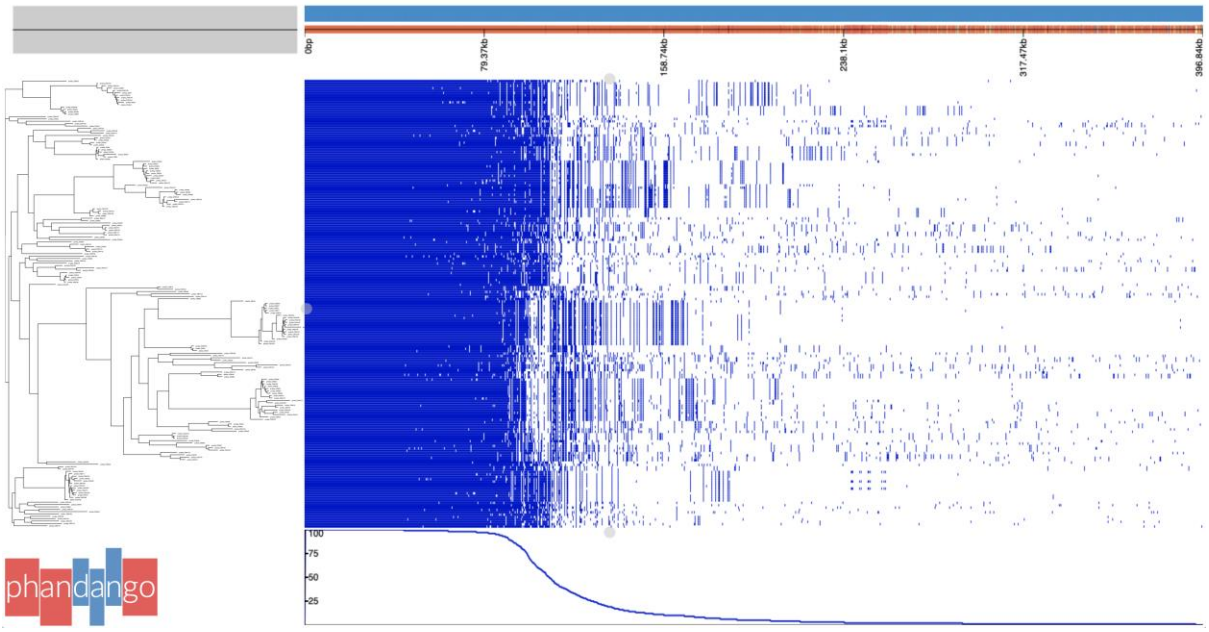


Figure 5.8: *Visualisation of pan-genome diversity amongst 190 P. aeruginosa. Tree at left provides a neighbour joining tree based on accessory genome genetic distances. Blue heatmap provides the presence and absence of genes in the pan-genome.*

SNP distances were extracted over the core genome alignment (3172 genes) – available as external file *core_gene_alignment.SNPdist.xls*. A heatmap of the pairwise SNP differences is provided in **Figure 5.9**.

Figure 5.9: *Pairwise SNP differences between 190 included P. aeruginosa strains. Strains are ordered according to simple hierarchical clustering as given by the dendrograms. The top colour bar provides the sampling source and the colour bar at left provides the room location (see legend at bottom right). Colour scale provides a visual representation of the degree of SNP differences between isolates.*

5.3.2.1.2.4. Phylogenetic Analysis

Core genome maximum likelihood phylogenetic trees were reconstructed from the concatenated core gene alignments using IQTree v1.6.12 [192] using GTR specification and otherwise default parameters. The topology of the tree including *P. composti* was used to root the tree of 190 isolates. The phylogeny was annotated by room/ward of the hospital (**Figure 5.10**), AMR phenotype measured by disc-diffusion assay (**Figure 5.11**) and by AMR gene presence and absence (using a 98% threshold to call gene presence) (**Figure 5.12**).

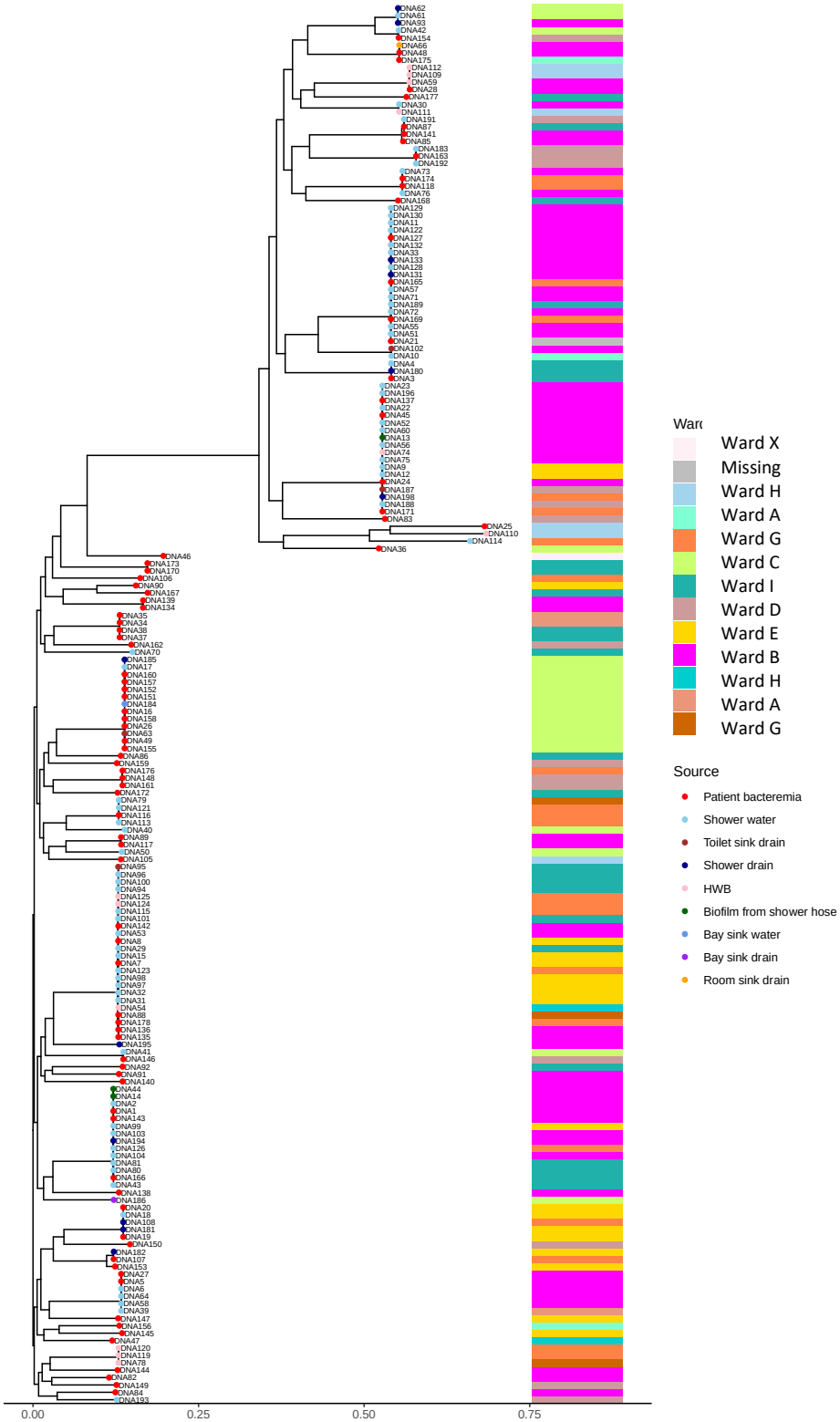


Figure 5.10: *Core genome (3172 genes, 132,140 SNPs) phylogenetic tree over 190 P. aeruginosa isolates. (Colour of tip points provides the sampling scheme for the isolate in question with the hospital room/ward provided by the coloured bar at side, as given in the legend.)*

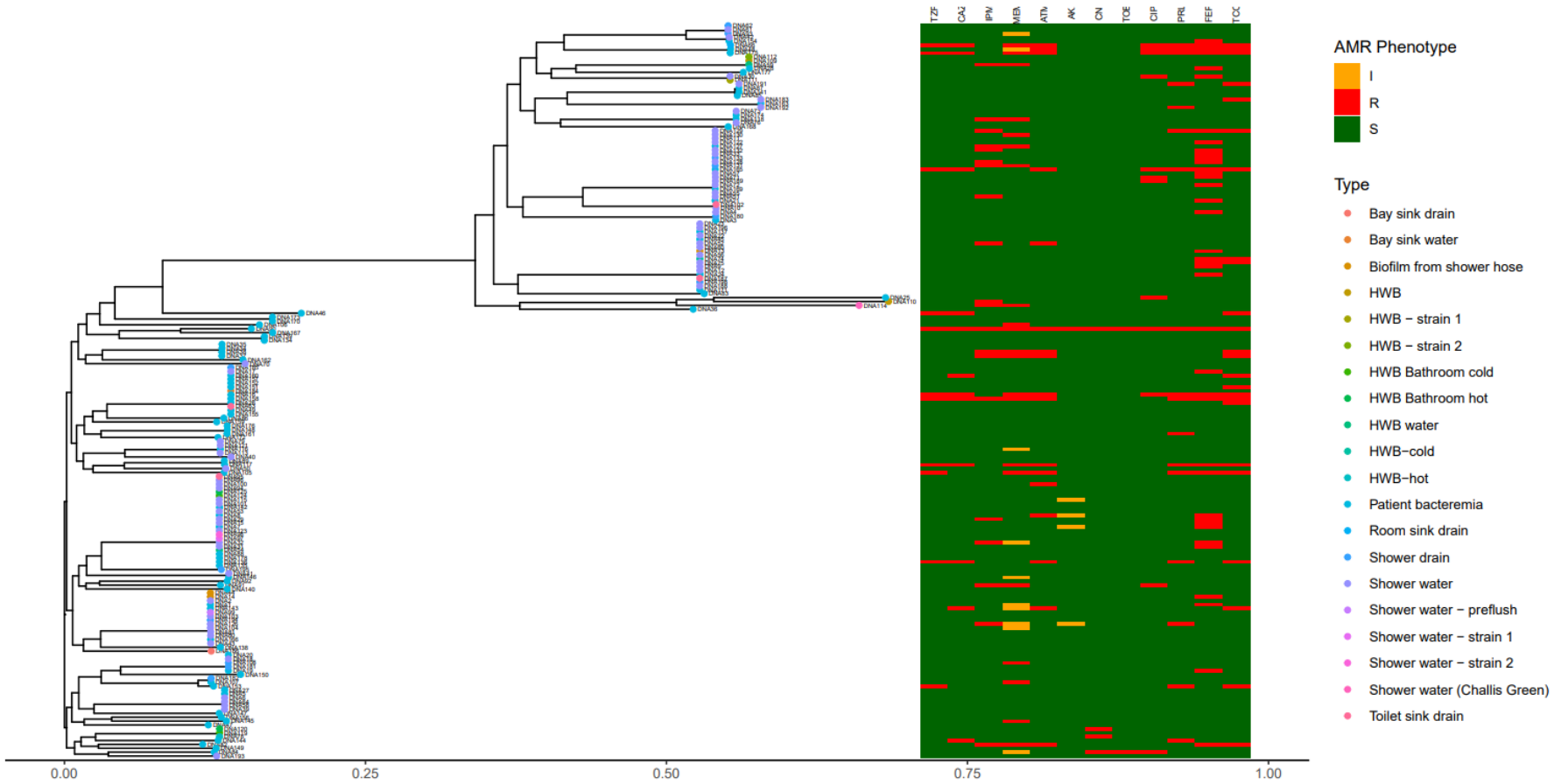


Figure 5.11: *Core genome) phylogenetic tree over 190 P. aeruginosa isolates. (NB: this is a high diversity alignment and not conducive to transmission chain reconstruction). Colour of tip points provides the sampling scheme for the isolate in question. Heatmap at right provides, for each tested antibiotic (column names at top), the phenotypic status (R=resistant, S=susceptible, I=intermediate) as per disc-diffusion assay values provided by Ozge Yetis.*

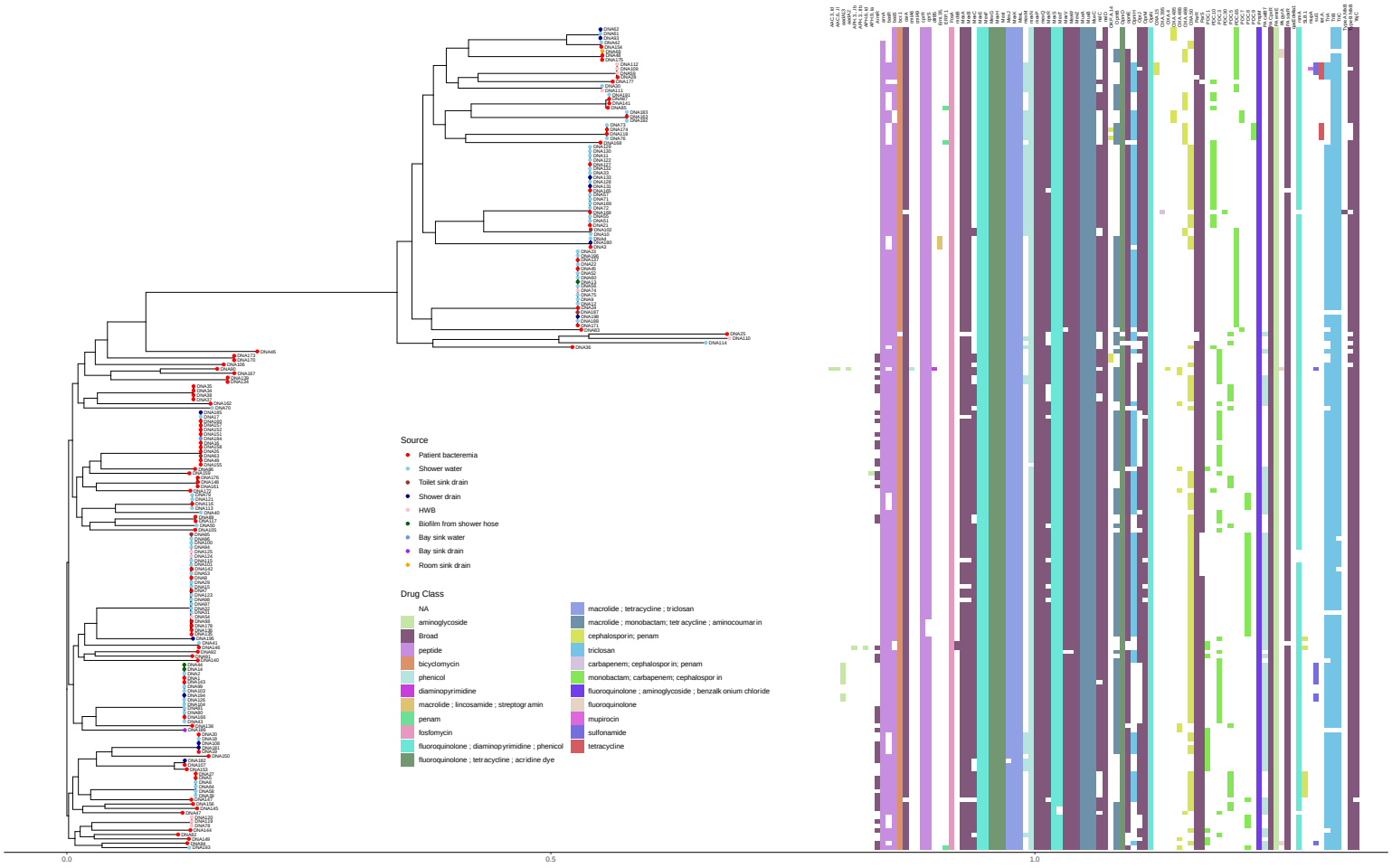


Figure 5.12: *Core genome (3172 genes, 132,140 SNPs) phylogenetic tree over 190 P. aeruginosa isolates. Colour of tip points provides the sampling scheme for the isolate in question. Heatmap at right provides the presence (colour) and absence of AMR genes annotated by CARD Resistance Gene Identifier with 98% identity to the reference gene database. Genes are loosely grouped (and coloured) by the class of drugs they confer resistance to. Those genes which confer resistance to >four classes as annotated by CARD ontology are classified as 'Broad' in their resistance profile*

Phylogenetic trees given above demonstrated that these are really highly diverse strains suggesting multiple co-circulating but otherwise unrelated isolates in nosocomial settings. There are two very divergent groupings.

5.3.2.1.2.5. Cup genes

In addition to the well studied *pel*, *psl* and *alg* genes, a special focus was given to *cup* genes (especially cupB and cupC) for biofilm formation given their well documented role in literature [193,194].

Out of 190 isolates sequenced, only six isolates had all *cupB* and *cupC* genes, as given in the below Table 5.14:

Table 5.14. Isolates having *cupB* and *cupC* genes

Name	DNA extractions	Room Location	Type	DOSC	Category	Room Location	cupB1	cupC1
DNA18	DNA isolate 18	Ward E-30	Shower water	09/12/2018	Shower water	Ward E	cupB1	cupC1
DNA19	DNA isolate 19	Ward E-30	Patient bacteraemia	02/07/2018	Patient bacteraemia	Ward E	cupB1	cupC1
DNA20	DNA isolate 20	Ward E-30	Patient bacteraemia	04/09/2017	Patient bacteraemia	Ward E	cupB1	cupC1
DNA108	DNA isolate 108	Ward G-38	Shower drain	16/07/2019	Shower drain	Ward G	cupB1	cupC1
DNA150	DNA isolate 150	Ward D	Patient bacteraemia	09/10/2018	Patient bacteraemia	Ward D	cupB1	cupC1
DNA181	DNA isolate 181	Ward E-30	Shower drain	29/01/2019	Shower drain	Ward E	cupB1	cupC1

*DOSC: Date of Sample Collection

5.3.2.1.2.6. Isolates having some *cupB* genes but not *cupC* or vice versa:

Table 5.15. Isolates having cupC but not cupB1 (has other cupB genes)

Name	DNA extractions	Room Location	Type	DOSC	Category	Room Location	cupB1	cupC1
DNA92	DNA isolate 92	Ward I-11	Patient bacteraemia	19/08/2020	Patient bacteraemia	Ward I	No	cupC1
DNA195	DNA isolate 195	WARD B-5	Shower drain	11/12/2018	Shower drain	WARD B	No	cupC1

*DOSC: Date of Sample Collection

Table 5.16. Isolates having cupC but not cupB1, cupB2 and cupB5(has other cupB genes):

Name	DNA extractions	Room Location	Type	DOSC	Category	Room Location	cupB1 cupB2 and cupB5	cupC1
DNA25	DNA isolate 25	Ward H-3	Patient bacteraemia	09/10/2019	Patient bacteraemia	Ward H	No	cupC1
DNA110	DNA isolate 110	Ward H-02	HWB	23/09/2015	HWB	Ward H	No	cupC1
DNA114	DNA isolate 114	Ward G - Bay 14-17	Shower water (Anonymised 1 Green)	09/09/2020	Shower water	Ward G	No	cupC1

*DOSC: Date of Sample Collection

17 isolates are listed having all *cupB* genes and *cupC3* but not *cupC1* and *cupC2*. Through these combinations, 6 isolates having all *cupB* and *cupC* genes were selected for Oxford Nanopore Technology sequencing in future studies. Cup genes and their involvement in biofilm formation and urinary tract infections are well studied in *E. coli*. These genes are included in WGS studies as case control study as detailed in Chapter 6 showed its importance in *P. aeruginosa* bacteraemia.

5.3.3. Focus studies comparing VNTR and WGS

13 environmental isolates from Chapter 3 study period 21.11.2018 - 06.06.2019 from Ward A and Ward B were typed by VNTR as shown in Table 5.17.1. One patient isolate from Ward B during this period was also typed by VNTR, results as shown in below Table 5.17.2

Table 5.17.1. Comparison of VNTR and WGS results of environmental and patient strains. Y: Yes. N: No

WGS	Sender ref no	Name/environmental site	Date of collection	t1	t2	t3	t4	t5	t6	t7	t8	t9	Cluster	Ward	Bed
Y-DNA6	19E15	BED 27 SHOWER HEAD WATER	21/11/2018	9	-	5	2	4	3	3	1	9	B	Ward B	27
N	19E22	BED 27 SHOWER HEAD WATER	11/12/2018	9	2	5	2	4	3	3	1	9	B	Ward B	27
N	19E12	BED 3 SHOWER HEAD WATER	21/11/2018	11	-	3	3	2	3	10	4	13	E	Ward B	3
N	19E20	BED 3 SHOWER HEAD WATER	11/12/2018	11	8	3	3	2	3	10	4	13	E	Ward B	3
N	19E35	Bed 3 SHOWER DRAIN	11/12/2018	11	8	3	3	2	3	10	4	13	E	Ward B	3
N	19E18	BED 41 SHOWER HEAD WATER	21/11/2018	12	3	3	6	6	3	8	2	9	G	Ward B	41
Y-DNA60	19E17	BED 34 SHOWER HEAD WATER	21/11/2018	12	-	3	6	6	3	8	2	9	G	Ward B	34
Y-DNA22	19E23	BED 34 SHOWER HEAD WATER	11/12/2018	12	3	3	6	6	3	8	2	9	G	Ward B	34
N	19E43	Bed 35 SHOWER HEAD WATER	19/12/2018	12	3	5	5	2	3	7	2	14	Ga	Ward B	35
Y-DNA2	19E42	Bed 35 SHOWER HEAD WATER	11/12/2018	12	3	5	5	2	3	7	2	14	Ga	Ward B	35
N	19E5	BED BAY 1-4 SHOWER HEAD WATER	11/12/2018	12	3	6	3	2	4	13	5	7	H	Ward A	1 to 4
Y-DNA11	19E13	BED 5 SHOWER HEAD WATER	21/11/2018	12	5	3	2	6	1	7	4	13	J	Ward B	5
N	19E16	BED 32 SHOWER HEAD WATER	21/11/2018	12	-	-	2	6	1	7	4	13	J	Ward B	32

Table 5.17.2 Comparison of VNTR and WGS results of patient strains. Y: Yes

WGS	Sender ref no	Site	Date of collection	t1	t2	t3	t4	t5	t6	t7	t8	t9	Cluster	Ward	Bed
Y-DNA1	19P41	Bacteraemia	14/12/2018	12	3	5	5	2	3	7	2	14	Ga	Ward B	35

From these 14 isolates, 6 of them were also typed by WGS.

Two shower water samples isolated 20 days apart (19E17 and 19E23) showed highly similar VNTR profiles (same profile except one missing repeat of 19E17 at the second location). In WGS analysis, they showed 14 SNP differences in the core genome alignment. It is interpreted to be unlikely that one is a direct descendant of the other given the SNP differences since 14 SNPs in 20 days is too high a rate of evolution.

Shower water – patient isolate pair (shower water strain isolated 3 days before the sample collection from the patient staying in same room) showed the same VNTR profile. SNP difference analysis following WGS showed two isolates being highly similar (0 SNPs apart in the SNP distance matrix of all 190 isolates sequenced), suggesting direct transmission given the mutations and time period involved.

5.3.4. SNP Distance Matrix Analyses

From the 190 isolates sequenced, isolates from the same rooms were analysed for their SNP differences as an approximation to identifying possible transmission events. In total, 25 same room environmental - patient pairs were analysed. Overall, 15 patient bacteraemia - shower water matches were found to be less than 6 core SNPs apart and were found in the same rooms, close in time (time differences detailed as below - although this was not selected as a cut-off, it was found to be relevant when compared to nearest closest match is 37 SNPs). When in the case of more than one bacteraemia/shower water which are the same, only one of those pairs were taken into account as if the same strain was isolated multiple times over a period of few months, counting them as individual different strains could mean recounting/duplicating the potential transmission events. 15 patient bacteraemia - shower water pairs from same rooms, isolated in close dates showed no matches (>36 SNPs). When in the case of patient strains different from each other, but still not matching with the environmental strains, they were counted as different pairs.

In 5/25 locations, there were matching and non-matching environmental – clinical pairs.

SNP matches (<6 SNPs) on same shower locations:

A. Shower water preceding patient bacteraemia

3 occasions where shower water preceding patient bacteraemia 3 days – 3 years:

Ward B

Table 5.18. Ward B-35 isolate clusters

DNA isolate 1	Ward B-35	Patient bacteraemia	14/12/2018
DNA isolate 2	Ward B-35	Shower water	11/12/2018
DNA isolate 194	Ward B-35	Shower drain	03/12/2018

DNA1 and DNA2 has 0 SNPs difference (**shower water preceding patient bacteraemia by 3 days)**.

Table 5.19. Ward B-32 isolate clusters

DNA isolate 127	Ward B-32	Patient bacteraemia	07/03/2018
DNA isolate 128	Ward B-32	Shower water	10/04/2015
DNA isolate 129	Ward B-32	Shower water	09/07/2019
DNA isolate 130	Ward B-32	Shower water	13/02/2019
DNA isolate 131	Ward B-32	Shower drain	13/02/2019
DNA isolate 132	Ward B-32	Shower water	03/12/2018
DNA isolate 133	Ward B-32	Shower drain	03/12/2018

DNA127 and DNA128 has 1 SNPs difference (**shower water preceding patient bacteraemia by 3 years**).

Table 5.20. Ward B-34 isolate clusters

DNA isolate 13	Ward B-34	Biofilm from shower hose	06/12/2018
DNA isolate 22	Ward B-34	Shower water	11/12/2018
DNA isolate 45	Ward B-34	Patient bacteraemia	10/11/2019
DNA isolate 55	Ward B-34	Shower water	10/04/2015
DNA isolate 56	Ward B-34	Shower water	09/07/2019
DNA isolate 60	Ward B-34	Shower water	21/11/2018
DNA isolate 140	Ward B-34	Patient bacteraemia	14/05/2018
DNA isolate 196	Ward B-34	Shower water	19/12/2018
DNA isolate 197	Ward B-34	Shower drain	19/12/2018

DNA45 and DNA56&DNA60 have 2 SNPs difference (**shower water preceding patient bacteraemia by 1 year**). DNA45 and DNA196 has 0 SNPs difference (**same shower water strain isolated for 8 months).**

B. Patient bacteraemia preceding shower water

Patient bacteraemia preceding shower water on 9 occasions (3 days – 25 months). When two bacteraemia are the same, even they are different patients it is counted as one by taking the most historical patient into account.

Ward B

Table 5.21. Ward B-27 isolate clusters

DNA isolate 5	Ward B-27	Patient bacteraemia	12/02/2018
DNA isolate 6	Ward B-27	Shower water	21/11/2018

DNA5 and DNA6 have 3 SNPs difference (**patient bacteraemia preceding shower water by 9 months**).

Ward E

Table 5.22. Ward E-29 isolate clusters

DNA isolate 7	Ward E-29	Patient bacteraemia	20/10/2018
DNA isolate 8	Ward E-29	Patient bacteraemia	28/01/2018
DNA isolate 15	Ward E-29	Shower water	18/02/2020
DNA isolate 31	Ward E-29	Shower water	17/04/2015

DNA7-8 and DNA 15 have 0 SNPs difference (**patient bacteraemia preceding shower water by 25 months**) & (**patient bacteraemia preceding shower water by 16 months**).

Table 5.23. Ward E-30 isolate clusters

DNA isolate 18	Ward E-30	Shower water	09/12/2018
DNA isolate 19	Ward E-30	Patient bacteraemia	02/07/2018
DNA isolate 20	Ward E-30	Patient bacteraemia	04/09/2017
DNA isolate 32	Ward E-30	Shower water	17/04/2015
DNA isolate 147	Ward E-30	Patient bacteraemia	11/09/2018
DNA isolate 181	Ward E-30	Shower drain	29/01/2019

DNA18, DNA19 and DNA20 have 0 SNPs difference (**patient bacteraemia preceding shower water by 17 months**) & (**patient bacteraemia preceding shower water by 5 months**).

Ward D

Table 5.24. Ward D-36 isolate clusters

DNA isolate 163	Ward D-36	Patient bacteraemia	23/06/2019
DNA isolate 183	Ward D-36	Shower water	29/07/2019
DNA isolate 187	Ward D-36	Toilet sink drain	29/07/2019
DNA isolate 192	Ward D-36	Shower water	13/08/2019

DNA163 and DNA192 are 0 SNPs different (**patient bacteraemia preceding shower water by 2 months**),

Ward I

Table 5.25. Ward I-15 isolate clusters

DNA isolate 3	Ward I-15	Patient bacteraemia	10/12/2018
DNA isolate 4	Ward I-15	Shower water	13/12/2018
DNA isolate 29	Ward I-15	Shower water	02/07/2014
DNA isolate 180	Ward I-15	Shower drain	13/12/2018

DNA3 and DNA4 have 0 SNPs difference (**patient bacteraemia preceding shower water by 3 days**)

Table 5.26. Ward I-1 isolate clusters

DNA isolate 37	Ward I-1	Patient bacteraemia	23/08/2019
DNA isolate 38	Ward I-1	Patient bacteraemia	23/08/2019
DNA isolate 43	Ward I-1	Shower water	18/07/2019
DNA isolate 166	Ward I-1	Patient bacteraemia	01/10/2017

DNA43 and DNA166 have 2 SNPs difference (**patient bacteraemia preceding shower water by 21 months**).

Ward C

Table 5.27. Ward C-11 isolate clusters

DNA extractions	Room Location	Type	DOSC
DNA isolate 16	Ward C-11	Patient bacteraemia - NS	31/10/2019
DNA isolate 17	Ward C-11	Shower water	30/04/2019
DNA isolate 26	Ward C-20	Patient bacteraemia - CL	16/10/2019
DNA isolate 36	Ward C-9	Patient bacteraemia -BG	18/09/2019
DNA isolate 40	Ward C-(7-10)	Shower water	14/05/2015
DNA isolate 41	Ward C-(7-10)	Shower water	30/04/2019
DNA isolate 42	Ward C-(7-10)	Shower water	17/06/2019
DNA isolate 49	Ward C-20	Patient bacteraemia - PW	01/02/2020
DNA isolate 50	Ward C-(20-24)	Shower water	14/05/2015
DNA isolate 61	Ward C-20	Shower water	12/02/2020
DNA isolate 62	Ward C-20	Shower drain	12/02/2020
DNA isolate 63	Ward C-20	Toilet sink drain	12/02/2020
DNA isolate 151	Ward C-2	Patient bacteraemia -NS	28/09/2018
DNA isolate 152	Ward C-2	Patient bacteraemia -AS	18/10/2018
DNA isolate 155	Ward C -12	Patient bacteraemia -CR	16/06/2018
DNA isolate 157	Ward C -5	Patient bacteraemia -EB	06/07/2018
DNA isolate 158	Ward C -8	Patient bacteraemia -NS	12/02/2019
DNA isolate 160	Ward C - 4	Patient bacteraemia - CL	29/03/2019
DNA isolate 184	Ward C - (7-10)	Bay sink water	02/12/2019
DNA isolate 185	Ward C - (7-10)	Shower drain	19/08/2019
DNA isolate 186	Ward C - (7-10)	Bay sink drain	19/08/2019

Same room analysis:

Ward C - (7-10)
DNA158 and DNA184-185 have 2 SNPs difference **(patient bacteraemia preceding shower water by 6 months).**

Ward C-11

DNA16 and DNA17 have 5 SNPs difference (**shower water preceding patient bacteraemia by 6 months**

Same patient: DNA151 is the most historical strain typed from this patient, isolated at 28/09/2018. Strain isolated from the same patient 5 months after at 12/02/2019 (DNA158) has 2 SNPs difference; third time strain isolated from this patient is 8 further months after (DNA16) at 31/10/2019 is 5 SNPs different. On this three sampling points this patient was staying at three different places at Ward C (one bed bay and two single isolation rooms).

Same ward analysis

DNA17, DNA26, DNA63, DNA151**,** DNA152, DNA155, DNA157**,** DNA160, DNA184 and DNA185 have 0 SNP differences. (**Patient strain preceding shower water by 9 months**)

Five patient bacteraemia – shower water match (Ward C-2, Ward C-4, Ward C-5, Ward C-11, Ward C-20) over 15 months.

Ward G

Table 5.28. Ward G-34 isolate clusters

DNA isolate 116	Ward G-34	Patient bacteraemia	22/04/2018
DNA isolate 119	Ward G-34	HWB Bathroom cold	06/08/2019
DNA isolate 120	Ward G-34	HWB Bathroom hot	06/08/2019
DNA isolate 121	Ward G-34	Shower water	06/08/2019

DNA116 and DNA 121 have 0 SNPs difference (**patient bacteraemia preceding shower water by 4 months**)

SNP not matching (>36 SNPs) on same shower locations:

There were 17 occasions where shower water patient bacteraemia isolates did not show close relatedness in SNP matrix even though they were isolated from the same shower locations SNP>37 (collected between 3 days up to 4 years).**Ward B**

Table 5.29. Ward B-41 isolate clusters

DNA isolate 14	Ward B-41	Biofilm from shower hose	06/12/2018
DNA isolate 23	Ward B-41	Shower water	03/12/2018
DNA isolate 24	Ward B-41	Patient bacteraemia	18/05/2018
DNA isolate 44	Ward B-41	Biofilm from shower hose	06/12/2018

DNA23 and DNA 24 have 37 SNPs difference (**patient bacteraemia – shower water strains collected 7 months apart did not match**)

Table 5.30. Ward B-29 isolate clusters

DNA isolate 71	Ward B-29	Shower water	10/04/2015
DNA isolate 74	Ward B-29	HWB-cold	09/07/2019
DNA isolate 75	Ward B-29	Shower water	09/07/2019
DNA isolate 82	Ward B-29	Patient bacteraemia	01/12/2019
DNA isolate 89	Ward B-29	Patient bacteraemia	08/07/2020

DNA75 is 24477 and 24657 SNPs different than DNA82 and DNA89 **(patient bacteraemia – shower water strains collected 5 months and 1 year apart did not match)**

DNA75 when compared with DNA82&DNA89 ~24497 SNPs (**patient bacteraemia – shower water collected 4 months and 1 year apart did not match**)

Table 5.31. Ward B-36 isolate clusters

DNA isolate 27	Ward B-36	Patient bacteraemia	26/11/2019
DNA isolate 51	Ward B-36	Shower water	10/04/2015
DNA isolate 52	Ward B-36	Shower water	09/07/2019

DNA27 against DNA51 and DNA52 ~24k SNPs **(patient bacteraemia – shower water strains collected 4 months and 4 years apart did not match)**

Table 5.32. Ward B-17 isolate clusters

DNA isolate 91	Ward B-17	Patient bacteraemia	19/08/2020
DNA isolate 93	Ward B-17	Shower drain	17/04/2019
DNA isolate 102	Ward B-17	Toilet sink drain	17/09/2019
DNA isolate 103	Ward B-17	Shower water	27/06/2019
DNA isolate 104	Ward B-17	Shower water	27/06/2019
DNA isolate 135	Ward B-17	Patient bacteraemia	24/08/2018
DNA isolate 136	Ward B-17	Patient bacteraemia	15/09/2018

DNA91 against all >10k SNPs DNA135 – DNA103 >10k SNPs **(patient bacteraemia – shower water strains collected in 2 years did not match)**

Ward E

Table 5.33. Ward E-30 isolate clusters

DNA isolate 18	Ward E-30	Shower water	09/12/2018
DNA isolate 19	Ward E-30	Patient bacteraemia	02/07/2018
DNA isolate 20	Ward E-30	Patient bacteraemia	04/09/2017
DNA isolate 32	Ward E-30	Shower water	17/04/2015
DNA isolate 147	Ward E-30	Patient bacteraemia	11/09/2018
DNA isolate 181	Ward E-30	Shower drain	29/01/2019

DNA18 against DNA32 and DNA147 – ~12358 SNPs **(patient bacteraemia – shower water strains collected 3 months apart did not match)**

Table 5.34. Ward E-15 isolate clusters

DNA isolate 12	Ward E-15	Shower water	10/12/2018
DNA isolate 153	Ward E-15	Patient bacteraemia	07/12/2018
DNA isolate 182	Ward E-15	Shower drain	10/12/2018

DNA12 has more than 20k SNP differences than DNA153 and DNA182 **(patient bacteraemia – shower water strains collected 3 days apart did not match)**

Table 5.35. Ward E-18 isolate clusters

DNA isolate 90	Ward E-18	Patient bacteraemia	21/07/2020
DNA isolate 97	Ward E-18	Shower water - strain 1	11/05/2015
DNA isolate 98	Ward E-18	Shower water - strain 2	11/05/2015
DNA isolate 99	Ward E-18	Shower water - preflush	25/11/2019

DNA90 against DNA97-99 >10k SNPs different **(patient bacteraemia – shower water strains collected 4 months and 4 years apart did not match)**

Ward I

Table 5.36. Ward I-1 isolate clusters

DNA isolate 37	Ward I-1	Patient bacteraemia	23/08/2019
DNA isolate 38	Ward I-1	Patient bacteraemia	23/08/2019
DNA isolate 43	Ward I-1	Shower water	18/07/2019
DNA isolate 166	Ward I-1	Patient bacteraemia	01/10/2017

DNA37-DNA38 has 0 SNPs, they have 11993 SNPs with DNA43 and DNA166 (these two have 2 SNPs) **(patient bacteraemia – shower water strains collected 5 days apart did not match)**

Table 5.37. Ward I-11 isolate clusters

DNA isolate 92	Ward I-11	Patient bacteraemia	19/08/2020
DNA isolate 94	Ward I-11	Shower water	29/07/2019
DNA isolate 95	Ward I-11	Toilet sink drain	29/07/2019
DNA isolate 96	Ward I-11	Shower water	29/07/2019
DNA isolate 100	Ward I-11	Shower water	18/07/2019
DNA isolate 101	Ward I-11	Shower water	24/08/2020

DNA92 – DNA94-96 have 68 SNPs **(patient bacteraemia – shower water strains collected 11 months apart did not match)**

Table 5.38. Ward I-6 isolate clusters

DNA isolate 70	Ward I-6	Shower water	10/06/2014
DNA isolate 87	Ward I-6	Patient bacteraemia	26/02/2020

~25k SNPs different **(patient bacteraemia – shower water strains collected 6 years apart did not match)**

Ward C

Table 5.39. Ward C-11 isolate clusters

DNA extractions	Room Location	Type	DOSC
DNA isolate 16	Ward C-11	Patient bacteraemia - NS	31/10/2019
DNA isolate 17	Ward C-11	Shower water	30/04/2019
DNA isolate 26	Ward C-20	Patient bacteraemia - CL	16/10/2019
DNA isolate 36	Ward C-9	Patient bacteraemia -BG	18/09/2019
DNA isolate 40	Ward C-(7-10)	Shower water	14/05/2015
DNA isolate 41	Ward C-(7-10)	Shower water	30/04/2019
DNA isolate 42	Ward C-(7-10)	Shower water	17/06/2019
DNA isolate 49	Ward C-20	Patient bacteraemia - PW	01/02/2020
DNA isolate 50	Ward C-(20-24)	Shower water	14/05/2015
DNA isolate 61	Ward C-20	Shower water	12/02/2020
DNA isolate 62	Ward C-20	Shower drain	12/02/2020
DNA isolate 63	Ward C-20	Toilet sink drain	12/02/2020
DNA isolate 151	Ward C-2	Patient bacteraemia -NS	28/09/2018
DNA isolate 152	Ward C-2	Patient bacteraemia -AS	18/10/2018
DNA isolate 155	Ward C -12	Patient bacteraemia -CR	16/06/2018
DNA isolate 157	Ward C -5	Patient bacteraemia -EB	06/07/2018
DNA isolate 158	Ward C -8	Patient bacteraemia -NS	12/02/2019
DNA isolate 160	Ward C - 4	Patient bacteraemia - CL	29/03/2019
DNA isolate 184	Ward C - (7-10)	Bay sink water	02/12/2019
DNA isolate 185	Ward C - (7-10)	Shower drain	19/08/2019
DNA isolate 186	Ward C - (7-10)	Bay sink drain	19/08/2019

Ward C-(20-24)

DNA49-DNA61 have 25308 SNPs **(patient bacteraemia – shower water strains collected 10 days apart did not match)**

DNA26-61 have 16541 SNPs **(patient bacteraemia – shower water strains collected 4 months apart did not match)**

Ward C-(7-10): DNA36 and DNA40-41-42 have 25388, 25673, 17079 SNPs **(patient bacteraemia – shower water strains collected 3 months, 5 months and 4 years apart did not match)**

Ward G

Table 5.40. Ward G-38 isolate clusters

DNA isolate 107	Ward G-38	Patient bacteraemia	09/10/2019
DNA isolate 108	Ward G-38	Shower drain	16/07/2019
DNA isolate 115	Ward G-38	Shower water	06/08/2019
DNA isolate 198	Ward G-38	Shower drain	30/07/2019

They all have > 10929 SNPs **(patient bacteraemia – shower water strains collected 2 months apart did not match)**.

Table 5.41. Ward G - Bay 14-17 isolate clusters

DNA isolate 106	Ward G- Bay 4 , Bed 15	Patient bacteraemia	18/09/2020
DNA isolate 113	Ward G - Bay 14-17	Shower water	29/07/2015
DNA isolate 114	Ward G - Bay 14-17	Shower water (Anonymised 1 Green)	09/09/2020

DNA106 against DNA113-114 have >10k SNPs **(patient bacteraemia – shower water strains collected 9 days and 5 years apart did not match)**

Table 5.42. Ward G-39 isolate clusters

DNA isolate 118	Ward G-39	Patient bacteraemia	20/07/2018
DNA isolate 123	Ward G-39	Shower water	22/07/2014
DNA isolate 124	Ward G-39	HWB Bathroom cold	06/08/2019
DNA isolate 125	Ward G-39	HWB Bathroom hot	06/08/2019
DNA isolate 126	Ward G-39	Shower water	06/08/2019

DNA118 against all >20k SNPs **(patient bacteraemia – shower water strains collected 11 months and 4 years apart did not match)**

5.3.5. Spatial/temporal distribution of *P. aeruginosa* from clinical and environmental (drain and shower water) sources – inferences through WGS analysis

Table 5.43. Ward B, C, E, G and I isolate clusters

Ward-Room	Isolates	SNP difference	Explanation
Ward B-35	DNA1 and DNA194	1	Shower drain isolate preceding patient bacteraemia by 11 days
Ward B-32	DNA127, DNA 131 and DNA 133	2	DNA127 has 2 SNPs between DNA131 (patient bacteraemia preceding shower drain by 11 months) and DNA133 (patient bacteraemia preceding shower drain by 9 months)
Ward B-34	DNA45 and DNA13	3	Biofilm from shower hose strain preceding patient bacteraemia by 9 months
Ward B-28	DNA48 and DNA66	1	Patient bacteraemia preceding room sink drain by 3 days)
Ward E-30	DNA18 and	0	Shower water and shower drain from different wards

& Ward G-38	DNA108		match
Ward I-15	DNA3 and DNA180	0	Patient bacteraemia preceding shower drain by 3 days
Ward I-11	DNA94 and DNA95-96	2	Shower water and– toilet sink drain matches
Ward C-(20-24)	DNA26 and DNA63	0	Patient bacteraemia preceding toilet sink drain isolate by 4 months

5.4. Discussion

5.4.1. VNTR analysis of environmental and clinical isolates of *P. aeruginosa*

One of the previous outbreak investigations on *P. aeruginosa* showed that there are usually one or two strain isolated in both patient and environmental sites [94]. However strains in UCH were found to have very diverse VNTR profiles. Another study from literature searching the source of *P. aeruginosa* in a newly opened hospital showed eight distinct clades of 141 *P. aeruginosa* isolates typed by WGS, as well as identical pairs from patients and environment where they hypothesised diversity generated by water transmission or by seeding from the wider environment [96]. Our results suggest that seeding from the wider environment could be a contributor for diverse *P. aeruginosa* dissemination. Another route of introduction to new strains could be via suppliers where there is no or little standard guidance on supply waters or when the guidance is not properly followed [195].

Even though there are some exact/similar VNTR profile results in shower water and patient isolates, to understand the transmission route of *P. aeruginosa*, the timing and the site of locations of the isolates should match. However, it was not feasible to screen all shower waters for presence of *P. aeruginosa* all the time and it is not known when the patient used a shower. In the isolate set typed so far (111 patient and 77 environmental strains) only four pairs were found to be matching in terms of time and location, suggesting the difficulty of isolating related strains.

The VNTR method has some limitations because repeat numbers in the same loci might change through time (especially ms61). Previous studies have showed that even if the strains showed highly similar VNTR profiles, the resolution offered by full WGS concluded that they were different strains. This was observed for the ms61 loci in particular where strains

having the same repeats showed different bands in PFGE. Therefore to avoid misleading environmental – clinical links, WGS is required for accurate investigation of outbreaks [94]. Since in our study only 9 loci were analysed out of a total 15 possible loci for *P. aeruginosa*, discriminatory power could have increased by adding more loci [89]. In other studies, researchers added ms212 loci to increase discriminatory power for instance [94].

When analysing dates of sample collections from patients, it is suggested to check patients' locations over a longer time covering environmental sample collection dates in future study. Patients might have moved to different beds/wards just after or before their blood sample was taken.

5.4.2. WGS analysis of environmental and clinical isolates of *P. aeruginosa*

In this chapter, emphasis for sequencing results was given to AMR and biofilm properties of *P. aeruginosa* by analysing the genomes of strains from patients/hospital shower water and shower hoses. Presence of AMR genes were compared with phenotypic antibiotic susceptibility disc-diffusion assay results. Phenotypic and genotypic antibiotic resistance results did not necessarily match since the presence of an AMR gene does not mean that it will be phenotypically expressed. It could also be because presence of unknown resistance genes/mechanisms or epistasis between co-occurring gene sets.

Presence of known biofilm genes were also analysed. *P. aeruginosa* establishes its biofilm forming capacity through three main exopolysaccharides, *Psl, Pel* and alginate which allows them to form stable structures and makes it difficult to eradicate[42]. *Pel* (for producing glucose-rich matrix) and *psl* (for producing mannose-rich matrix) gene clusters are shown to be essential for biofilm formation by many studies [196]. Alginate is also suggested to take part in biofilm development, although it is shown to be not essential in *in-vitro* studies, it shows significant features especially in CF patients by contributing to antibiotic resistance such as against tobramycin [46] In addition to those well studied genes for biofilm production, *cup* gene clusters (cupA-E) were found to be contributing attachment to abiotic surfaces, hence biofilm formation by encoding fimbrial structures. These fimbrial gene clusters are especially well studied in *E. coli*, as Fim or Pap fimbirae [186,197,198]. Presence/absence of those genes were shown by sequencing results, however a more detailed study on their regulatory systems such as two-component systems is needed [186]. In the analysis done so far, we did not see any marked differences in biofilm gene presence and absence - this may suggest other mechanisms could be taking part.

Another important aspect of this sequencing study was to search for relatedness of the isolates, especially relatedness of clinal and environmental strains but as well their relatedness between each group, in the hope of demonstrating potential transmission events and understanding the persistence of the same strain in same/similar environments. VNTR results as well as metadata (from where and when the isolates were collected) were used as preliminary study to select isolates for WGS, by keeping into account VNTR's lower discriminatory power [90,94]. To search for relatedness of isolates, a core genome SNP distance matrix was used. In the literature, there is no set SNP cut-off used to demonstrate related isolates are part of a transmission chain. The first study on wgSNP analysis of *P. aeruginosa* strains used 4 SNPs as their cutoff value calculated by using Youden indicators which is reported to be a reliable method by other studies [110]. Another study looking at *P. aeruginosa* outbreak strains used 0-14 SNP difference as an indicator for relatedness [111]. Other group comparing MLST and SNP analysis for investigating epidemiological links of *P. aeruginosa* used less than 26 SNPs or 13 allele differences to show relatedness [112]. Given that SNP threshold used highly depends on metadata of isolates, when the SNP difference of strains isolated from the same wards in this thesis analysed, it was observed that <6 SNPs may be a relevant cutoff. In the same ward analysis, the closest SNP to 6 cutoff value was found as 37 SNPs, which was then used to define isolates which were not closely related. Future work could explore more formal estimates of transmission chains which take into account phylogenetic uncertainty and provided epidemiological data [199].

Genomic sequence analysis was undertaken to determine relatedness and identify AMR and biofilm-forming markers/determinants of these isolates. Although more detailed analysis is needed, preliminary results showed the importance of environmental isolates in onset of hospital-acquired *P. aeruginosa* bacteraemia. As *P. aeruginosa* is an opportunistic pathogen, characteristics of patient population are also important. The following chapter explains a case control study undertaken to understand the risk factors for developing hospital-acquired *P. aeruginosa* bacteraemia.

Chapter 6. Case Control Study

The Case control study was performed under the supervision of Pietro Coen, UCH epidemiologist and included a background study, and selection of case and controls groups.

6.1. Introduction

This chapter focuses on the risks of getting hospital-acquired *P. aeruginosa* bacteraemia through a case-control study. Case control studies can be retrospective and designed to understand if a historical exposure is associated with an outcome, e.g., disease or a condition. The case list represents the set of subjects who have the outcome and the controls represent the set of subjects who do not have the outcome.

Apart from the outcome of interest, case and control subjects should be as similar as possible to make the comparison meaningful, so we tend to match controls to cases by age group, sex, duration of hospital stay, specialty etc. Matching is important because it minimizes the danger of observed differences in outcome being also due to factors we did not measure. Matching hence maximizes the validity and the statistical power of the comparison. Statistical power to detect risk factors with case control studies can be enhanced by matching more than one control per case[200].

In a case control study the odds ratio (OR) is a natural measure of association between exposure and outcome. If the probability of an event is *p* given a specific type of exposure, then the 'odds' of that event given that exposure is o = p/(1-p). The OR is the ratio of odds with different exposures. If *a* and *b* are the numbers of exposed and unexposed cases, and *c* and *d* are the numbers of exposed and unexposed controls, the mathematical equivalent of the OR is given by (a/c) / (b/d) = (a x d) / (b x c) [201].

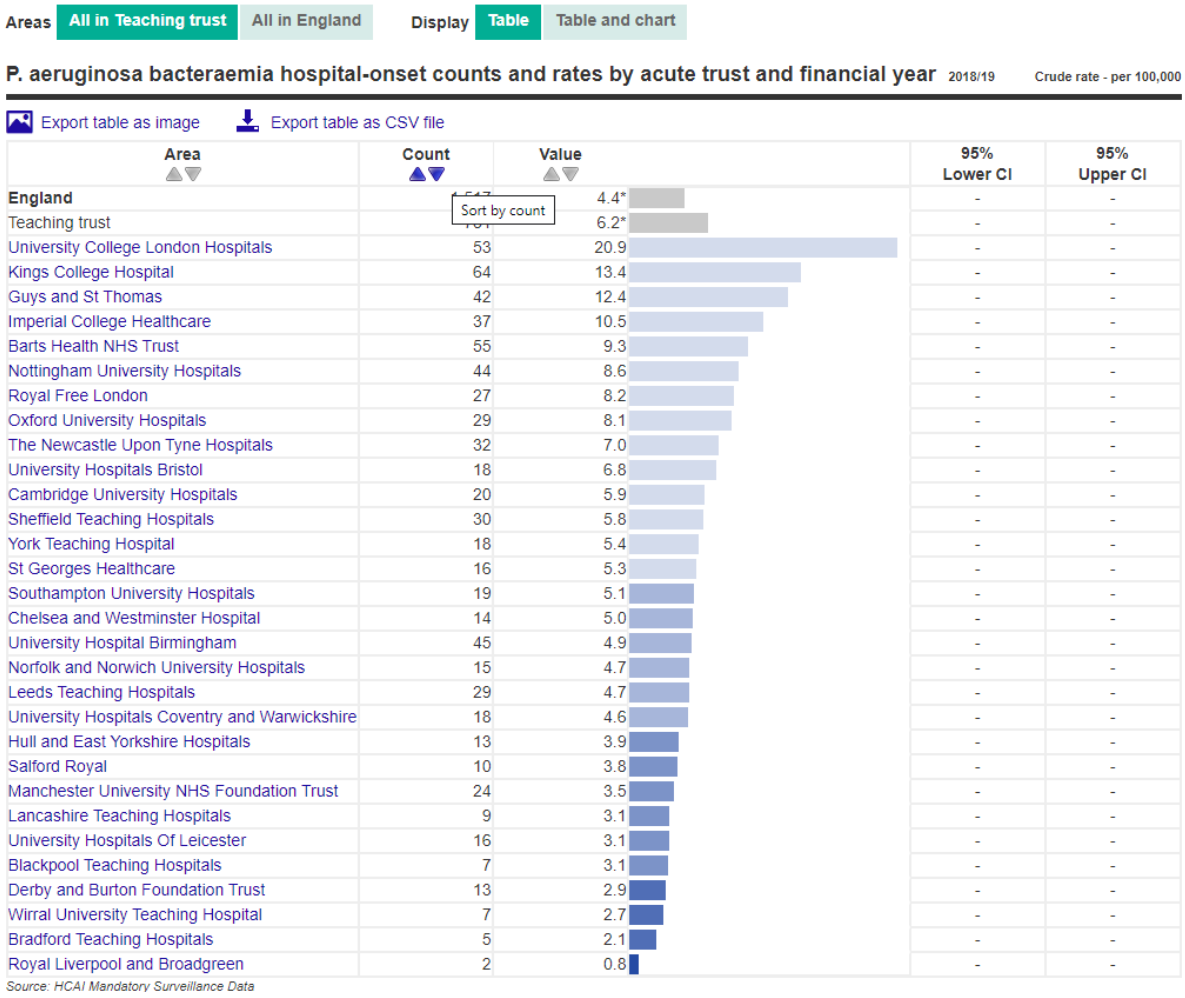
Taking 1 as a cut-off;

- OR = 1 means exposure is not associated with the disease or condition
- OR > 1 means exposure may be a risk factor for the disease or condition
- OR < 1 means exposure may be protective against the disease or condition [202]

Regression is used by statisticians to test the relationship between one dependent variable and other independent variables. Logistic regression is used when the dependent variable is binary [203]. Conditional logistic regression is the equivalent standard method for matched case control studies [204].

Using two types of control set allows us to investigate two aspects of the risk of acquiring *P. aeruginosa*: a) the risk of acquiring the organism per se, vs. b) the risk of suffering from the invasive disease aspect of the *P. aeruginosa* (i.e. the bacteraemia) associated with *P. aeruginosa*.

The case control methodology was undertaken to investigate the exposures associated with the onset of *P. aeruginosa* in UCH inpatients. At the time of the study UCH had the highest hospital rates of bacteraemia when compared to other acute trusts (Figure 6.1), so that this study had scope for helping the organization to identify control measures.



Areas: All in Teaching trust | All in England Display: Table | Table and chart

P. aeruginosa bacteraemia hospital-onset counts and rates by acute trust and financial year 2018/19 Crude rate - per 100,000

Export table as image Export table as CSV file

Area	Count	Value	95% Lower CI	95% Upper CI
England		4.4*	-	-
Teaching trust		6.2*	-	-
University College London Hospitals	53	20.9	-	-
Kings College Hospital	64	13.4	-	-
Guys and St Thomas	42	12.4	-	-
Imperial College Healthcare	37	10.5	-	-
Barts Health NHS Trust	55	9.3	-	-
Nottingham University Hospitals	44	8.6	-	-
Royal Free London	27	8.2	-	-
Oxford University Hospitals	29	8.1	-	-
The Newcastle Upon Tyne Hospitals	32	7.0	-	-
University Hospitals Bristol	18	6.8	-	-
Cambridge University Hospitals	20	5.9	-	-
Sheffield Teaching Hospitals	30	5.8	-	-
York Teaching Hospital	18	5.4	-	-
St Georges Healthcare	16	5.3	-	-
Southampton University Hospitals	19	5.1	-	-
Chelsea and Westminster Hospital	14	5.0	-	-
University Hospital Birmingham	45	4.9	-	-
Norfolk and Norwich University Hospitals	15	4.7	-	-
Leeds Teaching Hospitals	29	4.7	-	-
University Hospitals Coventry and Warwickshire	18	4.6	-	-
Hull and East Yorkshire Hospitals	13	3.9	-	-
Salford Royal	10	3.8	-	-
Manchester University NHS Foundation Trust	24	3.5	-	-
Lancashire Teaching Hospitals	9	3.1	-	-
University Hospitals Of Leicester	16	3.1	-	-
Blackpool Teaching Hospitals	7	3.1	-	-
Derby and Burton Foundation Trust	13	2.9	-	-
Wirral University Teaching Hospital	7	2.7	-	-
Bradford Teaching Hospitals	5	2.1	-	-
Royal Liverpool and Broadgreen	2	0.8	-	-

Sort by count

Source: HCAI Mandatory Surveillance Data

Figure 6.1. Ranking of UCH when compared to other acute trusts in the UK, data taken from Fingertips UKHSA website for 2018 – 2019 financial year [205].

6.2. Methods

6.2.1. Demographic Study

The aim of the case-control study was to determine if there were pre-existing risk factors for the onset of *P. aeruginosa* among hospital inpatients, many of whom were immunocompromised. In 2017, there had been an increase in the number of patients with *P. aeruginosa* in the hospital, coinciding with an outbreak of Parainfluenza virus. Hence, I found it necessary to carry out a demographic study comparing 185 patients before and after April 2017. I focussed on gender, age, admission specialities, sensitivities to antibiotics, admission methods, ward locations and blood test values (CRP, WCC, NEU). The Chi-squared test was applied for statistical significance.

6.2.2. Case Control Study

Case data of a total of 137 patients with hospital acquired *P. aeruginosa* and a total of 845 patients from two control groups were analysed for the following variables: admission method, blood test results (white cell count (WCC), neutrophil count (NEU), creatinine (CRE), albumin (ALB), glomerular filtration rate (GFR) and C-reactive protein (CRP)), respiratory syncytial virus (RSV), parainfluenza, rhinovirus, number of viruses, any virus, number of lines, a central line, surgery, urinary tract infection, *P. aeruginosa* detected in urine, wound, respiratory, or a combination there of (urine/wound/respiratory). Controls were selected and matched with case patients by the trust epidemiology lead. Control patients were of the same age group as case patients when first detected. Ages were grouped as: 0 to 1, 2 to 5, 6 to 18, 19 to 24, 25 to 29, 30-35 and so on (in 5 year age groups until 110 years old). We scored the matching quality as shown in the Table 6.1 as below. More details on how quality of matching was scored (matching by age, sex, specialty, ward and detection of viral infection in the 14 days prior to bacteraemia) are given in Table 6.1. SPSS and R were used to do the statistical analysis.

Table 6.1. Matching quality of controls with case patients

Matching quality	**Same specialty**	**Same ward**	**Same sex**	**In hospital when the case was first detected**
1	Yes	Maybe	Yes	Yes
2	Yes	Maybe	No	Yes
3	Yes	Maybe	Yes	No

4	Yes	Maybe	No	No
5	No	Yes	Yes	Yes
6	No	Yes	No	Yes
7	No	Yes	Yes	No
8	No	Yes	No	No

Hospital-acquired *P. aeruginosa* bacteraemia patients were investigated retrospectively from 15 April 2015 – 28 July 2018. A total of 845 patients constituted two control sets. Set 1 included 357 were from patients who suffered from a bacteraemia by causal organisms other than *P. aeruginosa*; and set 2 included 488 were from patients who never had bacteraemia whilst in UCH care. The rationale of using two different control sets lies in the hypothesis that comparison of cases against set 1 will yield risk factors for acquiring the organism (rather than the disease), whereas comparison against set 2 will yield risk factors for the invasive disease aspect of *P. aeruginosa*. Analysis with set 1 was designed as a future study as bacteraemia database included contaminant microorganism which would affect the regression analysis.

Presence or absence of shower water contamination with *P. aeruginosa* was considered as a potential variable. The hospital bed locations of patients during their stay in the wards were mapped on hospital floor plans as given in Appendix C.1. Note that in and Ward B and Ward H, some of the rooms had four to five bacteraemia patients throughout the study period.

Shower contamination data and locations of patients were compiled to determine correlation. Graphs representing these are given in Appendix C.2. There were four practice changes in the study period: Introduction of Anonymised 1 Ag+ showers (July 2015), Royal Free Hospital patients moving to UCH (June 2016), shortening shower hoses from 1.2 m to 0.8m (April 2019) and introduction of Anonymised 2 filter showers (July 2019).

P. aeruginosa isolated in shower waters captured during routine sampling of augmented care wards during the period that case patients were staying in the hospital rooms were compared.

Case and control patient data were categorized and analysed on SPSS. R was used for conditional logistic regression (Commands used and programme outcomes are given in detail in Appendix C.3). From the variables listed above, blood test values were first analysed by

using the actual test results and then analysed by categorizing them with cut-off values given in Table 6.3. Correlation analyses were done so that correlated variables will not be included in the analyses. Dummy variables were created where relevant; univariate analysis was run to see the effect of each variable listed above followed by multivariable analyses for the full model.

6.3. Results

6.3.1. Background: Demographics Study

Comparison of P. aeruginosa bacteraemia patient demographics before and after April 2017

Background study comparing *P. aeruginosa* bacteraemia patient demographics in terms of gender, age group, patient speciality, admission method, antibiotic sensitivity pattern, ward location, white cell count and neutrophil levels. It included a short description of patients who had both Parainfluenza and *P. aeruginosa* bacteraemia.

The first set constituted of 55 cases detected between 16 April 2015 and 17 March 2017. The second set included 130 cases occurred between 2 April 2017 and 26 July 2019. A total of 185 *P. aeruginosa* patient demographics were analysed.

Chi-squared tests for statistical significance:

Figure 6.2. shows the comparison of *P. aeruginosa* bacteraemia patient genders before and after April 2017.

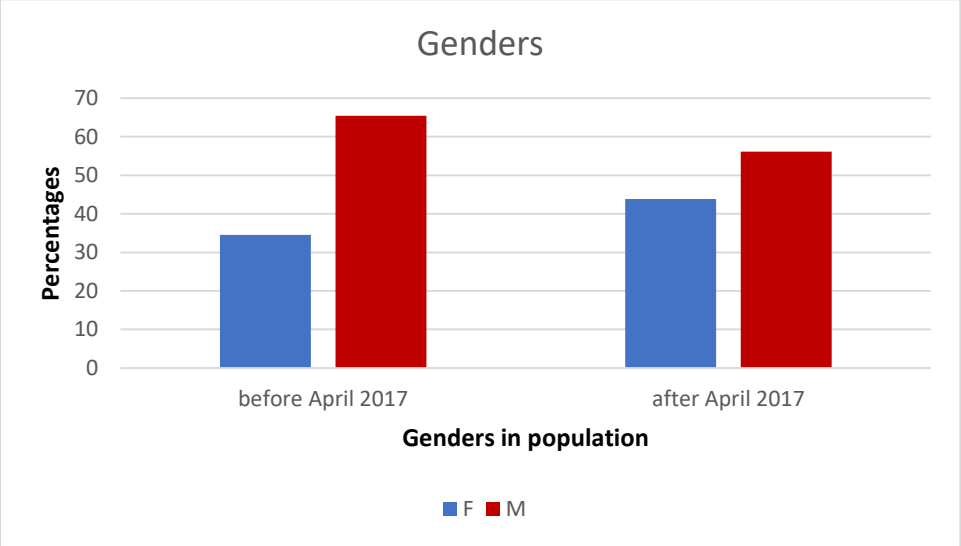


Figure 6.2. Comparison of *P. aeruginosa* bacteraemia patient gender.†

†X^2 (1, N = 185) = 1.38, p = 0.24, not statistically significant at p<.05.

Figure 6.3 shows the comparison of *P. aeruginosa* bacteraemia patient ages before and after April 2017.

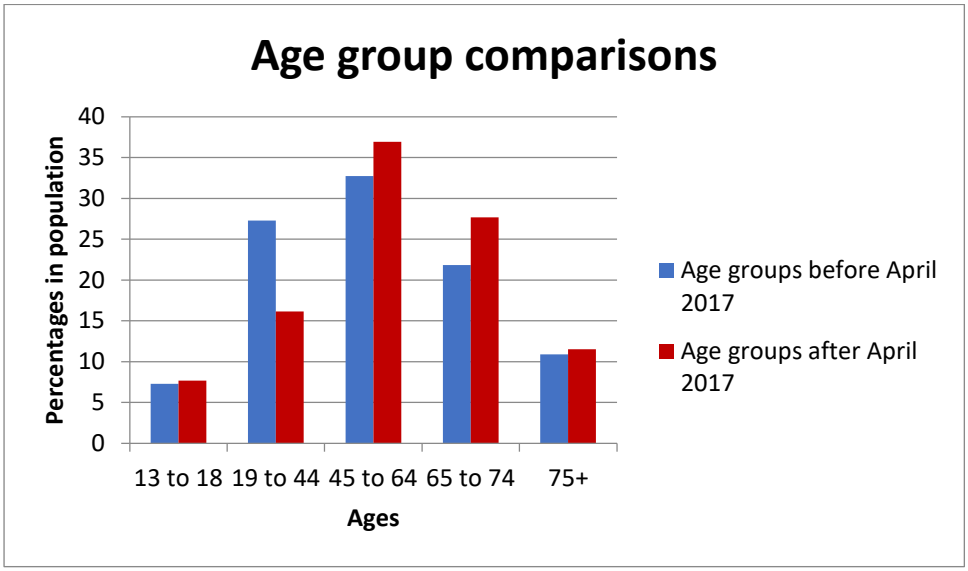


Figure 6.3. Comparison of *P. aeruginosa* bacteraemia patient age groups. †

†X^2 (4, N = 185) = 3.18, p = 0.53, not statistically significant at p<.05.

Table 6.2. shows the distribution of treatment specialties in *P. aeruginosa* cases before and after April 2017. Comparison of the two distributions do not show a significant difference (X^2 (20, N = 185) = 17.3, p = 0.63, not statistically significant at p<.05.).

Table 6.2 shows the number of cases and percentages in different specialities before and after April 2017.

Table 6.2. Number and percentage of *P. aeruginosa* bacteraemia patient specialities before and April 2017.

Specialty	Before April 2017		After April 2017	
	#Cases	%Cases	#Cases	%Cases
Clinical haematology	30	54.5	86	66.2
Medical oncology	5	9.1	6	4.6
Neurology	3	5.5	7	5.4
Gastroenterology	3	5.5	6	4.6
Urology	2	3.6	5	3.8
Neurosurgery	2	3.6	3	2.3
Critical care medicine	3	5.5	3	2.3
Colorectal surgery	2	3.6	N/A	N/A
ENT	1	1.8	2	1.5
Geriatric medicine	N/A	N/A	2	1.5
Infectious diseases	1	1.8	2	1.5
Upper GI surgery	N/A	N/A	1	0.8
Rehabilitation	N/A	N/A	1	0.8
Paediatrics	N/A	N/A	1	0.8
Gynaecology	N/A	N/A	1	0.8
Trauma and orthopaedics	1	1.8	N/A	N/A
Thoracic surgery	1	1.8	N/A	N/A
Thoracic medicine	N/A	N/A	1	0.8
Rheumatology	N/A	N/A	1	0.8
Clinical pharmacology	N/A	N/A	1	0.8
A&E	N/A	N/A	1	0.8
Rehabilitation	1	1.8	N/A	N/A

Figure 6.4 shows the comparison of *P. aeruginosa* bacteraemia patient blood isolates antibiotic resistance patterns before and after April 2017.

Resistance to piperacillin-tazobactam (PTZ), ceftazidime (CAZ), meropenem (MEM), gentamicin (CN), amikacin (AK) and ciprofloxacin (CIP) are given. Some of the patient antibiotic sensitivity data is missing, this might result into changes in percentages (Patient numbers missing particular antibiotic data listed as follows: PTZ: 13, CAZ: 10, MEM: 12, CN: 10, AK: 13 and CIP: 10).

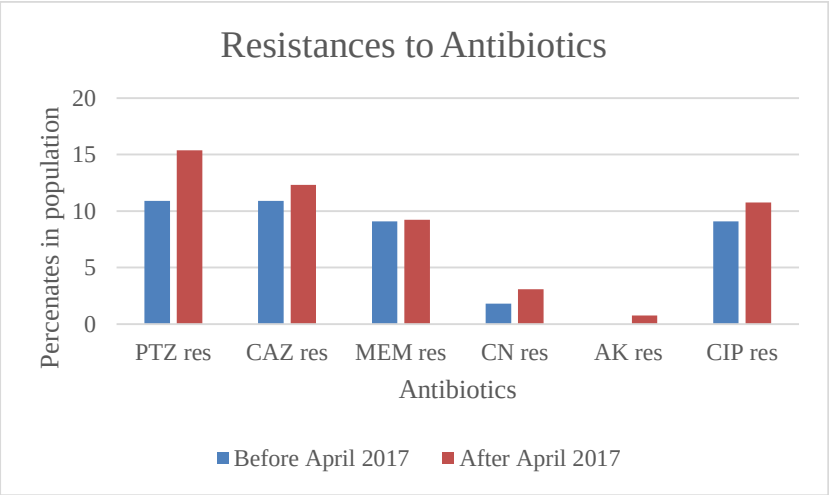


Figure 6.4. Comparison of *P. aeruginosa* bacteraemia patient blood isolates' antibiotic resistance patterns.†

†None of the before-after Chi-squared tests showed statistical significance; PTZ: X^2 (3, N = 185) = 4.3, p = 0.23; **CAZ:** X^2 (3, N = 185) = 5.17, p = 0.16; **MEM:** X^2 (3, N = 185) = 6.59; **CN:** X^2 (2 N = 185) = 4.79, p = 0.09; **AK:** X^2 (3, N = 185) = 7.41, p = 0.06; **CIP:** X^2 (3, N = 185) = 7.26, p = 0.06.

Figure 6.5 shows the comparison of *P. aeruginosa* bacteraemia patients' admission methods. Patients coming to hospital with an appointment are considered as elective admission. Non-elective admission is when patients come through A&E. Transfer stands for the patients who were transferred from different hospitals.

Chi-squared test for statistical significance:

X^2 (2, N = 185) = 2.367, p = 0.306205. It is not statistically significant at p<.05.

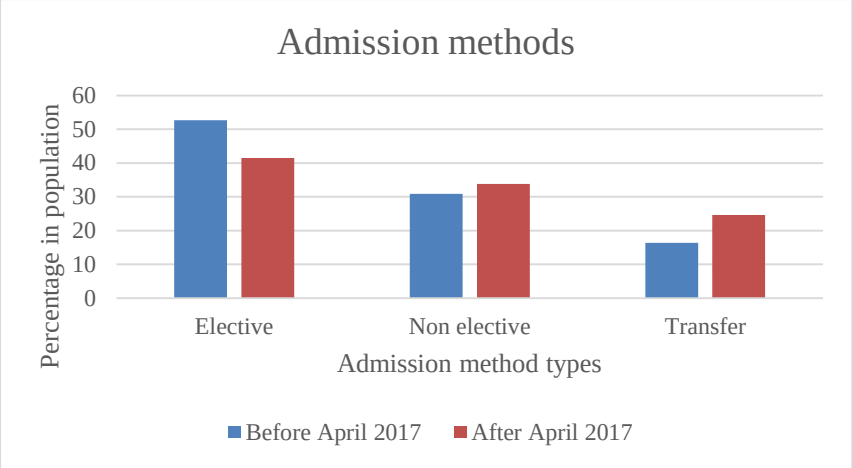


Figure 6.5. Comparison of *P. aeruginosa* bacteraemia patients' admission methods before and after April 2017.†

†X^2 (12, N = 185) = 13.6, p = 0.33, not statistically significant at p<.05.

Figure 6.6 shows the comparison of *P. aeruginosa* bacteraemia patients' ward locations considering the number of cases.

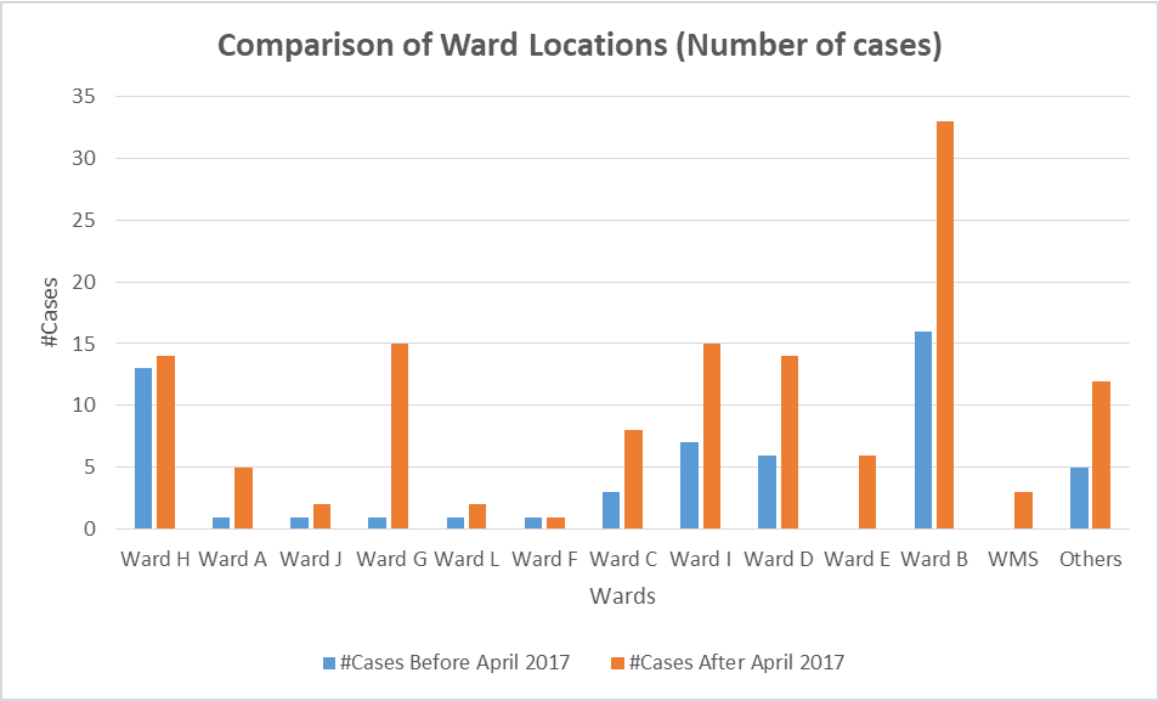


Figure 6.6. Comparison of *P. aeruginosa* bacteraemia patient numbers in each ward

Note the rise in case numbers of *P. aeruginosa* cases in individual wards. This is consistent with the overall increase in numbers of cases from 55 to 155 before and after April 2017. In contrast, comparison of the distribution by ward using percentages within each time period shows a much a smaller difference between time periods (Figure 6.7).

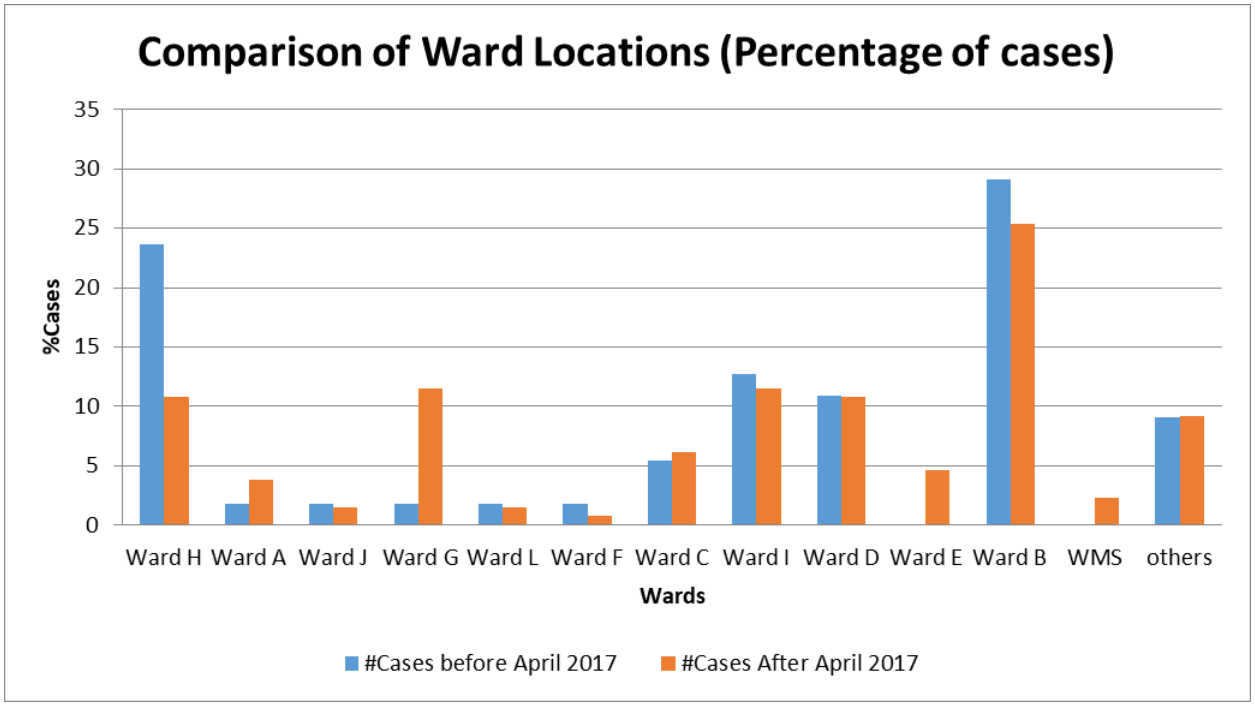


Figure 6.7. Comparison of *P. aeruginosa* bacteraemia patient percentages in each ward.

Figure 6.8 shows the blood test data of *P. aeruginosa* bacteraemia patients. CRP stands for C-reactive protein; Level 10 was selected as a cut off measure. WCC stands for white cell count; Level 2 was selected as a cut off measure. NEU stands for neutrophil; Level 1 was selected as a cut off measure. All data belongs to either one day or two days prior to the date of sample collection for *Pseudomonas aeruginosa.*

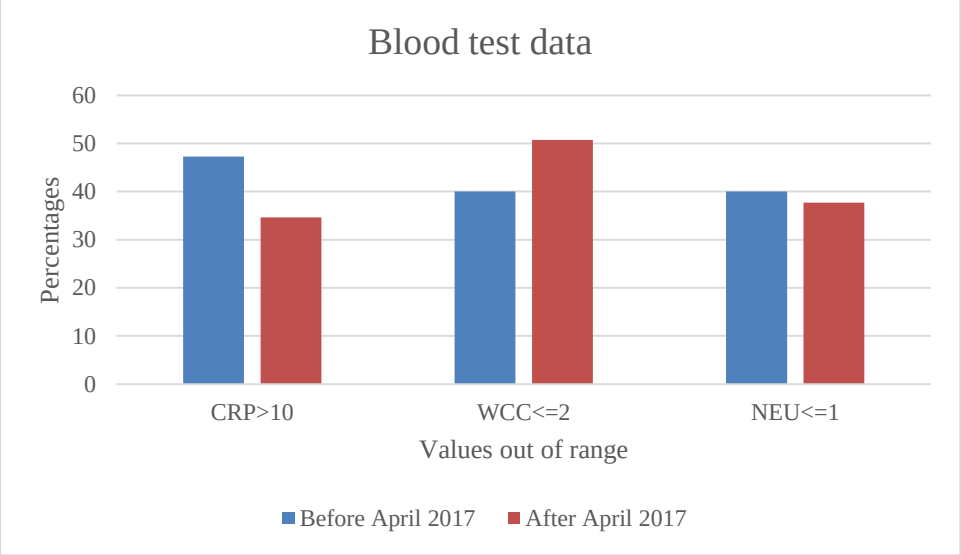


Figure 6.8. Blood test data of *P. aeruginosa* bacteraemia patients.†

† None of the before-after Chi-squared tests showed statistical significance: CRP: X^2 (2, N = 185) = 2.66, p = 0.26; WCC: X^2 (2, N = 185) = 1.80, p = 0.41; NEU: X^2 (2, N = 185) = 0.29, p = 0.87.

Parainfluenza

Parainfluenza cases showed similar incidence trends as *P. aeruginosa* cases. Six out of 185 *P. aeruginosa* cases patients were also Parainfluenza positive. As although there were likely to have been other undiagnosed Parainfluenza cases we should not rule out a connection between the two organisms. Their ward locations were: Ward H (1 case), Ward G (4 cases) and Ward B (1 case). One of them belonged to the period before April 2017.

Four out of six patients having both parainfluenza & pseudomonas infections were admitted in Ward G.

6.3.2. Case Control Study

Case-control study using the never-had-a-bacteraemia control set (seeking invasive disease risk factors)

Conditional logistic regression analysis was conducted using R statistics programme for the case (*P. aeruginosa* bacteraemia) and "no bacteraemia" control groups (488 control patients).

According to correlation analysis, WCC-NEU are correlated (as expected since NEU is included in WCC), other variables are not correlated, or their correlations are not statistically significant, details given in Appendix C.3.

6.3.2.1. Univariate Analyses

Table 6.3. Univariate analysis of conditional logistic regression results.

Variable	Categories	OR	95%LL	95%UL	p-value
Admission method	1=Elective 0=Others (Non-elective, Transfer)	2.90	1.83	4.58	<0.001
Admission method	1=Non-elective 0=Others (Elective, Transfer)	0.638	0.410	0.996	0.048
Admission method	1=Transfer 0=Others (Elective, Non-Elective)	5.44	2.84	10.4	<0.001
WCC Groups	1=0-2 0 = >=2	6.14	3.28	11.5	<0.001
WCC Blood Test Values	Actual blood test values	0.882	0.829	0.939	<0.001
NEU Groups†	1 = <1 0 = >=1	4.69	2.49	8.84	<0.001
NEU Blood Test Values	Actual blood test values	0.902	0.840	0.968	0.005
GFR Groups	1 = <90%, 0 = >=90%	0.786	0.484	1.28	0.33
GFR Blood Test Values	Actual blood test values	1.000	0.989	1.01	0.94
CRP	Actual blood test values	2.30	0.764	6.93	0.14
Parainfluenza	Detected:1 Not detected:0	2.14	0.602	7.63	0.24
RSV	Detected:1 Not detected:0	1.98	0.350	11.2	0.44
Rhinovirus	Detected:1 Not detected:0	0.681	0.138	3.35	0.64
Number of viruses	Numbers	1.45	0.733	2.87	0.29
Any virus	Detected:1 Not detected:0	1.49	0.699	3.17	0.30
Number of lines	Numbers	4.20	2.50	7.06	<0.001
A central line	Inserted:1 Not inserted:0	5.16	2.87	9.26	<0.001
Surgery	Performed:1 Not performed:0	0.361	0.139	0.938	0.036
Urinary tract infection	Yes:1 No:0	2.151	1.07	4.32	0.032
P. aeruginosa in urine	Yes:1 No:0	64.7	7.15	585	<0.001
P. aeruginosa in wound	Yes:1 No:0	5.44	1.66	17.9	0.005
P. aeruginosa in respiratory	Yes:1 No:0	7.05	2.13	23.3	0.001
P. aeruginosa any site (urine/wound/respiratory)	Yes:1 No:0	11.2	4.62	27.3	<0.001

†Neutropenia is defined as a neutrophil count <1.5 * 10^9 /L (Normal range in the UK 1.5 – 8.0 * 10^9 /L: , so we used the 1.5 cut-off and the analysis was rerun, but this did not significantly change the results as there were only 3/137 cases and 13/488 controls having NEU counts between 1 and 1.5. (When 1.5 cut off is used, OR is 4.478 whereas when 1.0 cut off is used OR is 4.687)

Odds ratios should be interpreted in relation with the confidence intervals and p values. Results shown in Table 6.3 revealed that admission method, WCC, NEU, presence of a central line, *P. aeruginosa* presence in urine or in any site had odds ratio greater than 1 with statistical significance ($p<0.05$).

6.3.2.2. Multivariable Analyses

Univariate analysis is a useful starting point for approaching multivariable analyses. Factors that at the univariate stage were significant (at the $p<0.05$ level) and having odds ratios significantly different from 1 were included in the multivariable model except NEU because NEU count is already included in WCC count. In addition, other variables (admission method, presence of any virus and parainfluenza and surgery) were included in multivariable model because they were clinically significant (Table 6.4). This was to be on the side of caution as confounding effects make it possible for a variable to be not significant at the univariate stage but significant at the multivariable stage [206].

In order to better understand the effect of *P. aeruginosa* at site of urine/wound/respiratory, multivariable analyses was performed for individual sites as well as at any site as shown in Table 6.5 and Table 6.6.

(non-bacteraemia controls)

Table 6.4. Multivariable analysis.

Variable	Categories	OR	95%LL	95%UL	p-value
Admission method	1 is Non-elective 0 is Others (Elective, Transfer)	0.798	0.421	1.51	0.49
Admission method	1 is Transfer 0 is Others (Elective, Non-Elective)	3.77	1.50	9.47	0.005
WCC Groups	1: 0-2 0: >=1	5.36	2.61	11.0	<0.001

Any virus	Detected:1 Not detected:0	1.26	0.409	3.85	0.69
Parainfluenza	Detected:1 Not detected:0	1.98	0.314	12.5	0.47
Number of lines	Numbers	3.17	1.74	5.77	<0.001
Surgery	Performed:1 Not performed:0	0.198	0.0527	0.745	0.017
P. aeruginosa in urine	Yes:1 No:0	64.1	6.77	607	<0.001
P. aeruginosa in wound	Yes:1 No:0	9.62	1.68	55.2	0.011
P. aeruginosa in respiratory	Yes:1 No:0	5.54	1.37	22.3	0.016

Table 6.5. Multivariable analysis for *P. aeruginosa* presence any site + urine/wound/respiratory

Variable	Categories	OR	95%LL	95%UL	p-value
P. aeruginosa any site (urine/wound/ respiratory)	Yes:1 No:0	4.343	1.4172	13.307	0.0102
P. aeruginosa	Yes:1	13.097	1.2872	133.258	0.0298

in urine	No:0				
P. aeruginosa in wound	Yes:1 No:0	2.7746	0.7869	9.783	0.1125
P. aeruginosa in respiratory	Yes:1 No:0	1.846	0.4120	8.275	0.4229

Table 6.6. Multivariable analysis for *P. aeruginosa* presence in urine/wound/respiratory

Variable	Categories	OR	95%LL	95%UL	p-value
P. aeruginosa in urine	Yes:1 No:0	42.4503	4.571	394.26	0.000979
P. aeruginosa in wound	Yes:1 No:0	4.7935	1.388	16.56	0.013214
P. aeruginosa in respiratory	Yes:1 No:0	4.7947	1.373	16.74	0.014016

Multivariable analysis showed WCC count, number of lines, and *P. aeruginosa* in urine to have odds ratio greater than 1 with statistical significance ($p<0.05$) (Table 6.3). To better understand the importance of *P. aeruginosa* in wound which had significance lower than others, analyses was run for *P. aeruginosa* in different sites which showed only *P. aeruginosa* in urine having odds ratio greater than 1 with statistical significance (Table 6.5).

6.4. Discussion

Conditional regression analysis comparing case and without bacteraemia control group showed that WCC value, number of lines, and *P. aeruginosa* in urine are risk factors for having *P. aeruginosa* bacteraemia. WCC number is a measure of immune system. Chemotherapy as well as some viral diseases may lower WCC. Increased level of is a sign of immune system fighting with disease causing agents [207]. Patients having chemotherapy induced neutropenia are at risk for pseudomonal bloodstream infections [208]. Hence, WCC (where neutrophil count included) being a risk factor for *P. aeruginosa* is expected. Haematology and immunosuppressed patient groups are at risk. Intravascular lines can be ports of microorganism entry to the body leading to infection and bacteraemia. Number of intravascular lines being a risk factor was therefore expected.

A study focusing on community-acquired *P. aeruginosa* upon hospital admission ran a retrospective case control study with 151 case patients with *P. aeruginosa* bacteraemia and 152 control patients with Enterobacteriaceae bacteraemia and found immunodeficiency, age, receiving antimicrobial therapy and presence of central venous catheter/urinary device were predictors for *P. aeruginosa* bacteraemia (conditional logistic regression, multivariate analysis by including all variables having p<=0.1 after univariate analysis) [209].

One of the most important outcomes of this study revealed that presence of *P. aeruginosa* in urine is a predisposing factor for *P. aeruginosa*. As *E. coli* is the leading cause for UTIs, its mechanisms are well studied which can provide the basis for *P. aeruginosa*. UTI usually starts with an uropathogen residing in gut which leads to periurethral contamination and hence urethra and bladder. Flagella, pili and adhesins take important role in this colonization and migration. Once the host immune system is overcome by toxins and proteases, uropathogens travel to the kidneys and attach to the renal epithelium. Tissue damaging toxins enable them to cross the epithelial barrier and go into bloodstream, hence causing bacteraemia [210]. In *P. aeruginosa*, chaperone usher pathways (cup genes) are important in fimbrial attachment and in biofilm formation [194]. This gene group was included in WGS analyses to better understand their role in relation with UTIs.

One of the early studies investigating risk factors for *P. aeruginosa* bacteraemia showed urinary tract infection with catheter as a high risk as well as other factors such as neutropenia and being in ICU (outcomes of multivariate logistic regression) [211]. There are other studies

showing urinary catheters as a significant risk factor for *P. aeruginosa* bacteraemia. One retrospective observational study focussed on 85 patients over 3 years and demonstrated the most frequent *P. aeruginosa* source as urinary tract (43.5%), majority of them being associated with presence of urinary catheter, showing its importance in both hospital and community by focusing on all patients [212].

Another group using a multivariable logistic regression approach for a case-control study focusing on *P. aeruginosa* bacteraemia (54 cases) in Emergency Department found respiratory tract infection as an independent risk factor for *P. aeruginosa* bacteraemia but not urinary tract infection. However, this group used *E. coli* bacteraemia patients as controls (n=108). As mentioned above, *E. coli* is one of the leading causes of UTIs which could explain the difference in our study results where non bacteraemia patients are used[210,213]. Running the conditional regression analysis for the bacteraemia other than *P. aeruginosa* was attempted but not included as some issues regarding control patient data were detected. Patients in this group often did not have clinical blood infection but the organisms were contaminant. Study was planned to run the analyses for all control patients in this list and then discarding the ones listed with potential commensal microorganisms to see if that affected the outcomes but this was designed as a future study. Future recommendation is to better select bacteraemia control patients to avoid this confusion.

Our study focused on hospital-acquired *P. aeruginosa*, but community-onset bloodstream infections are important as shown by a recent group using logistic regression for a predictive score. They used univariate analysis to search for variables having p value <0.1 but used other potentially important predictors in their multivariate analysis showing male gender, haematological malignancy and source of infection other than urinary/biliary tract to be predictors for community-acquired *P. aeruginosa* bacteraemia. Other community-acquired bacteraemia sources included in this study were caused by Enterobacterales (*E. coli*, Klebsiella spp., Salmonella spp. etc.) [214]. Outcomes of this study highlights the importance of comparison group when using logistic regression to search for potential predictors or risk factors of a certain disease/infection. As an example, urinary tract infection not being found as predictor for *P. aeruginosa* bacteraemia should be analysed in comparison with Enterobacterales group patients where UTIs caused by Enterobacterales family is common especially in outpatients [215].

Given the importance of *P. aeruginosa* in UTIs, one study group focused on *P. aeruginosa* urinary sepsis by conducting a retrospective case control study including 83 case patients older than 65 years with *P. aeruginosa* urinary sepsis with 1:3 controls having urinary sepsis caused by other microorganisms. This showed being male, having urinary catheter and HAI were predictive factors for *P. aeruginosa* urinary sepsis[216].

In our study, shower contamination data may be an important risk factor but could not be included in the regression analysis due to limited environmental data. Especially in ICU, however, the population of bacteraemia patients concentrating in certain rooms (as given in hospital floor plans – Appendix C.1) suggest exposure to environmental *P. aeruginosa* should still be considered as a risk factor in a future study.

Shower contamination data followed the same pattern through different wards. *P. aeruginosa* contamination was absent after introduction of Anonymised 1 Ag+ showers in July 2015 and remained negative until early 2018. Sampling performed in 2019 showed that *P. aeruginosa* contamination reappeared in most showers (up to 90% of the showers for Ward B). Comparison with case locations revealed that they did not necessarily overlap the shower contamination data. As shown in shower contamination data and patient ward locations graphs in Appendix C.2., limited environmental sampling data and not being sure whether the patients used the showers were limiting factors to determine correlation.

86 out of 137 (~63%) patient cases were resident in the augmented care wards of UCH where shower contamination data was available from in-house hospital test laboratory. (Remaining patients were from Westmoreland Street, non-augmented care wards or Ward H (ICU) where there are no showers near patient beds. On four occasions *P. aeruginosa* was not present in the environment (shower water) before or after the occupying patient was diagnosed with *P. aeruginosa* bacteraemia. 28 out of 86 showers were found to be free of *P. aeruginosa* before the occupying patient was diagnosed with bacteraemia but found to be contaminated in the following sampling. 11 of them found to be contaminated before the occupying patient was diagnosed with bacteraemia but not found to be contaminated the following sampling. Seven of them - room numbers changed/not clear which room's sampling result to take into account. Six of them could not be concluded as the water test was missing. Hence shower contamination data was not included as a variable in the case control study.

Another study showed a bloodstream infection was caused by a contaminated peripheral venous catheter and the same *P. aeruginosa* strain found in preoperative shower of the immune suppressed patient [217]. Infection can also happen without the presence of catheter, for example, shower or drain water could contaminate damaged mucous membranes in patients receiving chemotherapy providing a portal of entry of *P. aeruginosa* into the blood stream.

Other studies focusing on risk factors for mortality associated with *P. aeruginosa* bacteraemia also used the logistic regression model through multivariable analysis. A retrospective cohort study through multiple logistic regression analyses of 136 *P. aeruginosa* bacteraemia patients admitted to Seoul National Hospital showed septic shock, pneumonia and receiving ineffective antibiotics as risk factors for *P. aeruginosa* bacteraemia mortality [218].

In conclusion, although choice and selection of controls could be problematic due to need for matching schemes, case-control studies are helpful for rare disease analysis such as bacteraemia cases. Further variables can be analysed with a bigger sample size for a broader study.

In the thesis, the case control study demonstrated risk factors of getting *P. aeruginosa* bacteraemia; environmental sample collection showed high levels of contamination of shower waters by *P. aeruginosa*. As detailed in the introduction and Chapter 5, biofilm forming properties of *P. aeruginosa* gives it a survival advantage to colonize surfaces. Biofilm formation in shower hoses and two different methods to isolate biofilm are explained in the following chapter.

Chapter 7. Investigation of biofilm in shower hoses and waters

7.1. Introduction

Biofilm forming properties of *P. aeruginosa* enables it to firmly attach to different environmental surfaces and medical materials as well as cystic fibrosis patient lungs [219]. General biofilm properties, genes taking part in biofilm development, removal of biofilms from both clinical and environmental surfaces are given in detail in Chapter 1, Section 1.2.3.

Water stagnation and accumulation of organic nutrients serving as nutrients are known factors for enhancing biofilm formation in pipeworks of buildings [67,73]. First aspect of this chapter analyses the effect of water stagnation in hospital shower hoses on *P. aeruginosa* biofilm formation.

Laboratory-based studies for biofilm formation includes static biofim systems such as microtiter plate biofilm assay, air-liquid interface assay and colony biofilm assay [220]. In this chapter, microtiter plate biofilm assay in other words 96-well plate assay was applied. Derivatives of this assay has been widely used since 1990s on *P. aeruginosa*, *E. coli*, *S. aureus*, etc. Protocol relies on monitoring bacterial attachment to an abiotic surface and staining, solubilizing and measuring biofilms [139,220].

7.2. Methods

Biofilm studies included two aspects:

1. To recover biofilm strains from shower hoses used in hospital, compare the counts throughout the hose as detailed in Chapter 7.

2. To measure biofilm formation of strains recovered from shower hoses and hospital water strains via crystal violet assay and compare the results with WGS outcomes. Biofilm formation through microtiter well plate assay is adopted from O'Toole et al [221]. Strains were grown in nutrient broth for 18 hours at 37°C. They were then transferred to microtiter-well plates as 12 replicates per isolate, incubating for 24 hours at 37°C. Cells were stained by crystal violet (1%) and dissolved by acetic acid (30%). OD measurements were done at 570 nm and 595 nm.

7.3. Results

7.3.1. Comparison of *P. aeruginosa* biofilm formation in shower hoses

To compare biofilm formation throughout the shower hose, hoses were collected during shower changes and biofilm protocol detailed in Chapter 2 was applied. In total, 30 shower hoses were analysed in three sets. The first set included old showers, second set Anonymised 1 long-hosed showers and third set Anonymised 1 short-hosed showers. Details of shower types historically and presently used in the hospital are given in Appendix A.

Biofilm procedure was applied to ten old shower heads and hoses (details given in Appendix D.2). *P. aeruginosa* biofilm was recovered from three of ten shower hoses, two of them belonging to the same ward (all showers were from non-augmented care units). *P. aeruginosa* numbers (CFU) are given in Figure 7.1, middle of the hose having the highest counts.

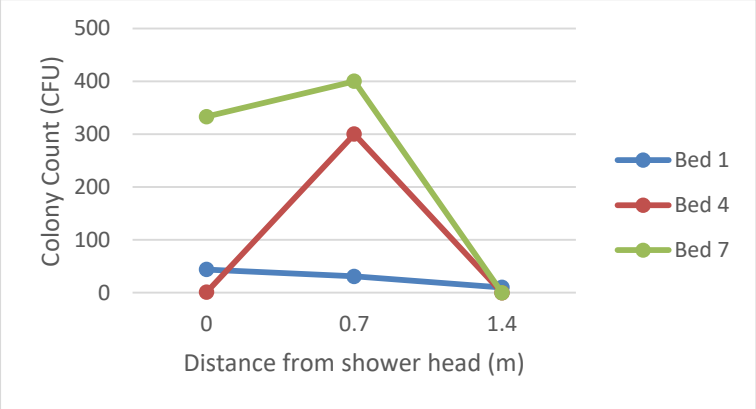
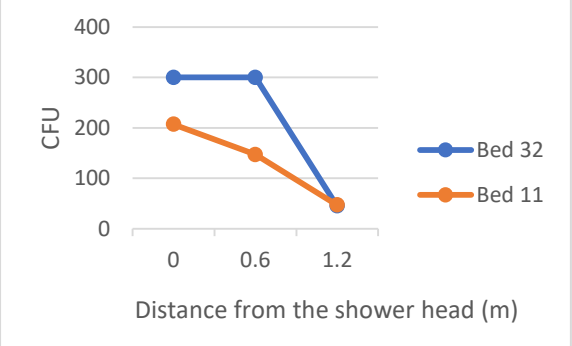
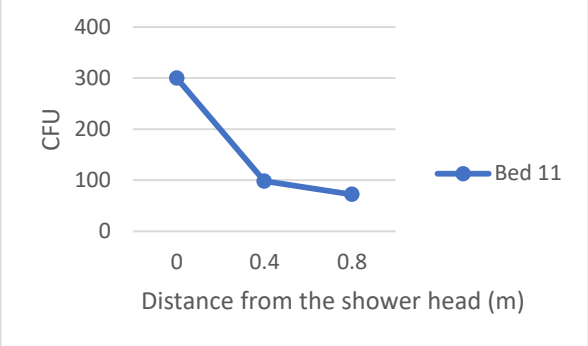
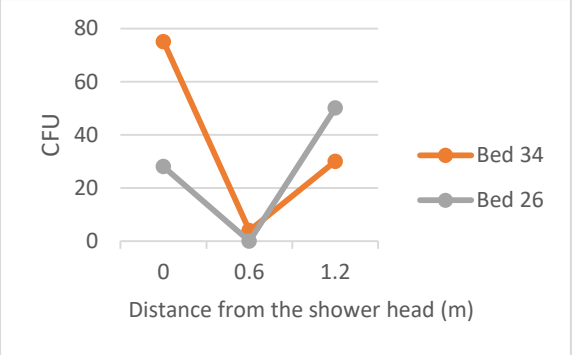
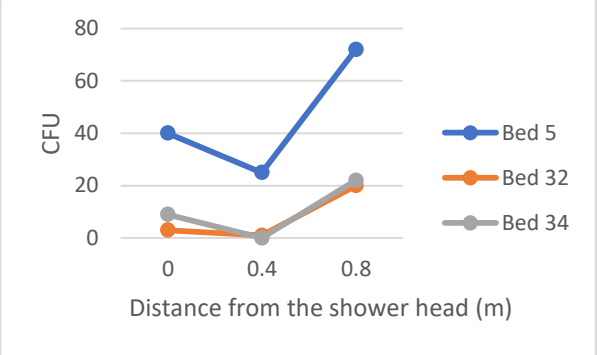
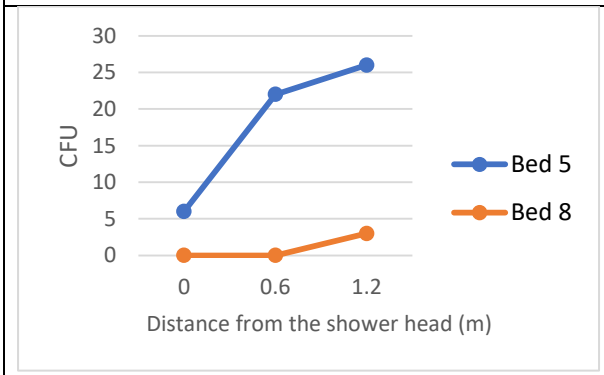
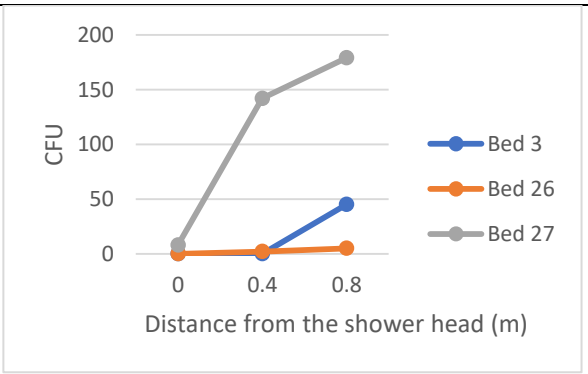


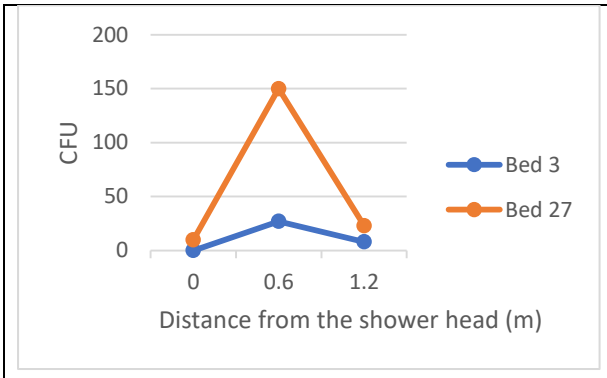
Figure 7.1. *P. aeruginosa* biofilm growth CFU's along the shower hose.

Biofilm procedure was applied to twenty new showers (Anonymised 1 Ag+ shower heads and hoses). Two sets of showers (ten in each group) were collected on 6 December 2018 and 26 February 2019 respectively during the three-monthly shower change. The first group included long hosed showers (1.2m) whereas the second group included short hosed ones (0.8m). CFU count patterns are given in Figure 7.2. Shower photos at the times of sample collection are given in Appendix D.2.

Similar patterns at both sampling times were observed for shower hoses of: Bed 7 and Bed 9 did not show any *P. aeruginosa* growth. Bed 11 showed a decreasing colony count from proximal to distant at both rounds. Bed 34 showed lowest colony count in the centre at both

times. Different patterns between sampling times were observed for the rest of shower hoses as given in Figure 7.2.

Anonymised 1 showers – 1st round biofilm patterns	**Anonymised 1 showers – 2nd round biofilm patterns**
Decreasing counts from proximal to distant	
	
Lowest count in the centre	
	
Increasing counts from proximal to distant	
	
Highest count in the centre:	



No Example

Figure 7.2. Comparison of *P. aeruginosa* biofilm growth patterns between two rounds of showers (long and short hoses)

In order to better see the patterns, counts from long and short hosed showers are combined and demonstrated as shown in Figure 7.3. No clear pattern along the hose is observed.

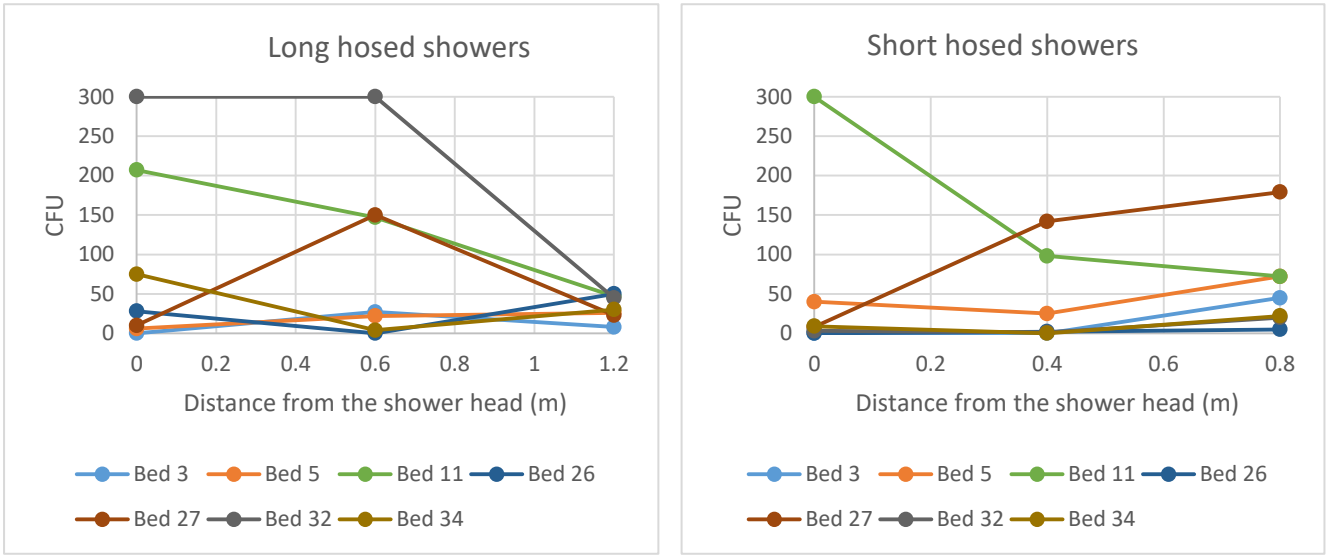


Figure 7.3. Comparison of *P. aeruginosa* biofilm growth patterns between long and short hosed showers

7.3.2. Crystal violet assay for biofilm quantification

7.3.2.1. Isolate selection criteria

In order to select the strains from hospital water for biofilm assay, VNTR results were checked. Three isolates having the most common VNTR clusters in the hospital were selected

as they are colonising many showers and might have an advantage in biofilm formation. Also the ones having similar VNTR profiles with the patients were prioritised:

19E8 (VNTR profile:12,3,6,3,2,4,13,5,7) is the most common VNTR profile found so far in the hospital – the same/very similar VNTR profiles were isolated from 7 different shower waters from 3 different wards. However, this isolate did not match any patient bacteraemia isolates typed.

19E21 (VNTR profile:12,5,3,2,6,1,7,4,13) was commonly found in shower waters (4 different shower waters from 3 different wards). This isolate had the same/highly similar VNTR profiles as two patient bacteraemia isolates.

19E44 (VNTR profile:12,3,3,6,6,3,8,2,8) was found in 5 different shower waters from 3 different wards and one handwash basin water sample. This isolate had the same/highly similar VNTR profiles as 3 patient bacteraemia isolates.

(For comparison VNTR profile of PAO1 is: 12,5,5,3,2,2,7,6,12)

Two isolates selected from the strains recovered from biofilm assay (Chapter 2, Section 2.2.7), as they were already growing biofilms in shower hoses (19B20 and 19B25).

Table 7.1. Metadata of DNA10-14.

Name	**Reference**	**DOSC**	**Source**	**Location**
DNA10	19E 8	27/02/2019	Shower water	Ward A - 12
DNA11	19E 21	11/12/2018	Shower water	Ward B – 5
DNA12	19E 44	10/12/2018	Shower water	Ward E - 15
DNA13	19B 20	06/12/2018	Biofilm from shower hose	Ward B – 34
DNA14	19B 25	06/12/2018	Biofilm from shower hose	Ward B – 41

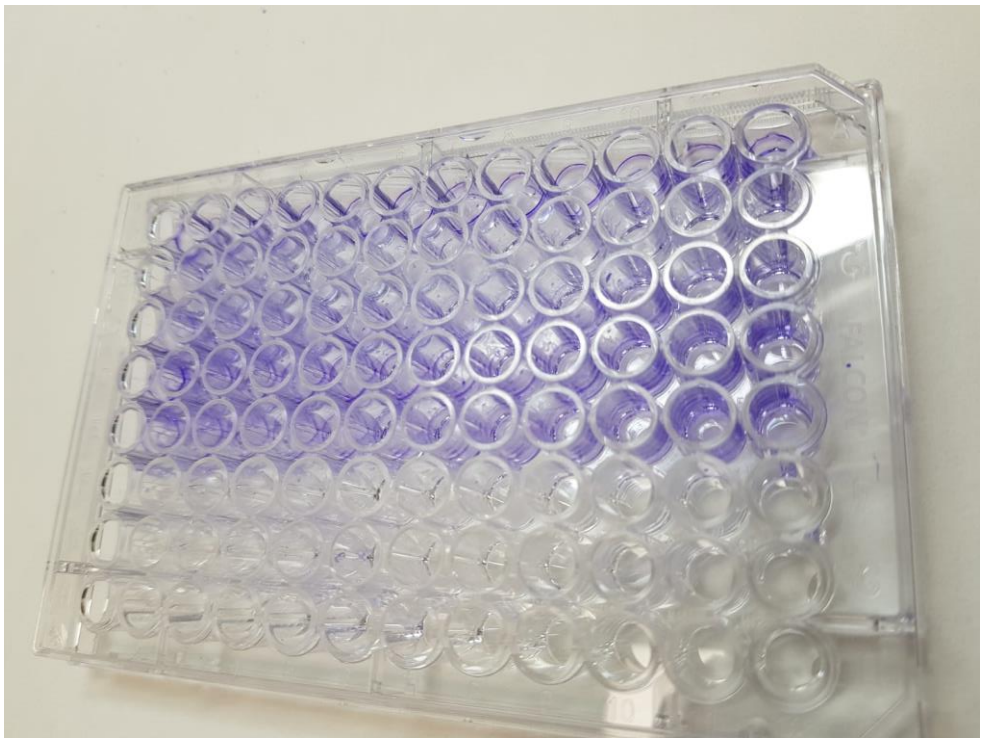


Figure 7.4. Biofilm formation in air-liquid interphase

Table 7.2. OD-570nm measurements

Descriptive Statistics

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
DNA10	12	.35	.76	.4595	.03197	.11075	.012
DNA11	12	.08	.26	.1688	.01534	.05314	.003
DNA12	12	.19	.33	.2296	.01372	.04753	.002
DNA14	12	.26	.53	.3154	.02584	.08952	.008
DNA15	12	.22	.63	.3266	.03995	.13839	.019
Valid N (listwise)	12						

Table 7.3. OD-595nm measurements

Descriptive Statistics

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
DNA10	12	.40	.88	.5274	.03721	.12890	.017
DNA11	12	.09	.29	.1907	.01786	.06186	.004
DNA12	12	.21	.38	.2612	.01573	.05448	.003
DNA13	12	.29	.61	.3595	.03004	.10405	.011
DNA14	12	.25	.72	.3718	.04640	.16072	.026
Valid N (listwise)	12						

Normality test was applied to check the distribution:

7.3.2.2. Normality test for 12 replicates of 5 strains, microtiter assay

Two of the well-known normality tests (Kolmogorov–Smirnov test and Shapiro–Wilk test) were applied to test the normality of results through SPSS [222]. Shapiro-Wilk's test ($p>.05$) showed that only DNA-11 OD_{595} measurements were normally distributed (results given in Table 7.4 below).

Table 7.4. Test of Normality Results of 12 replicates

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
DNA10	.210	12	.151	.802	12	.010
DNA11	.089	12	.200*	.969	12	.905
DNA12	.238	12	.059	.824	12	.018
DNA13	.357	12	.000	.620	12	.000
DNA14	.348	12	.000	.656	12	.000

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

DNA-10: Skewness: 1.998 Kurtosis: 4.996

DNA-11: Skewness: .028 Kurtosis: -.586

DNA-12: Skewness: 1.255 Kurtosis: .467

DNA-13: Skewness: 2.006 Kurtosis: 2.808

DNA-14: Skewness: 1.882 Kurtosis: 1.232

After assessing the high variability in results, potential reasons were investigated. Due to edge-effect (which can result in different readings of corners of the plate when compared to wells in the middle [223]) on 96 well plates, wells on the edges were left empty and 10 replicates were included for each isolate instead of 12.

7.3.2.3. Normality test for 10 replicates of 5 strains, microtiter assay

First and last column OD measurements were not included as they were observed to be outliers.

Shapiro-Wilk's test ($p>.05$) showed all OD_{595} measurements were normally distributed (results given in Table 7.5 below).

Table 7.5. Test of Normality and Descriptive Statistics Results of 10 Replicates

Table 7.5.1. Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
DNA10	.185	10	.200*	.919	10	.347
DNA11	.154	10	.200*	.965	10	.837
DNA12	.215	10	.200*	.856	10	.069
DNA13	.312	10	.006	.864	10	.086
DNA14	.198	10	.200*	.939	10	.541

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table 7.5.2. Descriptive Statistics

	N	Range	Minimum	Maximum	Mean		Std. Deviation	Variance	Skewness		Kurtosis	
	Statistic	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic	Statistic	Std. Error	Statistic	Std. Error
DNA10	10	.17	.40	.57	.4839	.01929	.06100	.004	.276	.687	-1.372	1.334
DNA11	10	.20	.09	.29	.1778	.01857	.05873	.003	.365	.687	.319	1.334
DNA12	10	.09	.21	.31	.2411	.00936	.02959	.001	1.332	.687	1.250	1.334
DNA13	10	.06	.29	.35	.3159	.00586	.01855	.000	1.112	.687	.852	1.334
DNA14	10	.12	.25	.37	.3044	.01136	.03592	.001	.512	.687	.028	1.334
Valid N (listwise)	10											

Box plots of 12 replicates in comparison with 10 replicates where edge effect (peripheral variations in optical quality) deleted is given in Appendix D.1.

In terms of biofilm formation from crystal violet assay (based on OD measurements) is as below:

DNA10 (19E8) > DNA13 (19B20) > DNA14 (19B25) > DNA12 (19E44) > DNA11 (19E21)

These 5 isolates were included in WGS analysis detailed in Chapter 5. SNP comparison:

For environmental - clinical isolate comparisons were made according to SNP difference matrix as below:

- DNA10 did not show close relatedness with our clinical isolates typed so far.
- DNA11 had <26 SNPs when compared with four of our *P. aeruginosa* bacteraemia isolates.

- DNA12 and DNA13 had <26 SNPs when compared with two of our *P. aeruginosa* bacteraemia isolates.
- DNA14 had <26 SNPs when compared with two of our *P. aeruginosa* bacteraemia isolates.

cup, *psl* and *pel* gene family (taking part in biofilm synthesis) presence and absences were studied through WGS data as shown in Table 7.6: WGS biofilm gene presence:

Table 7.6. *cup*, *psl* and *pel* gene family absence/presence

Name	**Biofilm gene characterisation**																				
	algD	**cupA1**	**cupA2**	**cupA3**	**cupA4**	**cupA5**	**cupB1**	**cupB2**	**cupB3**	**cupB4**	**cupB5**	**cupC1**	**cupC2**	**cupC3**	**pelA**	**pelB**	**pelC**	**pelD**	**pelE**	**pelF**	**pelG**	**pslD**
DNA10	algD	cupA1	cupA2	cupA3	cupA4	cupA5	NA	cupB2	cupB3	cupB4	cupB5	NA	NA	cupC3	pelA	pelB	pelC	pelD	pelE	pelF	pelG	pslD
DNA11	algD	cupA1	cupA2	cupA3	cupA4	cupA5	NA	cupB2	cupB3	cupB4	cupB5	NA	NA	cupC3	pelA	pelB	pelC	pelD	pelE	pelF	pelG	NA
DNA12	algD	cupA1	cupA2	cupA3	cupA4	cupA5	NA	cupB2	cupB3	NA	cupB5	NA	NA	cupC3	pelA	pelB	pelC	pelD	pelE	pelF	pelG	pslD
DNA13	algD	cupA1	cupA2	cupA3	cupA4	cupA5	NA	cupB2	cupB3	NA	cupB5	NA	NA	cupC3	pelA	pelB	pelC	pelD	pelE	pelF	pelG	pslD
DNA14	algD	cupA1	cupA2	cupA3	cupA4	cupA5	NA	cupB2	cupB3	cupB4	cupB5	NA	NA	cupC3	pelA	pelB	pelC	pelD	pelE	pelF	pelG	pslD

NA: Gene not available/not found

As seen in Table 7.6, all five strains had all the studied genes except *cupB1*, *cupC1* and *cupC2*; DNA11 was missing *pslD*; DNA12 and DNA13 were missing *cupB4* in addition.

SNP differences of these isolates were also studied to see if they were related. SNP distance matrix is given in Table 7.7.

Table 7.7. SNP distance matrix for isolates DNA10-14

SNP Distances	DNA10	DNA11	DNA12	DNA13	DNA14
DNA10	0	12984	16408	16409	24878
DNA11	12984	0	16422	16422	25176
DNA12	16408	16422	0	24	24666
DNA13	16409	16422	24	0	24665
DNA14	24878	25176	24666	24665	0

It was seen in Table 7.7 that DNA12 and DNA13 had 24 SNP differences, suggesting they are closely related. When their metadata in Table 7.1 is analysed, they were found to be from different location and origin: DNA12 is isolated from shower water in Ward E; DNA13 is isolated from the shower hose in Ward B. All the other isolates have >10k SNP difference, showing that they are genetically distant.

7.4. Discussion

7.4.1. Comparison of *P. aeruginosa* biofilm formation in shower hoses

Water stagnation in the hose might promote bacterial growth throughout the shower hose and this can lead to biofilm formation. For this purpose, biofilm was measured on 1 cm^2 pieces cut from proximal, central and distant parts of the shower hose. *P. aeruginosa* biofilm was obtained but no particular pattern was observed in amount of *P. aeruginosa* biofilm formed since patient usage of the showers cannot be controlled and the positioning of the U-loop where water stagnation occurs changes regarding to which position patients leave the showers. Although no clear outcomes relating U-loop water stagnation and biofilm growth was observed, it is well known that water and nutrient accumulation promotes biofilm growth, hence it is still suggested for patients not to hang the shower heads to the holders but let them freely suspend, allowing water to drain off. Laboratory-based controlled experiment searching further this water stagnation- biofilm growth was planned as future study.

7.4.2. Crystal violet assay for biofilm quantification

Different studies in literature on microtiter assay used 550nm, 570nm and 600nm for OD measurements [39,58,139]. In this study both 570nm and 595 nm OD measurements were done, 595 nm measures were used for normality tests, referring to literature and to make the results comparable through a collaboration with Dingdan Cui, PhD student from Eastman Dental Institute [196,224]. In the washing steps, O'Toole suggested turning the plate over, shaking the liquid out, submerging the plate in a tub of water, shaking the liquid out (many other groups followed this technique) whereas our collaborators in Eastman and some other groups pipette-wash the plate [139,225]. In this study, both of the techniques were tried. In the crystal violet pipette wash steps, it was visually observed that washed away liquid had further crystal violet dye even after 3-4 washes. The second technique was to wash the wells

several times with running PBS, shaking the liquid out, blotting the plate on paper towels until excess dye is washed off. When results of these two wash step changes were analysed through microplate readings, it was seen that method including pipette-wash gave higher optical density results with a greater variation than the O'Toole method. It was concluded that pipette wash might not have been enough to wash out the excess crystal violet and hence the dye attached to planktonic *P. aeruginosa* interfering the biofilm measurements and O'Toole method was chosen.

Another optimization of the results were done through eliminating edge effect of the optical readings from 96-well plate. It is known that wells of four corners of the microplate give different readings when compared to the middle wells [223]. Some groups put sterile water to outside wells to eliminate edge effect [45], we discarded the results on the outside wells which reduced the deviation in replicates as detailed in the Results.

7.4.3. VNTR – WGS – Crystal Violate Assay Comparison

Selection of five isolates for crystal violet assay was based on VNTR results as WGS results were not obtained at the start of the study. VNTR results put the importance of these five isolates by being the most prevalent environmental strains in the hospital and the WGS results supplemented this finding by showing their clinical importance through highly similar *P. aeruginosa* bacteraemia isolates. However, the isolate which gave the highest biofilm formation in crystal violet assay (DNA10) did not show any relatedness with 88 *P. aeruginosa* bacteraemia isolates typed. This could be because DNA10 is well adapted to produce biofilm in water systems but did not infect patients typed in this study. Further isolates DNA11-DNA14 all had <26 SNPs with at least four of the *P. aeruginosa* bacteraemia isolates typed, suggesting they might not be as good biofilm producers in the environment as DNA10 but are more important clinically in terms of transmitting to the patients. Although DNA12 and DNA13 came from different wards and origins (hose and water), their genetic relatedness could suggest transmission between ward water and shower systems.

Chapter 8. Main Discussion

8.1. Clinical importance of environmental *P. aeruginosa*

P. aeruginosa outbreaks associated with water in healthcare settings have been reported since 1983. The first healthcare water related cases reported were *P. aeruginosa* folliculitis associated with health spa and physiotherapy pools [226,227]. In 1990s, *P. aeruginosa* outbreaks associated with contaminated tap water in intensive care, paediatric and oncology units, water-baths and bath toys were reported [228–231]. In 2000s, *P. aeruginosa* outbreaks associated with healthcare waters continued to be reported and focus was put in risk assessment of healthcare water [142].

In the UK, environmental sources were investigated following *P. aeruginosa* outbreaks in neonatal units in Northern Ireland in 2011 and 2012 [83,232]. In 2013, HTM 04-01 published an addendum on advice for augmented care units for *P. aeruginosa* giving overview about the bacteria, different types of water outlets in healthcare settings and guidance on water sampling [233]. This led to detailed examination of water source contamination and risk assessments. When molecular analyses of clinical and environmental strains were performed, many transmission links were shown [94].

The Fingertips website of UKHSA makes public health data collection available online [234]. As detailed in Chapter 6, UCH had the highest hospital rates of *P. aeruginosa* bacteraemia when compared to other acute trusts in England in the financial year 2018-2019, which prompted this PhD. As of the completion of this thesis, Fingertips website showing the latest measures (updated on January 2023) shows that UCH still had the highest hospital onset *P. aeruginosa* bacteraemia in the financial year 2021-2022 [235]. Reasons of this high hospital-onset *P. aeruginosa* bacteraemia rate were investigated through epidemiological as well as microbiological studies. This thesis puts the focus on hospital shower waters and *P. aeruginosa* bacteraemia isolates. Selection of samples mainly relied on the samples taken for different purposes. Criteria of selection of samples could be improved by power calculation. Results of this studies suggest weekly environmental sampling which could improve the understanding of relatedness between environmental and clinical isolates. Previous environmental sampling from Environmental Research Laboratory showed high level contamination of hospital shower waters by *P. aeruginosa*; taps and sinks were rarely contaminated and only at low level. *P. aeruginosa* bacteraemia isolates were included because these are the only isolates stored by the reference laboratory (not wound, UTI, etc.

isolates). With closer sampling times, more pairs of clinical and environmental isolates were found; handwash basin water and drain, sink water and drain and shower drain isolates were collected and included in the analyses where possible.

8.2. Molecular Analysis of Environmental *P. aeruginosa* using VNTR and WGS

Water sampling data of hospital outlets was available from 2014 in the Environmental Research Laboratory database. Weekly/once in two weeks sampling was performed through longitudinal sampling of twenty randomly selected showers over seven months (Chapter 3), which showed presence and persistence of *P. aeruginosa* despite remedial actions. Once a shower is colonised, the organism is difficult to eradicate. In total, 12/20 showers were contaminated at the beginning of the study, and, regardless of the remedial actions taken, 14/20 showers were found to be colonized at the last sampling day of the study. Sampling every six-months as given in HTM guidelines was not sufficiently frequent [69]. Showers can be colonized shortly after one sampling date and patients exposed to *P. aeruginosa* until the next routine sampling date. VNTR analysis of these isolates showed strains having same VNTR profiles were isolated 3 months apart from the same shower. WGS including a broader timeline of isolates revealed that the same shower water strain from a haematology ward (<3 SNP differences) was isolated for 39 months, spanning 2015-2019. Between those years, antimicrobial shower heads were introduced to this ward, they were changed with pristine ones once in every quarter year; drains were thought to be the contamination source; and hence shower hoses were shortened. Many disinfection strategies were tried as well as daily cleaning procedures, but none was effective in eradicating persistent *P. aeruginosa* strains. In cases where some of these interventions were helpful, they were effective for only short periods.

8.2.1. SNP cut-off for *P. aeruginosa* relatedness

WGS not only showed persistence of the same *P. aeruginosa* strain with higher confidence than VNTR but also showed clinical – shower water pairs of the same strain isolated years apart [94]. Although there is no agreed SNP cut-off in literature to report relatedness, when the SNP distance matrix of 190 isolates was analysed by matching the water samples collected from rooms/bathrooms and *P. aeruginosa* bacteraemia patients stayed in those rooms, <6 SNPs was a epidemiologically relevant cut-off. The rest of the strains isolated from same room had >36 SNP differences so were not closely related. This is a preliminary analysis and further work by statistical probability methods is suggested. In literature, Youden's index is used for SNP cut off calculations and has been reported to be a sensitive,

accurate and specific method [110]. Other groups working on relatedness of clinical and environmental *P. aeruginosa* isolates reported 4 SNPs, 14 SNPs and 26 SNPs as relatedness cut-offs [110–112].

When analysing SNP differences from subsets of the main dataset, it was observed that when the analysis is run for different number of isolates, pairwise SNP differences changed. Different sets of samples were inferring from core genomic regions of different length. To avoid this, main SNP matrix of 190 isolates were used for all of the analyses. When subsets of the same wards and sample type (shower water, sink water, drain, etc.) were analysed, they were filtered from the main matrix.

Outcomes drawn from phylogenetic trees in Chapter 5 and SNP differences explained above did not take into account the effect of recombination. The development of new tools (e.g. BEAST, TreeTime, etc.) can make accurate estimates of evolutionary rates and are designed as future study.

8.2.2. Quality and Quantity of DNA for WGS Purposes

One of the factors affecting WGS analysis is the quality and quantity (yield) of DNA extraction. Pathogens Genomic Unit (PGU) required a total input of 101-200 ng DNA in 50 µL starting volume which was measured by Qubit. Before sending the isolates to PGU, specifications of DNA extraction were checked by Nanodrop. The reference value for primary measure pure DNA Absorbance 260/280 ratio is ~1.8 and, 260/230 ratio is within the range 1.8 – 2.2 [236]. Random selection of 16 DNA extract from different batches of extractions had absorbance 260/280 ratio within 1.44 – 1.77 range which were deemed satisfactory. However, absorbance 260/230 values were found to be within 0.1 – 1.35 range which were lower than the reference value and within a diverse range. When the reasons were investigated, it was found that when the elution buffer used at the final stage of DNA extraction was a saline solution, the stability of absorbance 260/230 value was affected by increasing the salt concentration [237]. Zymo DNA elution buffer used in these DNA extractions contained 10 mM Tris, pH 8.5, 0.1 mM EDTA. Disodium salt used in preparation of EDTA could be a reason for an increase in salt concentration affecting absorbance 260/230 ratio. A further study listing EDTA as a common contaminant also showed that absorbance 260/230 measurements are unreliable as EDTA was already present in the DNA elution buffer used, hence cannot be classified as a contaminant [238].

8.2.3. ONT Sequencing

After Illumina sequencing, ONT sequencing was run on a subset of isolates (n=28) which were found to be important in terms of transmission, AMR and biofilm gene carriage. Advantages of ONT sequencing is to enable long reads through real-time analysis and to further assemble the complete genome of the strains. Analysis of ONT sequencing results is planned as future study [239].

8.3. Shower head filter intervention to prevent *P. aeruginosa* exposure

Whole genome sequencing showed the importance of environmental strains in hospital-acquired infections. One of the many interventions to prevent exposure of patients to *P. aeruginosa* was the implementation of hollow fibre filtered shower heads to augmented wards (Chapter 4). At the beginning of this PhD, during 2018, some showers had membrane filters (Chapter 3). The disadvantage of filters is their cost and having relatively short shelf lives (up to 120 days). Hollow fibre filters were reported to have several advantages against membrane filters such as being more effective for microbe recovery; having extended lifespan and low operational cost [175,176]. There are various companies producing hollow-fibre filters with slightly different specifications [171,173,174,240,241]. Even during the expected shelf-life membrane pores or fibres can get blocked, such that filters need to be replaced before their expiry.

Filters in general have specific requirements such as minimum pressure and maximum amount of bacterial load to work efficiently. However, in UCH, showers in higher floors were found to have <1 bar water pressure, below the lower limit of the shower filter in place. The pressure was insufficient to enable enough water flowrate for good showering practice for patients. Patients tended to remove the shower heads, hoping to get greater water flow, resulting in exposing themselves to unfiltered shower water. If a filtered shower head is removed, it should not be placed back but replaced with a new filtered head (as per manufacturer guidelines) [24]. However, this was difficult to control in a hospital setting as patients / untrained personnel can put the same shower head back in place and it would not be known during the next sampling visit if the shower heads had been removed during usage. Removing and placing shower heads back can also result in cross-contamination.

When assessing sampling frequency of HTM guidelines, operational costs should also be considered. According to the current guidelines, when a healthcare water source is found to

be contaminated with >10CFU/100 mL, risk assessment should be made, outlet removed from service, retested with pre-/post flush, remedial action taken, and the outlet retested at 3 days, 2 and 4 weeks. If any of these steps is not effective in decontaminating *P. aeruginosa*, another remediation should be done, and re-tested to see if *P. aeruginosa* is cleared [69]. When the number of healthcare water sources such as showers, sinks, handwash basins, etc. and the bed capacity of large hospitals are taken into account, it will be appreciated that operational costs are high. In order to justify the costs of remedial actions and shower replacements, clear evidence demonstrating the role of hospital water (e.g. sinks and showers) in hospital acquired *P. aeruginosa* bacteraemia needs to be shown. Studies comparing relatedness of environmental and clinical *P. aeruginosa* showed important evidence of transmission by molecular analyses such as PFGE, VNTR and WGS [82,94–96,163]. Although the costs are not high when judged by patient survival, this thesis suggests more attention and financial resource should be put in this area.

8.4. Antimicrobial Resistance Properties of *P. aeruginosa*

Another important aspect of environmental and clinical *P. aeruginosa* isolates are their antimicrobial resistance properties. As described in Chapter 3 and Chapter 5, phenotypic antibiotic susceptibility tests were tested by disc diffusion assay and genotypes were analysed by WGS. EUCAST set breakpoints for phenotypic antibiotic susceptibility tests which used to place results in this thesis into Sensitive/Intermediate/Resistant categories. At the start of this thesis, EUCAST breakpoints version 8.1 (valid from 18.05.2018) was in use. However, EUCAST breakpoints were revised every year [15]. A major change in breakpoints and susceptibility definitions occurred in version 10.0 (valid from 01.01.2020). The previous sensitive and intermediate categories were combined, and definition was changed to 'susceptible, increased exposure'. This affected interpretation of TZP, CAZ, IMI, CIP, PRL, FEP and TCC susceptibilities by moving 'S' category to 'I' as well as CN as no breakpoints were given for this antibiotic due to insufficient evidence for *P. aeruginosa*. The new 'I' definition was put in place to clear out the ambiguity of the old intermediate category [242]. However, usage of the same 'I' letter for a different definition led to confusion at the beginning and made it difficult to compare the results from different years especially because many papers report the antibiotic susceptibility test outcomes as S, I, R but not with the measured disc zones. In the ASTs included in this thesis, all the disc-zone were measured and then S, I, R interpretations were made. Hence, when the EUCAST breakpoints were changes,

interpretations were revised accordingly. Using EUCAST breakpoints is in alignment with the data presented by ECDC (European Centre for Disease Prevention and Control). CLSI (Clinical and Laboratory Standards Institute) is widely used in the United States and outside Europe [150].

8.5. Biofilm Properties of *P. aeruginosa*

In this thesis, biofilm formed on clinical shower hoses was extracted through a novel protocol (designed previously in-house by our research laboratory) which included three wash steps and vigorously shaking with glass beads (Chapter 2). Further optimization of this method was possible by adjusting the wash steps and vortex durations to compare if the resulting CFUs change. This method used to compare the counts of *P. aeruginosa* along the length of the shower hose with the assumption that water stagnation promoted biofilm growth. Three sets of ten showers were included in the study, but no clear patterns of count comparisons were found. Although the positioning of the shower heads at the time of the hose collection were recorded (Appendix D.2) , these might not be representative of how the shower heads were left by the patients . Hence, a laboratory-based controlled study was suggested for future study. The study suggested quarterly shower head & hose changes were appropriate remediation actions (Chapter 3) but should be accompanied by disinfection strategies.

In the laboratory-based studies for quantification of biofilm, a microplate assay was used. However due to limited time, only five environmental strains were included in the analysis. Future work is planned to include more isolates, especially clinical ones. Further trials of the protocol could have been tried to better optimize the methods, as many groups used different incubation platforms to grow *P. aeruginosa* (rocking or stabile) and times, different types and concentration of solvents to solubilize crystal violet (acetic acid or ethanol) and different spectrophotometer wavelengths (550nm, 570nm and 600nm) [39,60,139,243]. Initial outcomes regarding biofilm gene presence (*alg*, *pel*, *psl*, and *cup* genes) through WGS data were completed but further detailed analysis including more genes involved in biofilm formation is planned as future study.

8.6. Recommendations

Overall, findings of this thesis suggested that Hospital Water Safety Groups should not rely on 6 monthly water sampling based on local risk assessment as suggested in HTM01 guidelines, but more frequent sampling is needed. Where products such as the antimicrobial silver-impregnated shower head/hose units used in this study are adopted, quarterly shower head and hose changes should be accompanied by disinfection and to be closely monitored to see if the showers were cleared and remained free of contamination. As suggested by the case-control study, enhanced and regular disinfection (e.g. using peracetic acid) and monitoring of high risk areas more frequently should be considered to minimise contamination of wounds and damaged mucous membranes. Neutropenic patients should be checked on admission and during their hospital stay for presence of *P. aeruginosa* in urine as this was found to be a risk factor for acquisition of *P. aeruginosa* bacteraemia.

Although pre and post flush water sample testing was beyond the scope of this thesis, previous studies of Environmental Research Laboratory showed significant reduction in *P. aeruginosa* numbers in post flush sampling. Hence recommendation for patients or staff run the shower water for at least two minutes before use to ensure patient safety.

During water sample collection, the upper floors of the hospital were found to have slower water flow rates due to low water pressure (<1 bar). The main disadvantage was that shower manufacturer guidelines, e.g. for filtered shower heads, only guarantee effective filtering at pressures higher than 1 bar. In conclusion, unless the local water pressure can be mechanically boosted, the findings in this thesis indicate that immune-suppressed patients should not be accommodated in wards with less than 1 bar water pressure as the showers cannot be filtered.

At the start of the PhD, we hypothesised that the increase in *P. aeruginosa* bacteraemia in the hospital in 2017/8 was caused by spread of contamination of showers. Sequencing analysis following environmental and clinical sample collection demonstrated the presence of indistinguishable isolates of *P. aeruginosa* in hospital showers and in patient's blood. In some cases, the showers were colonized prior to the development of bacteraemia and plausible routes of transmission such as development of mucous membrane and urinary tract infection have been identified through case control study. In some other cases, clinical isolates were collected prior to environmental contamination. However, the evidence remains circumstantial and gaps in sampling makes the analysis difficult.

In terms of shower types, we hypothesised that the type of shower, arrangement of hose, shower head holder and shower can minimize the formation of *P. aeruginosa* biofilm in the hose and reduce the spread of microorganisms from the drain. Shower heads and hoses were collected during quarterly changes, biofilm studies were conducted alongside the hose, however there was no evidence to support the hypothesis. This thesis findings did not show changes in arrangement of the shower head and hose were beneficial but it indicated that disinfection methods should be investigated further. As controlled experiments cannot be conducted in the hospital environment and patient and staff behaviour have effect on shower usage and cleaning procedures; laboratory-based experiments to test the hypothesis is recommended for future study.

When shower contamination and locations of *P. aeruginosa* bacteraemia patients were taken into account, we hypothesised that the risk of *P. aeruginosa* infection is dependent on patient characteristics and the proximity of the patient bed to contaminated sites such as showers and drains in the hospital. Case control study was conducted for this purpose which identified urinary tract infection as a significant risk factor that could be helpful in prevention. Acquisition from the showering was thought to be a plausible link but it could not be analysed in the regression analyses due to gaps in historical water sample collection.

Studies undertaken in this thesis demonstrated that neither antimicrobial nor filtered shower heads could be assumed to provide safe hospital water. The Environmental Research Laboratory continued providing results of water sampling which showed persistence of shower water contamination. After the completion of sample collection for this thesis, thermal disinfection showers were put in place in haematology wards. This included a thermal bypass valve to flush hot water (60°C) to shower outlets to remove *P. aeruginosa* [52].

9. Strengths and Weaknesses

This thesis investigates many aspects of *P. aeruginosa* in hospital environment by putting the focus on shower water and its relatedness to clinical *P. aeruginosa* isolates. End to end data is provided from sample collection to sequencing (by VNTR and WGS) of relevant isolates. AMR and biofilm forming properties of the isolates are investigated by both phenotypic methods and genotypic analyses. When doing the detailed analysis of *P. aeruginosa* strains by laboratory-based methods as well as bioinformatics tools, the previous and current *P. aeruginosa* infections and bacteraemia rates in the hospital were not overlooked. Detailed demographics analysis was performed as the basis of case control study. Effects of interventions and remedial actions on water contamination were analysed and inferences made by comparing *P. aeruginosa* bacteraemia rates.

The main limitation in searching for relatedness between environmental and clinical isolates is that the main water sample collection had been every six months as per HTM 04-01 guidelines. Many of the environmental samples had a long difference in time of collection when compared to their clinical pairs. As it is not feasible to collect samples from all hospital outlets continuously, the sampling was targeted, e.g. collecting water samples from vicinity of patient locations where *P. aeruginosa* bacteraemia was reported. This led to environmental – clinical pairs where the clinical isolate preceded the environmental isolate, but this could also be because no earlier environmental sample was collected. This limitation was also the main reason not being able to use shower water contamination data as a variable in case control study.

Recombination rate was not measured or estimated in this study. If there is a high rate of recombination, number of mutations over a long time would be higher than with other species which introduces uncertainty into the analysis of strain differences. Analysis of the samples by using TreeTime tool (using maximum likelihood approach[131]) is planned as a future study and discussed with bioinformaticians. Once completed, phylogenetic trees obtained by both methods (the current analysis described in Chapter 5 and the future analysis considering evolutionary rates and recombination [130]) will be compared.

Time limits and delays due to Covid19 pandemic affected the following aspects of this thesis: Analysis of Illumina sequencing data through SNP differences, AMR and biofilm gene markers were completed but more detailed analysis is needed (Chapter 5). Analysis of ONT sequencing is also planned. The second part of case control study which included analysis of

bacteraemia control group was not completed within PhD timeframe. Analysing recombination events especially in drains were planned at the beginning of the PhD but as the initial projects opened up new questions which were investigated in detail, there was insufficient time for recombination analysis.

References

[1] Kelly KN, Monson JRT. Hospital-acquired infections. Surg 2012;30:640–4. https://doi.org/10.1016/j.mpsur.2012.10.005.

[2] G. Ducel, J. Fabry LN. Prevention of hospital-acquired infections. A practical guide. 2nd edition. World Heal Organ 2002. https://doi.org/WHO/CDS/CSR/EPH/2002.12.

[3] Battaglia CC, Hale K. Hospital-Acquired Infections in Critically Ill Patients With Cancer. J Intensive Care Med 2019;34:523–36. https://doi.org/10.1177/0885066618788019.

[4] Suetens C, Hopkins S, Kolman J, Diaz Högberg L. Point prevalence survey of healthcare-associated infections and antimicrobial use in European acute care hospitals 2011. https://doi.org/10.2900/86011.

[5] Khan HA, Baig FK, Mehboob R. Nosocomial infections: Epidemiology, prevention, control and surveillance. Asian Pac J Trop Biomed 2017. https://doi.org/10.1016/j.apjtb.2017.01.019.

[6] Kramer A, Schwebke I, Kampf G. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. BMC Infect Dis 2006;6:1–8. https://doi.org/10.1186/1471-2334-6-130/TABLES/3.

[7] Otter JA, Yezli S, Salkeld JAG, French GL. American Journal of Infection Control Evidence that contaminated surfaces contribute to the transmission of hospital pathogens and an overview of strategies to address contaminated surfaces in hospital settings. Am J Infect Control 2013;41:S6–11. https://doi.org/10.1016/j.ajic.2012.12.004.

[8] Pokharna H, Chen AY. The Hospital and Ambulatory Care Environment. Mol. Tech. Study Hosp. Infect., John Wiley and Sons; 2011, p. 1–21. https://doi.org/10.1002/9781118063842.ch1.

[9] Wu W, Jin Y, Bai F, Jin S. Chapter 41 – Pseudomonas aeruginosa. Mol. Microbiol, 2015, p. 753–67. https://doi.org/10.1016/B978-0-12-397169-2.00041-X.

[10] Moradali MF, Ghods S, Rehm BHA. Pseudomonas aeruginosa lifestyle: A paradigm for adaptation, survival, and persistence. Front Cell Infect Microbiol 2017;7. https://doi.org/10.3389/fcimb.2017.00039.

[11] Jenny M, Kingsbury J, Edu M. iMedPub Journals Journal of Biology and Medical Research Properties and Prevention: A Review of Pseudomonas aeruginosa. vol. 2. 2018.

[12] Bennett, John E., Dolin, Raphael, Blaser MJ. Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. 8th ed. Philadelphia:United States: Elsevier; 2015.

[13] Cutruzzolà F, Arese M, Ranghino G, van Pouderoyen G, Canters G, Brunori M. Pseudomonas aeruginosa cytochrome C(551): probing the role of the hydrophobic patch in electron transfer. J Inorg Biochem 2002;88:353–61. https://doi.org/10.1016/s0162-0134(01)00390-7.

[14] Sagar A. Oxidase Test- Principle, Uses, Procedure, Types, Result Interpretation, Examples and Limitations. Microbiol Notes 2014;5:1–6.

[15] European Committee on Antimicrobial Susceptibility Testing - Breakpoint tables for interpretation of MICs and zone diameters. 2019.

[16] Palzkill T. Metallo-β-lactamase structure and function. Ann N Y Acad Sci 2013;1277:91–104. https://doi.org/10.1111/j.1749-6632.2012.06796.x.

[17] Cho H, Uehara T, Bernhardt TG. Beta-lactam antibiotics induce a lethal malfunctioning of the bacterial cell wall synthesis machinery. Cell 2014;159:1300–11.

https://doi.org/10.1016/j.cell.2014.11.017.

[18] Zhao WH, Hu ZQ. β-Lactamases identified in clinical isolates of Pseudomonas aeruginosa. Crit Rev Microbiol 2010;36:245–58. https://doi.org/10.3109/1040841X.2010.481763.

[19] Laudy AE, Róg P, Smolinska-Król K, Ćmiel M, Søoczynska A, Patzer J, et al. Prevalence of ESBL-producing Pseudomonas aeruginosa isolates in Warsaw, Poland, detected by various phenotypic and genotypic methods. PLoS One 2017;12. https://doi.org/10.1371/journal.pone.0180121.

[20] Strateva T, Yordanov D. Pseudomonas aeruginosa - A phenomenon of bacterial resistance. J Med Microbiol 2009;58:1133–48. https://doi.org/10.1099/jmm.0.009142-0.

[21] Bassetti M, Vena A, Croxatto A, Righi E, Guery B. How to manage Pseudomonas aeruginosa infections. Drugs Context 2018;7. https://doi.org/10.7573/dic.212527.

[22] Poulikakos P, Falagas ME. Aminoglycoside therapy in infectious diseases. Expert Opin Pharmacother 2013;14:1585–97. https://doi.org/10.1517/14656566.2013.806486.

[23] Speltini A, Sturini M, Maraschi F, Profumo A. Fluoroquinolone antibiotics in environmental waters: Sample preparation and determination. J Sep Sci 2010;33:1115–31. https://doi.org/10.1002/jssc.200900753.

[24] Gellatly SL, Hancock REW. Pseudomonas aeruginosa: New insights into pathogenesis and host defenses. Pathog Dis 2013;67:159–73. https://doi.org/10.1111/2049-632X.12033.

[25] Giltner CL, Habash M, Burrows LL. Pseudomonas aeruginosa Minor Pilins Are Incorporated into Type IV Pili. J Mol Biol 2010;398:444–61. https://doi.org/10.1016/J.JMB.2010.03.028.

[26] Alhazmi A. Pseudomonas aeruginosa-Pathogenesis and Pathogenic Mechanisms. Int J Biol 2015;7. https://doi.org/10.5539/ijb.v7n2p44.

[27] Engel J, Balachandran P. Role of Pseudomonas aeruginosa type III effectors in disease. Curr Opin Microbiol 2009;12:61–6. https://doi.org/10.1016/j.mib.2008.12.007.

[28] Casilag F, Lorenz A, Krueger J, Klawonn F, Weiss S, Häussler S. LasB elastase of Pseudomonas aeruginosa acts in concert with alkaline protease AprA to prevent flagellin-mediated immune recognition. Infect Immun 2015;84:162–71. https://doi.org/10.1128/IAI.00939-15.

[29] Köhler T, Donner V, Van Delden C. Lipopolysaccharide as Shield and Receptor for R-Pyocin-Mediated Killing in Pseudomonas aeruginosa †. J Bacteriol 2010;192:1921–8. https://doi.org/10.1128/JB.01459-09.

[30] Fothergill JL, Winstanley C, James CE. Novel therapeutic strategies to counter Pseudomonas aeruginosa infections. Expert Rev Anti Infect Ther 2012;10:219–35. https://doi.org/10.1586/eri.11.168.

[31] Michel-Briand Y, Baysse C. The pyocins of Pseudomonas aeruginosa. Biochimie 2002;84:499–510. https://doi.org/10.1016/S0300-9084(02)01422-0.

[32] Mojesky AA. Ecological, evolutionary, and molecular mechanisms driving pyocin diversity in pseudomonas aeruginosa. 2021. https://doi.org/10.18297/etd/3598.

[33] Di Lorenzo F, Silipo A, Bianconi I, Lore' NI, Scamporrino A, Sturiale L, et al. Persistent cystic fibrosis isolate Pseudomonas aeruginosa strain RP73 exhibits an under-acylated LPS structure responsible of its low inflammatory activity. Mol Immunol 2015. https://doi.org/10.1016/j.molimm.2014.04.004.

[34] Streeter K, Katouli M. Pseudomonas aeruginosa: A review of their Pathogenesis and Prevalence in Clinical Settings and the Environment. Infect Epidemiol Med 2016 Winter 2016;2:25–32. https://doi.org/10.18869/modares.iem.2.1.25.

[35] Lam JS, Taylor VL, Islam ST, Hao Y, Kocíncová D. Genetic and functional diversity of Pseudomonas aeruginosa lipopolysaccharide. Front Microbiol 2011;2:118. https://doi.org/10.3389/FMICB.2011.00118/BIBTEX.

[36] Minandri F, Imperi F, Frangipani E, Bonchi C, Visaggio D, Facchini M, et al. Role of Iron Uptake Systems in Pseudomonas aeruginosa Virulence and Airway Infection 2016. https://doi.org/10.1128/IAI.00098-16.

[37] Nguyen AT, Oglesby-Sherrouse AG. Spoils of war: iron at the crux of clinical and ecological fitness of Pseudomonas aeruginosa. BioMetals 2015 283 2015;28:433–43. https://doi.org/10.1007/S10534-015-9848-6.

[38] Yu S, Wei Q, Zhao T, Guo Y, Ma LZ. A survival strategy for Pseudomonas aeruginosa that uses exopolysaccharides to sequester and store iron to stimulate psl-dependent biofilm formation. Appl Environ Microbiol 2016;82:6403–13. https://doi.org/10.1128/AEM.01307-16/ASSET/2A986370-24AA-4E40-A492-31A357F16FD4/ASSETS/GRAPHIC/ZAM9991174980008.JPEG.

[39] Berlutti F, Morea C, Battistoni A, Sarli S, Cipriani P, Superti F, et al. Iron availability influences aggregation, biofilm, adhesion and invasion of Pseudomonas aeruginosa and Burkholderia cenocepacia. Int J Immunopathol Pharmacol 2005;18:661–70. https://doi.org/10.1177/039463200501800407.

[40] Rasamiravaka T, Labtani Q, Duez P, El Jaziri M. The formation of biofilms by pseudomonas aeruginosa: A review of the natural and synthetic compounds interfering with control mechanisms. Biomed Res Int 2015;2015. https://doi.org/10.1155/2015/759348.

[41] Schultz G. Schultz, G. WUWHS 2008. https://www.researchgate.net/figure/Five-stages-of-biofilm-development-1-initial-attachment-2-irreversible_fig1_314257428 (accessed December 12, 2019).

[42] Colvin KM, Irie Y, Tart CS, Urbano R, Whitney JC, Ryder C, et al. The Pel and Psl polysaccharides provide Pseudomonas aeruginosa structural redundancy within the biofilm matrix. Environ Microbiol 2012;14:1913–28. https://doi.org/10.1111/j.1462-2920.2011.02657.x.

[43] Tseng BS, Reichhardt C, Merrihew GE, Araujo-Hernandez SA, Harrison JJ, MacCoss MJ, et al. A biofilm matrix-associated protease inhibitor protects Pseudomonas aeruginosa from proteolytic attack. MBio 2018;9. https://doi.org/10.1128/mBio.00543-18.

[44] De Kievit TR. Quorum sensing in Pseudomonas aeruginosa biofilms. Environ Microbiol 2009;11:279–88. https://doi.org/10.1111/j.1462-2920.2008.01792.x.

[45] Baker P, Hill PJ, Snarr BD, Alnabelseya N, Pestrak MJ, Lee MJ, et al. Exopolysaccharide biosynthetic glycoside hydrolases can be utilized to disrupt and prevent Pseudomonas aeruginosa biofilms. Sci Adv 2016;2:1–21. https://doi.org/10.1126/sciadv.1501632.

[46] Hentzer M, Teitzel GM, Balzer GJ, Heydorn A, Molin S, Givskov M, et al. Alginate overproduction affects pseudomonas aeruginosa biofilm structure and function. J Bacteriol 2001;183:5395–401. https://doi.org/10.1128/JB.183.18.5395-5401.2001.

[47] Cao B, Christophersen L, Kolpen M, Jensen PØ, Sneppen K, Høiby N, et al. Diffusion Retardation by Binding of Tobramycin in an Alginate Biofilm Model 2016.

https://doi.org/10.1371/journal.pone.0153616.

[48] Harmsen M, Yang L, Pamp SJ, Tolker-Nielsen T. An update on Pseudomonas aeruginosa biofilm formation, tolerance, and dispersal. FEMS Immunol Med Microbiol 2010;59:253–68. https://doi.org/10.1111/j.1574-695X.2010.00690.x.

[49] Díaz De Rienzo MA, Stevenson PS, Marchant R, Banat IM. Pseudomonas aeruginosa biofilm disruption using microbial surfactants. J Appl Microbiol 2016;120:868–76. https://doi.org/10.1111/JAM.13049.

[50] Qu L, She P, Wang Y, Liu F, Zhang D, Chen L, et al. Effects of norspermidine on Pseudomonas aeruginosa biofilm formation and eradication. Microbiologyopen 2016;5:402–12. https://doi.org/10.1002/MBO3.338.

[51] Parkes LO, Hota SS. Sink-Related Outbreaks and Mitigation Strategies in Healthcare Facilities. Curr Infect Dis Rep 2018;20:1–14. https://doi.org/10.1007/S11908-018-0648-3/FIGURES/1.

[52] Yui S, Karia K, Ali S, Muzslay M, Wilson P. Thermal disinfection at suboptimal temperature of Pseudomonas aeruginosa biofilm on copper pipe and shower hose materials. J Hosp Infect 2021;117:103–10.

[53] Fusch C, Pogorzelski D, Main C, Meyer CL, El Helou S, Mertz D. Self-disinfecting sink drains reduce the Pseudomonas aeruginosa bioburden in a neonatal intensive care unit. Acta Paediatr 2015;104:e344–9. https://doi.org/10.1111/APA.13005.

[54] Guillon A, Fouquenet D, Morello E, Henry C, Georgeault S, Si-Tahar M, et al. Treatment of Pseudomonas aeruginosa Biofilm Present in Endotracheal Tubes by Poly-L-Lysine. Antimicrob Agents Chemother 2018;62. https://doi.org/10.1128/AAC.00564-18/ASSET/2446BDD1-D8C8-4A33-8E79-D00CEB565BDC/ASSETS/GRAPHIC/ZAC0111875490004.JPEG.

[55] Lee SW, Phillips KS, Gu H, Kazemzadeh-Narbat M, Ren D. How microbes read the map: Effects of implant topography on bacterial adhesion and biofilm formation. Biomaterials 2021;268:120595. https://doi.org/10.1016/J.BIOMATERIALS.2020.120595.

[56] Moore G, Walker J. Presence and Control of Legionella pneumophila and Pseudomonas aeruginosa Biofilms in Hospital Water Systems. Biofilms Infect. Prev. Control A Healthc. Handb., Elsevier Inc.; 2014, p. 311–37. https://doi.org/10.1016/B978-0-12-397043-5.00017-7.

[57] Ruhal R, Antti H, Rzhepishevska O, Boulanger N, Barbero DR, Wai SN, et al. A multivariate approach to correlate bacterial surface properties to biofilm formation by lipopolysaccharide mutants of Pseudomonas aeruginosa. Colloids Surfaces B Biointerfaces 2015;127:182–91. https://doi.org/10.1016/j.colsurfb.2015.01.030.

[58] Rzhepishevska O, Limanska N, Galkin M, Lacoma A, Lundquist M, Sokol D, et al. Characterization of clinically relevant model bacterial strains of Pseudomonas aeruginosa for anti-biofilm testing of materials. Acta Biomater 2018;76:99–107. https://doi.org/10.1016/j.actbio.2018.06.019.

[59] Guo K, Freguia S, Dennis PG, Chen X, Donose BC, Keller J, et al. Effects of surface charge and hydrophobicity on anodic biofilm formation, community composition, and current generation in bioelectrochemical systems. Environ Sci Technol 2013;47:7563–70. https://doi.org/10.1021/ES400901U/SUPPL_FILE/ES400901U_SI_001.PDF.

[60] Rzhepishevska O, Hakobyan S, Ruhal R, Gautrot J, Barbero D, Ramstedt M. The surface charge of anti-bacterial coatings alters motility and biofilm architecture †. Cite This Biomater Sci 2013;1:589. https://doi.org/10.1039/c3bm00197k.

[61] Kao WK, Gagnon PM, Vogel JP, Chole RA. Surface charge modification decreases Pseudomonas aeruginosa adherence in vitro and bacterial persistence in an in vivo implant model. Laryngoscope 2017;127:1655–61. https://doi.org/10.1002/lary.26499.

[62] Flickinger ST, Copeland MF, Downes EM, Braasch AT, Tuson HH, Eun Y-J, et al. Quorum Sensing between Pseudomonas aeruginosa Biofilms Accelerates Cell Growth. J Am Chem Soc 2011;133:5966–75. https://doi.org/10.1021/ja111131f.

[63] Maura D, Hazan R, Kitao T, Ballok AE, Rahme LG. Evidence for direct control of virulence and defense gene circuits by the pseudomonas aeruginosa quorum sensing regulator, MvfR. Sci Rep 2016;6. https://doi.org/10.1038/srep34083.

[64] Mukherjee S, Moustafa D, Smith CD, Goldberg JB, Bassler BL. The RhlR quorum-sensing receptor controls Pseudomonas aeruginosa pathogenesis and biofilm development independently of its canonical homoserine lactone autoinducer. PLOS Pathog 2017;13:e1006504. https://doi.org/10.1371/journal.ppat.1006504.

[65] Bodelón G, Montes-García V, López-Puente V, Hill EH, Hamon C, Sanz-Ortiz MN, et al. Detection and imaging of quorum sensing in Pseudomonas aeruginosa biofilm communities by surface-enhanced resonance Raman scattering. Nat Mater 2016;15:1203–11. https://doi.org/10.1038/nmat4720.

[66] Stoodley P, Cargo R, Rupp CJ, Wilson S, Klapper I. Biofilm material properties as related to shear-induced deformation and detachment phenomena 2002;29:361–7.

[67] Exner M, Kramer A, Lajoie L, Gebel J, Engelhart S, Hartemann P. Prevention and control of health care-associated waterborne infections in health care facilities. Am J Infect Control 2005;33:S26–40. https://doi.org/10.1016/j.ajic.2005.04.002.

[68] WHO. Guidelines for Drinking-water Quality Third Edition. 2008.

[69] Department of Health. Health Technical Memorandum 04-01: Safe water in healthcare premises Part B: Operational management. 2016.

[70] Department of Health. Health Technical Memorandum 04-01: Safe water in healthcare premises. Part C: Pseudomonas aeruginosa – advice for augmented care units. 2016.

[71] Walker J, Moore G. Pseudomonas aeruginosa in hospital water systems: Biofilms, guidelines, and practicalities. J Hosp Infect 2015. https://doi.org/10.1016/j.jhin.2014.11.019.

[72] Ortolano GA, McAlister MB, Angelbeck JA, Schaffer J, Russell RL, Maynard E, et al. Hospital water point-of-use filtration: A complementary strategy to reduce the risk of nosocomial infection. Am J Infect Control 2005. https://doi.org/10.1016/j.ajic.2005.03.014.

[73] Loveday HP, Wilson JA, Kerr K, Pitchers R, Walker JT, Browne J. Association between healthcare water systems and Pseudomonas aeruginosa infections: A rapid systematic review. J Hosp Infect 2014. https://doi.org/10.1016/j.jhin.2013.09.010.

[74] Walker JT, Jhutty A, Parks S, Willis C, Copley V, Turton JF, et al. Investigation of healthcare-acquired infections associated with Pseudomonas aeruginosa biofilms in taps in neonatal units in Northern Ireland. J Hosp Infect 2014;86:16–23. https://doi.org/10.1016/j.jhin.2013.10.003.

[75] Durojaiye OC, Carbarns N, Murray S, Majumdar S. Outbreak of multidrug-resistant Pseudomonas aeruginosa in an intensive care unit. J Hosp Infect 2011;78:154–5. https://doi.org/10.1016/J.JHIN.2011.02.007.

[76] Rice SA, Van Den Akker B, Pomati F, Roser D. A risk assessment of Pseudomonas aeruginosa

in swimming pools: A review. J Water Health 2012;10:181–96. https://doi.org/10.2166/wh.2012.020.

[77] Roser DJ, Van Den Akker B, Boase S, Haas CN, Ashbolt NJ, Rice SA. Pseudomonas aeruginosa dose response and bathing water infection. Epidemiol Infect 2014;142:449–62. https://doi.org/10.1017/S0950268813002690.

[78] Price D, Ahearn DG. Incidence and persistence of Pseudomonas aeruginosa in whirlpools. J Clin Microbiol 1988;26:1650. https://doi.org/10.1128/JCM.26.9.1650-1654.1988.

[79] Chattopadhyay S, Perkins SD, Shaw M, Nichols TL. Evaluation of exposure to Brevundimonas diminuta and Pseudomonas aeruginosa during showering n.d. https://doi.org/10.1016/j.jaerosci.2017.08.008.

[80] Perkins SD, Mayfield J, Fraser V, Angenent LT. Potentially pathogenic bacteria in shower water and air of a stem cell transplant unit. Appl Environ Microbiol 2009;75:5363–72. https://doi.org/10.1128/AEM.00658-09.

[81] Blanc DS, Nahimana I, Petignat C, Wenger A, Bille J, Francioli P, et al. Faucets as a reservoir of endemic Pseudomonas aeruginosa colonization/infections in intensive care units. Intensive Care Med 2004;30:1964–8. https://doi.org/10.1007/s00134-004-2389-z.

[82] Aumeran C, Paillard C, Robin F, Kanold J, Baud O, Bonnet R, et al. Pseudomonas aeruginosa and Pseudomonas putida outbreak associated with contaminated water outlets in an oncohaematology paediatric unit. J Hosp Infect 2007. https://doi.org/10.1016/j.jhin.2006.08.009.

[83] Troop P. Independent Review of Incidents of Pseudomonas aeruginosa Infection in Neonatal Units in Northern Ireland. 2012.

[84] Moritz MM, Flemming HC, Wingender J. Integration of Pseudomonas aeruginosa and Legionella pneumophila in drinking water biofilms grown on domestic plumbing materials. Int J Hyg Environ Health 2010;213:190–7. https://doi.org/10.1016/j.ijheh.2010.05.003.

[85] Moore G, Stevenson D, Thompson K-A, Parks S, Ngabo D, Bennett AM, et al. Biofilm formation in an experimental water distribution system: the contamination of non-touch sensor taps and the implication for healthcare. Biofouling 2015;31:677–87. https://doi.org/10.1080/08927014.2015.1089986.

[86] Hutchins CF, Moore G, Webb J, Walker JT. Investigating alternative materials to EPDM for automatic taps in the context of Pseudomonas aeruginosa and biofilm control. J Hosp Infect 2020;106:429–35. https://doi.org/10.1016/J.JHIN.2020.09.013.

[87] Tenover FC, Arbeit RD, Goering R V, Mickelsen PA, Murray BE, Persing DH, et al. GUEST COMMENTARY Interpreting Chromosomal DNA Restriction Patterns Produced by Pulsed-Field Gel Electrophoresis: Criteria for Bacterial Strain Typing. vol. 33. 1995.

[88] Shokoohizadeh L. Molecular Methods for Bacterial Strain Typing. Med Lab J 2015;10. https://doi.org/10.18869/acadpub.mlj.10.2.1.

[89] Vu-Thien H, Corbineau G, Hormigos K, Fauroux B, Corvol H, Clément A, et al. Multiple-locus variable-number tandem-repeat analysis for longitudinal survey of sources of Pseudomonas aeruginosa infection in cystic fibrosis patients. J Clin Microbiol 2007;45:3175–83. https://doi.org/10.1128/JCM.00702-07.

[90] Turton JF, Turton SE, Yearwood L, Yarde S, Kaufmann ME, Pitt TL. Evaluation of a nine-locus variable-number tandem-repeat scheme for typing of Pseudomonas aeruginosa. Clin

Microbiol Infect 2010;16:1111–6. https://doi.org/10.1111/j.1469-0691.2009.03049.x.

[91] Bosch A, Miñán A, Vescina C, Degrossi J, Gatti B, Montanaro P, et al. Fourier transform infrared spectroscopy for rapid identification of nonfermenting gram-negative bacteria isolated from sputum samples from cystic fibrosis patients. J Clin Microbiol 2008;46:2535–46. https://doi.org/10.1128/JCM.02267-07/ASSET/126D6A8D-8858-4D8D-9E4B-C7CDDB7FB518/ASSETS/GRAPHIC/ZJM0080882730006.JPEG.

[92] Martak D, Valot B, Sauget M, Cholley P, Thouverez M, Bertrand X, et al. Fourier-transform infra red spectroscopy can quickly type gram-negative bacilli responsible for hospital outbreaks. Front Microbiol 2019;10:1440. https://doi.org/10.3389/FMICB.2019.01440/BIBTEX.

[93] Balloux F, Brønstad Brynildsrud O, van Dorp L, Shaw LP, Chen H, Harris KA, et al. From Theory to Practice: Translating Whole-Genome Sequencing (WGS) into the Clinic. Trends Microbiol 2018;26:1035–48. https://doi.org/10.1016/j.tim.2018.08.004.

[94] Parcell BJ, Oravcova K, Pinheiro M, Holden MTG, Phillips G, Turton JF, et al. Pseudomonas aeruginosa intensive care unit outbreak: winnowing of transmissions with molecular and genomic typing. J Hosp Infect 2018;98:282–8. https://doi.org/10.1016/j.jhin.2017.12.005.

[95] Halstead F D , Quick J, Niebel M, Garvey M, Cumley N, Smith R, Neal T, Roberts P, Hardy K, Shabir S, Walker J T, Hawkey P LNJ. Pseudomonas aeruginosa infection in augmented care: the molecular ecology and transmission dynamics in four large UK hospitals. J Hosp Infect 2021;111:162–8. https://doi.org/10.1016/J.JHIN.2021.01.020.

[96] Quick J, Cumley N, Wearn CM, Niebel M, Constantinidou C, Thomas CM, et al. Seeking the source of Pseudomonas aeruginosa infections in a recently opened hospital: an observational study using whole-genome sequencing. BMJ Open 2014;4:e006278. https://doi.org/10.1136/BMJOPEN-2014-006278.

[97] Quainoo S, Coolen JPM, van Hijum SAFT, Huynen MA, Melchers WJG, van Schaik W, et al. Whole-genome sequencing of bacterial pathogens: The future of nosocomial outbreak analysis. Clin Microbiol Rev 2017;30:1015–63. https://doi.org/10.1128/CMR.00016-17/ASSET/989378D2-603A-4C05-9DCC-5A3FE2E19CFD/ASSETS/GRAPHIC/ZCM0041726050003.JPEG.

[98] Joensen KG, Scheutz F, Lund O, Hasman H, Kaas RS, Nielsen EM, et al. Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic Escherichia coli. J Clin Microbiol 2014;52:1501–10. https://doi.org/10.1128/JCM.03617-13.

[99] Kwong JC, Mccallum N, Sintchenko V, Howden BP. Whole genome sequencing in clinical and public health microbiology. Pathology 2015;47:199. https://doi.org/10.1097/PAT.0000000000000235.

[100] Freschi L, Vincent AT, Jeukens J, Emond-Rheault JG, Kukavica-Ibrulj I, Dupont MJ, et al. The Pseudomonas aeruginosa Pan-Genome Provides New Insights on Its Population Structure, Horizontal Gene Transfer, and Pathogenicity. Genome Biol Evol 2019;11:109–20. https://doi.org/10.1093/gbe/evy259.

[101] Redfern J, Enright MC. Further understanding of *Pseudomonas aeruginosa* 's ability to horizontally acquire virulence: possible intervention strategies. Expert Rev Anti Infect Ther 2020;18:539–49. https://doi.org/10.1080/14787210.2020.1751610.

[102] van Belkum A, Soriaga LB, LaFave MC, Akella S, Veyrieras JB, Barbu EM, et al. Phylogenetic distribution of CRISPR-Cas systems in antibiotic- resistant pseudomonas aeruginosa. MBio

2015;6. https://doi.org/10.1128/mBio.01796-15.

[103] Kos VN, Déraspe M, McLaughlin RE, Whiteaker JD, Roy PH, Alm RA, et al. The resistome of Pseudomonas seudomonas aeruginosa in relationship to phenotypic susceptibility. Antimicrob Agents Chemother 2015;59:427–36. https://doi.org/10.1128/AAC.03954-14.

[104] Poulsen BE, Yang R, Clatworthy AE, White T, Osmulski SJ, Li L, et al. Defining the core essential genome of Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 2019;116:10072–80. https://doi.org/10.1073/pnas.1900570116.

[105] Redfern J, Enright MC. Further understanding of Pseudomonas aeruginosa's ability to horizontally acquire virulence: possible intervention strategies. Https://Doi-OrgLibproxyUclAcUk/101080/1478721020201751610 2020;18:539–49. https://doi.org/10.1080/14787210.2020.1751610.

[106] Marttinen P, Hanage WP, Croucher NJ, Connor TR, Harris SR, Bentley SD, et al. Detection of recombination events in bacterial genomes from large population samples. Nucleic Acids Res 2012;40:e6–e6. https://doi.org/10.1093/nar/gkr928.

[107] Jolley KA, Bray JE, Maiden MCJ. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications [version 1; referees: 2 approved]. Wellcome Open Res 2018;3. https://doi.org/10.12688/wellcomeopenres.14826.1.

[108] Pathogenwatch | A Global Platform for Genomic Surveillance n.d. https://pathogen.watch/ (accessed October 16, 2023).

[109] Schürch AC, Arredondo-Alonso S, Willems RJL, Goering R V. Whole genome sequencing options for bacterial strain typing and epidemiologic analysis based on single nucleotide polymorphism versus gene-by-gene–based approaches. Clin Microbiol Infect 2018;24:350–4. https://doi.org/10.1016/j.cmi.2017.12.016.

[110] Goyal M, Pelegrin AC, Jaillard M, Saharman YR, Klaassen CHW, Verbrugh HA, et al. Whole Genome Multi-Locus Sequence Typing and Genomic Single Nucleotide Polymorphism Analysis for Epidemiological Typing of Pseudomonas aeruginosa From Indonesian Intensive Care Units. Front Microbiol 2022;13. https://doi.org/10.3389/fmicb.2022.861222.

[111] Goering R V, H Abdelbary MM, Magalhães B, Valot B, Prod G, Greub G, et al. Combining Standard Molecular Typing and Whole Genome Sequencing to Investigate Pseudomonas aeruginosa Epidemiology in Intensive Care Units. Front Public Heal | WwwFrontiersinOrg 2020;1. https://doi.org/10.3389/fpubh.2020.00003.

[112] Blanc DS, Magalhães B, Koenig I, Senn L, Grandbastien B. Comparison of Whole Genome (wg-) and Core Genome (cg-) MLST (BioNumericsTM) Versus SNP Variant Calling for Epidemiological Investigation of Pseudomonas aeruginosa. Front Microbiol 2020;11:1729. https://doi.org/10.3389/fmicb.2020.01729.

[113] Oliver A, Mulet X, López-Causapé C, Juan C. The increasing threat of Pseudomonas aeruginosa high-risk clones. Drug Resist Updat 2015;21–22:41–59. https://doi.org/10.1016/j.drup.2015.08.002.

[114] Orlek A, Stoesser N, Anjum MF, Doumith M, Ellington MJ, Peto T, et al. Plasmid classification in an era of whole-genome sequencing: Application in studies of antibiotic resistance epidemiology. Front Microbiol 2017;8:182. https://doi.org/10.3389/fmicb.2017.00182.

[115] Treepong P, Kos VN, Guyeux C, Blanc DS, Bertrand X, Valot B, et al. Global emergence of the widespread Pseudomonas aeruginosa ST235 clone. Clin Microbiol Infect 2018;24:258–66. https://doi.org/10.1016/J.CMI.2017.06.018.

[116] Miyoshi-Akiyama T, Tada T, Ohmagari N, Hung NV, Tharavichitkul P, Pokhrel BM, et al. Emergence and Spread of Epidemic Multidrug-Resistant Pseudomonas aeruginosa n.d. https://doi.org/10.1093/gbe/evx243.

[117] Maatallah M, Cheriaa J, Backhrouf A, Iversen A, Grundmann H, Do T, et al. Population Structure of Pseudomonas aeruginosa from Five Mediterranean Countries: Evidence for Frequent Recombination and Epidemic Occurrence of CC235. PLoS One 2011;6:e25617. https://doi.org/10.1371/JOURNAL.PONE.0025617.

[118] Wong-Beringer A, Wiener-Kronish J, Lynch S, Flanagan J. Comparison of type III secretion system virulence among fluoroquinolone-susceptible and -resistant clinical isolates of Pseudomonas aeruginosa. Clin Microbiol Infect 2008;14:330–6. https://doi.org/10.1111/J.1469-0691.2007.01939.X.

[119] Kim DW, Cha CJ. Antibiotic resistome from the One-Health perspective: understanding and controlling antimicrobial resistance transmission. Exp Mol Med 2021;53:301–9. https://doi.org/10.1038/s12276-021-00569-z.

[120] López-Causapé C, Cabot G, del Barrio-Tofiño E, Oliver A. The versatile mutational resistome of Pseudomonas aeruginosa. Front Microbiol 2018;9:685. https://doi.org/10.3389/FMICB.2018.00685/BIBTEX.

[121] Su M, Satola SW, Read TD. Genome-based prediction of bacterial antibiotic resistance. J Clin Microbiol 2019;57. https://doi.org/10.1128/JCM.01405-18/ASSET/3C5F9705-12BC-4A6F-8535-AD7DCC47BE98/ASSETS/GRAPHIC/JCM.01405-18-F0001.JPEG.

[122] Ellington MJ, Ekelund O, Aarestrup FM, Canton R, Doumith M, Giske C, et al. The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee. Clin Microbiol Infect 2017;23:2–22. https://doi.org/10.1016/J.CMI.2016.11.012.

[123] Buhl M, Kästle C, Geyer A, Autenrieth IB, Peter S, Willmann M. Molecular Evolution of Extensively Drug-Resistant (XDR) Pseudomonas aeruginosa Strains From Patients and Hospital Environment in a Prolonged Outbreak. Front Microbiol 2019;10:1742. https://doi.org/10.3389/FMICB.2019.01742.

[124] Breathnach AS, Cubbon MD, Karunaharan RN, Pope CF, Planche TD. Multidrug-resistant Pseudomonas aeruginosa outbreaks in two hospitals: Association with contaminated hospital waste-water systems. J Hosp Infect 2012;82:19–24. https://doi.org/10.1016/j.jhin.2012.06.007.

[125] Witney AA, Gould KA, Pope CF, Bolt F, Stoker NG, Cubbon MD, et al. Genome sequencing and characterization of an extensively drug-resistant sequence type 111 serotype O12 hospital outbreak strain of Pseudomonas aeruginosa. Clin Microbiol Infect 2014;20:O609–18. https://doi.org/10.1111/1469-0691.12528.

[126] Willmann M, Bezdan D, Zapata L, Susak H, Vogel W, Schröppel K, et al. Analysis of a long-term outbreak of XDR Pseudomonas aeruginosa: a molecular epidemiological study. J Antimicrob Chemother 2015;70:1322–30. https://doi.org/10.1093/JAC/DKU546.

[127] Didelot X, Falush D. Bacterial Recombination in vivo. Horiz Gene Transf Evol Pathog 2009:23–46. https://doi.org/10.1017/cbo9780511541520.003.

[128] Didelot X, Maiden MCJ. Impact of recombination on bacterial evolution. Trends Microbiol 2010;18:315–22. https://doi.org/10.1016/j.tim.2010.04.002.

[129] Hanage WP. Not so simple after all: Bacteria, their population genetics, and recombination.

Cold Spring Harb Perspect Biol 2016;8. https://doi.org/10.1101/cshperspect.a018069.

[130] Didelot X, Croucher NJ, Bentley SD, Harris SR, Wilson DJ. Bayesian inference of ancestral dates on bacterial phylogenetic trees. Nucleic Acids Res 2018;46:e134. https://doi.org/10.1093/NAR/GKY783.

[131] Sagulenko P, Puller V, Neher RA. TreeTime: Maximum-likelihood phylodynamic analysis n.d. https://doi.org/10.1093/ve/vex042.

[132] Didelot X, Achtman M, Parkhill J, Thomson NR, Falush D. A bimodal pattern of relatedness between the Salmonella Paratyphi A and Typhi genomes: Convergence or divergence by homologous recombination? Genome Res 2007;17:61. https://doi.org/10.1101/GR.5512906.

[133] King SJ, Whatmore AM, Dowson CG. NanA, a neuraminidase from Streptococcus pneumoniae, shows high levels of sequence diversity, at least in part through recombination with Streptococcus oralis. J Bacteriol 2005;187:5376–86. https://doi.org/10.1128/JB.187.15.5376-5386.2005.

[134] Vos M, Didelot X. A comparison of homologous recombination rates in bacteria and archaea. ISME J 2009;3:199–208. https://doi.org/10.1038/ismej.2008.93.

[135] Spencer DH, Kas A, Smith EE, Raymond CK, Sims EH, Hastings M, et al. Whole-genome sequence variation among multiple isolates of Pseudomonas aeruginosa. J Bacteriol 2003;185:1316–25. https://doi.org/10.1128/JB.185.4.1316-1325.2003.

[136] Muzslay M, Moore G, Alhussaini N, Wilson APRR. ESBL-producing Gram-negative organisms in the healthcare environment as a source of genetic material for resistance in human infections. J Hosp Infect 2017;95:59–64. https://doi.org/10.1016/J.JHIN.2016.09.009.

[137] Hota S, Hirji Z, Stockton K, Lemieux C, Dedier H, Wolfaardt G, et al. Outbreak of Multidrug-Resistant Pseudomonas aeruginosa Colonization and Infection Secondary to Imperfect Intensive Care Unit Room Design . Infect Control Hosp Epidemiol 2009;30:25–33. https://doi.org/10.1086/592700.

[138] Sørensen SJ, Bailey M, Hansen LH, Kroer N, Wuertz S. Studying plasmid horizontal transfer in situ: A critical review. Nat Rev Microbiol 2005;3:700–10. https://doi.org/10.1038/nrmicro1232.

[139] O'Toole GA. Microtiter Dish Biofilm Formation Assay 2011:e2437. https://doi.org/10.3791/2437.

[140] Zymo R. Quick DNA Miniprep Plus Kit. 2017 2017:0–10.

[141] Facciolà A, Pellicanò GF, Visalli G, Paolucci IA, Rullo EV, Ceccarelli M, et al. The role of the hospital environment in the healthcare-associated infections: A general review of the literature. Eur Rev Med Pharmacol Sci 2019;23:1266–78. https://doi.org/10.26355/EURREV_201902_17020.

[142] Mena KD, Gerba CP. Risk Assessment of in Water. Rev Environ Contam Toxicol 2009;201:71–115. https://doi.org/10.1007/978-1-4419-0032-6_3.

[143] Balfour-Lynn IM. Environmental risks of Pseudomonas aeruginosa-What to advise patients and parents. J Cyst Fibros 2021;20:17–24. https://doi.org/10.1016/j.jcf.2020.12.005.

[144] Dean K, Mitchell J. Reverse QMRA for Pseudomonas aeruginosa in Premise Plumbing to Inform Risk Management. J Environ Eng 2019;146:04019120. https://doi.org/10.1061/(ASCE)EE.1943-7870.0001641.

[145] De Geyter D, Vanstokstraeten R, Crombé F, Tommassen J, Wybo I, Piérard D. Sink drains as reservoirs of VIM-2 metallo-β-lactamase-producing Pseudomonas aeruginosa in a Belgian intensive care unit: relation to patients investigated by whole-genome sequencing. J Hosp Infect 2021;115:75–82. https://doi.org/10.1016/J.JHIN.2021.05.010.

[146] Bédard E, Prévost M, Déziel E. Pseudomonas aeruginosa in premise plumbing of large buildings. Microbiologyopen 2016;5:937. https://doi.org/10.1002/MBO3.391.

[147] C Jefferies JM, Cooper T, Yam T, Clarke SC, Jefferies JJefferies J. Pseudomonas aeruginosa outbreaks in the neonatal intensive care unit-a systematic review of risk factors and environmental sources n.d. https://doi.org/10.1099/jmm.0.044818-0.

[148] Moloney EM, Deasy EC, Swan JS, Brennan GI, O'Donnell MJ, Coleman DC. Whole-genome sequencing identifies highly related Pseudomonas aeruginosa strains in multiple washbasin U-bends at several locations in one hospital: evidence for trafficking of potential pathogens via wastewater pipes. J Hosp Infect 2019;104:484–91. https://doi.org/10.1016/j.jhin.2019.11.005.

[149] European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters Version 11.0, valid from 2021-01-01 2021. http://www.eucast.org.

[150] Cusack TP, Ashley EA, Ling CL, Roberts T, Turner P, Wangrangsimakul T, et al. Time to switch from CLSI to EUCAST? A Southeast Asian perspective 2019. https://doi.org/10.1016/j.cmi.2019.03.007.

[151] Shard AG. Detection limits in XPS for more than 6000 binary systems using Al and Mg Kα X-rays. Surf Interface Anal 2014;46:175–85. https://doi.org/10.1002/SIA.5406.

[152] Johansson G, Hedman J, Berndtsson A, Klasson M, Nilsson R. Calibration of electron spectra. J Electron Spectros Relat Phenomena 1973;2:295–317. https://doi.org/10.1016/0368-2048(73)80022-2.

[153] Berrouane YF, McNutt LA, Buschelman BJ, Rhomberg PR, Sanford MD, Hollis RJ, et al. Outbreak of severe Pseudomonas aeruginosa infections caused by a contaminated drain in a Whirlpool Bathtub. Clin Infect Dis 2000;31:1331–7. https://doi.org/10.1086/317501/2/31-6-1331-FIG002.GIF.

[154] Hopman J, Meijer C, Kenters N, Coolen JPM, Ghamati MR, Mehtar S, et al. Risk Assessment After a Severe Hospital-Acquired Infection Associated With Carbapenemase-Producing Pseudomonas aeruginosa. JAMA Netw Open 2019;2:e187665–e187665. https://doi.org/10.1001/JAMANETWORKOPEN.2018.7665.

[155] Vianelli N, Giannini MB, Quarti C, Sabattini MAB, Fiacchini M, De Vivo A, et al. Resolution of a Pseudomonas aeruginosa outbreak in a hematology unit with the use of disposable sterile water filters. Haematologica 2006;91:983–5.

[156] Challis Ag + Shower Head & Hose n.d. www.challisagplus.com.

[157] Betteridge T, Merlino J, Natoli J, Cheong EYL, Gottlieb T, Stokes HW. Plasmids and bacterial strains mediating multidrug-resistant hospital-acquired infections are coresidents of the hospital environment. Microb Drug Resist 2013;19:104–9. https://doi.org/10.1089/MDR.2012.0104/ASSET/IMAGES/LARGE/FIGURE1.JPEG.

[158] Nurjadi D, Scherrer M, Frank U, Mutters NT, Heininger A, Späth I, et al. Genomic Investigation and Successful Containment of an Intermittent Common Source Outbreak of OXA-48-Producing Enterobacter cloacae Related to Hospital Shower Drains. Microbiol Spectr 2021;9.

https://doi.org/10.1128/SPECTRUM.01380-21/SUPPL_FILE/REVIEWER-COMMENTS.PDF.

[159] Stoodley P, Jacobsen A, Dunsmore BC, Purevdorj B, Wilson S, Lappin-Scott HM, et al. The influence of fluid shear and AICI3 on the material properties of Pseudomonas aeruginosa PAO1 and Desulfovibrio sp. EX265 biofilms. Water Sci Technol 2001;43:113–20. https://doi.org/10.2166/wst.2001.0353.

[160] Buckingham-Meyer K, Goeres DM, Hamilton MA. Comparative evaluation of biofilm disinfectant efficacy tests. J Microbiol Methods 2007;70:236–44. https://doi.org/10.1016/J.MIMET.2007.04.010.

[161] Thames Water Utilities Limited Water Quality Report 2021:1–12. https://www.thameswater.co.uk/help/water-quality/check-your-water-quality#/search.

[162] Cholley P, Thouverez M, Floret N, Bertrand X, Talon D. The role of water fittings in intensive care rooms as reservoirs for the colonization of patients with Pseudomonas aeruginosa. Intensive Care Med 2008;34:1428–33. https://doi.org/10.1007/S00134-008-1110-Z.

[163] Coppry M , Leroyer C , Saly M , Venier A-G , Slekovec C , Bertrand X , Parer S , Alfandari S , Cambau E , Megarbane B , Lawrence C , Clair B , Lepape A , Cassier P , Trivier D , Boyer A , Boulestreau H, Asselineau J , Dubois V , Thiébaut R RA-M. Exogenous acquisition of Pseudomonas aeruginosa in intensive care units: a prospective multi-centre study (DYNAPYO study). J Hosp Infect 2020;104:40–5. https://doi.org/10.1016/J.JHIN.2019.08.008.

[164] Jorth P, McLean K, Ratjen A, Secor PR, Bautista GE, Ravishankar S, et al. Evolved aztreonam resistance is multifactorial and can produce hypervirulence in Pseudomonas aeruginosa. MBio 2017;8. https://doi.org/10.1128/mBio.00517-17.

[165] Mapipa Q, Digban TO, Nnolim NE, Nwodo UU. Antibiogram profile and virulence signatures of Pseudomonas aeruginosa isolates recovered from selected agrestic hospital effluents. Sci Reports 2021 111 2021;11:1–11. https://doi.org/10.1038/s41598-021-91280-6.

[166] Hardiman CA, Weingarten RA, Conlan S, Khil P, Dekker JP, Mathers AJ, et al. Horizontal Transfer of Carbapenemase-Encoding Plasmids and Comparison with Hospital Epidemiology Data. Antimicrob Agents Chemother 2016;60:4910. https://doi.org/10.1128/AAC.00014-16.

[167] Carling PC. Wastewater drains: epidemiology and interventions in 23 carbapenem-resistant organism outbreaks. Infect Control Hosp Epidemiol 2018;39:972–9. https://doi.org/10.1017/ICE.2018.138.

[168] Sasahara T, Ogawa M, Fujimura I, Ae R, Kosami K, Morisawa Y. Efficacy and Effectiveness of Showerheads Attached with Point-of-use (POU) Filter Capsules in Preventing Waterborne Diseases in a Japanese Hospital. Biocontrol Sci 2020;25:223–30. https://doi.org/10.4265/BIO.25.223.

[169] Trautmann M, Halder S, Hoegel J, Royer H, Haller M. Point-of-use water filtration reduces endemic Pseudomonas aeruginosa infections on a surgical intensive care unit. Am J Infect Control 2008;36:421–9. https://doi.org/10.1016/j.ajic.2007.09.012.

[170] Florentin A, Lizon J, Asensio E, Forin J, Rivier A. American Journal of Infection Control Water and surface microbiologic quality of point-of-use water filters : A comparative study. AJIC Am J Infect Control 2016;44:1061–2. https://doi.org/10.1016/j.ajic.2016.02.028.

[171] Medical Shower Filter 92 Day - T-safe | Delivering Safe Water n.d. https://t-safe.com/en-gb/products/medical-shower-filter-92-day/ (accessed July 22, 2022).

[172] Pall-Aquasafe™ Disposable Shower Head Filter for up to 31 Days Use, 12 per case - Products

n.d. https://shop.pall.co.uk/uk/en/products/zidAQF4 (accessed April 23, 2023).

[173] Germlyser® D n.d. https://www.aqua-free.com/en/products/product/shower-filter/germlyser-d (accessed March 10, 2023).

[174] H2OK MSF Medical shower filter n.d. https://www.gapswater.co.uk/acatalog/Pentair-X-Flow-MSF-SLSS---PENTAIR-Medical-Shower-Filter-Sterilised-Set-31.2.106-107377BOX.html (accessed March 10, 2023).

[175] Totaro M, Valentini P, Casini B, Miccoli M, Costa AL, Baggiani A. Experimental comparison of point-of-use filters for drinking water ultrafiltration. J Hosp Infect 2017;96:172–6. https://doi.org/10.1016/j.jhin.2016.11.017.

[176] Smith CM, Hill VR. Dead-end hollow-fiber ultrafiltration for recovery of diverse microbes from water. Appl Environ Microbiol 2009;75:5284–9. https://doi.org/10.1128/AEM.00456-09.

[177] Alla Schmittel, Massimo Basagni, Eric Gaulle TK. Point - of - use Water Purifier With Polysulfone Hollow Fibres. United States Pat Appl Publ 2017;1.

[178] Jun Kamo, Takayuki Hirai, Hiroshi Takahashi KK. Porous Polyethylene Hollow Fiber Membrane Of Large Pore Diameter, Production Process Thereof, And Hydrophilized Porous Polyethylene Hollow Fiber Membranes., 2017.

[179] RIFFENBURGH RH. Statistical Testing, Risks, and Odds in Medical Decisions. Stat Med 2006:93–114. https://doi.org/10.1016/B978-012088770-5/50045-9.

[180] Hydreion L. Microbiological Testing of the Sawyer 7/6B Filter. Report No S05-03. 2005.

[181] Barna Z, Antmann K, Paszti J, Banfi R, Kadar M, Szax A, Nemeth M, Szego E VM. Infection control by point-of-use water filtration in an intensive care unit - a Hungarian case study. J Water Health 2014;12:858–67. https://doi.org/10.2166/WH.2014.052.

[182] Cervia JS, Farber B, Armellino D, Klocke J, Bayer RL, McAlister M, Stanchfield I, Canonica FP OG. Point-of-use water filtration reduces healthcare-associated infections in bone marrow transplant recipients. Transpl Infect Dis 2010;12:238–41. https://doi.org/10.1111/J.1399-3062.2009.00459.X.

[183] Chia PY, Sengupta S, Kukreja A, S.l. Ponnampalavanar S, Ng OT, Marimuthu K. The role of hospital environment in transmissions of multidrug-resistant gram-negative organisms. Antimicrob Resist Infect Control 2020;9:1–11. https://doi.org/10.1186/S13756-020-0685-1/TABLES/1.

[184] Alvarez-Ortega C, Wiegand I, Olivares J, Hancock REW, Martínez JL. The intrinsic resistome of Pseudomonas aeruginosa to β-lactams. Virulence 2011;2. https://doi.org/10.4161/viru.2.2.15014.

[185] Ghafoor A, Hay ID, Rehm BHA. Role of Exopolysaccharides in Pseudomonas aeruginosa Biofilm Formation and Architecture. Appl Environ Microbiol 2011;77:5238. https://doi.org/10.1128/AEM.00637-11.

[186] Mikkelsen H, Sivaneson M, Filloux A. Key two-component regulatory systems that control biofilm formation in Pseudomonas aeruginosa. Environ Microbiol 2011;13:1666–81. https://doi.org/10.1111/j.1462-2920.2011.02495.x.

[187] Pope CF, O'sullivan DM, Mchugh TD, Gillespie SH. MINIREVIEW A Practical Guide to Measuring Mutation Rates in Antibiotic Resistance. Antimicrob Agents Chemother 2008;52:1209–14. https://doi.org/10.1128/AAC.01152-07.

[188] Carballo-Pacheco M, Nicholson ID MD, Lilja EE, AllenID RJ, WaclawID B. Phenotypic delay in the evolution of bacterial antibiotic resistance: Mechanistic models and their implications 2020. https://doi.org/10.1371/journal.pcbi.1007930.

[189] Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics 2014:btu153.

[190] Gomila M, Peña A, Mulet M, Lalucat J, García-Valdés E. Phylogenomics and systematics in Pseudomonas. Front Microbiol 2015;6:214. https://doi.org/10.3389/fmicb.2015.00214.

[191] Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MTG, et al. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics 2015;31:3691–3.

[192] Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. Mol Biol Evol 2015;32:268–74. https://doi.org/10.1093/molbev/msu300.

[193] Vallet I, Diggle SP, Stacey RE, Cámara M, Ventre I, Lory S, et al. Biofilm Formation in Pseudomonas aeruginosa: Fimbrial cup Gene Clusters Are Controlled by the Transcriptional Regulator MvaT. J Bacteriol 2004;186:2880–90. https://doi.org/10.1128/JB.186.9.2880-2890.2004/SUPPL_FILE/JB01665-03_TABLE5_SUPPLEMENTAL.DOC.

[194] Vallet I, Olson JW, Lory S, Lazdunski A, Filloux A. The chaperone/usher pathways of Pseudomonas aeruginosa: Identification of fimbrial gene clusters (cup) and their involvement in biofilm formation. Proc Natl Acad Sci U S A 2001;98:6911–6. https://doi.org/10.1073/PNAS.111551898.

[195] Kelsey M. Pseudomonas in augmented care: should we worry? J Antimicrob Chemother 2013;68:2697–700. https://doi.org/10.1093/JAC/DKT288.

[196] Friedman L, Kolter R. Two genetic loci produce distinct carbohydrate-rich structural components of the Pseudomonas aeruginosa biofilm matrix. J Bacteriol 2004;186:4457–65. https://doi.org/10.1128/JB.186.14.4457-4465.2004.

[197] Qaisar U, Luo L, Haley CL, Brady SF, Carty NL, Colmer-Hamood JA, et al. The pvc Operon Regulates the Expression of the Pseudomonas aeruginosa Fimbrial Chaperone/Usher Pathway (Cup) Genes. PLoS One 2013;8:e62735. https://doi.org/10.1371/journal.pone.0062735.

[198] Hasman H, Chakraborty T, Klemm P. Antigen-43-mediated autoaggregation of Escherichia coli is blocked by fimbriation. J Bacteriol 1999;181:4834–41. https://doi.org/10.1128/jb.181.16.4834-4841.1999.

[199] Didelot X, Kendall M, Xu Y, White PJ, McCarthy N. Genomic Epidemiology Analysis of Infectious Disease Outbreaks Using TransPhylo. Curr Protoc 2021;1:e60. https://doi.org/10.1002/CPZ1.60.

[200] Lewallen S, Courtright P. Epidemiology in Practice: Case-Control Studies. Community Eye Heal 1998;11:57.

[201] Martin Bland. An Introduction to Medical Statistics - Martin Bland - Oxford University Press 2015.

[202] Centre for Disease Control. Interpreting Results of Case-Control Studies. CDC USA GOV 2013.

[203] Logistic Regression — Detailed Overview | by Saishruthi Swaminathan | Towards Data Science n.d. https://towardsdatascience.com/logistic-regression-detailed-overview-46c4da4303bc (accessed October 16, 2022).

[204] Kuo CL, Duan Y, Grady J. Unconditional or Conditional Logistic Regression Model for Age-Matched Case–Control Data? Front Public Heal 2018;6:57. https://doi.org/10.3389/FPUBH.2018.00057/BIBTEX.

[205] Fingertips. AMR local indicators. P. aeruginosa bacteraemia all counts and rates by acute trust - Public Health England n.d. https://fingertips.phe.org.uk/profile/amr-local-indicators/data#page/3/gid/1938132910/pat/158/par/TE_trust/ati/118/are/RGT/iid/93399/age/1/sex/4/cid/4/tbm/1 (accessed January 10, 2022).

[206] Hosmer DW, Lemeshow S, Sturdivant RX. Applied Logistic Regression: Third Edition. Appl Logist Regres Third Ed 2013:1–510. https://doi.org/10.1002/9781118548387.

[207] White Blood Count (WBC): MedlinePlus Medical Test n.d. https://medlineplus.gov/lab-tests/white-blood-count-wbc/ (accessed October 18, 2022).

[208] Kerr KG, Snelling AM. Pseudomonas aeruginosa: a formidable and ever-present adversary. vol. 73. Elsevier; 2009. https://doi.org/10.1016/j.jhin.2009.04.020.

[209] Schechner V, Nobre V, Kaye KS, Leshno M, Giladi M, Rohner P, et al. Gram-negative bacteremia upon hospital admission: When should Pseudomonas aeruginosa be suspected? Clin Infect Dis 2009;48:580–6. https://doi.org/10.1086/596709/2/48-5-580-TBL003.GIF.

[210] Flores-Mireles AL, Walker JN, Caparon M, Hultgren SJ. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat Rev Microbiol 2015 135 2015;13:269–84. https://doi.org/10.1038/nrmicro3432.

[211] Gransden WR, Leibovici L, Eykyn SJ, Pitlik SD, Samra Z, Konisberger H, et al. Risk factors and a clinical index for diagnosis of Pseudomonas aeruginosa bacteremia. Clin Microbiol Infect 1995;1:119–23. https://doi.org/10.1111/j.1469-0691.1995.tb00455.x.

[212] Enoch DA, Kuzhively J, Sismey A, Grynik A, Karas JA. Pseudomonas Aeruginosa Bacteraemia in Two UK District Hospitals. Infect Dis Rep 2013;5:e4. https://doi.org/10.4081/IDR.2013.E4.

[213] Choi Y, Paik JH, Kim JH, Han SB, Durey A. Clinical Predictors of Pseudomonas aeruginosa Bacteremia in Emergency Department. Emerg Med Int 2018;2018:1–6. https://doi.org/10.1155/2018/7581036.

[214] Pérez-Crespo PMM, Rojas Á, Lanz-García JF, Retamar-Gentil P, Reguera-Iglesias JM, Lima-Rodríguez O, et al. Pseudomonas aeruginosa Community-Onset Bloodstream Infections: Characterization, Diagnostic Predictors, and Predictive Score Development—Results from the PRO-BAC Cohort. Antibiotics 2022;11:707. https://doi.org/10.3390/ANTIBIOTICS11060707/S1.

[215] Dunne MW, Aronin SI, Yu KC, Watts JA, Gupta V. A multicenter analysis of trends in resistance in urinary Enterobacterales isolates from ambulatory patients in the United States: 2011-2020 2021. https://doi.org/10.1186/s12879-022-07167-y.

[216] Esparcia A, Madrazo M, Alberola J, López-Cruz I, Eiros JM, Nogueira JM, et al. Community-onset Pseudomonas aeruginosa urinary sepsis in elderly people: Predictive factors, adequacy of empirical therapy and outcomes. Int J Clin Pract 2019;73. https://doi.org/10.1111/IJCP.13425.

[217] Fertelli D, Cadnum JL, Nerandzic MM, Sitzlar B, Kundrapu S, Donskey CJ. Peripheral Venous Catheter and Bloodstream Infection Caused by Pseudomonas aeruginosa after a Contaminated Preoperative Shower. Infect Control Hosp Epidemiol 2013;34:543–4. https://doi.org/10.1086/670226.

[218] Kang CI, Kim SH, Kim H Bin, Park SW, Choe YJ, Oh MD, et al. Pseudomonas aeruginosa bacteremia: Risk factors for mortality and influence of delayed receipt of effective antimicrobial therapy on clinical outcome. Clin Infect Dis 2003;37:745–51. https://doi.org/10.1086/377200/2/37-6-745-FIG001.GIF.

[219] Thi MTT, Wibowo D, Rehm BHA. Pseudomonas aeruginosa Biofilms. Int J Mol Sci 2020;21:1–25. https://doi.org/10.3390/IJMS21228671.

[220] Merritt JH, Kadouri DE, O'Toole GA. Growing and Analyzing Static Biofilms. Curr Protoc Microbiol 2011;22:1B.1.1-1B.1.18. https://doi.org/10.1002/9780471729259.MC01B01S22.

[221] O'Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, Kolter R. [6] Genetic approaches to study of biofilms. Methods Enzymol 1999;310:91–109. https://doi.org/10.1016/S0076-6879(99)10008-9.

[222] Mishra P, Pandey CM, Singh U, Gupta A, Sahu C, Keshri A. Descriptive Statistics and Normality Tests for Statistical Data. Ann Card Anaesth 2019;22:67. https://doi.org/10.4103/ACA.ACA_157_18.

[223] Metrani R, Jayaprakasha GK, Patil BS. Optimized method for the quantification of pyruvic acid in onions by microplate reader and confirmation by high resolution mass spectra. Food Chem 2018;242:451–8. https://doi.org/10.1016/J.FOODCHEM.2017.08.099.

[224] Mulet X, Cabot G, Ocampo-Sosa AA, Domínguez MA, Zamorano L, Juan C, et al. Biological markers of Pseudomonas aeruginosa epidemic high-risk clones. Antimicrob Agents Chemother 2013;57:5527–35. https://doi.org/10.1128/AAC.01481-13.

[225] Sanchez CJ, Mende K, Beckius ML, Akers KS, Romano DR, Wenke JC, et al. Biofilm formation by clinical isolates and the implications in chronic infections. BMC Infect Dis 2013;13. https://doi.org/10.1186/1471-2334-13-47.

[226] Gustafson TL, Band JD, Hutcheson RH, Schaffner W. Pseudomonas Folliculitis: An Outbreak and Review. Rev Infect Dis 1983;5:1–8. https://doi.org/10.1093/CLINIDS/5.1.1.

[227] Schlech WF, Simonsen N, Sumarah R, Martin RS. Nosocomial outbreak of Pseudomonas aeruginosa folliculitis associated with a physiotherapy pool. C Can Med Assoc J 1986;134:909.

[228] Bert F, Maubec E, Bruneau B, Berry P, Lambert-Zechovsky N. Multi-resistant Pseudomonas aeruginosa outbreak associated with contaminated tap water in neurosurgery intensive care unit. J Hosp Infect 1998;39:53–62. https://doi.org/10.1016/S0195-6701(98)90243-2.

[229] Ferroni A, Nguyen L, Pron B, Quesne G, Brusset MC, Berche P. Outbreak of nosocomial urinary tract infections due to Pseudomonas aeruginosa in a paediatric surgical unit associated with tap-water contamination. J Hosp Infect 1998;39:301–7. https://doi.org/10.1016/S0195-6701(98)90295-X.

[230] Muyldermans G, De Smet F, Pierard D, Steenssens L, Stevens D, Bougatef A, et al. Neonatal infections with Pseudomonas aeruginosa associated with a water-bath used to thaw fresh frozen plasma. J Hosp Infect 1998;39:309–14. https://doi.org/10.1016/S0195-6701(98)90296-1.

[231] Buttery JP, Alabaster SJ, Heine RG, Scott SM, Crutchfield RA, Garland SM. Multiresistant Pseudomonas aeruginosa outbreak in a pediatric oncology ward related to bath toys. Pediatr Infect Dis J 1998;17:509–13. https://doi.org/10.1097/00006454-199806000-00015.

[232] Walker J, Anjeet M, Mr J, Parks S, Willis C, Copley V, et al. Investigation of Pseudomonas aeruginosa on biofilms in water tap assemblies from neonatal units in Northern Ireland

Version Number 2.0-Final Copy Title: Principal Investigator Biosafety Title: Head of Biosafety. 2012.

[233] Department of Health. Water systems Health Technical Memorandum 04-01: Addendum 2013.

[234] AMR local indicators - produced by the UKHSA - Data - OHID n.d. https://fingertips.phe.org.uk/profile/amr-local-indicators/data#page/3/gid/1938132910/pat/158/par/TE_trust/ati/118/are/RRV/iid/93403/age/1/sex/4/cat/-1/ctp/-1/yrr/1/cid/4/tbm/1/page-options/car-do-0 (accessed April 10, 2023).

[235] AMR local indicators - produced by the UKHSA - OHID n.d. https://fingertips.phe.org.uk/profile/amr-local-indicators (accessed October 24, 2022).

[236] Desjardins P, Conklin D. NanoDrop microvolume quantitation of nucleic acids. J Vis Exp 2010. https://doi.org/10.3791/2565.

[237] Lucena-Aguilar G, Sánchez-López AM, Barberán-Aceituno C, Carrillo-Ávila JA, López-Guerrero JA, Aguilar-Quesada R. DNA Source Selection for Downstream Applications Based on DNA Quality Indicators Analysis. Biopreserv Biobank 2016;14:264. https://doi.org/10.1089/BIO.2015.0064.

[238] Koetsier G, Cantor E, Biolabs E. A Practical Guide to Analyzing Nucleic Acid Concentration and Purity with Microvolume Spectrophotometers n.d.

[239] Chen Z, Erickson DL, Meng J. Benchmarking Long-Read Assemblers for Genomic Analyses of Bacterial Pathogens Using Oxford Nanopore Sequencing. Int J Mol Sci 2020, Vol 21, Page 9161 2020;21:9161. https://doi.org/10.3390/IJMS21239161.

[240] AS-Wallshower - RENTACS n.d. https://rentacs.com/product/as-wallshower/ (accessed March 10, 2023).

[241] Baclyser® S n.d. https://www.aqua-free.com/en/products/product/shower-filter/baclyser-s (accessed March 10, 2023).

[242] Turnidge J, Abbott IJ. EUCAST breakpoint categories and the revised “I”: a stewardship opportunity for “I”mproving outcomes. Clin Microbiol Infect 2022;28:475–6. https://doi.org/10.1016/j.cmi.2021.12.007.

[243] Hwang GB, Page K, Patir A, Nair SP, Allan E, Parkin IP. The Anti-Biofouling Properties of Superhydrophobic Surfaces are Short-Lived. ACS Nano 2018;12:59. https://doi.org/10.1021/acsnano.8b02293.

Division of labour/ Author contribution statement
Progress reports/ Thesis committee meetings

Progress reports created during this PhD were written by Özge Yetiş and reviewed by Thesis Committee Members; Professor Asterios Gavriilidis, Dr Elaine Allan and Dr Lena Ciric

Thesis committee meetings were led by Professor Peter Wilson and Dr Shanom Ali; meetings were chaired and minutes documented by Dr Shanom Ali

Laboratory/ Scientific activities

The following activities i) Water sample collection and processing, ii) collection and in-house validation of point-of-use filters, iii) hospital water pressure measurements, iv) efficacy testing of antimicrobial shower materials, and v)biofilm methodology in Chapters 3, 4 and 7 were undertaken with the support of Kush Karia and Dr Shanom Ali, of the Environmental Research Laboratory.

All results and interpretations and statistical analyses (unless specified) were undertaken by Özge Yetiş. All microbiological assessments, identifications, examinations and interpretations were undertaken by Özge Yetiş

Thesis Chapters

Thesis chapters were written, revised and edited by Özge Yetiş. Reviews of all chapters were undertaken by Professor Peter Wilson and Dr Shanom Ali

Chapter 3

Statistical analysis and interpretation was provided by Paul Bassett. XPS measurements, resulting graphs and conclusions were provided by Sanjayan Sathasivam.

Chapter 5

VNTR analysis was provided by Jane Turton and Zoe Payne, UKHSA.

WGS analysis of the pilot study was provided by the team at Quadram Institute (Justin O Grady, Andrew Page and Leonardo de Oliveira Martins).

WGS analysis of the main study is provided by Lucy van Dorp, UCL Genetics Institute

Chapter 6

The Case control study was performed under the supervision of Pietro Coen, UCH epidemiologist and included a background study, and selection of case and controls groups.

Papers/Posters

Papers/posters produced in relation to this thesis were undertaken and reviewed by the authors as named in the corresponding manuscript/poster.

Appendix

Appendix A. Shower head types

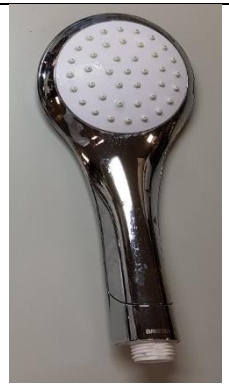
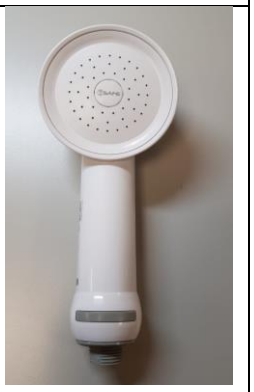
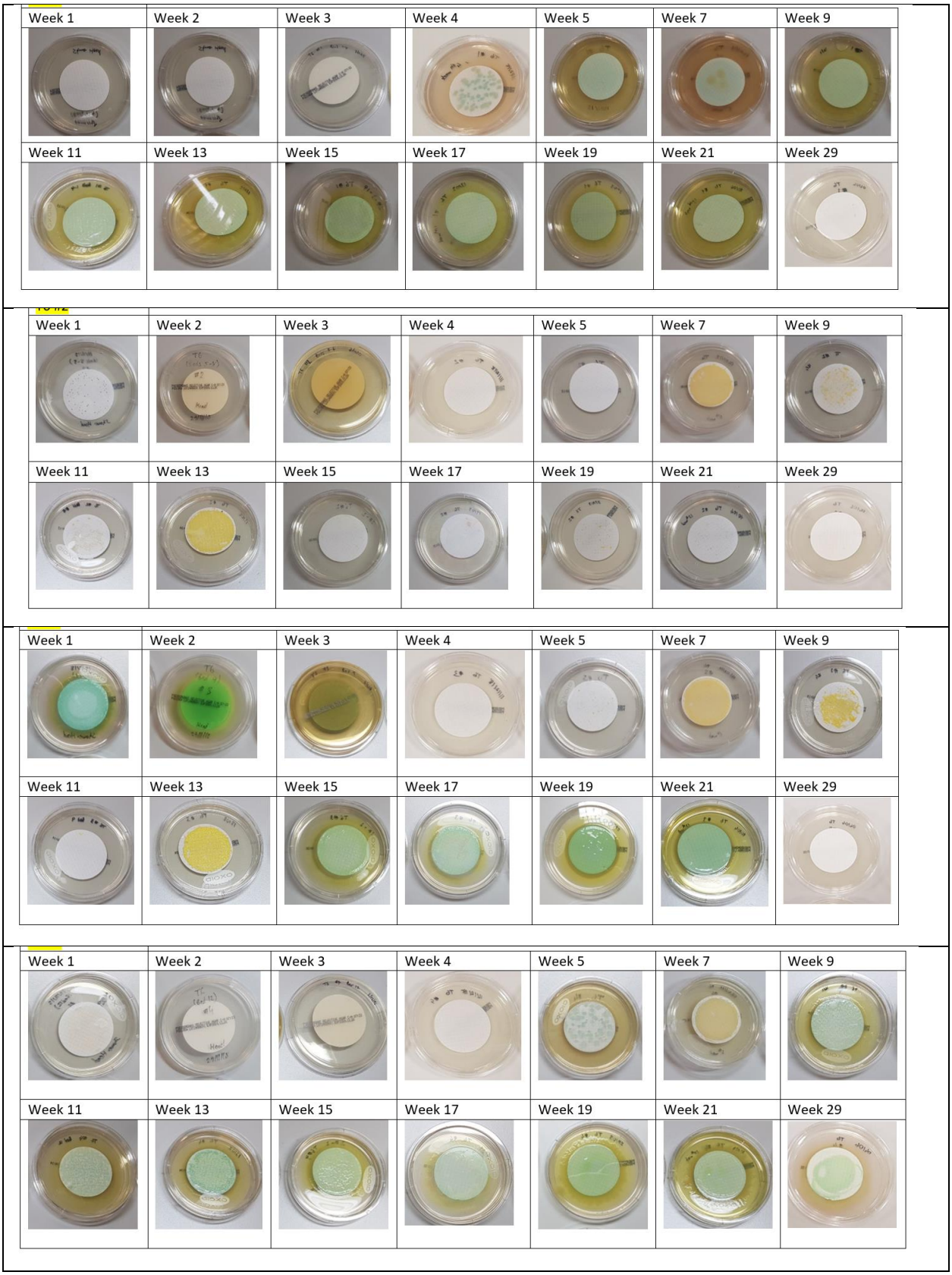
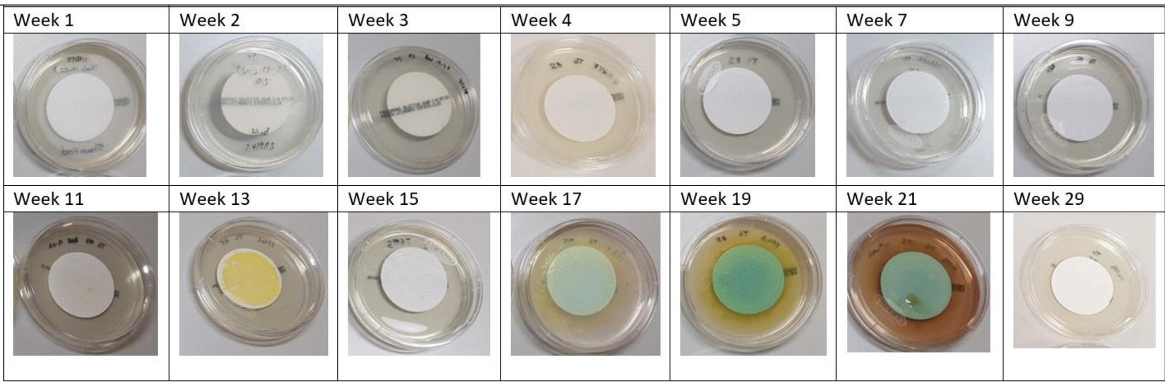
Shower head Types	Old small head (non-antimicrobial)	Wide flat head (non-antimicrobial)	Small flat head (non-antimicrobial)	Anonymised 3 (POU membrane filter; non-antimicrobial)	Anonymised 1 Ag+ (antimicrobial shower head; non-filter type)	Anonymised 2 (POU hollow-fibre filter; antimicrobial)
						

Table A. Old shower head and hose types

Number	Shower head type	Shower hose material
1	Old small	Plastic
2	Old small	Metal
3	Old small	Plastic
4	Big flattened	Plastic
5	Big flattened	Metal
6	Big flattened	Metal
7	Small flattened	Plastic
8	Small flattened	Metal
9	Anonymised 3	Metal
10	Anonymised 3	Metal

Appendix A.1. Environmental sampling of shower waters and drains from Ward A
Shower water filter membranes on PCN plates after 48 hours of incubation at 37ºC from Ward A showers #1-10

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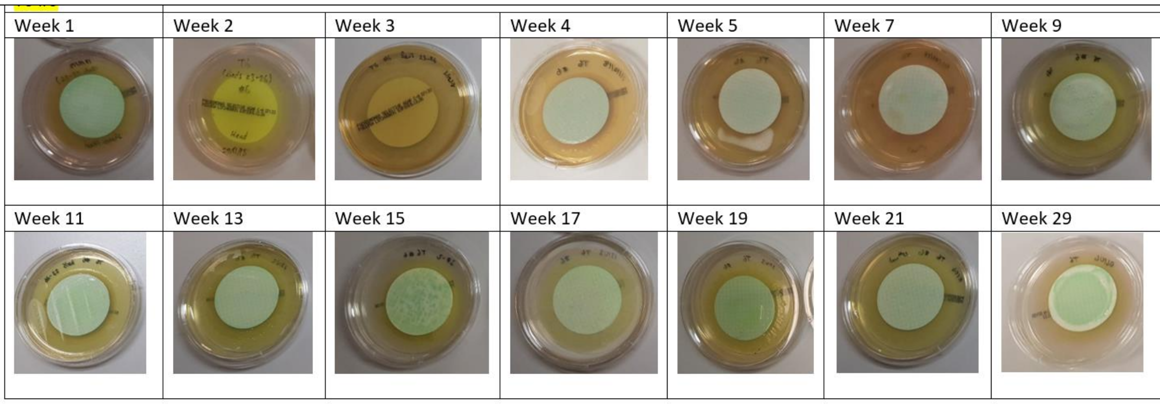
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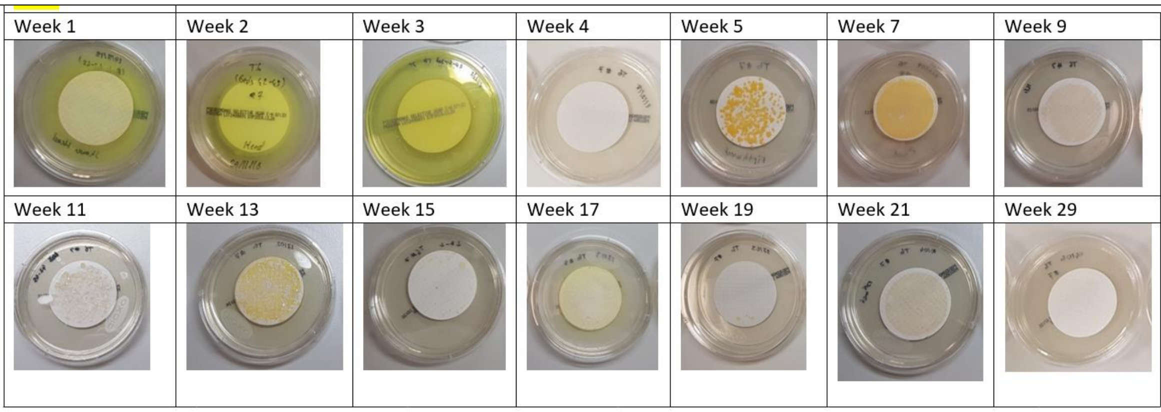
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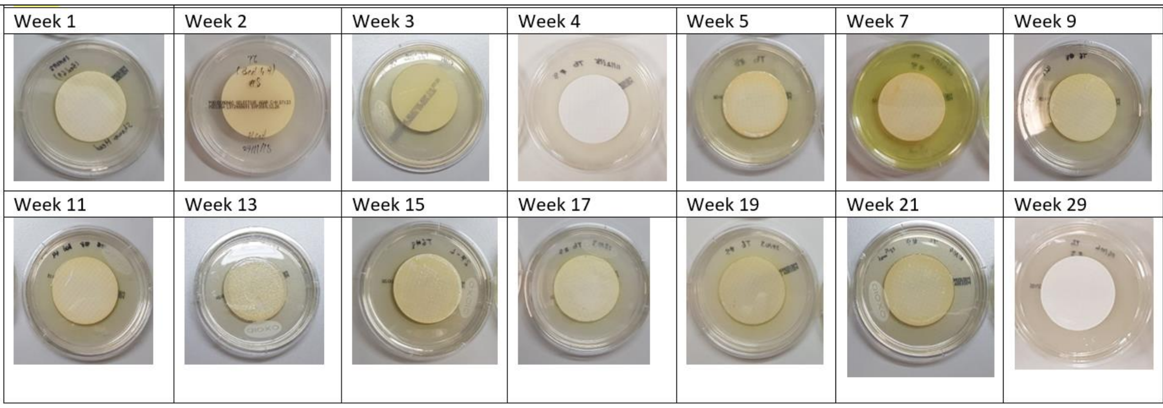
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		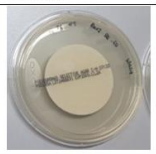		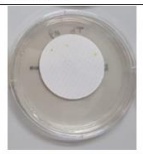	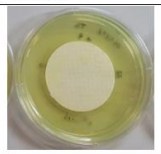	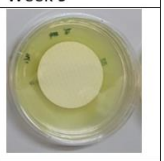
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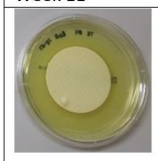	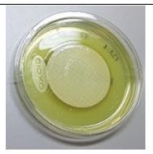	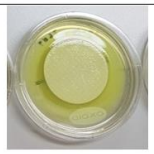	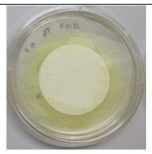		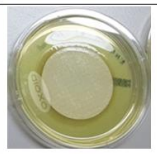	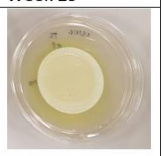

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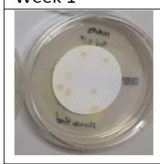	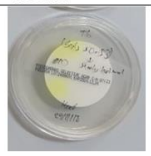	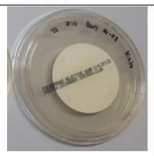	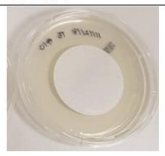	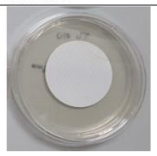	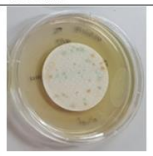	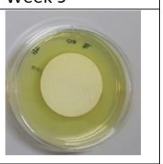
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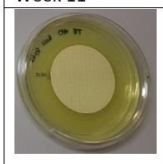
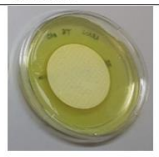
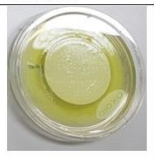
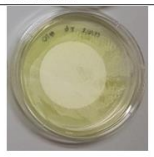
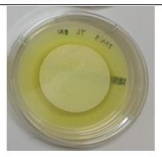
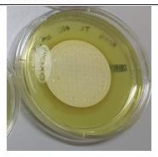
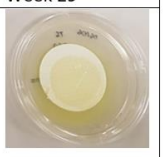
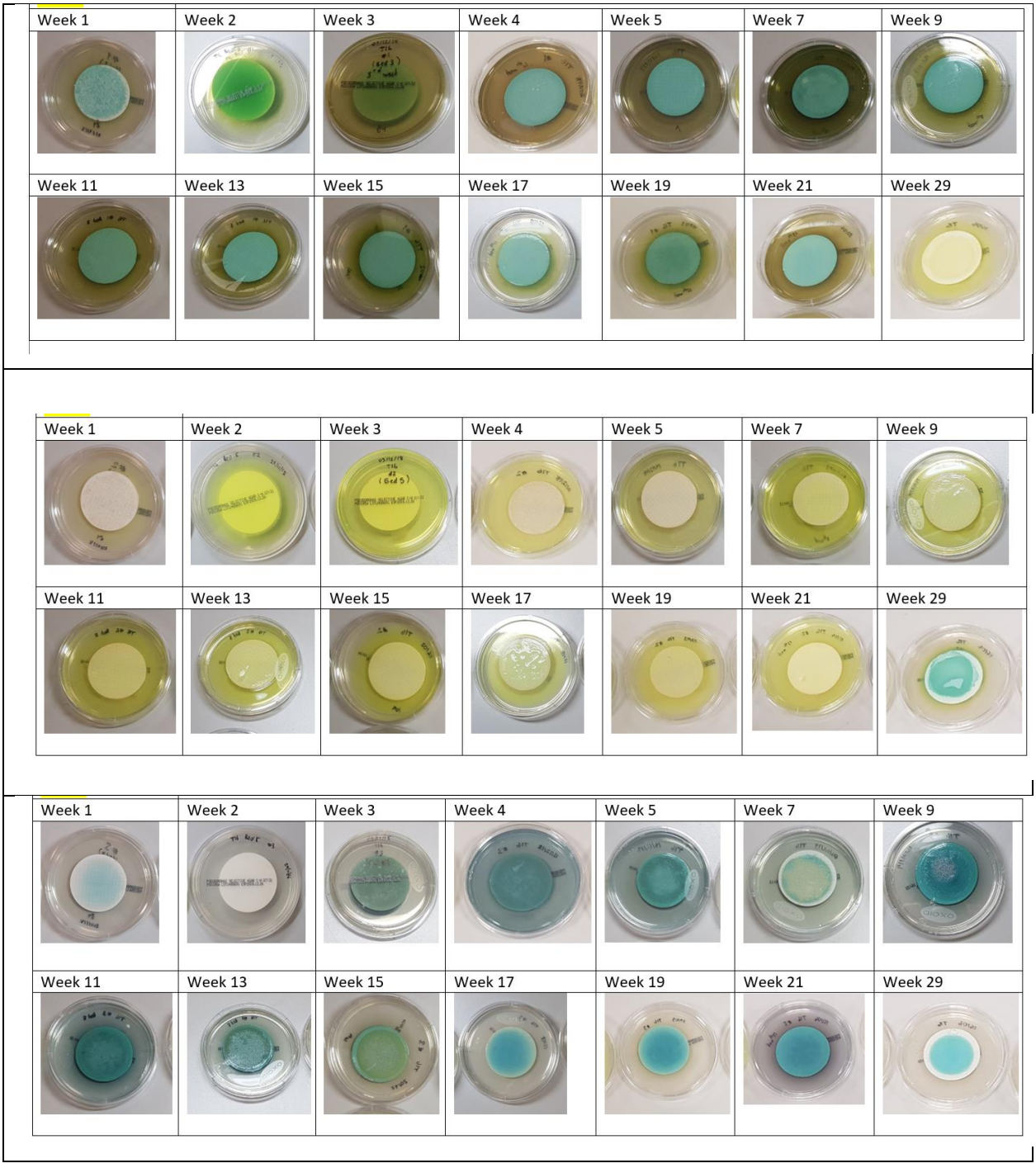
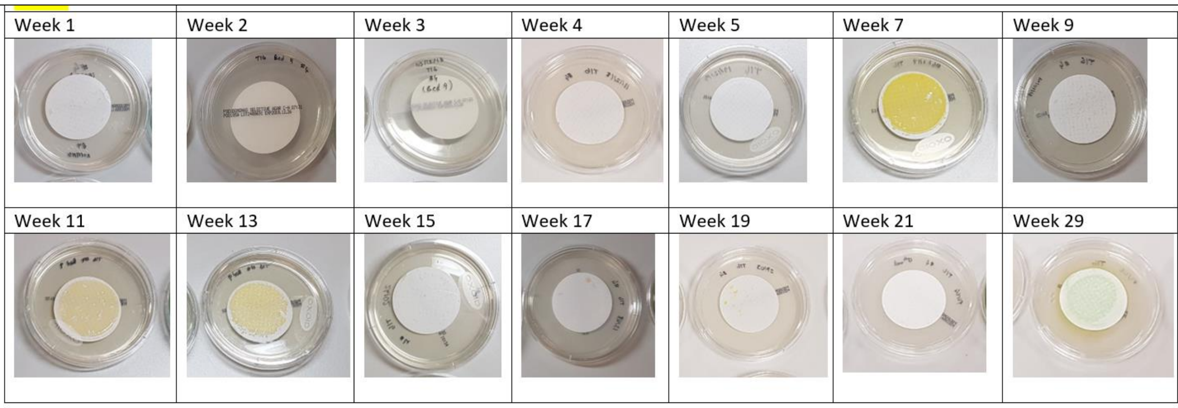
						

Note: Green/yellow colours on filter papers represent presumptive *P. aeruginosa* contamination. Clear filter papers show no microbial growth on PCN agar plates.

Appendix A.2. Environmental sampling of shower waters and drains from Ward B Shower water filter membranes on PCN plates after 48 hours of incubation at 37ºC from Ward B showers #1-10

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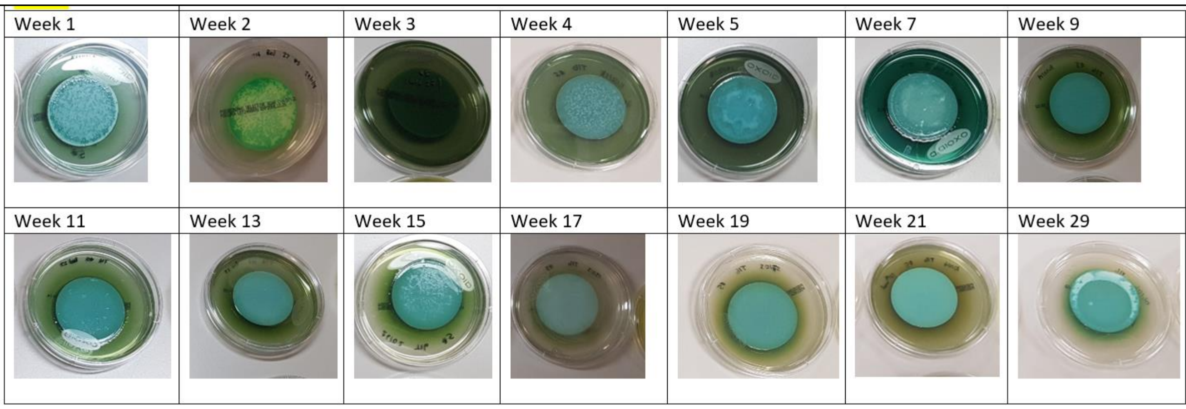
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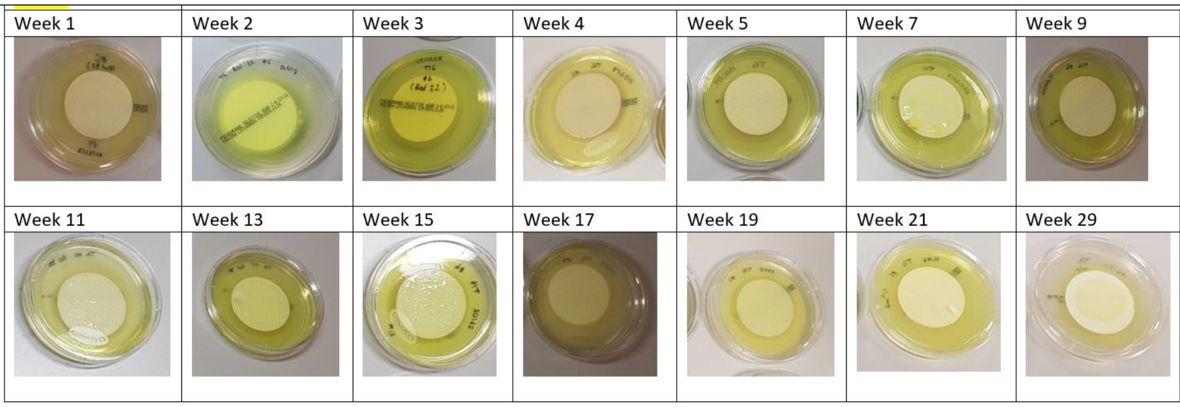
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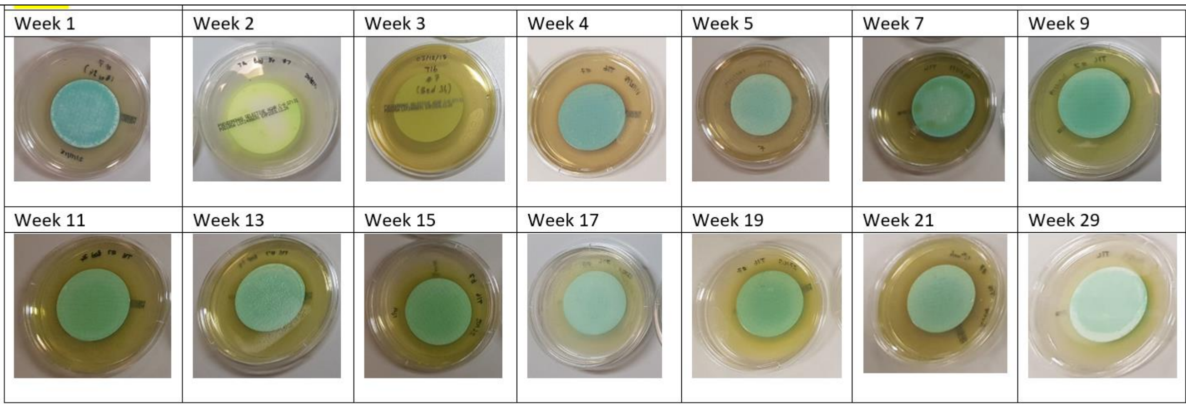
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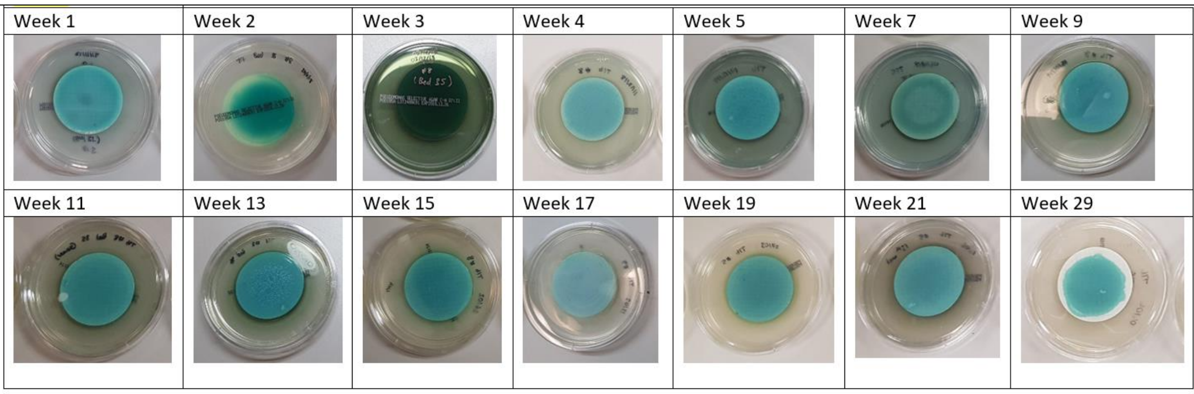
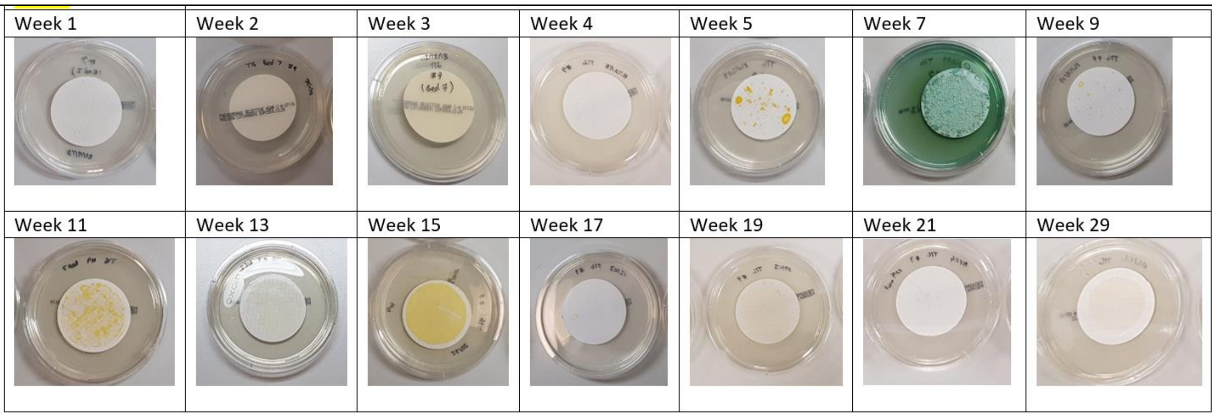
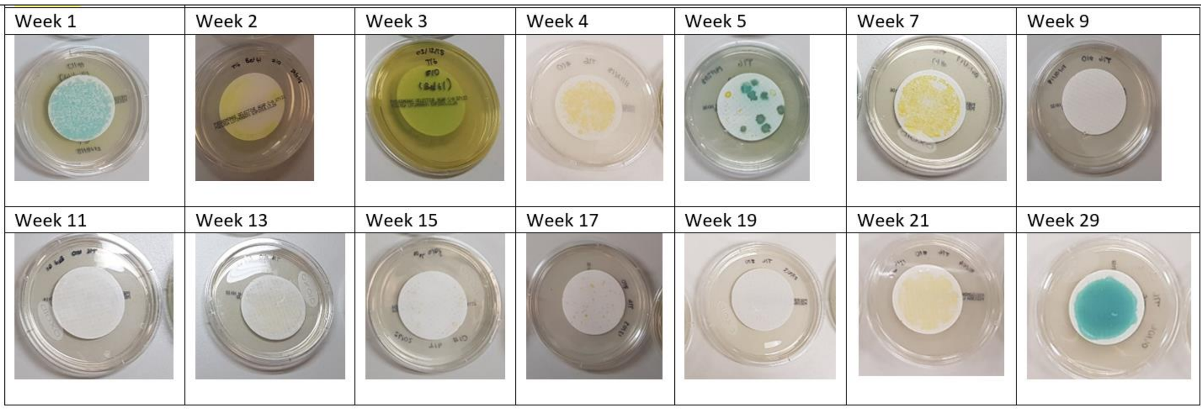
Week 9
Week 11
Week 13
Week 15
Week 17
Week 19
Week 21
Week 29







Note: Green/yellow colours on filter papers represent presumptive *P. aeruginosa* contamination. Clear filter papers show no microbial growth on PCN agar plates.

Appendix A.3. Maldi scores of confirmed *P. aeruginosa* strains from Ward A

No	ID	Score	No	ID	Score	No	ID	Score
A1	*P. aeruginosa*	2.45	A36	*P. aeruginosa*	2.32	A58	*P. aeruginosa*	2.28
A2	*P. aeruginosa*	2.37	A37	*P. aeruginosa*	2.27	A59	*P. aeruginosa*	2.45
A3	*P. aeruginosa*	2.46	A38	*P. aeruginosa*	2.41	A60	*P. aeruginosa*	2.32
A5	*P. aeruginosa*	2.34	A39	*P. aeruginosa*	2.35	A61	*P. aeruginosa*	2.47
A6	*P. aeruginosa*	2.45	A40	*P. aeruginosa*	2	A86	*P. aeruginosa*	2.37
A7	*P. aeruginosa*	2.34	A41	*P. aeruginosa*	2.42	A88	*P. aeruginosa*	2.28
A8	*P. aeruginosa*	2.24	A42	*P. aeruginosa*	2.51	A89	*P. aeruginosa*	2.4
A9	*P. aeruginosa*	2.2	A72	*P. aeruginosa*	2.44	A90	*P. aeruginosa*	2.41
A10	*P. aeruginosa*	2.33	A43	*P. aeruginosa*	2.45	A92	*P. aeruginosa*	2.52
A11	*P. aeruginosa*	2.43	A44	*P. aeruginosa*	2.46	A95	*P. aeruginosa*	2.35
A12	*P. aeruginosa*	2.34	A73	*P. aeruginosa*	2.33	A97	*P. aeruginosa*	2.49
A13	*P. aeruginosa*	2.43	A74	*P. aeruginosa*	2.3	A96	*P. aeruginosa*	2.44
A14	*P. aeruginosa*	2.34	A75	*P. aeruginosa*	2.34	A63	*P. aeruginosa*	2.37
A15	*P. aeruginosa*	2.32	A45	*P. aeruginosa*	2.43	A64	*P. aeruginosa*	2.38
A16	*P. aeruginosa*	2.42	A46	*P. aeruginosa*	2.39	A65	*P. aeruginosa*	2.39
A17	*P. aeruginosa*	2.39	A47	*P. aeruginosa*	2.41	A66	*P. aeruginosa*	2.35
A18	*P. aeruginosa*	2.39	A78	*P. aeruginosa*	2.31	A67	*P. aeruginosa*	2.24
A19	*P. aeruginosa*	2.36	A80	*P. aeruginosa*	2.28	A68	*P. aeruginosa*	2.47
A21	*P. aeruginosa*	2.43	A48	*P. aeruginosa*	2.33	A69	*P. aeruginosa*	2.33
A22	*P. aeruginosa*	2.39	A49	*P. aeruginosa*	2.33	A70	*P. aeruginosa*	2.37
A23	*P. aeruginosa*	2.38	A50	*P. aeruginosa*	2.45	A71	*P. aeruginosa*	2.38
A24	*P. aeruginosa*	2.43	A51	*P. aeruginosa*	2.49	A98	*P. aeruginosa*	2.42
A25	*P. aeruginosa*	2.37	A52	*P. aeruginosa*	2.53	A99	*P. aeruginosa*	2.37
A26	*P. aeruginosa*	2.4	A53	*P. aeruginosa*	2.35	A100	*P. aeruginosa*	2.4
A27	*P. aeruginosa*	2.47	A81	*P. aeruginosa*	2.41	A101	*P. aeruginosa*	2.43
A28	*P. aeruginosa*	2.33	A82	*P. aeruginosa*	2.31	A102	*P. aeruginosa*	2.45
A29	*P. aeruginosa*	2.42	A83	*P. aeruginosa*	2.38	A103	*P. aeruginosa*	2.35
A30	*P. aeruginosa*	2.34	A84	*P. aeruginosa*	2.41	A104	*P. aeruginosa*	2.42
A31	*P. aeruginosa*	2.33	A85	*P. aeruginosa*	2.34	A105	*P. aeruginosa*	2.37
A32	*P. aeruginosa*	2.37	A54	*P. aeruginosa*	2.25	A106	*P. aeruginosa*	2.36
A33	*P. aeruginosa*	2.39	A55	*P. aeruginosa*	2.22	A107	*P. aeruginosa*	2.39
A34	*P. aeruginosa*	2.37	A56	*P. aeruginosa*	2.35	A108	*P. aeruginosa*	2.44
A35	*P. aeruginosa*	2.49	A57	*P. aeruginosa*	1.97	A109	*P. aeruginosa*	2.35

Appendix A.4. Maldi scores of confirmed *P. aeruginosa* strains from Ward B

No	ID	Score	No	ID	Score	No	ID	Score
B1	*P. aeruginosa*	2.27	B33	*P. aeruginosa*	2.42	B64	*P. aeruginosa*	2.44
B2	*P. aeruginosa*	2.28	B34	*P. aeruginosa*	2.48	B117	*P. aeruginosa*	2.54
B3	*P. aeruginosa*	2.26	B35	*P. aeruginosa*	2.39	B119	*P. aeruginosa*	2.58
B4	*P. aeruginosa*	2.42	B36	*P. aeruginosa*	2.41	B120	*P. aeruginosa*	2.34
B5	*P. aeruginosa*	2.21	B37	*P. aeruginosa*	2.47	B122	*P. aeruginosa*	2.4
B6	*P. aeruginosa*	2.38	B38	*P. aeruginosa*	2.38	B124	*P. aeruginosa*	2.41
B7	*P. aeruginosa*	2.32	B39	*P. aeruginosa*	2.19	B65	*P. aeruginosa*	2.4
B8	*P. aeruginosa*	2.38	B161	*P. aeruginosa*	2.53	B66	*P. aeruginosa*	2.32
B10	*P. aeruginosa*	2.33	B40	*P. aeruginosa*	2.27	B67	*P. aeruginosa*	2.42
B11	*P. aeruginosa*	2.4	B41	*P. aeruginosa*	2.43	B68	*P. aeruginosa*	2.44
B12	*P. aeruginosa*	2.37	B42	*P. aeruginosa*	2.42	B69	*P. aeruginosa*	2.42
B13	*P. aeruginosa*	2.2	B43	*P. aeruginosa*	2.38	B70	*P. aeruginosa*	2.49
B14	*P. aeruginosa*	2.37	B44	*P. aeruginosa*	2.44	B71	*P. aeruginosa*	2.46
B15	*P. aeruginosa*	2.35	B104	*P. aeruginosa*	2.43	B72	*P. aeruginosa*	2.35
B16	*P. aeruginosa*	2.38	B45	*P. aeruginosa*	2.47	B73	*P. aeruginosa*	2.32
B17	*P. aeruginosa*	2.39	B46	*P. aeruginosa*	2.48	B74	*P. aeruginosa*	2.41
B18	*P. aeruginosa*	2.4	B47	*P. aeruginosa*	2.46	B160	*P. aeruginosa*	2.32
B19	*P. aeruginosa*	2.13	B48	*P. aeruginosa*	2.31	B75	*P. aeruginosa*	2.32
B20	*P. aeruginosa*	2.44	B49	*P. aeruginosa*	2.5	B76	*P. aeruginosa*	2.43
B21	*P. aeruginosa*	1.94	B50	*P. aeruginosa*	2.38	B77	*P. aeruginosa*	2.11
B22	*P. aeruginosa*	2.35	B51	*P. aeruginosa*	2.55	B78	*P. aeruginosa*	2.37
B116	*P. aeruginosa*	2.39	B52	*P. aeruginosa*	2.46	B79	*P. aeruginosa*	2.41
B23	*P. aeruginosa*	2.2	B53	*P. aeruginosa*	2.47	B80	*P. aeruginosa*	2.52
B24	*P. aeruginosa*	2.46	B54	*P. aeruginosa*	2.45	B81	*P. aeruginosa*	2.48
B25	*P. aeruginosa*	2.6	B55	*P. aeruginosa*	2.32	B82	*P. aeruginosa*	2.42
B26	*P. aeruginosa*	2.23	B56	*P. aeruginosa*	2.41	B128	*P. aeruginosa*	2.51
B27	*P. aeruginosa*	2.44	B57	*P. aeruginosa*	2.31	B130	*P. aeruginosa*	2.49
B28	*P. aeruginosa*	2.42	B58	*P. aeruginosa*	2.32	B185	*P. aeruginosa*	2.46
B89	*P. aeruginosa*	2.33	B59	*P. aeruginosa*	2.35	B83	*P. aeruginosa*	2.42
B29	*P. aeruginosa*	2.31	B60	*P. aeruginosa*	2.51	B135	*P. aeruginosa*	2.33
B30	*P. aeruginosa*	2.28	B61	*P. aeruginosa*	2.45	B84	*P. aeruginosa*	2.53
B31	*P. aeruginosa*	2.47	B62	*P. aeruginosa*	2.46	B85	*P. aeruginosa*	2.46
B32	*P. aeruginosa*	2.44	B63	*P. aeruginosa*	2.25	B86	*P. aeruginosa*	2.42

Cont'd Appendix A.4. - Maldi scores of confirmed *P. aeruginosa* strains from Ward B

No	ID	Score	No	ID	Score	No	ID	Score
B87	*P. aeruginosa*	2.5	B101	*P. aeruginosa*	2.41	B111	*P. aeruginosa*	2.43
B88	*P. aeruginosa*	2.41	B150	*P. aeruginosa*	2.38	B112	*P. aeruginosa*	2.49
B138	*P. aeruginosa*	2.5	B151	*P. aeruginosa*	2.41	B113	*P. aeruginosa*	2.32
B139	*P. aeruginosa*	2.31	B152	*P. aeruginosa*	2.37	B131	*P. aeruginosa*	2.4
B140	*P. aeruginosa*	2.55	B153	*P. aeruginosa*	2.41	B132	*P. aeruginosa*	2.25
B103	*P. aeruginosa*	2.29	B154	*P. aeruginosa*	2.45	B133	*P. aeruginosa*	2.3
B90	*P. aeruginosa*	2.29	B157	*P. aeruginosa*	2.38	B134	*P. aeruginosa*	2.39
B91	*P. aeruginosa*	2.45	B105	*P. aeruginosa*	2.37	B136	*P. aeruginosa*	2.45
B92	*P. aeruginosa*	2.38	B158	*P. aeruginosa*	2.39	B137	*P. aeruginosa*	2.39
B93	*P. aeruginosa*	2.27	B115	*P. aeruginosa*	2.35	B141	*P. aeruginosa*	2.46
B94	*P. aeruginosa*	2.29	B118	*P. aeruginosa*	2.34	B143	*P. aeruginosa*	2.36
B95	*P. aeruginosa*	2.36	B106	*P. aeruginosa*	2.38	B144	*P. aeruginosa*	2.44
B145	*P. aeruginosa*	2.52	B123	*P. aeruginosa*	2.27	B147	*P. aeruginosa*	2.28
B146	*P. aeruginosa*	2.48	B125	*P. aeruginosa*	2.45	B148	*P. aeruginosa*	2.16
B96	*P. aeruginosa*	2.33	B126	*P. aeruginosa*	2.31	B149	*P. aeruginosa*	2.41
B97	*P. aeruginosa*	2.34	B107	*P. aeruginosa*	2	B155	*P. aeruginosa*	2.36
B98	*P. aeruginosa*	2.11	B108	*P. aeruginosa*	2.32	B164	*P. aeruginosa*	2.45
B156	*P. aeruginosa*	2.38	B109	*P. aeruginosa*	2.46	B159	*P. aeruginosa*	2.44
B99	*P. aeruginosa*	2.5	B110	*P. aeruginosa*	2.51			
B100	*P. aeruginosa*	2.27	B114	*P. aeruginosa*	2.24			

Appendix A.5. AST results giving the number and percentage of responses in each AST category for each time period. The final column shows p-values from the analyses

Table 1: Ward A showers

Antibiotic	Antibiotic susceptibility	Period 1 n (%)	Period 2 n (%)	Period 3 n (%)	P-value(*)
Amikacin	Sensitive	7 (100%)	16 (100%)	11 (92%)	0.39
	Intermediate	0 (0%)	0 (0%)	0 (0%)	
	Resistant	0 (0%)	0 (0%)	1 (8%)	
Tobramycin	Sensitive	7 (100%)	15 (94%)	11 (92%)	0.75
	Intermediate	0 (0%)	0 (0%)	0 (0%)	
	Resistant	0 (0%)	1 (6%)	1 (8%)	
Piperacillin	Sensitive	0 (0%)	0 (0%)	0 (0%)	1.00
	Intermediate	7 (100%)	16 (100%)	12 (100%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	
Piperacillin-tazobactam	Sensitive	0 (0%)	0 (0%)	0 (0%)	1.00
	Intermediate	7 (100%)	16 (100%)	12 (100%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	
Ticarcillin-clavulanic acid	Sensitive	0 (0%)	0 (0%)	0 (0%)	1.00
	Intermediate	7 (100%)	16 (100%)	12 (100%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	
Meropenem	Sensitive	7 (100%)	16 (100%)	12 (100%)	1.00
	Intermediate	0 (0%)	0 (0%)	0 (0%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	
Imipenem	Sensitive	0 (0%)	0 (0%)	0 (0%)	0.74
	Intermediate	6 (86%)	13 (81%)	11 (92%)	
	Resistant	1 (14%)	3 (19%)	1 (8%)	
Ciprofloxacin	Sensitive	0 (0%)	0 (0%)	0 (0%)	0.55
	Intermediate	7 (100%)	15 (94%)	12 (100%)	
	Resistant	0 (0%)	1 (6%)	0 (0%)	
Ceftazidime	Sensitive	0 (0%)	0 (0%)	0 (0%)	1.00
	Intermediate	7 (100%)	16 (100%)	12 (100%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	
Cefepime	Sensitive	0 (0%)	0 (0%)	0 (0%)	**0.03**
	Intermediate	4 (57%)	16 (100%)	10 (83%)	
	Resistant	3 (43%)	0 (0%)	2 (17%)	
Aztreonam	Sensitive	0 (0%)	0 (0%)	0 (0%)	0.39
	Intermediate	7 (100%)	16 (100%)	11 (92%)	
	Resistant	0 (0%)	0 (0%)	1 (8%)	

Table 2: Ward A drains

Antibiotic	Antibiotic susceptibility	Period 1 n (%)	Period 2 n (%)	Period 3 n (%)	P-value (*)
Amikacin	Sensitive	2 (100%)	8 (100%)	15 (100%)	1.00
	Intermediate	0 (0%)	0 (0%)	0 (0%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	
Tobramycin	Sensitive	2 (100%)	8 (100%)	15 (100%)	1.00
	Intermediate	0 (0%)	0 (0%)	0 (0%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	
Piperacillin	Sensitive	0 (0%)	0 (0%)	0 (0%)	1.00
	Intermediate	2 (100%)	8 (100%)	15 (100%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	
Piperacillin-tazobactam	Sensitive	0 (0%)	0 (0%)	0 (0%)	1.00
	Intermediate	2 (100%)	8 (100%)	15 (100%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	
Ticarcillin-clavulanic acid	Sensitive	0 (0%)	0 (0%)	0 (0%)	1.00
	Intermediate	2 (100%)	8 (100%)	15 (100%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	
Meropenem	Sensitive	2 (100%)	8 (100%)	13 (87%)	0.29
	Intermediate	0 (0%)	0 (0%)	2 (13%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	
Imipenem	Sensitive	0 (0%)	0 (0%)	0 (0%)	0.66
	Intermediate	2 (100%)	7 (87%)	12 (80%)	
	Resistant	0 (0%)	1 (13%)	3 (20%)	
Ciprofloxacin	Sensitive	0 (0%)	0 (0%)	0 (0%)	1.00
	Intermediate	2 (100%)	8 (100%)	15 (100%)	
	Resistant	0 (0%)	1 (6%)	0 (0%)	
Ceftazidime	Sensitive	0 (0%)	0 (0%)	0 (0%)	1.00
	Intermediate	2 (100%)	8 (100%)	15 (100%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	
Cefepime	Sensitive	0 (0%)	0 (0%)	0 (0%)	0.47
	Intermediate	2 (100%)	8 (100%)	14 (93%)	
	Resistant	0 (0%)	0 (0%)	1 (7%)	
Aztreonam	Sensitive	0 (0%)	0 (0%)	0 (0%)	1.00
	Intermediate	2 (100%)	8 (100%)	15 (100%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	

(*) P-value for comparison of periods 2 and 3 only

Table 3: Ward B showers

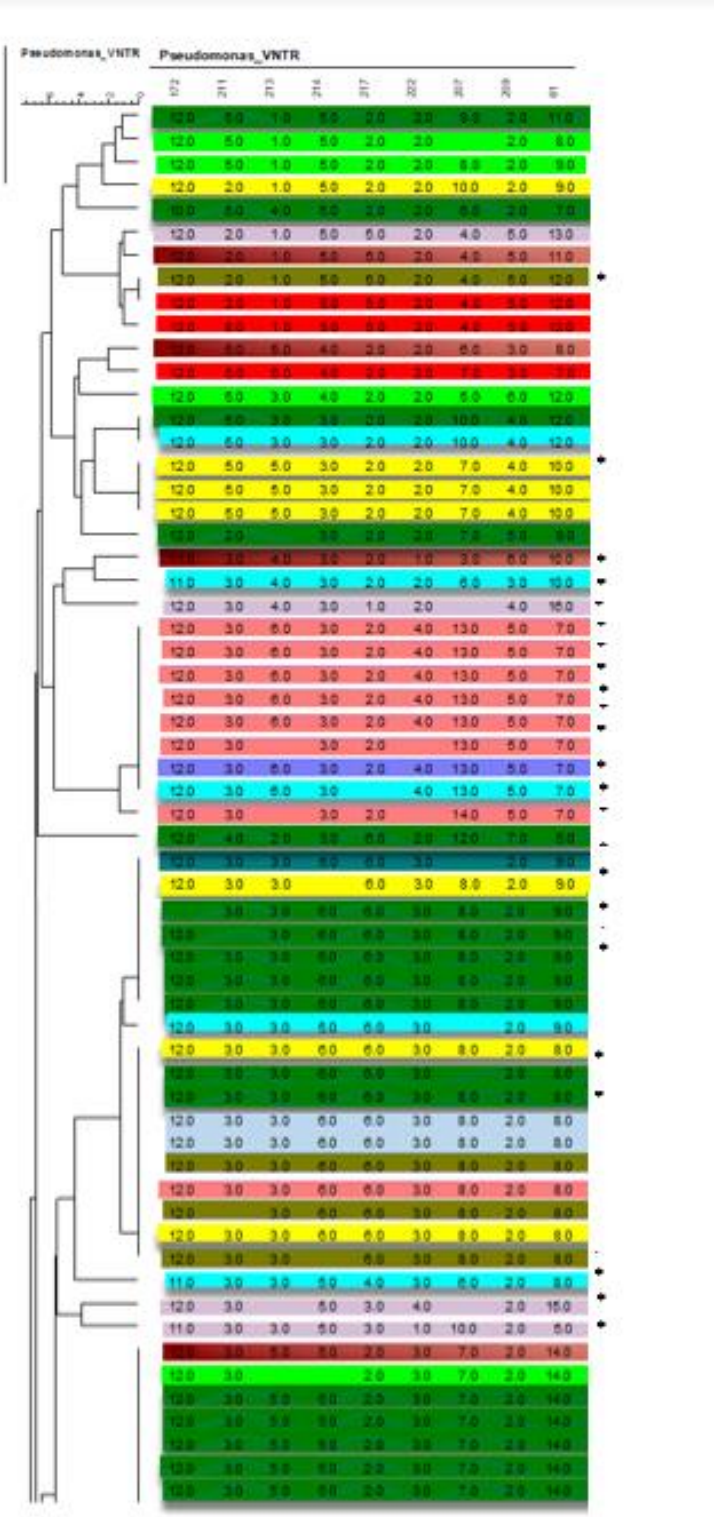
Antibiotic	Antibiotic susceptibility	Period 1 n (%)	Period 2 n (%)	Period 3 n (%)	P-value(*)
Amikacin	Sensitive	14 (100%)	25 (100%)	12 (92%)	0.58
	Intermediate	0 (0%)	0 (0%)	0 (0%)	
	Resistant	0 (0%)	1 (4%)	1 (8%)	
Tobramycin	Sensitive	14 (100%)	25 (96%)	13 (100%)	0.60
	Intermediate	0 (0%)	0 (0%)	0 (0%)	
	Resistant	0 (0%)	1 (4%)	0 (0%)	
Piperacillin	Sensitive	0 (0%)	0 (0%)	0 (0%)	0.43
	Intermediate	14 (100%)	23 (88%)	12 (92%)	
	Resistant	0 (0%)	3 (12%)	1 (8%)	
Piperacillin-tazobactam	Sensitive	0 (0%)	0 (0%)	0 (0%)	0.57
	Intermediate	14 (100%)	24 (92%)	12 (92%)	
	Resistant	0 (0%)	2 (8%)	1 (8%)	
Ticarcillin-clavulanic acid	Sensitive	0 (0%)	0 (0%)	0 (0%)	0.60
	Intermediate	13 (100%)	21 (81%)	11 (85%)	
	Resistant	1 (7%)	5 (19%)	2 (15%)	
Meropenem	Sensitive	13 (93%)	24 (192%)	13 (100%)	0.60
	Intermediate	1 (7%)	2 (8%)	0 (0%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	
Imipenem	Sensitive	0 (0%)	0 (0%)	0 (0%)	**0.03**
	Intermediate	7 (50%)	23 (88%)	9 (69%)	
	Resistant	7 (50%)	3 (12%)	4 (31%)	
Ciprofloxacin	Sensitive	0 (0%)	0 (0%)	0 (0%)	1.00
	Intermediate	14 (100%)	26 (100%)	13 (100%)	
	Resistant	0 (0%)	1 (6%)	0 (0%)	
Ceftazidime	Sensitive	0 (0%)	0 (0%)	0 (0%)	0.35
	Intermediate	14 (100%)	24 (92%)	13 (100%)	
	Resistant	0 (0%)	2 (8%)	0 (0%)	
Cefepime	Sensitive	0 (0%)	0 (0%)	0 (0%)	0.38
	Intermediate	12 (86%)	24 (92%)	13 (100%)	
	Resistant	2 (14%)	2 (8%)	0 (0%)	
Aztreonam	Sensitive	0 (0%)	0 (0%)	0 (0%)	0.72
	Intermediate	12 (100%)	24 (92%)	11 (85%)	
	Resistant	2 (14%)	2 (8%)	2 (15%)	

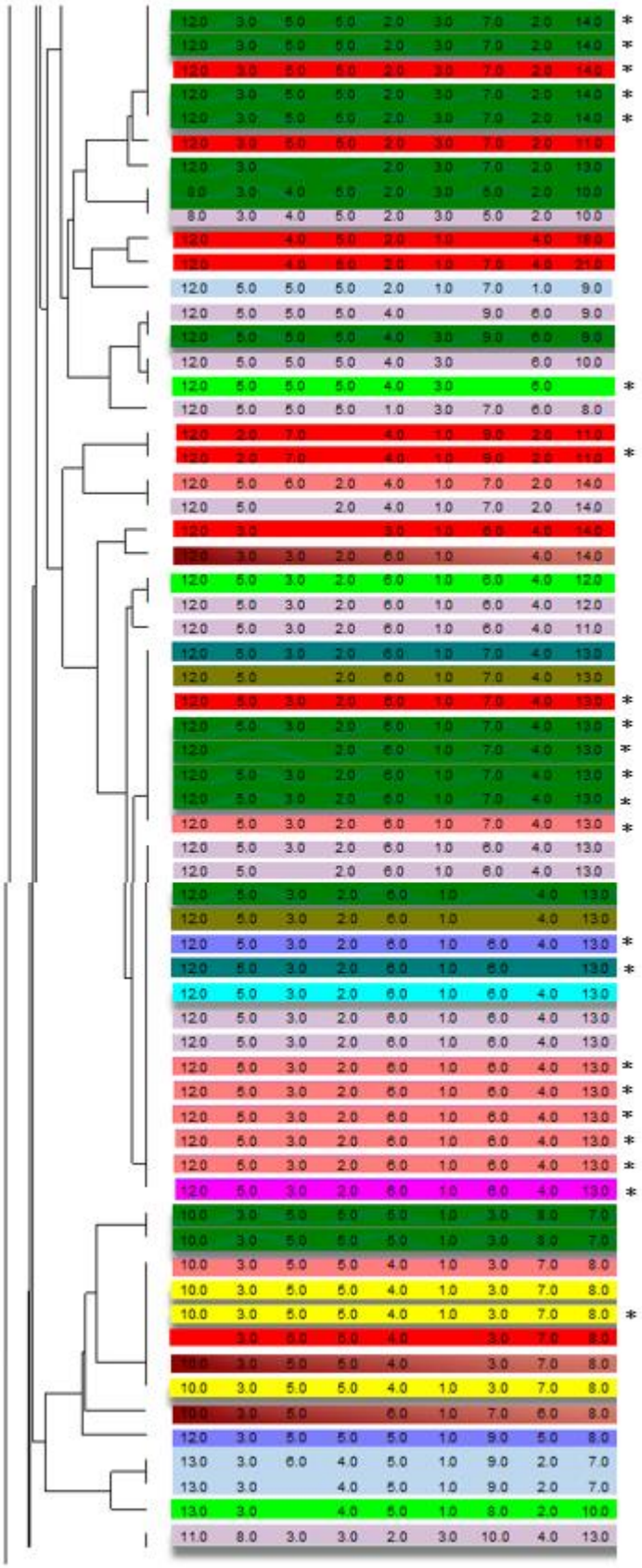
(*) P-value for comparison of periods 2 and 3 only

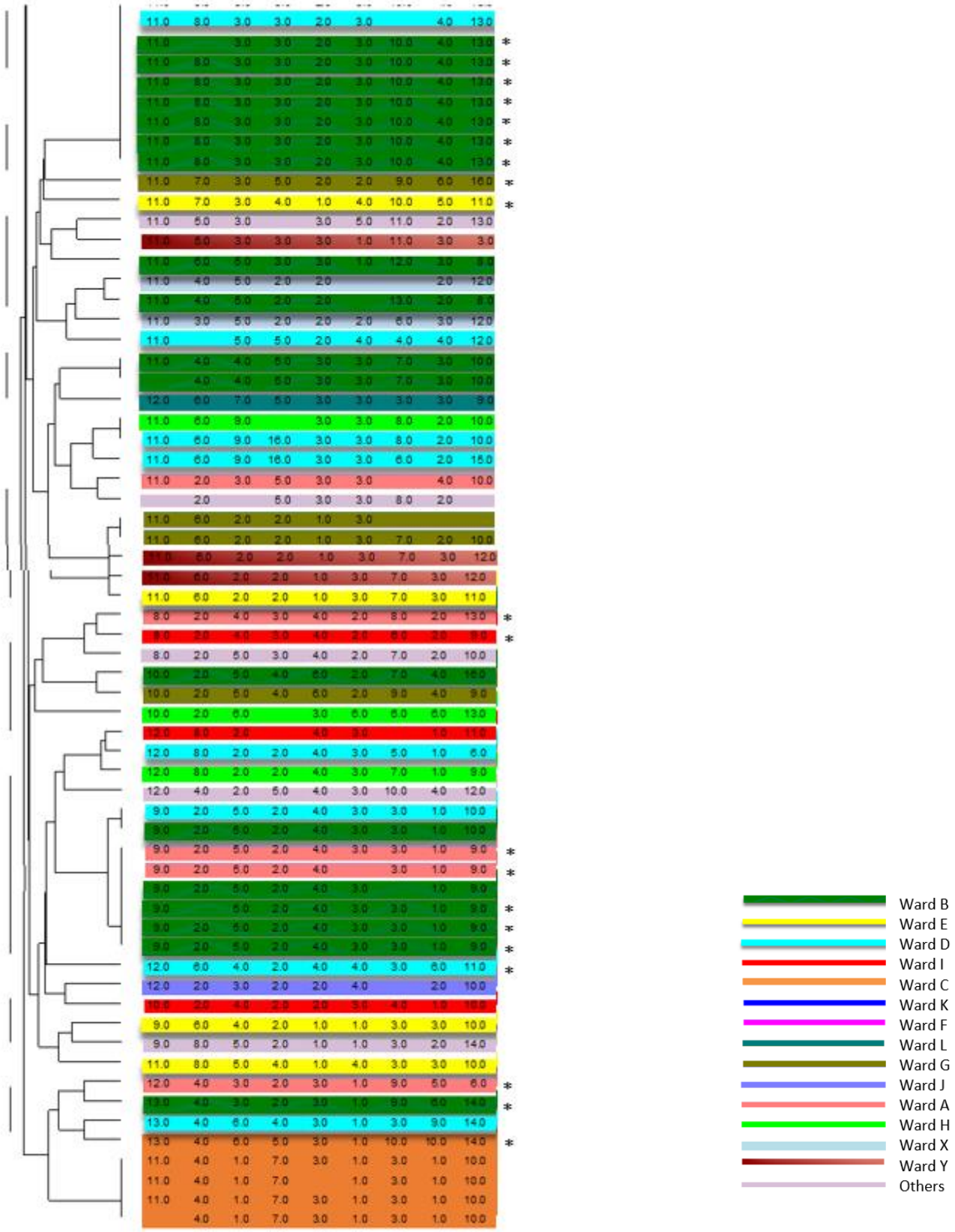
Table 4: Ward B drains

Antibiotic	Antibiotic susceptibility	Period 1 n (%)	Period 2 n (%)	Period 3 n (%)	P-value(*)
Amikacin	Sensitive	6 (75%)	10 (100%)	7 (87%)	0.27
	Intermediate	0 (0%)	0 (0%)	0 (0%)	
	Resistant	2 (25%)	0 (0%)	1 (13%)	
Tobramycin	Sensitive	8 (62%)	8 (80%)	8 (100%)	0.17
	Intermediate	0 (0%)	0 (0%)	0 (0%)	
	Resistant	3 (38%)	2 (20%)	0 (0%)	
Piperacillin	Sensitive	0 (0%)	0 (0%)	0 (0%)	1.00
	Intermediate	8 (100%)	10 (100%)	8 (100%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	
Piperacillin-tazobactam	Sensitive	0 (0%)	0 (0%)	0 (0%)	1.00
	Intermediate	8 (100%)	10 (100%)	8 (100%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	
Ticarcillin-clavulanic acid	Sensitive	0 (0%)	0 (0%)	0 (0%)	1.00
	Intermediate	8 (100%)	10 (100%)	8 (100%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	
Meropenem	Sensitive	8 (100%)	9 (90%)	8 (100%)	0.45
	Intermediate	0 (0%)	0 (0%)	0 (0%)	
	Resistant	0 (0%)	1 (10%)	0 (0%)	
Imipenem	Sensitive	0 (0%)	0 (0%)	0 (0%)	0.96
	Intermediate	6 (75%)	7 (70%)	6 (75%)	
	Resistant	2 (25%)	3 (30%)	2 (25%)	
Ciprofloxacin	Sensitive	0 (0%)	0 (0%)	0 (0%)	**0.03**
	Intermediate	5 (62%)	10 (100%)	8 (100%)	
	Resistant	3 (38%)	0 (0%)	0 (0%)	
Ceftazidime	Sensitive	0 (0%)	0 (0%)	0 (0%)	1.00
	Intermediate	8 (100%)	10 (100%)	8 (100%)	
	Resistant	0 (0%)	0 (0%)	0 (0%)	
Cefepime	Sensitive	0 (0%)	0 (0%)	0 (0%)	0.96
	Intermediate	6 (75%)	7 (70%)	6 (75%)	
	Resistant	2 (25%)	3 (30%)	2 (25%)	
Aztreonam	Sensitive	0 (0%)	0 (0%)	0 (0%)	**0.004**
	Intermediate	2 (25%)	8 (80%)	8 (100%)	
	Resistant	6 (75%)	2 (20%)	0 (0%)	

Appendix B. Phylogenetic tree depicting VNTR profiles of 185 P. aeruginosa isolates of clinical and environmental origin. Colour codes for individual wards are given in the key of the figure.



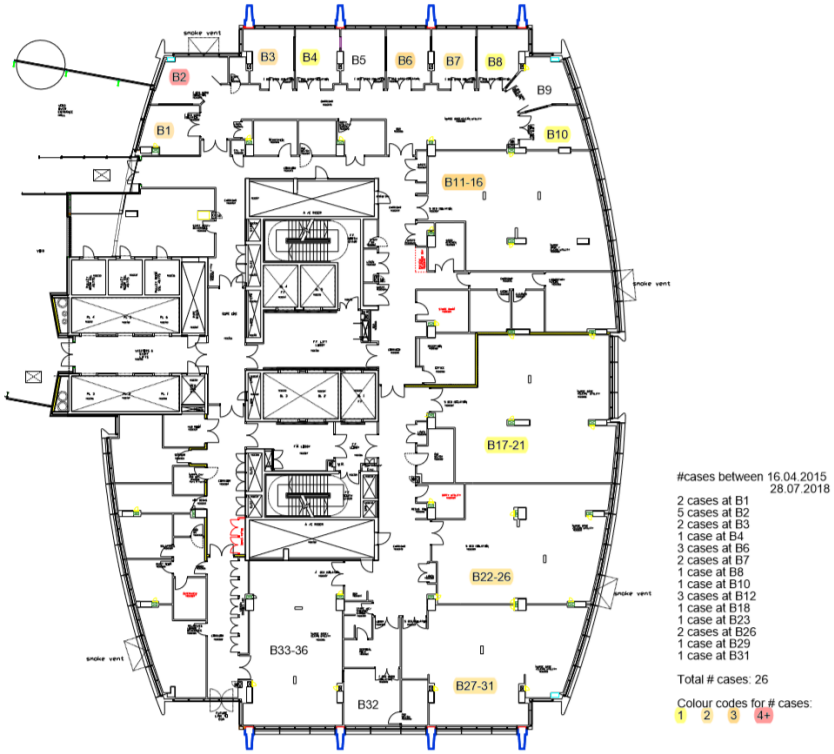



Ward B
Ward E
Ward D
Ward I
Ward C
Ward K
Ward F
Ward L
Ward G
Ward J
Ward A
Ward H
Ward X
Ward Y
Others

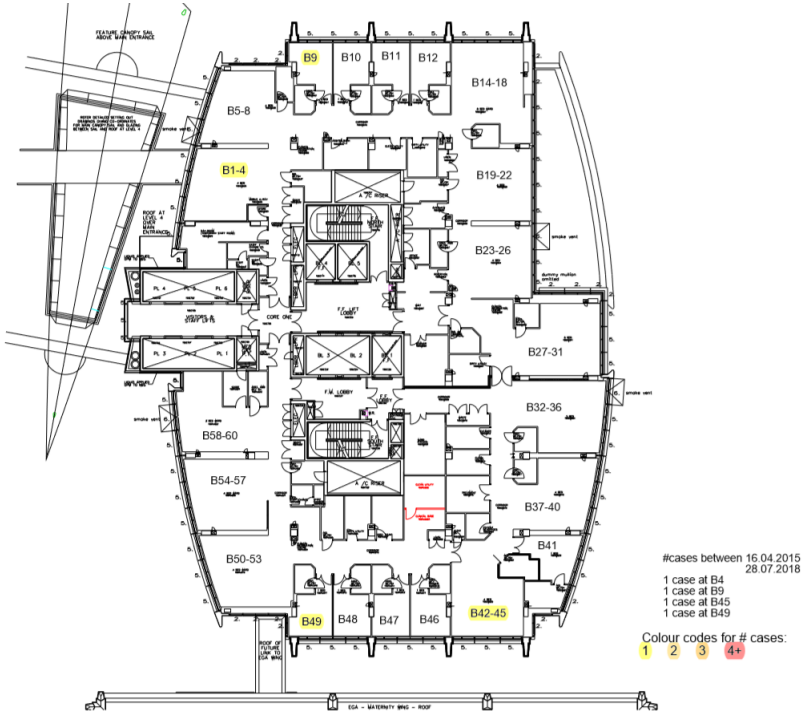
Appendix C. Case control study

Appendix C.1. Hospital floor plans of ten wards (A to J) sampled in this study and depicting *P. aeruginosa* bacteraemia patient locations (highlighted).

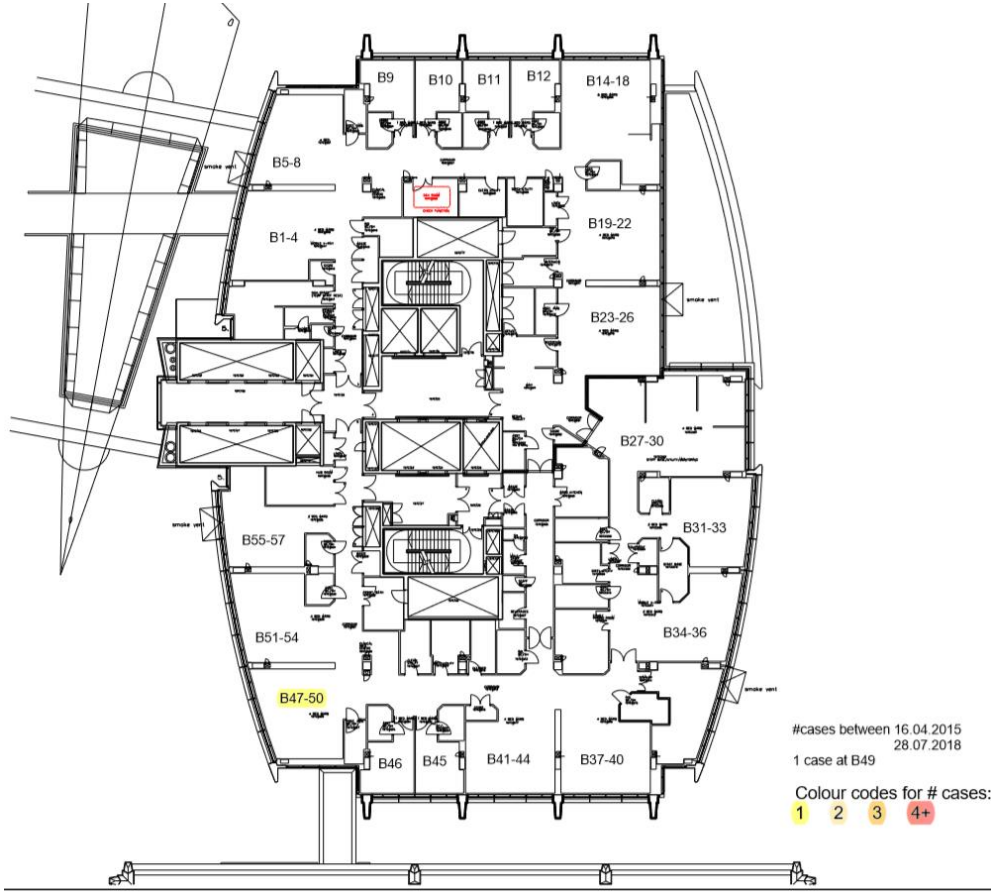
Ward H



Ward A



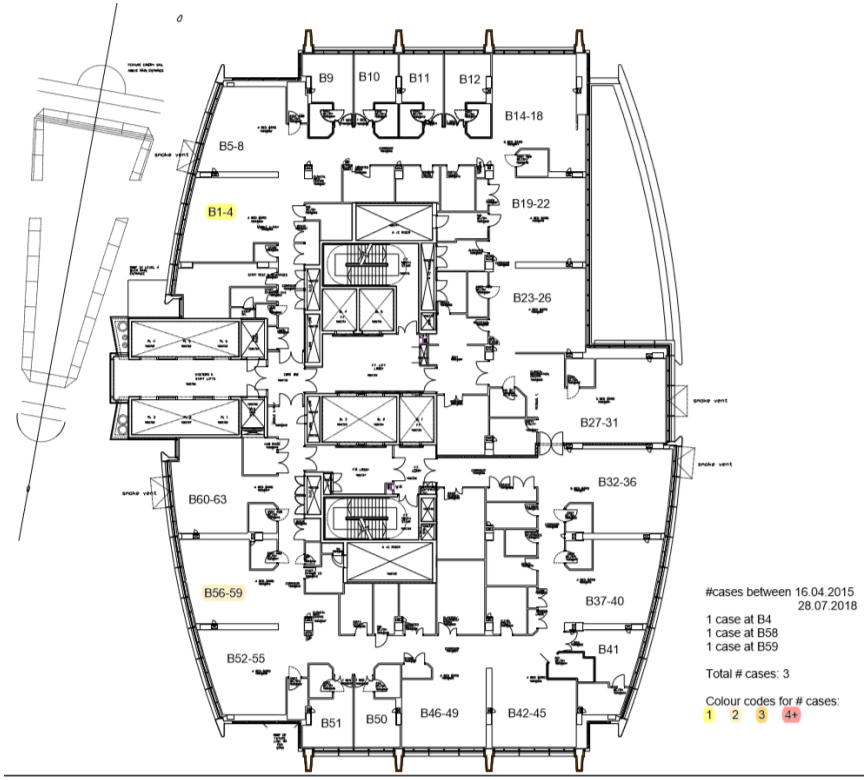
Ward J


B9
B10
B11
B12
B14-18
B5-8
B1-4
B19-22
B23-26
B27-30
B31-33
B55-57
B51-54
B34-36
B47-50
B46
B45
B41-44
B37-40
#cases between 16.04.2015
28.07.2018
1 case at B49
Colour codes for # cases:
1
2
3
4+

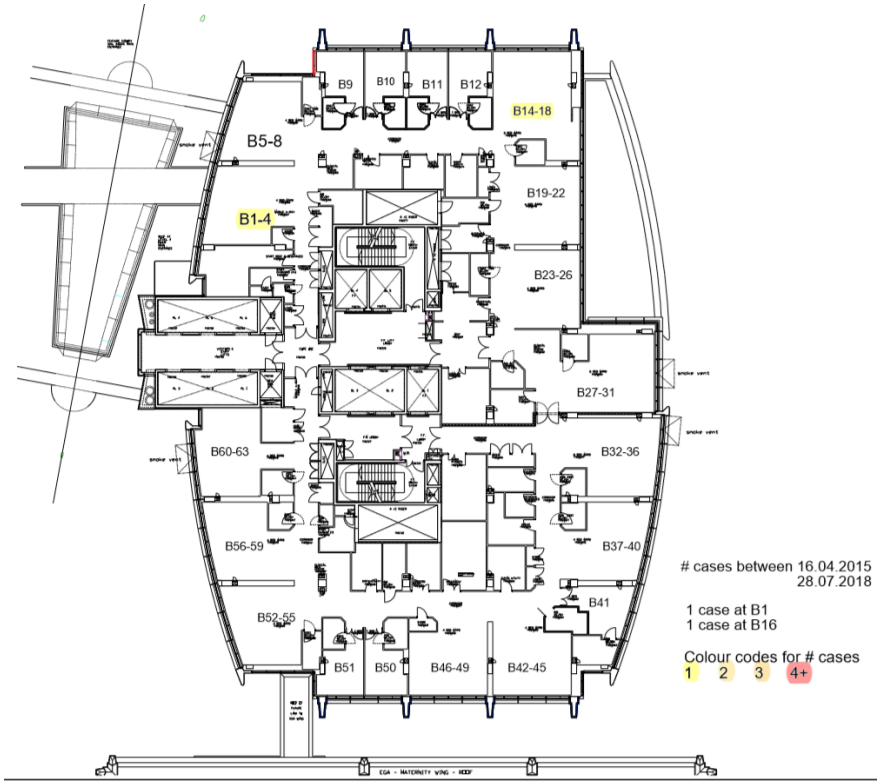
Ward G


B9-12
B14-17
B18
B19
B5-8
B1-4
B20
B21
B22
B23-27
B44
B28
B43
B29
B42
B30
B41
B31
B40
B32
B39
B38
B37
B36
B35
B34
B33
#cases between 16.04.2015
28.07.2018
1 case at B16
1 case at B17
1 case at B32
1 case at B33
2 cases at B34
1 case at B36
1 case at B37
1 case at B38
1 case at B39
2 cases at B40
Total # cases: 12
Colour codes for # cases:
1
2
3
4+

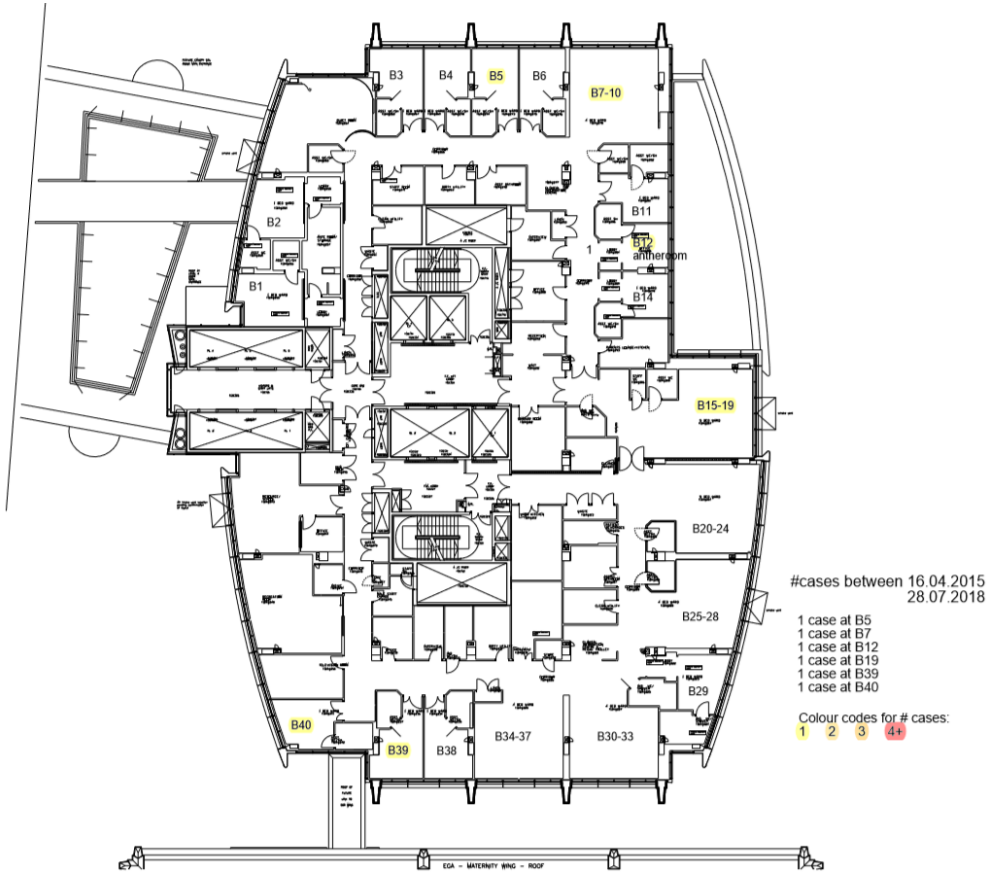
Ward L


B9
B10
B11
B12
B14-18
B5-8
B1-4
B19-22
B23-26
B27-31
B32-36
B60-63
B56-59
B37-40
B41
B52-55
B51
B50
B46-49
B42-45
#cases between 16.04.2015
28.07.2018
1 case at B4
1 case at B58
1 case at B59
Total # cases: 3
Colour codes for # cases:
1 2 3 4+

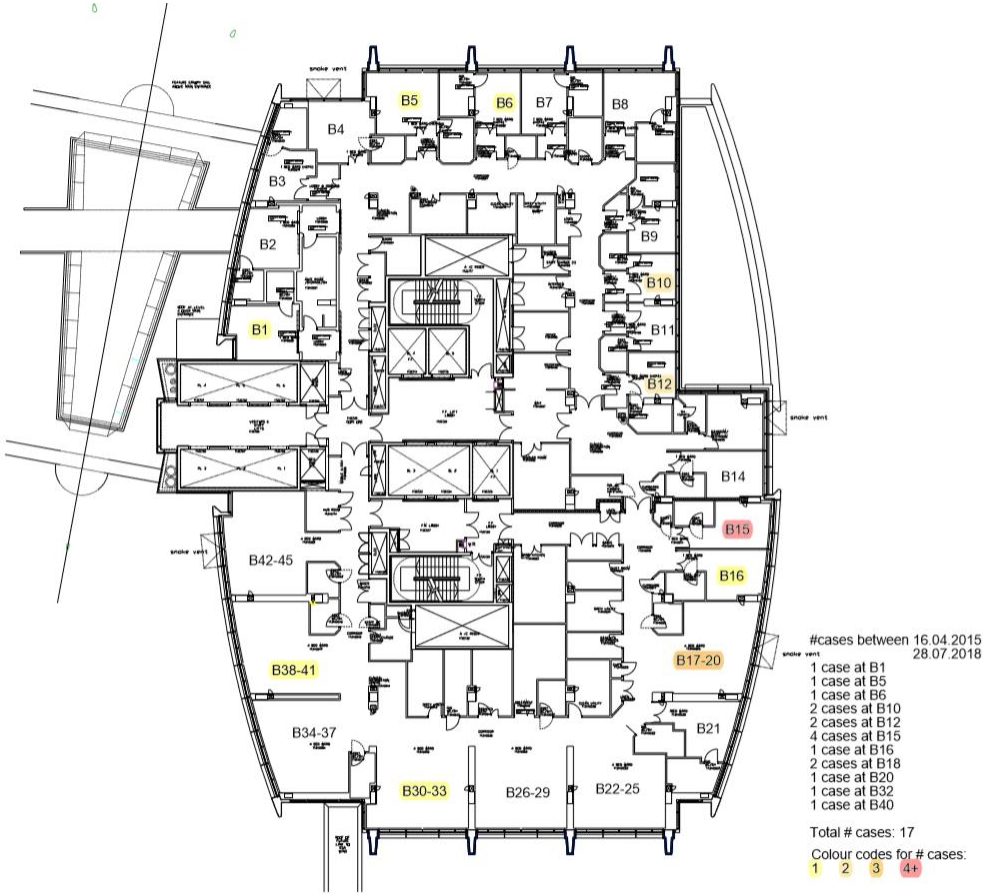
Ward F


B9
B10
B11
B12
B14-18
B5-8
B1-4
B19-22
B23-26
B27-31
B32-36
B60-63
B56-59
B37-40
B41
B52-55
B51
B50
B46-49
B42-45
cases between 16.04.2015
28.07.2018
1 case at B1
1 case at B16
Colour codes for # cases
1 2 3 4+

Ward C

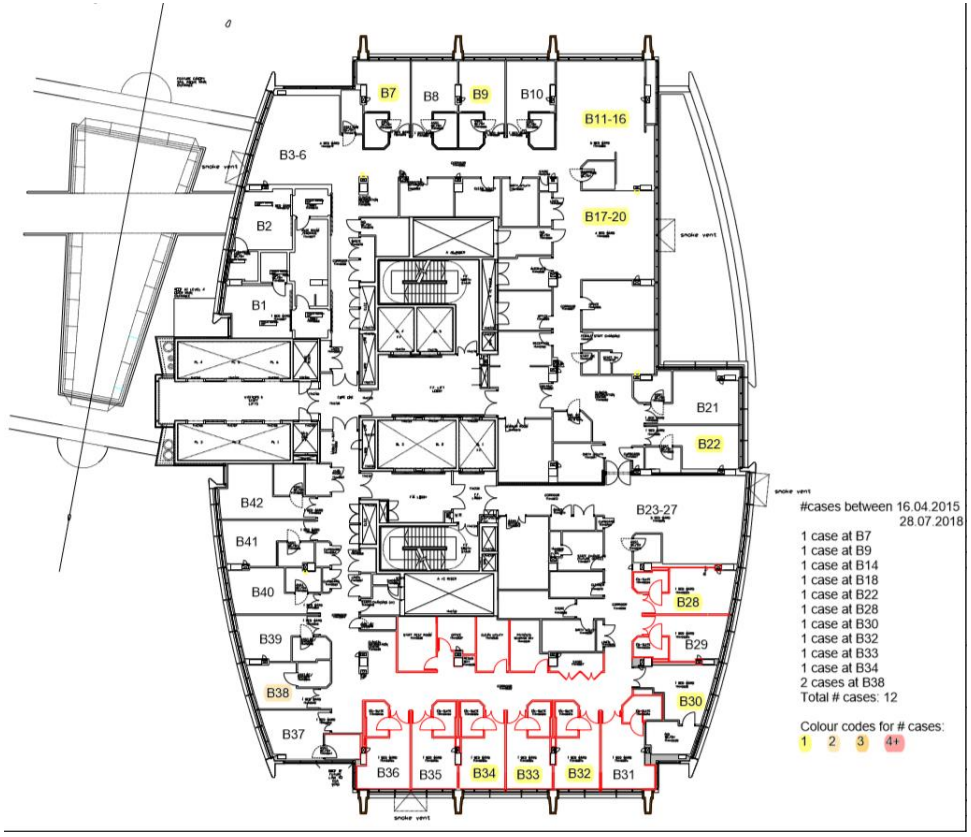


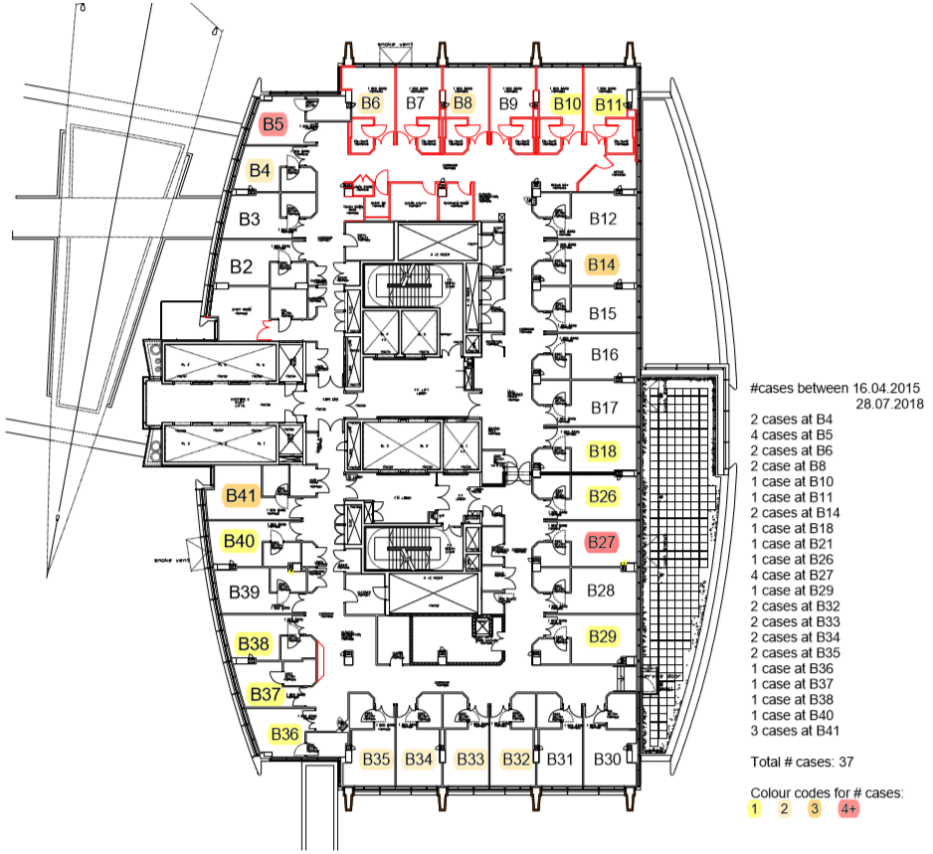
Ward I



Ward D

Ward B


B7
B8
B9
B10
B11-16
B3-6
B2
B1
B17-20
B21
B22
B42
B41
B40
B39
B38
B37
B23-27
B28
B29
B30
B36
B35
B34
B33
B32
B31
#cases between 16.04.2015
28.07.2018
1 case at B7
1 case at B9
1 case at B14
1 case at B18
1 case at B22
1 case at B28
1 case at B30
1 case at B32
1 case at B33
1 case at B34
2 cases at B38
Total # cases: 12
Colour codes for # cases:
1 2 3 4+


B5
B6
B7
B8
B9
B10
B11
B4
B3
B2
B12
B14
B15
B16
B17
B18
B41
B40
B39
B38
B37
B36
B26
B27
B28
B29
B35
B34
B33
B32
B31
B30
#cases between 16.04.2015
28.07.2018
2 cases at B4
4 cases at B5
2 cases at B6
2 case at B8
1 case at B10
1 case at B11
2 cases at B14
1 case at B18
1 case at B21
1 case at B26
4 cases at B27
1 case at B29
2 cases at B32
2 cases at B33
2 cases at B34
2 cases at B35
1 case at B36
1 case at B37
1 case at B38
1 case at B40
3 cases at B41
Total # cases: 37
Colour codes for # cases:
1 2 3 4+

Appendix C.2. Shower contamination and *P. aeruginosa* bacteraemia case locations per ward.

Following graphs show the prevalence of *P. aeruginosa* contamination in shower waters in comparison with *P. aeruginosa* bacteraemia patients between 2014 and 2019 at following wards of UCH: Ward A, B, C, D, E, F, G, H, I and K

Timeline of significant events are given as:

- Anonymised 1 showers are introduced to haematology wards at July 2015 (All wards except Ward A in this report).
- Royal Free Hospital haematology patients entered UCH from mid-2016.
- Shower hoses are shortened from 1.2m to 0.8m at all upper half of the tower at December 2018 (8^{th} -16^{th} floor). All remaining standard showers are changed to Anonymised 1 short hose.
- Anonymised 2 filter showers are applied to Ward B, Ward C, Ward D, Ward E, Ward F North, Ward H and Ward I North at July 2019.

Corresponding data and samples sizes are given at the end of the graphs in Appendix C.2 as Table 1.

Figure 1. Prevalence of *P. aeruginosa* contamination in showers in comparison with *P. aeruginosa* bacteraemia patients at Ward H.

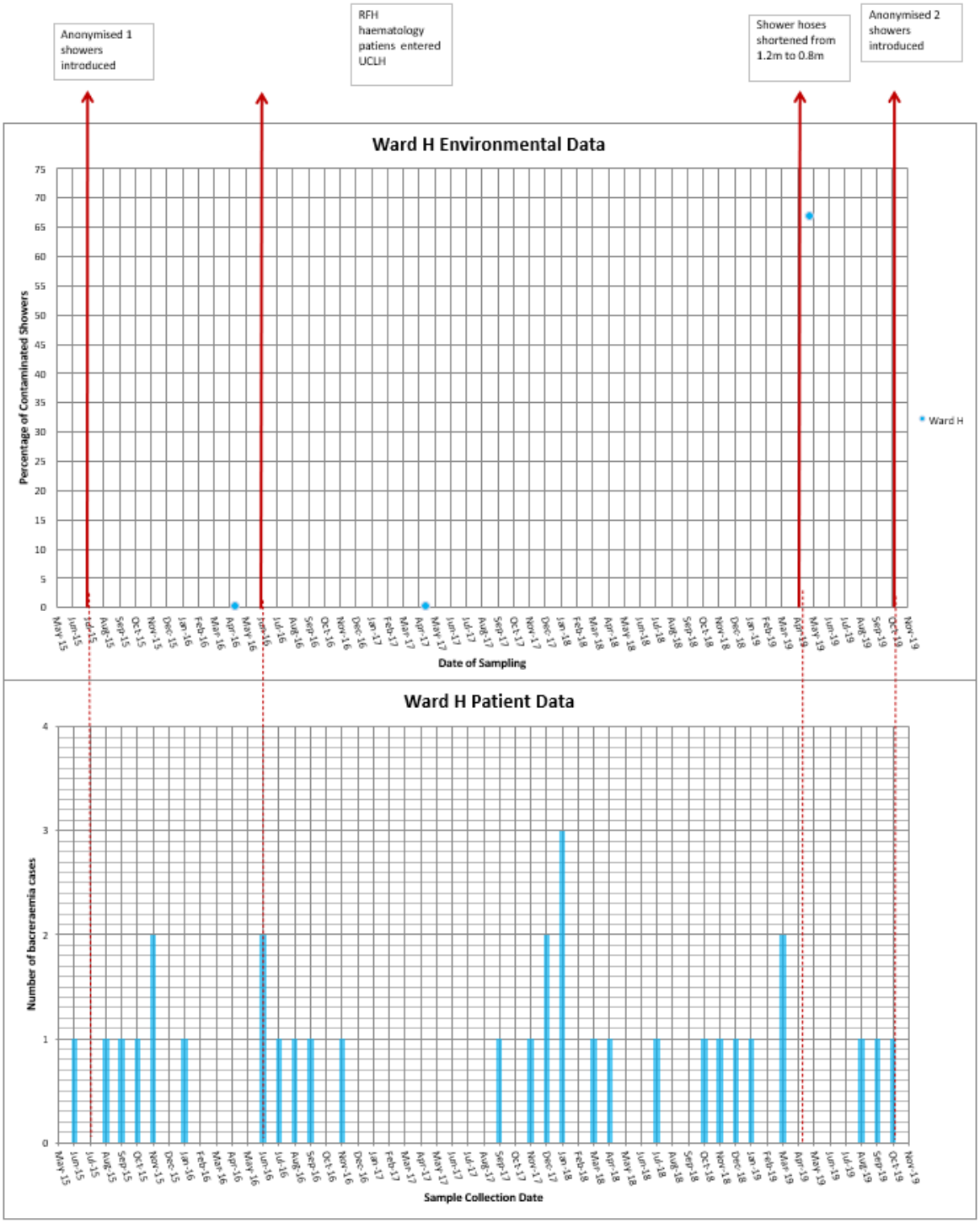


Figure 2. Prevalence of *P. aeruginosa* contamination in showers in comparison with *P. aeruginosa* bacteraemia patients at Ward A.

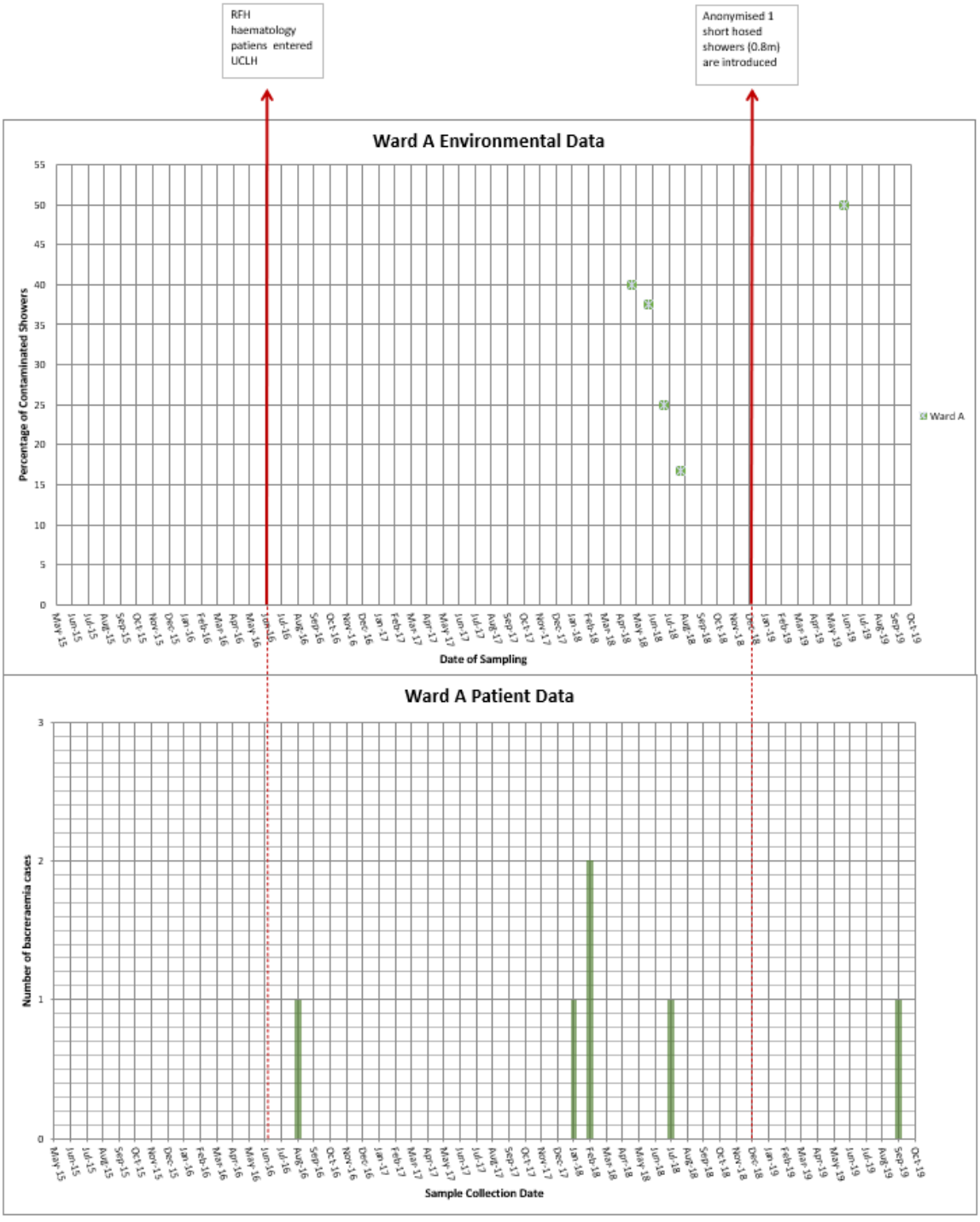


Figure 3. Prevalence of *P. aeruginosa* contamination in showers in comparison with *P. aeruginosa* bacteraemia patients at Ward G.

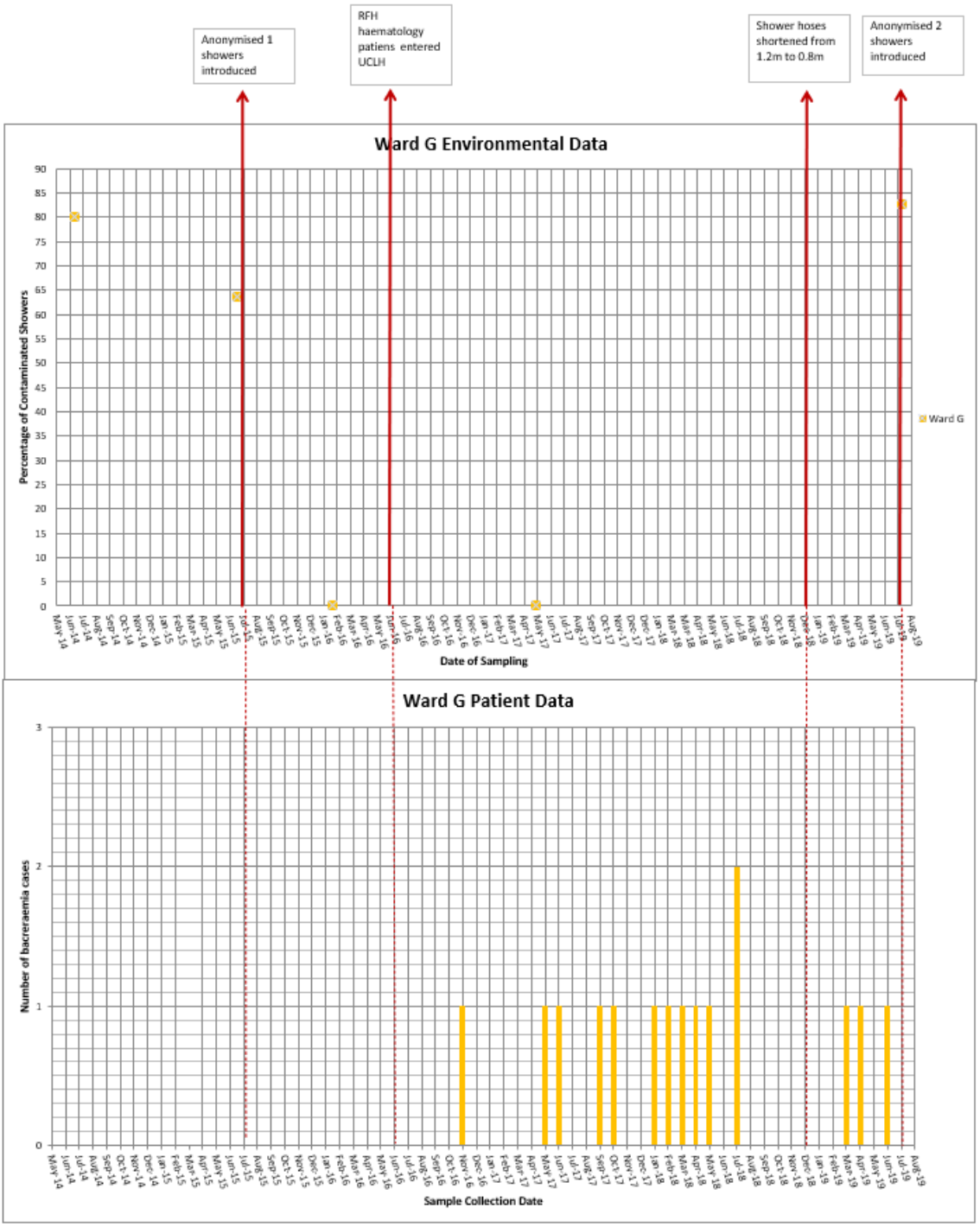


Figure 4. Prevalence of *P. aeruginosa* contamination in showers in comparison with *P. aeruginosa* bacteraemia patients at Ward F.

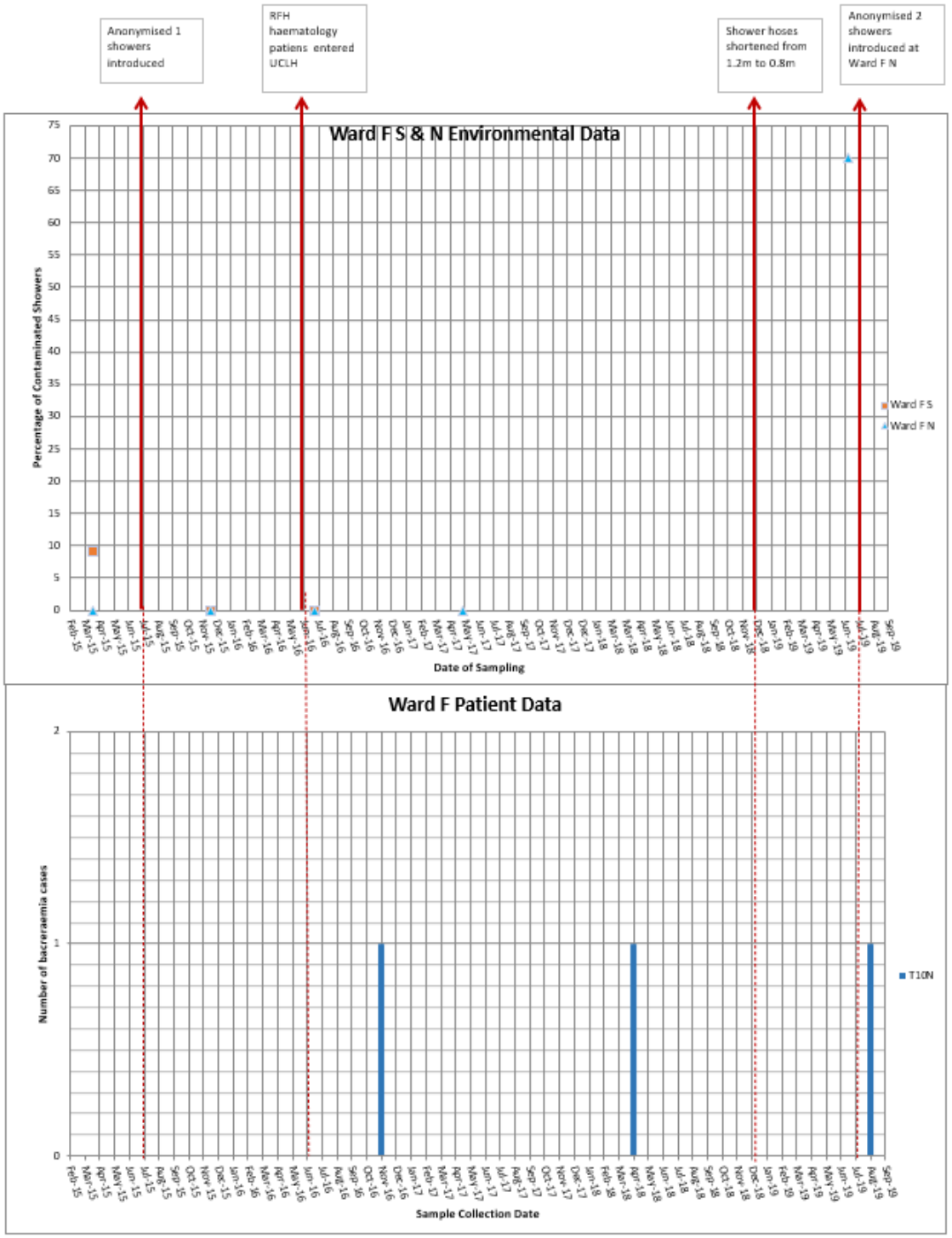


Figure 5. Prevalence of *P. aeruginosa* contamination in showers in comparison with *P. aeruginosa* bacteraemia patients at Ward C.

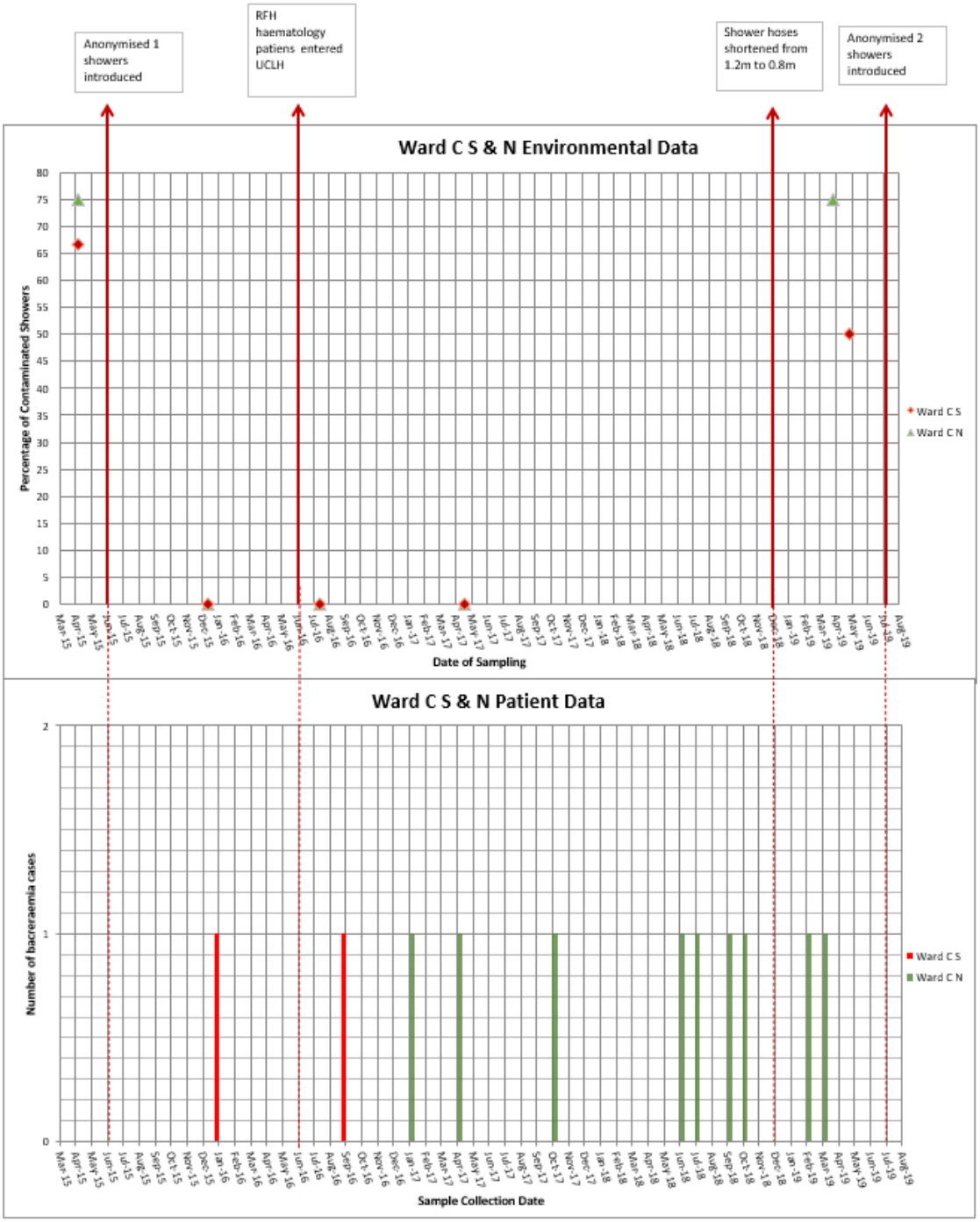


Figure 6. Prevalence of *P. aeruginosa* contamination in showers in comparison with *P. aeruginosa* bacteraemia patients at Ward I.

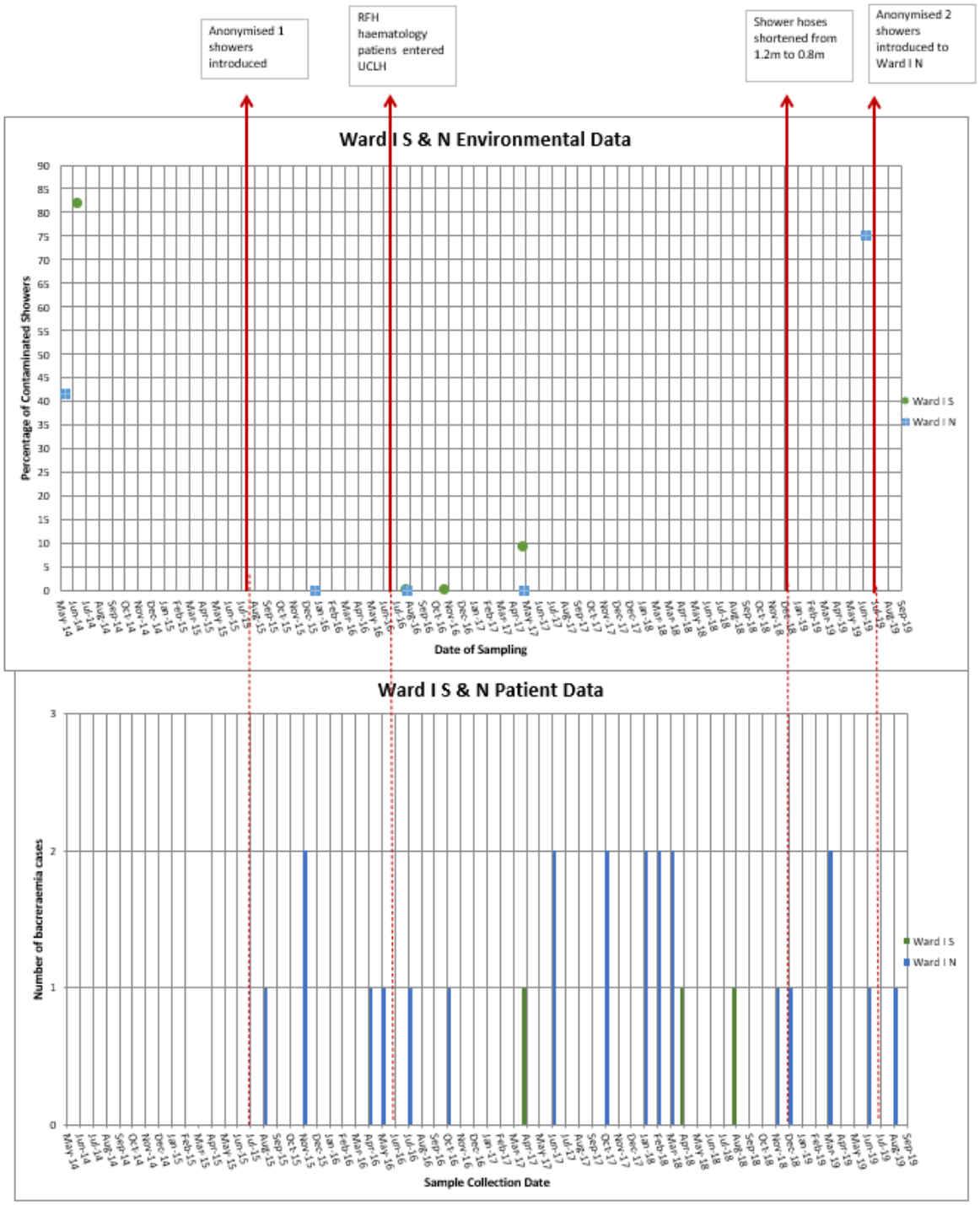


Figure 7. Prevalence of *P. aeruginosa* contamination in showers in comparison with *P. aeruginosa* bacteraemia patients at Ward D.

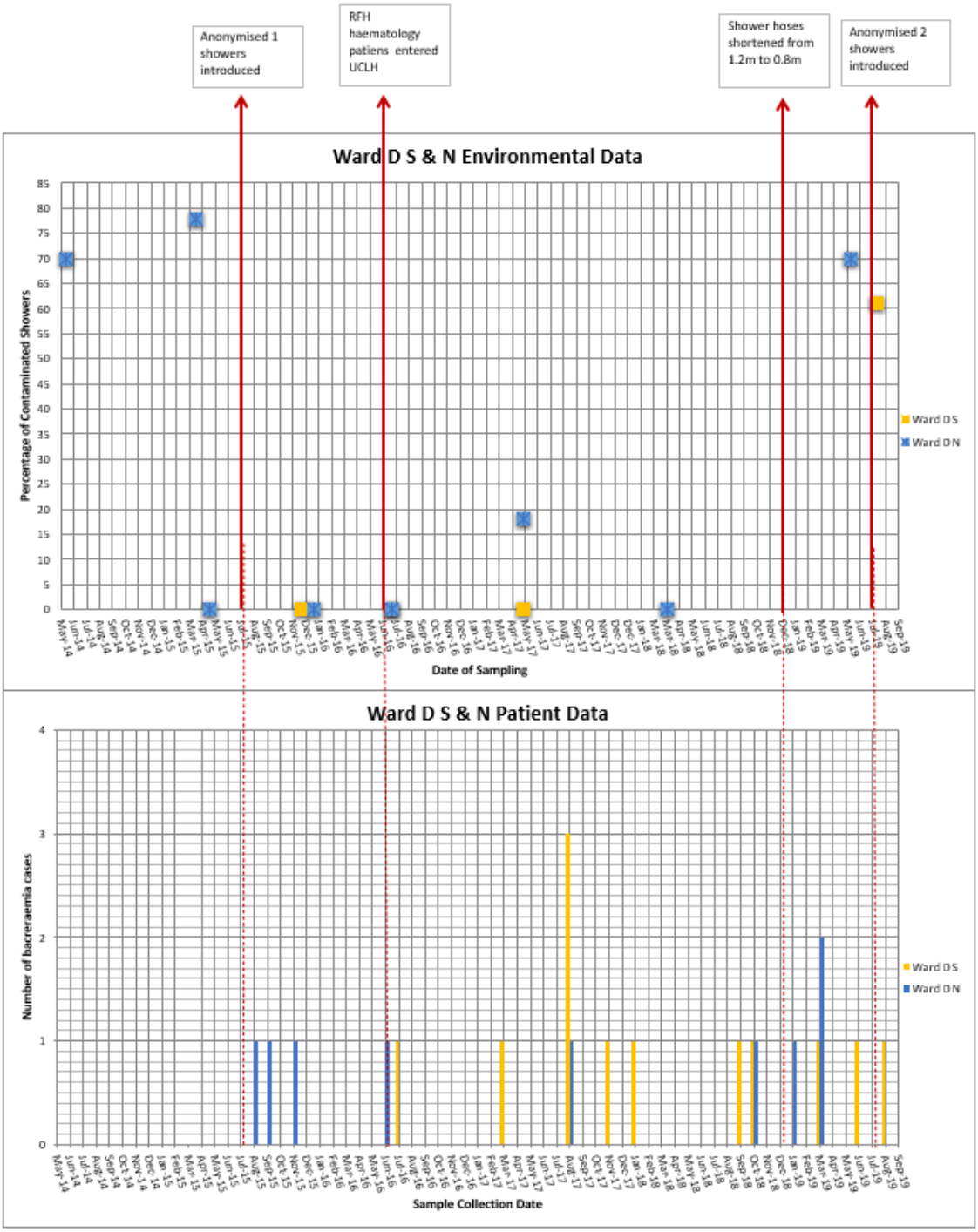


Figure 8. Prevalence of *P. aeruginosa* contamination in showers in comparison with *P. aeruginosa* bacteraemia patients at Ward E.

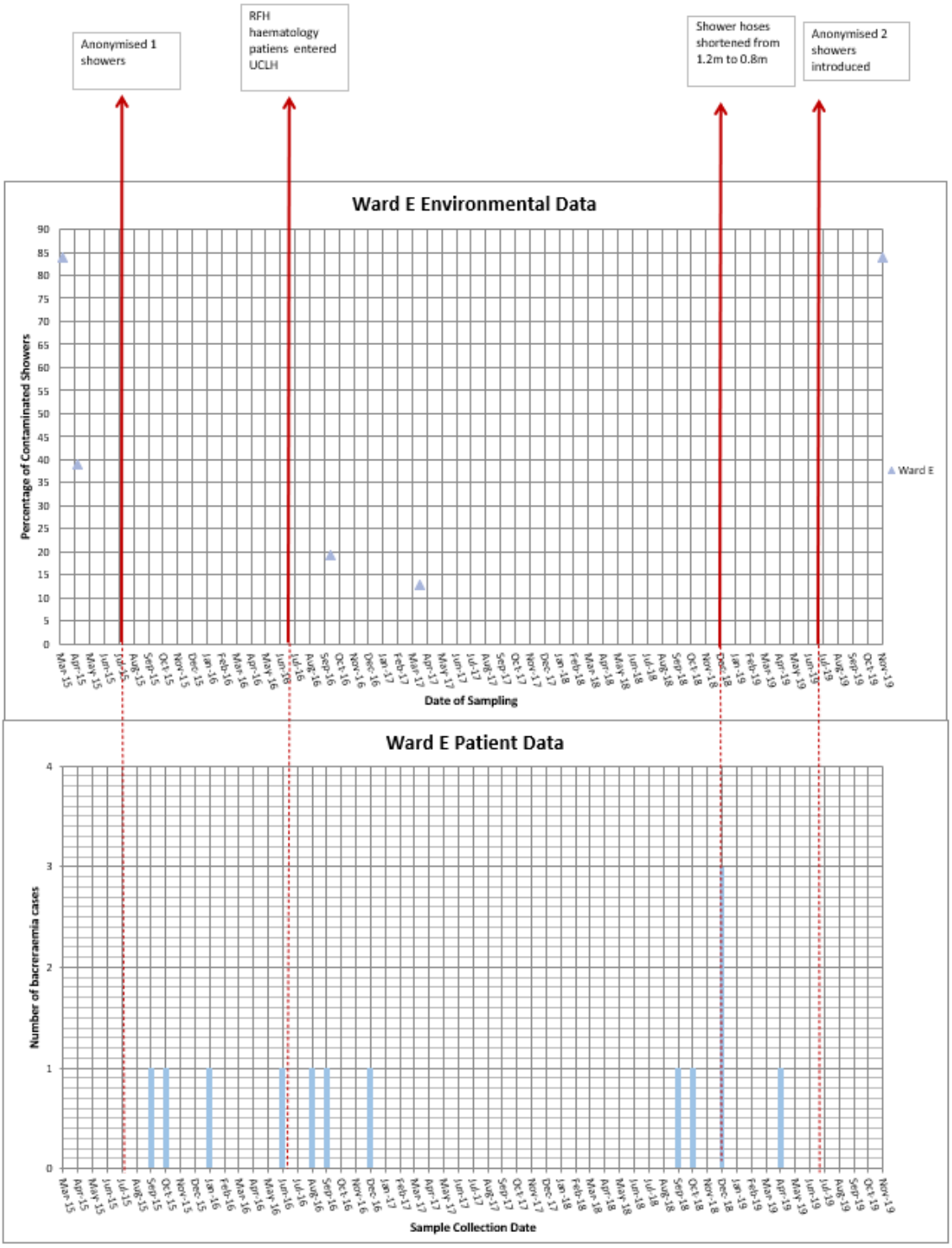


Figure 9. Prevalence of *P. aeruginosa* contamination in showers in comparison with *P. aeruginosa* bacteraemia patients at Ward B.

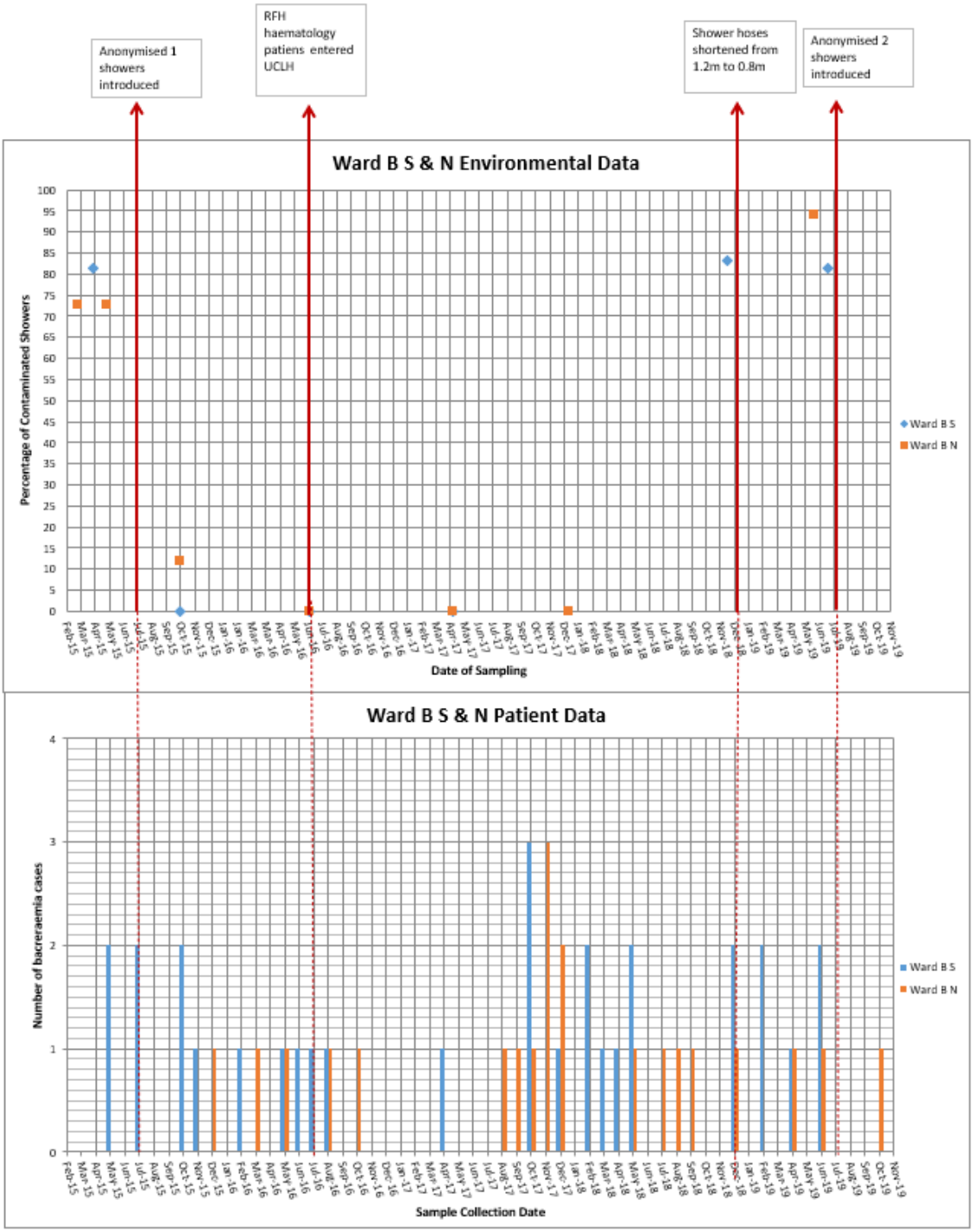


Table 1. Sample size of contaminated showers in accordance with total showers tested.

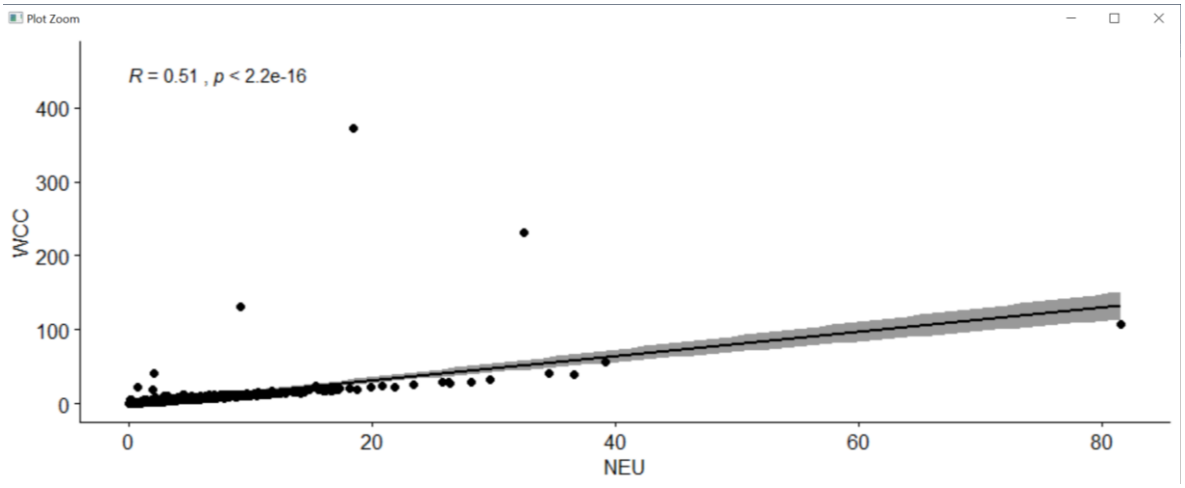
Ward B N	Mar-15	Ap-May 2(	Oct-15	Jul-16	May-17	Jan-18	Jun-19		Ward B S	Apr-15	Oct-15	Jul-16	May-17	Dec-18	Jul-19
pos	8	8	2	0	0	0	16		pos	13	0	0	0	5	13
tot	11	11	17	17	16	16	17		tot	16	16	16	16	6	16
%	72.72727	72.72727	11.76471	0	0	0	94.11765		%	81.25	0	0	0	83.33333	81.25
Ward E	Apr-15	May-15	Oct-16	Apr-17	Nov-19				Ward H	May-16	May-17	Jun-19			
pos	26	7	6	4	26				pos	0	0	2			
tot	31	18	31	31	31				tot	2	2	3			
%	83.87097	38.88889	19.35484	12.90323	83.87097				%	0	0	66.66667			
Ward D N	Jun-14	Apr-15	May-15	Jan-16	Jul-16	May-17	Apr-18	Jun-19		Ward D S	Dec-15	Jul-16	May-17	Aug-19	
pos	7	7	0	0	0	2	0	7		pos	0	0	0	11	
tot	10	9	8	11	11	11	12	10		tot	17	17	17	18	
%	70	77.77778	0	0	0	18.18182	0	70		%	0	0	0	61.11111	
Ward I N	Jun-14	Jan-16	Aug-16	May-17	Jul-19					Ward I S	Jul-14	Aug-16	Nov-16	May-17	
pos	5	0	0	0	9					pos	9	0	0	1	
tot	12	12	12	13	12					tot	11	11	11	11	
%	41.66667	0	0	0	75					%	81.81818	0	0	9.090909	
Ward C N	May-15	Jan-16	Aug-16	May-17	Apr-19					Ward C S	May-15	Jan-16	Aug-16	May-17	May-19
pos	9	0	0	0	9					pos	6	0	0	0	5
tot	12	12	12	12	12					tot	9	9	10	10	10
%	75	0	0	0	75					%	66.66667	0	0	0	50
Ward K N	May-15	Jan-16	May-17												
pos	0	0	0												
neg	8	8	11												
%	0	0	0												
Ward F N	Apr-15	Dec-15	Jul-16	May-17	Jun-19			Ward F S	Apr-15	Dec-15	Jul-16				
pos	0	0	0	0	7			pos	1	0	0				
tot	9	9	9	9	10			tot	11	11	11				
%	0	0	0	0	70			%	9.090909	0	0				
Ward G	Jul-14	Jul-15	Feb-16	May-17	Aug-19			Ward A	May-18	Jun-18	Jul-18	Aug-18	Jun-19		
pos	4	7	0	0	24			pos	2	3	1	1	5		
tot	5	11	28	28	29			tot	5	8	4	6	10		
%	80	63.63636	0	0	82.75862			%	40	37.5	25	16.66667	50		

Appendix C.3. Correlation Analysis

Full data set

```
> categorywccgroups
# A tibble: 625 x 14
   pairing outcome casecontrol  gender ageatdoc
     <dbl> <dbl+l>   <dbl+lbl> <dbl+l>    <dbl>
 1       0 1 [Pa ~ 1 [Case]    1 [Fem~       74
 2       0 0 [No ~ 2 [Control] 1 [Fem~       73
 3       0 0 [No ~ 2 [Control] 0 [Mal~       72
 4       0 0 [No ~ 2 [Control] 1 [Fem~       71
 5       0 0 [No ~ 2 [Control] 1 [Fem~       71
 6       0 0 [No ~ 2 [Control] 0 [Mal~       72
 7       0 0 [No ~ 2 [Control] 0 [Mal~       73
 8       0 0 [No ~ 2 [Control] 0 [Mal~       74
 9       0 0 [No ~ 2 [Control] 0 [Mal~       73
10       0 0 [No ~ 2 [Control] 0 [Mal~       73
# ... with 615 more rows, and 9 more variables:
#   losbeforedoc <dbl>, WCC <dbl>, ALB <dbl>,
#   CRE <dbl>, NEU <dbl>, CentralLines <dbl+lbl>,
#   OtherbacteriaincludingPapreviously <dbl+lbl>,
#   WCCGroups <dbl+lbl>, NEUGroups <dbl+lbl>
> cor(WCC, NEU, method = c("pearson", "kendall", "spearman"))
[1] NA
> cor.test(WCC, NEU, method = c("pearson", "kendall", "spearman"))

                Pearson's product-moment correlation

data:  WCC and NEU
t = 13.273, df = 513, p-value < 2.2e-16
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
 0.4383538 0.5672299
sample estimates:
      cor
0.5056069
```

```
> ggscatter(categorywccgroups, x='NEU', y='WCC', add="reg.line", conf.int = TRUE, cor.coef = TRUE, cor.method = "pearson")
```

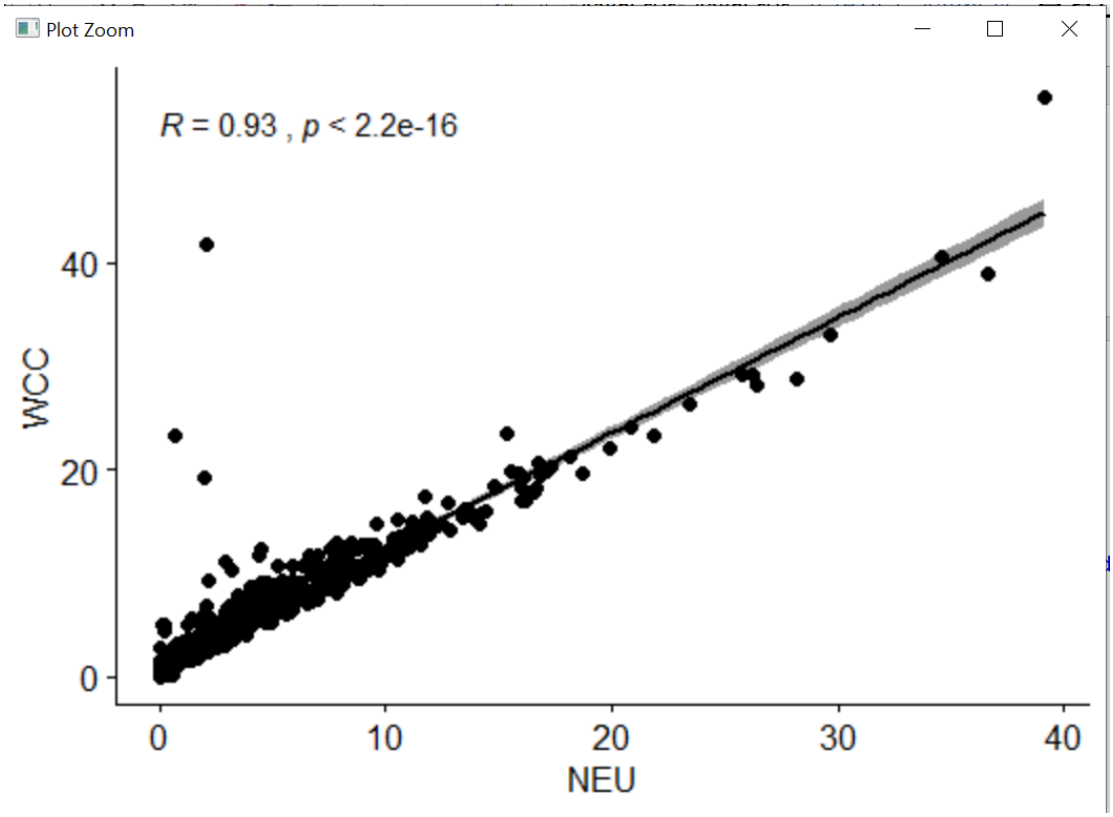


Odd values deleted - in order to better show the correlation for blood test results, five outlier WCC values were not included in the following graph.

WCC>>100 values deleted

```
> View(Spss_case_control_odd_values_deleted_v2)
> Spss_case_control_odd_values_deleted_v2
# A tibble: 620 x 12
   pairing outcome casecontrol  gender ageatdoc
```

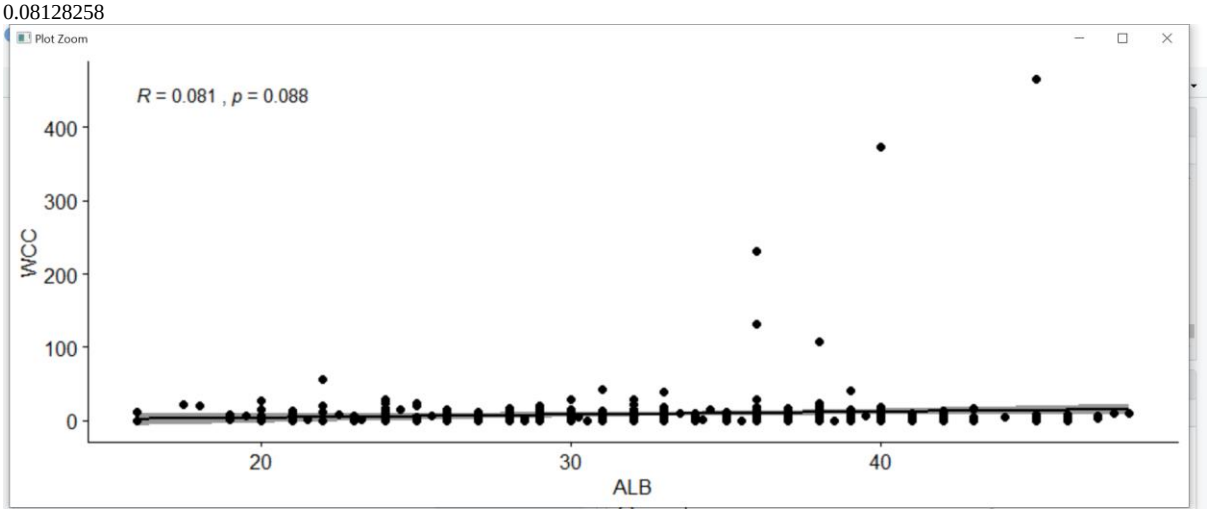
```
   <dbl> <dbl+l>   <dbl+lbl> <dbl+l>    <dbl>
 1     0 1 [Pa ~ 1 [Case]    1 [Fem~      74
 2     0 0 [No ~ 2 [Control] 1 [Fem~      73
 3     0 0 [No ~ 2 [Control] 0 [Mal~      72
 4     0 0 [No ~ 2 [Control] 1 [Fem~      71
 5     0 0 [No ~ 2 [Control] 1 [Fem~      71
 6     0 0 [No ~ 2 [Control] 0 [Mal~      72
 7     0 0 [No ~ 2 [Control] 0 [Mal~      73
 8     0 0 [No ~ 2 [Control] 0 [Mal~      74
 9     0 0 [No ~ 2 [Control] 0 [Mal~      73
10     0 0 [No ~ 2 [Control] 0 [Mal~      73
# ... with 610 more rows, and 7 more variables:
#   losbeforedoc <dbl>, WCC <dbl>, ALB <dbl>,
#   CRE <dbl>, NEU <dbl>, CentralLines <dbl+lbl>,
#   OtherbacteriaincludingPapreviously <dbl+lbl>
> ggscatter(Spss_case_control_odd_values_deleted_v2, x='NEU', y='WCC', add="reg.line", conf.int = TRUE, cor.coef = TRUE, cor.method
= "pearson")
```



Continued with raw data

```
> cor(WCC, ALB, method = c("pearson", "kendall", "spearman"))
[1] NA
> cor.test(WCC, ALB, method = c("pearson", "kendall", "spearman"))

        Pearson's product-moment correlation

data:  WCC and ALB
t = 1.7107, df = 440, p-value = 0.08785
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
 -0.01208109  0.17324124
sample estimates:
      cor
```

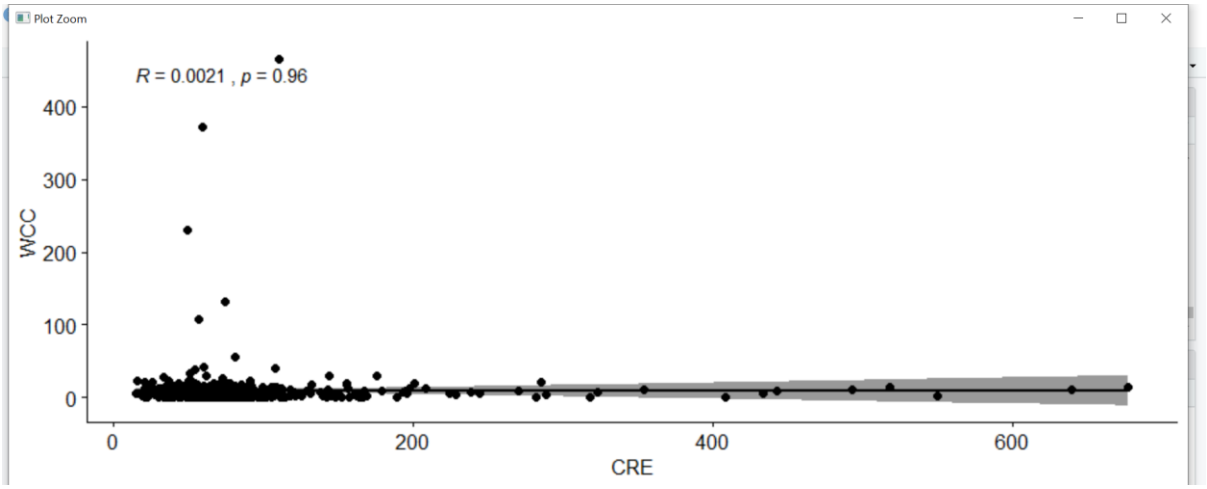
0.08128258



> cor.test(WCC, CRE, method = c("pearson", "kendall", "spearman"))

Pearson's product-moment correlation

data: WCC and CRE
t = 0.048422, df = 537, p-value = 0.9614
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
-0.08238088 0.08653015
sample estimates:
cor
0.002089538



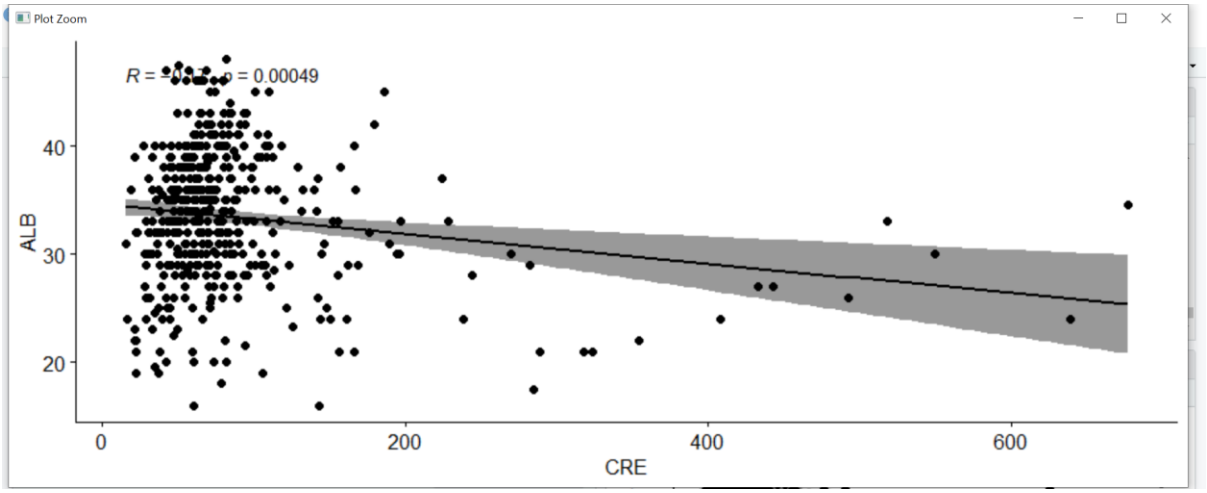
> cor.test(ALB, CRE, method = c("pearson", "kendall", "spearman"))

Pearson's product-moment correlation

data: ALB and CRE
t = -3.5125, df = 440, p-value = 0.0004897
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
-0.25450610 -0.07300706

sample estimates:
cor
-0.1651545

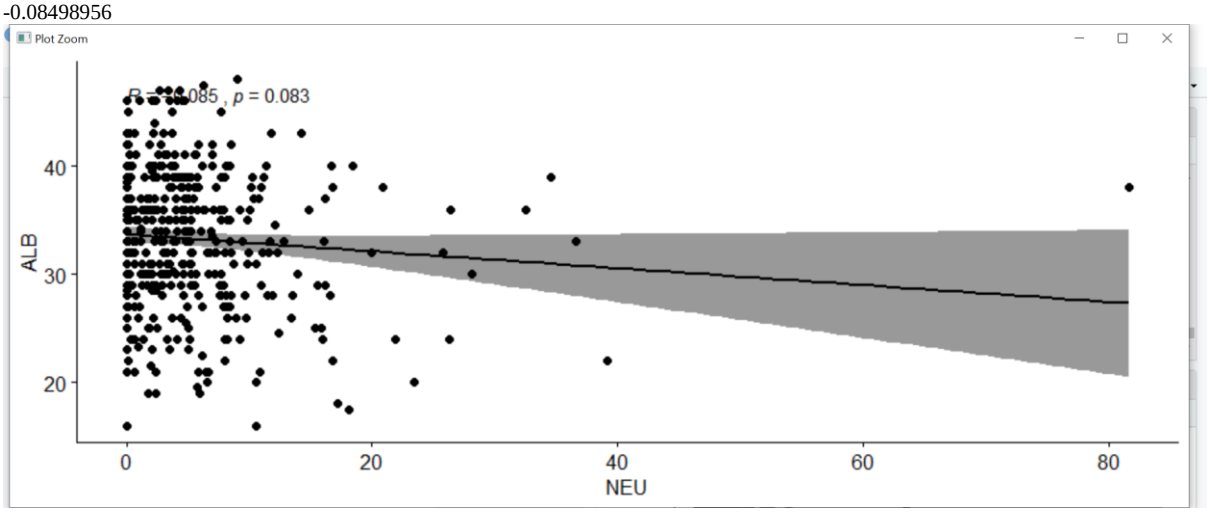
>



> cor.test(ALB, NEU, method = c("pearson", "kendall", "spearman"))

Pearson's product-moment correlation

data: ALB and NEU
t = -1.7356, df = 414, p-value = 0.08339
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
-0.17966705 0.01124796
sample estimates:
cor
-0.08498956

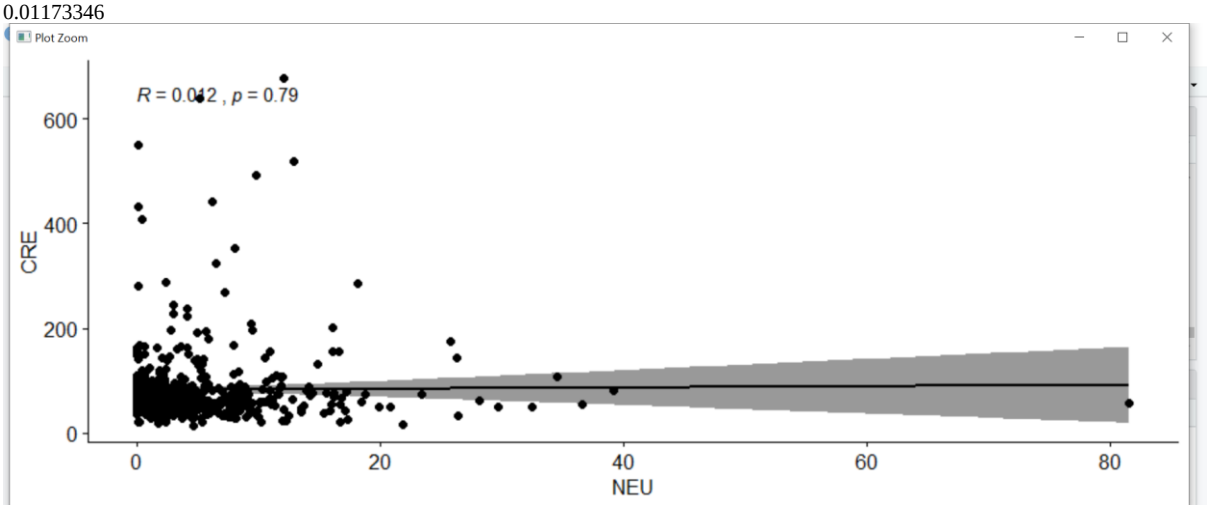


>
> cor.test(CRE, NEU, method = c("pearson", "kendall", "spearman"))

Pearson's product-moment correlation

data: CRE and NEU

t = 0.26343, df = 504, p-value = 0.7923
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
 -0.07551246 0.09880109
sample estimates:
       cor
0.01173346



Conditional Logistic Regression on R Output

Console window:

View(mydata)
> attach(mydata)
> mydata
> model <- clogit(outcome ~ gender + ageatdoc + losbeforedoc + WCC + ALB + CRE + NEU + CentralLines + OtherbacteriaincludingPapreviously + strata(pairing))
> summary(model)

Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ gender + ageatdoc +
    losbeforedoc + WCC + ALB + CRE + NEU + CentralLines + OtherbacteriaincludingPapreviously +
    strata(pairing), method = "exact")

  n= 410, number of events= 102
   (215 observations deleted due to missingness)

                                coef exp(coef) se(coef)   z
gender   -0.171163 0.842684 0.397278 -0.431
ageatdoc   0.489285 1.631150 0.141587 3.456
losbeforedoc   0.036753 1.037437 0.011006 3.339
WCC   -1.042558 0.352552 0.241211 -4.322
ALB   -0.005684 0.994332 0.031107 -0.183
CRE   0.001307 1.001308 0.002292 0.570
CentralLines   1.059498 2.884922 0.515037 2.057
OtherbacteriaincludingPapreviously 1.533604 4.634852 0.462382 3.317
                                Pr(>|z|)

```
gender                          0.666585
ageatdoc                         0.000549 ***
losbeforedoc                      0.000840 ***
WCC                             0.0000154 ***
ALB                             0.855015
CRE                             0.568470
CentralLines                      0.039674 *
OtherbacteriaincludingPapreviously  0.000911 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

                              exp(coef) exp(-coef) lower .95 upper .95
gender                          0.8427     1.1867    0.3868    1.8358
ageatdoc                         1.6312     0.6131    1.2359    2.1528
losbeforedoc                      1.0374     0.9639    1.0153    1.0601
WCC                              0.3526     2.8365    0.2197    0.5656
ALB                             0.9943     1.0057    0.9355    1.0568
CRE                             1.0013     0.9987    0.9968    1.0058
CentralLines                      2.8849     0.3466    1.0513    7.9165
OtherbacteriaincludingPapreviously    4.6349     0.2158    1.8726   11.4714

Concordance= 0.884  (se = 0.035 )
Likelihood ratio test= 95.54  on 9 df,   p=<2e-16
Wald test            = 40.5  on 9 df,   p=0.000006
Score (logrank) test = 59.17  on 9 df,   p=2e-09
```

Univariate Analyses

Admission methods

Elective vs non-elective or transfer

```
> library(survival)
> attach(nobacteremiadata)
> nobacteremiadata
> model <- clogit(outcome ~ am_1 + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ am_1 + strata(pairing),
    method = "exact")

  n= 625, number of events= 137

       coef exp(coef) se(coef)     z Pr(>|z|)
am_1 1.0637    2.8971   0.2332 4.561 5.09e-06 ***
---
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

     exp(coef) exp(-coef) lower .95 upper .95
am_1     2.897     0.3452     1.834     4.576

Concordance= 0.581  (se = 0.03 )
Likelihood ratio test= 21.69  on 1 df,   p=3e-06
Wald test            = 20.8  on 1 df,   p=5e-06
Score (logrank) test = 22.33  on 1 df,   p=2e-06
```

Non-elective vs elective or transfer

```
> model <- clogit(outcome ~ am_2 + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ am_2 + strata(pairing),
    method = "exact")

  n= 625, number of events= 137

       coef exp(coef) se(coef)      z Pr(>|z|)
am_2 -0.4482    0.6388   0.2266 -1.977    0.048 *
---
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

     exp(coef) exp(-coef) lower .95 upper .95
am_2    0.6388      1.565    0.4097     0.996

Concordance= 0.542  (se = 0.032 )
Likelihood ratio test= 4.02  on 1 df,   p=0.04
Wald test            = 3.91  on 1 df,   p=0.05
Score (logrank) test = 3.96  on 1 df,   p=0.05
```

Transfer vs elective or non-elective

```
> model <- clogit(outcome ~ am_3 + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ am_3 + strata(pairing),
    method = "exact")

  n= 625, number of events= 137

       coef exp(coef) se(coef)     z Pr(>|z|)
am_3 1.6928    5.4348   0.3314 5.108 3.25e-07 ***
---
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

     exp(coef) exp(-coef) lower .95 upper .95
am_3     5.435      0.184     2.839     10.41

Concordance= 0.602  (se = 0.029 )
Likelihood ratio test= 27.74  on 1 df,   p=1e-07
Wald test            = 26.09  on 1 df,   p=3e-07
Score (logrank) test = 31.22  on 1 df,   p=2e-08
```

WCC Groups

```
> library(survival)
> attach(nobacteremiadata)
> nobacteremiadata
> model <- clogit(outcome ~ wcc + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ wcc + strata(pairing),
    method = "exact")

  n= 546, number of events= 129
   (79 observations deleted due to missingness)

      coef exp(coef) se(coef)     z Pr(>|z|)
wcc 1.8145    6.1382   0.3196 5.677 1.37e-08 ***
---
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

    exp(coef) exp(-coef) lower .95 upper .95
```

```
wcc     6.138     0.1629     3.281     11.48

Concordance= 0.633  (se = 0.031 )
Likelihood ratio test= 41.5  on 1 df,   p=1e-10
Wald test            = 32.23  on 1 df,   p=1e-08
Score (logrank) test = 39.35  on 1 df,   p=4e-10
```

WCC Blood test values

```
> model <- clogit(outcome ~ wcc_min + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ wcc_min + strata(pairing),
    method = "exact")

  n= 546, number of events= 129
   (79 observations deleted due to missingness)

            coef exp(coef) se(coef)      z Pr(>|z|)
wcc_min -0.12548   0.88207  0.03164 -3.965 7.33e-05 ***
---
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

        exp(coef) exp(-coef) lower .95 upper .95
wcc_min    0.8821      1.134     0.829    0.9385

Concordance= 0.69  (se = 0.043 )
Likelihood ratio test= 23.09  on 1 df,   p=2e-06
Wald test            = 15.72  on 1 df,   p=7e-05
Score (logrank) test = 3.62  on 1 df,   p=0.06
```

NEU Groups

```
> model <- clogit(outcome ~ neutrophils + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ neutrophils + strata(pairing),
    method = "exact")

  n= 518, number of events= 113
   (107 observations deleted due to missingness)

              coef exp(coef) se(coef)     z Pr(>|z|)
neutrophils 1.5448    4.6869   0.3238 4.771 1.83e-06 ***
---
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

            exp(coef) exp(-coef) lower .95 upper .95
neutrophils     4.687     0.2134     2.485      8.84

Concordance= 0.601  (se = 0.035 )
Likelihood ratio test= 27.16  on 1 df,   p=2e-07
Wald test            = 22.77  on 1 df,   p=2e-06
Score (logrank) test = 26.31  on 1 df,   p=3e-07
```

NEU Blood test values

```
> model <- clogit(outcome ~ neutrophils_min + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ neutrophils_min +
    strata(pairing), method = "exact")
```

```
  n= 518, number of events= 113
   (107 observations deleted due to missingness)

                    coef exp(coef) se(coef)      z Pr(>|z|)
neutrophils_min -0.10358   0.90160  0.03643 -2.843  0.00447

neutrophils_min **
---
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

               exp(coef) exp(-coef) lower .95 upper .95
neutrophils_min    0.9016      1.109    0.8395    0.9683

Concordance= 0.654  (se = 0.047 )
Likelihood ratio test= 10.2  on 1 df,   p=0.001
Wald test            = 8.08  on 1 df,   p=0.004
Score (logrank) test = 8.16  on 1 df,   p=0.004
```

GFR Groups

```
> model <- clogit(outcome ~ gfr + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ gfr + strata(pairing),
    method = "exact")

  n= 522, number of events= 119
   (103 observations deleted due to missingness)

       coef exp(coef) se(coef)      z Pr(>|z|)
gfr -0.2405    0.7863   0.2476 -0.971    0.332

    exp(coef) exp(-coef) lower .95 upper .95
gfr    0.7863      1.272    0.4839     1.278

Concordance= 0.545  (se = 0.038 )
Likelihood ratio test= 0.95  on 1 df,   p=0.3
Wald test            = 0.94  on 1 df,   p=0.3
Score (logrank) test = 0.95  on 1 df,   p=0.3
```

GFR Blood test values

```
> model <- clogit(outcome ~ gfr_min + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ gfr_min + strata(pairing),
    method = "exact")

  n= 522, number of events= 119
   (103 observations deleted due to missingness)

              coef  exp(coef)   se(coef)      z Pr(>|z|)
gfr_min -0.0004491  0.9995510  0.0055566 -0.081    0.936

        exp(coef) exp(-coef) lower .95 upper .95
gfr_min    0.9996          1    0.9887      1.01

Concordance= 0.464  (se = 0.044 )
Likelihood ratio test= 0.01  on 1 df,   p=0.9
Wald test            = 0.01  on 1 df,   p=0.9
Score (logrank) test = 0.01  on 1 df,   p=0.9
```

CRP

```
> model <- clogit(outcome ~ crp + strata(pairing))
> summary(model)
Call:
```

```
coxph(formula = Surv(rep(1, 625L), outcome) ~ crp + strata(pairing),
    method = "exact")

  n= 305, number of events= 58
   (320 observations deleted due to missingness)

      coef exp(coef) se(coef)     z Pr(>|z|)
crp 0.8335    2.3013   0.5626 1.482    0.138

    exp(coef) exp(-coef) lower .95 upper .95
crp     2.301     0.4345    0.7641     6.932

Concordance= 0.557  (se = 0.035 )
Likelihood ratio test= 2.56  on 1 df,   p=0.1
Wald test            = 2.2  on 1 df,   p=0.1
Score (logrank) test = 2.31  on 1 df,   p=0.1
```

Parainfluenza

```
> model <- clogit(outcome ~ para + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ para + strata(pairing), method = "exact")

  n= 625, number of events= 137

       coef exp(coef) se(coef)     z Pr(>|z|)
para 0.7623    2.1431   0.6480 1.176    0.239

     exp(coef) exp(-coef) lower .95 upper .95
para     2.143     0.4666    0.6018     7.632

Concordance= 0.506  (se = 0.008 )
Likelihood ratio test= 1.34  on 1 df,   p=0.2
Wald test            = 1.38  on 1 df,   p=0.2
Score (logrank) test = 1.43  on 1 df,   p=0.2
```

RSV

```
> model <- clogit(outcome ~ rsv + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ rsv + strata(pairing),
    method = "exact")

  n= 625, number of events= 137

      coef exp(coef) se(coef)     z Pr(>|z|)
rsv 0.6845    1.9827   0.8850 0.773    0.439

    exp(coef) exp(-coef) lower .95 upper .95
rsv     1.983     0.5044    0.3499     11.24

Concordance= 0.502  (se = 0.006 )
Likelihood ratio test= 0.55  on 1 df,   p=0.5
Wald test            = 0.6  on 1 df,   p=0.4
Score (logrank) test = 0.62  on 1 df,   p=0.4
```

Rhinovirus

```
> model <- clogit(outcome ~ rhino + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ rhino + strata(pairing),
    method = "exact")

  n= 625, number of events= 137

        coef exp(coef) se(coef)      z Pr(>|z|)
rhino -0.3848    0.6806   0.8136 -0.473    0.636
```

```
      exp(coef) exp(-coef) lower .95 upper .95
rhino    0.6806      1.469    0.1382     3.352

Concordance= 0.506  (se = 0.005 )
Likelihood ratio test= 0.24  on 1 df,   p=0.6
Wald test            = 0.22  on 1 df,   p=0.6
Score (logrank) test = 0.23  on 1 df,   p=0.6
```

Number of viruses

```
> model <- clogit(outcome ~ nvir + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ nvir + strata(pairing),
    method = "exact")

  n= 625, number of events= 137

       coef exp(coef) se(coef)     z Pr(>|z|)
nvir 0.3707    1.4487   0.3479 1.066    0.287

     exp(coef) exp(-coef) lower .95 upper .95
nvir     1.449     0.6903    0.7326     2.865

Concordance= 0.501  (se = 0.012 )
Likelihood ratio test= 1.1  on 1 df,   p=0.3
Wald test            = 1.14  on 1 df,   p=0.3
Score (logrank) test = 1.15  on 1 df,   p=0.3
```

Any virus

```
> model <- clogit(outcome ~ virusany + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ virusany + strata(pairing),
    method = "exact")

  n= 625, number of events= 137

          coef exp(coef) se(coef)    z Pr(>|z|)
virusany 0.3969    1.4872   0.3853 1.03    0.303

         exp(coef) exp(-coef) lower .95 upper .95
virusany     1.487     0.6724    0.6989     3.165

Concordance= 0.502  (se = 0.012 )
Likelihood ratio test= 1.03  on 1 df,   p=0.3
Wald test            = 1.06  on 1 df,   p=0.3
Score (logrank) test = 1.07  on 1 df,   p=0.3
```

Number of lines

```
> model <- clogit(outcome ~ nlines + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ nlines + strata(pairing),
    method = "exact")

  n= 625, number of events= 137

        coef exp(coef) se(coef)     z Pr(>|z|)
nlines 1.4358    4.2031   0.2642 5.434  5.5e-08 ***
---
```

```
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

      exp(coef) exp(-coef) lower .95 upper .95
nlines     4.203     0.2379     2.504     7.055

Concordance= 0.634  (se = 0.029 )
Likelihood ratio test= 35.31  on 1 df,   p=3e-09
Wald test            = 29.53  on 1 df,   p=6e-08
Score (logrank) test = 36.84  on 1 df,   p=1e-09
```

A central line

```
> model <- clogit(outcome ~ line + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ line + strata(pairing),
    method = "exact")

  n= 625, number of events= 137

       coef exp(coef) se(coef)     z Pr(>|z|)
line 1.6409    5.1597   0.2986 5.495 3.91e-08 ***
---
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

     exp(coef) exp(-coef) lower .95 upper .95
line      5.16     0.1938     2.874     9.264

Concordance= 0.628  (se = 0.028 )
Likelihood ratio test= 34.62  on 1 df,   p=4e-09
Wald test            = 30.2  on 1 df,   p=4e-08
Score (logrank) test = 35.04  on 1 df,   p=3e-09
```

Surgery

```
> model <- clogit(outcome ~ op + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ op + strata(pairing),
    method = "exact")

  n= 625, number of events= 137

      coef exp(coef) se(coef)      z Pr(>|z|)
op -1.0184    0.3612   0.4867 -2.092   0.0364 *
---
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

   exp(coef) exp(-coef) lower .95 upper .95
op    0.3612      2.769    0.1391    0.9375

Concordance= 0.505  (se = 0.036 )
Likelihood ratio test= 4.88  on 1 df,   p=0.03
Wald test            = 4.38  on 1 df,   p=0.04
Score (logrank) test = 4.59  on 1 df,   p=0.03
```

Urinary tract infection

```
> model <- clogit(outcome ~ uti + strata(pairing))
```

```
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ uti + strata(pairing),
    method = "exact")

  n= 625, number of events= 137

      coef exp(coef) se(coef)     z Pr(>|z|)
uti 0.7658    2.1507   0.3564 2.149   0.0317 *
---
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

    exp(coef) exp(-coef) lower .95 upper .95
uti     2.151      0.465      1.07     4.324

Concordance= 0.554  (se = 0.03 )
Likelihood ratio test= 4.41  on 1 df,   p=0.04
Wald test            = 4.62  on 1 df,   p=0.03
Score (logrank) test = 4.77  on 1 df,   p=0.03
```

P. aeruginosa in urine

```
> model <- clogit(outcome ~ puti + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ puti + strata(pairing),
    method = "exact")

  n= 625, number of events= 137

       coef exp(coef) se(coef)     z Pr(>|z|)
puti  4.169    64.668    1.123 3.711 0.000206 ***
---
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

     exp(coef) exp(-coef) lower .95 upper .95
puti     64.67    0.01546     7.153     584.6

Concordance= 0.561  (se = 0.032 )
Likelihood ratio test= 24.34  on 1 df,   p=8e-07
Wald test            = 13.77  on 1 df,   p=2e-04
Score (logrank) test = 26.96  on 1 df,   p=2e-07
```

P. aeruginosa in wound

```
> model <- clogit(outcome ~ PsAwound + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ PsAwound + strata(pairing),
    method = "exact")

  n= 625, number of events= 137

          coef exp(coef) se(coef)     z Pr(>|z|)
PsAwound 1.6943    5.4426   0.6065 2.793  0.00522 **
---
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

         exp(coef) exp(-coef) lower .95 upper .95
PsAwound     5.443     0.1837     1.658     17.87

Concordance= 0.543  (se = 0.03 )
Likelihood ratio test= 8.38  on 1 df,   p=0.004
Wald test            = 7.8  on 1 df,   p=0.005
Score (logrank) test = 9.25  on 1 df,   p=0.002
```

P. aeruginosa in respiratory

```
> model <- clogit(outcome ~ PsArespiratory + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ PsArespiratory +
    strata(pairing), method = "exact")

  n= 625, number of events= 137

                coef exp(coef) se(coef)     z Pr(>|z|)
PsArespiratory 1.9528    7.0482   0.6095 3.204  0.00136 **
---
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

              exp(coef) exp(-coef) lower .95 upper .95
PsArespiratory     7.048     0.1419     2.134     23.27

Concordance= 0.565  (se = 0.035 )
Likelihood ratio test= 11.13  on 1 df,   p=8e-04
Wald test            = 10.27  on 1 df,   p=0.001
Score (logrank) test = 12.24  on 1 df,   p=5e-04
```

P. aeruginosa any site (urine/wound/respiratory)

```
> model <- clogit(outcome ~ PsAelsewhere + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ PsAelsewhere +
    strata(pairing), method = "exact")

  n= 625, number of events= 137

               coef exp(coef) se(coef)    z Pr(>|z|)
PsAelsewhere  2.4188   11.2322   0.4538 5.33 9.81e-08 ***
---
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

             exp(coef) exp(-coef) lower .95 upper .95
PsAelsewhere     11.23    0.08903     4.615     27.34

Concordance= 0.613  (se = 0.035 )
Likelihood ratio test= 36.2  on 1 df,   p=2e-09
Wald test            = 28.41  on 1 df,   p=1e-07
Score (logrank) test = 38.7  on 1 df,   p=5e-10
```

Multivariable Analyses

Multivariable analysis for *P. aeruginosa* presence any site + urine/wound/respiratory

```
> model <- clogit(outcome ~ PsAelsewhere + PsAurine + PsAwound + PsArespiratory + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ PsAelsewhere +
    PsAurine + PsAwound + PsArespiratory + strata(pairing), method = "exact")

  n= 625, number of events= 137

                coef exp(coef) se(coef)     z Pr(>|z|)
PsAelsewhere   1.4685    4.3427   0.5713 2.570   0.0102 *
PsAurine       2.5724   13.0968   1.1837 2.173   0.0298 *
PsAwound       1.0205    2.7746   0.6430 1.587   0.1125
PsArespiratory 0.6133    1.8464   0.7653 0.801   0.4229
---
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

              exp(coef) exp(-coef) lower .95 upper .95
```

```
PsAelsewhere      4.343    0.23027    1.4172    13.307
PsAurine         13.097    0.07635    1.2872   133.258
PsAwound          2.775    0.36042    0.7869     9.783
PsArespiratory    1.846    0.54158    0.4120     8.275

Concordance= 0.625  (se = 0.035 )
Likelihood ratio test= 45.06  on 4 df,   p=4e-09
Wald test            = 29.74  on 4 df,   p=6e-06
Score (logrank) test = 50.59  on 4 df,   p=3e-10
```

Multivariable for *P. aeruginosa* presence in urine/wound/respiratory

```
> model <- clogit(outcome ~  PsAurine + PsAwound + PsArespiratory + strata(pairing))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ PsAurine + PsAwound +
    PsArespiratory + strata(pairing), method = "exact")

  n= 625, number of events= 137

                coef exp(coef) se(coef)     z Pr(>|z|)
PsAurine       3.7483   42.4503   1.1371 3.296 0.000979
PsAwound       1.5673    4.7935   0.6325 2.478 0.013214
PsArespiratory 1.5675    4.7947   0.6380 2.457 0.014016

PsAurine       ***
PsAwound       *
PsArespiratory *
---
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

               exp(coef) exp(-coef) lower .95 upper .95
PsAurine         42.450    0.02356     4.571    394.26
PsAwound          4.794    0.20861     1.388     16.56
PsArespiratory    4.795    0.20856     1.373     16.74

Concordance= 0.607  (se = 0.036 )
Likelihood ratio test= 38.12  on 3 df,   p=3e-08
Wald test            = 23.31  on 3 df,   p=3e-05
Score (logrank) test = 45.81  on 3 df,   p=6e-10
```

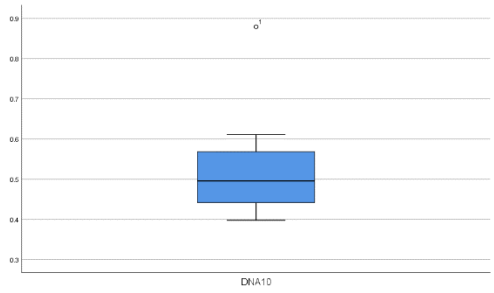
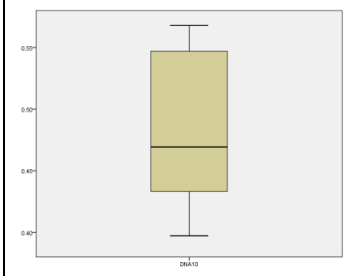
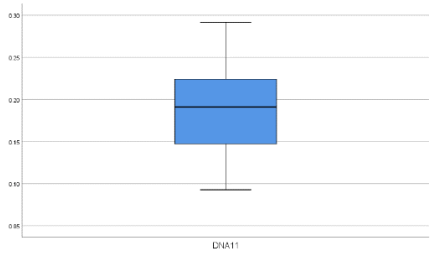
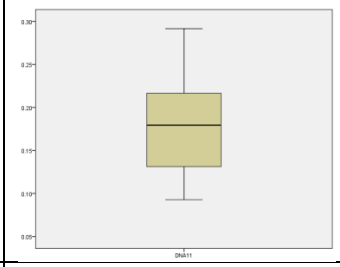
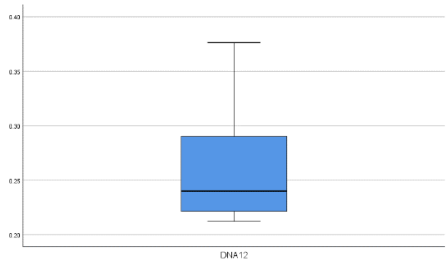
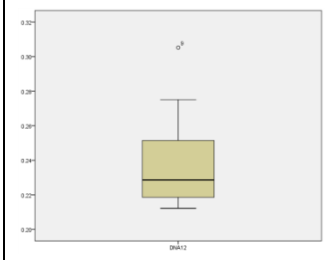
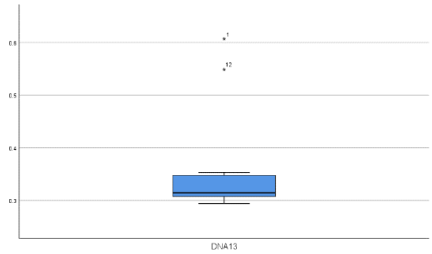
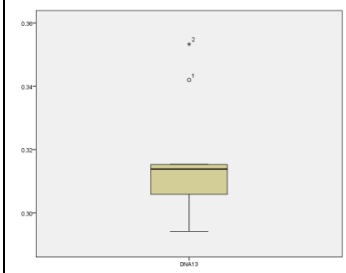
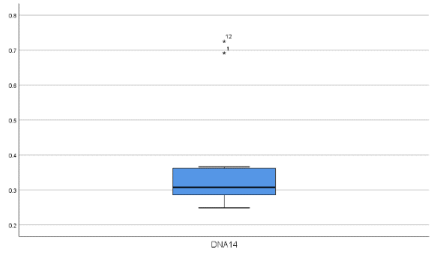
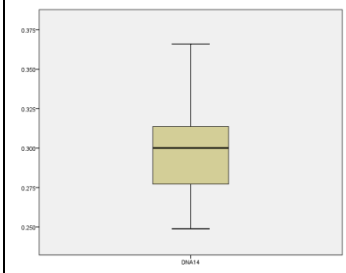
Full multivariable analysis

```
> model <- clogit(outcome ~ am_2 + am_3 + wcc + virusany + para + nlines + op +  PsAurine + PsAwound + PsArespiratory + strata(pairin
g))
> summary(model)
Call:
coxph(formula = Surv(rep(1, 625L), outcome) ~ am_2 + am_3 + wcc +
    virusany + para + nlines + op + PsAurine + PsAwound + PsArespiratory +
    strata(pairing), method = "exact")

  n= 546, number of events= 129
   (79 observations deleted due to missingness)

                coef exp(coef) se(coef)     z Pr(>|z|)
am_2         -0.2252    0.7984   0.3266 -0.690 0.490464
am_3          1.3263    3.7672   0.4703  2.820 0.004800
wcc           1.6788    5.3590   0.3669  4.576 4.75e-06
virusany      0.2269    1.2546   0.5715  0.397 0.691385
para          0.6835    1.9807   0.9396  0.727 0.466972
nlines        1.1530    3.1678   0.3058  3.771 0.000163
op           -1.6182    0.1983   0.6757 -2.395 0.016631
PsAurine      4.1608   64.1209   1.1468  3.628 0.000285
PsAwound      2.2638    9.6198   0.8909  2.541 0.011056
PsArespiratory 1.7121   5.5404   0.7114  2.407 0.016104
```

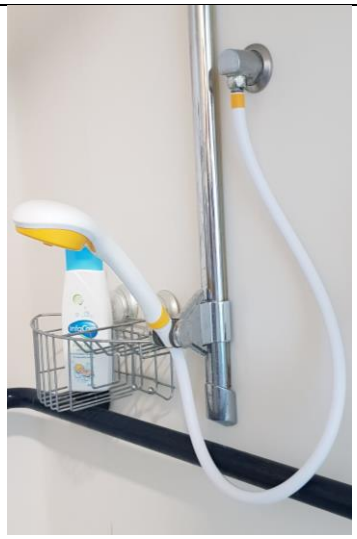
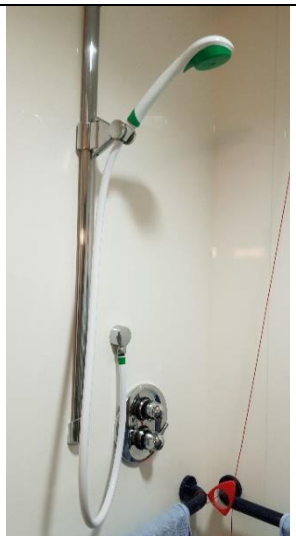
```
am_2
am_3          **
wcc           ***
virusany
para
nlines        ***
op            *
PsAurine      ***
PsAwound      *
PsArespiratory *
---
Signif. codes:
0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

              exp(coef) exp(-coef) lower .95 upper .95
am_2             0.7984     1.2526   0.42096    1.5142
am_3             3.7672     0.2654   1.49865    9.4699
wcc              5.3590     0.1866   2.61082   11.0002
virusany         1.2546     0.7970   0.40935    3.8455
para             1.9807     0.5049   0.31409   12.4910
nlines           3.1678     0.3157   1.73978    5.7680
op               0.1983     5.0439   0.05273    0.7454
PsAurine        64.1209     0.0156   6.77428  606.9273
PsAwound         9.6198     0.1040   1.67801   55.1495
PsArespiratory   5.5404     0.1805   1.37398   22.3412

Concordance= 0.823  (se = 0.033 )
Likelihood ratio test= 112  on 10 df,   p=<2e-16
Wald test            = 59.51  on 10 df,   p=4e-09
Score (logrank) test = 107.6  on 10 df,   p=<2e-16
```

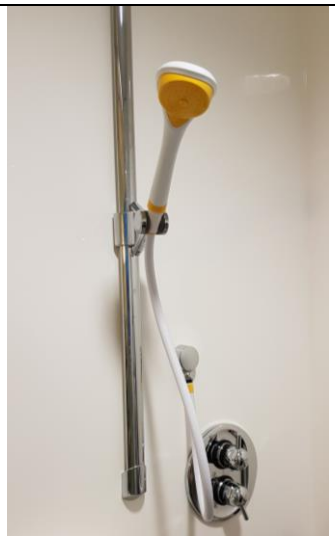
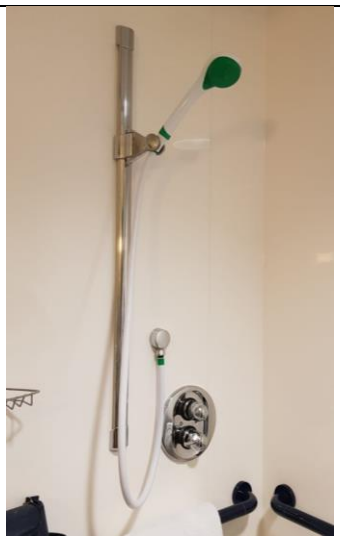
Appendix D. Microplate biofilm assay

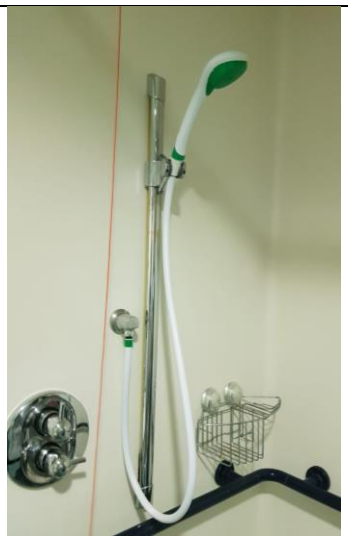
Appendix D.1. Box plots showing the OD measure distribution of replicates of 12 in comparison with 10 replicates.

12 replicates	10 replicates – edge effect deleted
DNA10 	
DNA11 	
DNA12 	
DNA13 	
DNA14 	

Appendix D.2. Shower photos at the dates of collection for biofilm assay

Beds	Photo – 6 December 2018	Photo – 28 February 2019
Bed 3		
Bed 5		
Bed 7		

Bed 8		
Bed 9		
Bed 11		No photo

Bed 26		
Bed 27		
Bed 32		

Bed 34		

Appendix E. Published papers from the thesis

E.1. Enhanced monitoring of healthcare shower water in augmented and non-augmented care wards showing persistence of *Pseudomonas aeruginosa* despite remediation work

JOURNAL OF MEDICAL MICROBIOLOGY

RESEARCH ARTICLE

Yetiş *et al.*, *Journal of Medical Microbiology* 2023;72:001698
DOI 10.1099/jmm.0.001698

MICROBIOLOGY SOCIETY

ACCESS

Enhanced monitoring of healthcare shower water in augmented and non-augmented care wards showing persistence of *Pseudomonas aeruginosa* despite remediation work

Özge Yetiş[1,*], Shanom Ali[2], Kush Karia[2], Paul Bassett[3] and Peter Wilson[4]

Abstract

Introduction. *Pseudomonas aeruginosa* in healthcare shower waters presents a high risk of infection to immune-suppressed patients; identifying the colonization-status of water outlets is essential in preventing acquisition.

Hypothesis/Gap Statement. Testing frequencies may be insufficient to capture presence/absence of contamination in healthcare waters between sampling and remediation activities. Standardization of outlets may facilitate the management and control of *P. aeruginosa*.

Aim. This study aims to monitor shower waters and drains for *P. aeruginosa* in augmented and non-augmented healthcare settings every 2 weeks for a period of 7 months during remedial actions.

Methodology. All shower facilities were standardized to include antimicrobial silver-impregnated showerhead/hose units, hose-length fixed to 0.8 m and replaced every 3 months. Standard hospital manual decontamination/disinfection occurred daily. Thermostatic-mixer-valves (TMVs) were replaced and disinfected if standard remediation unsuccessful.

Results. Of 560 shower and drain samples collected over 14 time-points covering 7 months, *P. aeruginosa* colonized 40% (4/10; non-augmented) and 80% (8/10; augmented-care) showers in the first week. For each week elapsed, new outlets became contaminated with *P. aeruginosa* by 18–19% ($P<0.001$) in shower waters (OR=1.19; CI=1.09–1.31) and drains (OR=1.18; CI=1.09–1.30). *P. aeruginosa* occurrence in shower water was associated with subsequent colonization of the corresponding drain and vice versa (chi-square; $P<0.001$) with simultaneous contamination present in 31% (87/280) of areas. TMV replacement was ineffective in eradicating colonisation in ~83% of a subset (6/20; three per ward) of contaminated showers.

Conclusions. We demonstrate the difficulties in eradicating *P. aeruginosa* from hospital plumbing, particularly when contamination is no longer sporadic. Non-augmented care settings are reservoirs of *P. aeruginosa* and should not be overlooked in outbreak investigations. Antimicrobial-impregnated materials may be ineffective once colonization with *P. aeruginosa* is established beyond the hose and head. Reducing hose-length insufficient to prevent cross-contamination from shower drains. *P. aeruginosa* colonization can be transient in both drain and shower hose/head. Frequent microbiological monitoring suggests testing frequencies following HTM04-01 guidelines are insufficient to capture the colonization-status of healthcare waters between samples. Disinfection/decontamination is recommended to minimize bioburden and the effect of remediation should be verified with microbiological monitoring. Where standard remediation did not remove *P. aeruginosa* contamination, intensive monitoring supported justifying replacement of showers and contiguous plumbing.

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Keywords: *Pseudomonas aeruginosa*; hospital environment; hospital shower water and drains; antimicrobial resistance.
Abbreviations: AST, antibiotic susceptibility test; CBA, Columbia Blood Agar; c.f.u, colony forming units; CI, confidence interval; EPDM, ethylene propylene diene monomer; h, hour; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MCA, milk cetrimide agar; MH, Muller–Hinton; OR, odds ratio; PBS, phosphate-buffered saline; POU, point of use; PsA, *P. aeruginosa*; PVC, polyvinyl chloride; TMV, thermostatic mixing valve; UV light, ultraviolet light; Wk, week.
Four supplementary tables are available with the online version of this article.

INTRODUCTION

Pseudomonas aeruginosa is a common cause of bacteraemia in immune-suppressed patients and is associated with a high mortality [1–3]. Hospitals with a large proportion of haematology/oncology patients have correspondingly high rates of *P. aeruginosa* bacteraemia [4]. *P. aeruginosa* may be present in sinks and faucets, showers and shower heads, ice machines, humidifiers, sink traps, and tap-outlet flow straighteners [1, 5]. The organism can grow in a wide range of temperatures and in nutrient-depleted environments [6]. Patients at increased risk of *P. aeruginosa* bacteraemia are often treated with broad-spectrum antibiotics, selecting resistant strains both in patient flora and in sanitary facilities, and any resulting infections carry a higher risk of mortality [3].

Water quality in hospitals and healthcare settings are regulated through guidelines set by local or national groups in European countries and USA [7]. In the UK, Health Technical Memoranda HTM 04–01C advises sampling of water outlets for *P. aeruginosa* at intervals determined by a local risk assessment in augmented care areas (i.e. areas with immune compromised or critically ill patients). The local Hospital Water Safety Group is required to risk assess operational practice to minimize contamination from incoming water supply, biofilms in the water supply in the building, wastewater and retrograde spread of contamination from the surface of the outlet [8]. In the USA, water system specifications are regulated by the American National Standards Institute (ANSI) in accordance with International Organization for Standardization (ISO) guidelines. These standards guide the water-quality measurement programmes [9, 10]. Control of *P. aeruginosa* colonization of hospital water systems is dependent upon the infrastructure of the setting and achieved through a holistic approach: this can include (i) treatment – chemical or thermal disinfection of the water system, (ii) physical barriers, i.e. point-of-use (POU) filtration devices, (iii) cleaning/education – training of cleaners and provision of appropriate disinfection product, (iv) material selection – use of easy-to-clean/antimicrobial surfaces and avoidance of materials, such as ethylene propylene diene monomer (EPDM), although alternatives are not necessarily superior [11, 12].

Showers are essential for patient care but provide a large surface area for the formation of biofilm. Shower outlets and drains may be contaminated with *P. aeruginosa* from the patient during ablutions, and biofilm present on the TMV and proximal surfaces (i.e. water supply behind the TMV) can spread via the faucet or showerhead. Subsequent spread to the adjacent plumbing presents a significant risk for development of *P. aeruginosa* reservoirs [2, 13, 14]. Drains in healthcare settings are interconnected and may cause transfer of pathogenic bacteria throughout the building. Disinfection, limescale removal and flushing of showers can reduce microbial colonization and persistence. Regular replacement of the shower head and hose units may reduce the risk of biofilm formation and colonization of the thermostatic mixing valve (TMV) and pipework by retrograde contamination. However, evidence for the efficacy of packages of standard remedial measures is poor in situations where contamination is already established or spreading.

Actions recommended to eradicate *P. aeruginosa* in a hospital setting can be disruptive to patient care and not be immediately effective. Where contaminated waters pose an immediate risk of cross-transfer to vulnerable patients, point of use filtration devices provide an effective - but temporary - measure while other corrective actions take place. However, bio-fouling of POU filters may restrict water flow, and the external surfaces of the showerhead remain prone to retrograde contamination [2]. Practical advice on remediation in situations where recommended interventions have failed is limited, for example, when water pressure is inadequate for POU filters. Routine monitoring of waters for *P. aeruginosa* in non-augmented care areas is not currently recommended in UK guidance [8] but patients may acquire the organisms during use of the shower.

We report the findings of a programme of enhanced monitoring to map *P. aeruginosa* colonization of a hospital shower-water system affecting an augmented and a non-augmented care ward whilst corrective measures were performed.

METHODS

Scope of enhanced monitoring programme

The aim of enhanced monitoring was to inform future policy in replacing showers and plumbing if colonization continued to spread despite corrective measures.

In the non-augmented care ward, there were initially various shower and shower-hose types, comprising either of EPDM or polyvinyl chloride (PVC) hose material and of 1.2 m length. A POU shower-filter was *in situ* in some bathrooms where patients had developed *P. aeruginosa* wound infection. After 3 weeks of enhanced monitoring, the Water Safety Group standardized all showers to an anti-microbial silver-impregnated shower head and hose unit with a hose-length of 0.8 m to prevent the showerhead from touching the drain. In both augmented and non-augmented care wards, the hose and showerhead units were replaced quarterly with pristine sets. These showerheads did not have POU filters.

Clinical setting, ward and room type

Two wards (Ward A and Ward B) on separate floors of an 18-floor tertiary teaching hospital (London, UK) were selected for enhanced environmental sampling of shower water and drains. Ten patient beds were selected at random from each floor.

Ward A was a non-augmented care unit accommodating surgical patients located in the middle level of the building. Beds in this ward were either for single-occupancy, i.e. within a single-isolation room (SIR), or multiple-occupancy in bays accommodating

up to four patients. SIRs provided a dedicated en-suite bathroom while bay areas provided a single bathroom facility shared between patients in that bay. This ward comprised 21 bathrooms in total of which nine were within the SIR en-suite unit and 12 were multiple occupancy access facilities.

Ward B was an augmented care unit caring for haematology patients. This ward was located on the top floor of the building and comprised of 34 SIRs, each with en-suite bathroom facilities exclusive to the occupying patient.

Routine cleaning procedure and protocol

Throughout this study, the existing hospital cleaning practices remained unchanged on both wards. Briefly, the routine (daily) cleaning of all bathroom surfaces consisted of manual application of a sporicidal disinfectant (peracetic acid; 0.1%, Diff X: MTP Innovations, UK) with a pre-wetted microfibre cloth. The cleaning technique employed involved folding of the cloth into half twice to create eight faces and using a new (unused) face of the cloth for each different surface.

The cleaning procedure was performed in the following order in all bathrooms: (i) shower outlet: (ii) sink/tap outlet; (iii) drains; (iv) toilet; and (v) floor surface. A new pair of nitrile gloves were used for each bathroom. A pristine pair of nitrile gloves and a new cloth was used to clean the shower exclusively.

The cleaning of outlets (showers and taps) involved visual inspection for limescale, and where present, dissolved by application of a descaler solution (WTP Showerhead Plus, UK) and removed after a dwell time (1 min) by mechanical scrubbing. The outlet was then opened fully for 2 min, and the water discharged into the corresponding drain. Drains were inspected for hair and debris and removed using a paper towel before discarding into a clinical waste receptacle. Floors were cleaned using microfibre-cloth mops pre-wetted with the same disinfectant solution; a new (unused) mop was used in each bathroom.

Sample collection timeline

Study phases and sampling frequency

The study was divided into three phases. At the outset there was a control period of 3 weeks, after which phases 1, 2 and 3 occurred at weeks 3–4, 13–15 and after week 21 (ward A)/week 29 (ward B).

Existing shower heads comprised of either one of (a) standard non-antimicrobial plastic, (b) antimicrobial silver-impregnated plastic or (c) point-of-use antimicrobial filtration units; the hose types at the start of the study were (i) ethylene propylene diene monomer (EPDM) or (ii) polyvinyl chloride (PVC) all of length 1.2 m

At each new phase, the showers were replaced with pristine antimicrobial silver-impregnated shower heads with 0.8 m antimicrobial silver-impregnated hoses.

In all shower areas, signage was affixed to walls to instruct users to avoid replacing the shower head onto its mount after use, i.e. to allow the shower head to drain freely under gravity into the shower tray.

The study was conducted over 7 months with environmental samples collected from a total of 14 time-points between 21 November 2018–6 June 2019 from both wards (Table 1). Monitoring of shower waters and drains surfaces (swabs) for *P. aeruginosa* was undertaken at weeks 3–4, 13–15 and after week 21 (Ward A) and week 29 (Ward B).

Measurement of shower-water pressure

As POU filters required 1 bar of water pressure to allow an adequate flow, water pressure in both wards was measured in a random sample of showers (eight augmented and six non-augmented care) using a Bourdon Pressure Gauge (0–4 bar range, RS Components, UK) that was attached to the shower TMV at the outlet. The pressure gauges were disinfected between use by spraying all surfaces and interior lumen with 70% isopropyl alcohol and wiping with an alcohol wipe before allowing the surfaces to dry prior to use.

Microbiological analysis of shower waters

Before and after collection of shower water, the outer surface of the shower head was disinfected with sterile alcohol wipes (Azo Wipes – 70% isopropyl alcohol). The outlet was opened and a volume of at least 100 ml of shower water was collected in sample bottles pre-dosed with 2 ml of a neutralising agent (0.1% Sodium thiosulfate, 0.3% Lecithin, 3% Tween 80) capturing the flow pre-flush (i.e. initial volume of water upon opening the shower valve).

To avoid cross contamination of the sample, a sterile water-sample collection bag was secured around the opening of the shower head and the opposite end of the bag was used to direct the water flow into the sample collection container via an incision to the bag using pre-disinfected scissors. Water was collected from the showers with the shower heads in place regardless of the shower head type (i.e. whether a point-of-use filter-shower was in place or not). Showers were flushed during morning cleaning before but not immediately preceding sampling.

Table 1. Presence and persistence of P. aeruginosa in shower waters and drain surfaces before (week 1–3) and after standardization of showerhead-hose units with 3-monthly replacement programme and during corrective actions to reduce contamination of patients

Ward A *(non-augmented care)*

Room #	Room type	Presence/Absence of *P. aeruginosa* in Shower waters or drains (per week) during successive showerhead+hose replacement																												
		Control Phase	Shower type	Hose length	Hose Material	Wk 1		Wk 2		Wk 3		Phase 1	Shower type / Hose Length/ Material	Wk 4	Wk 5	Wk 7	Wk 9	Wk 11	Wk 13	Phase 2	Wk 15	Wk 17	New TMV	Wk 19	Wk 21	Phase 3	Wk 29				
1	Bay		POU Filter	Long	EPDM	*		*		*			Ag+ /Short/PVC																	
2	Bay		POU Filter	Long	EPDM	*		*		*			Ag+ /Short/PVC																	
3	Side Room		Standard	Long	EPDM								Ag+ /Short/PVC																	
4	Side Room		POU Filter	Long	EPDM	*		*		*			Ag+ /Short/PVC																	
5	Bay		POU Filter	Long	EPDM	*		*		*			Ag+ /Short/PVC																	
6	Bay		Ag+	Short	PVC								Ag+ /Short/PVC																	
7	Bay		Ag+	Long	PVC								Ag+ /Short/PVC																	
8	Side Room		Standard	Long	EPDM								Ag+ /Short/PVC																	
9	Bay		Standard	Long	EPDM								Ag+ /Short/PVC																	
10	Bay		Standard	Long	EPDM								Ag+ /Short/PVC																	

Ward B *(augmented care)*

Room #	Room type	Presence/Absence of *P. aeruginosa* in Shower waters or drains (per week) during successive showerhead+hose replacement																												
		Control Phase	Shower type	Hose length	Hose Material	Wk 1		Wk 2		Wk 3		Phase 1	Shower type / Hose Length/ Material	Wk 4	Wk 5	Wk 7	Wk 9	Wk 11	Wk 13	Phase 2	Wk 15	Wk 17	New TMV	Wk 19	Wk 21	No shower head/hose change	Wk 29				
11	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																	
12	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																	
13	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																	
14	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																	
15	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																	
16	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																	
17	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																	
18	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																	
19	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																	
20	Side Room		Ag+	Long	PVC								Ag+ /Short/PVC																	

Table Key		
	POU Filter	Point-of-Use membrane filter device
	Standard	Non-antimicrobial plastic body
	Ag+	Antimicrobial Silver-impregnated materials
	Long	>>1.2m length hose
	Short	0.8m length hose
	EPDM	Ethylene Propylene Diene Monomer
	PVC	Poly-Vinyl-Chloride

PsA in Shower water
PsA in Drain
TMV not replaced
Drain sample not applicable
* Outlet with POU filter

Control Phase: Pre- showerhead+hose change
Phase 1: First shower head+hose change
Phase 2: Second shower head+hose change
Phase 3: Third shower head+hose change

EPDM, Ethylene Propylene Diene Monomer; PsA, *P. aeruginosa*; PVC, Polyvinyl Chloride; TMV, Thermostatic Mixing Valve; Wk, Week.

Samples were processed by membrane-concentration within 24 h of collection following HTM guidelines [15]. The water sample was concentrated by filtering 100 ml (±5 ml) through a 0.45 µm pore size nitrocellulose membrane mounted onto a filtration manifold and evacuated using a vacuum pump (65 kPa pressure limit). The membrane was transferred aseptically onto the surface of a Pseudomonas C-N selective agar plate (Oxoid, Basingstoke, UK). Plates were incubated at 37 °C for 48 h under aerobic conditions and inspected at 24 h to enumerate the presumptive *P. aeruginosa* colonies. The limit of detection of samples assayed in this way was 1 c.f.u./100 ml.

Microbiological analysis of shower drains

In sampling drains, sterile cotton-tipped swabs were pre-soaked by immersing in neutralizer solution (as above) before swabbing the drain exterior (in a left-to-right motion, followed by swabbing at 45° and 90° angles; with the process repeated three times) ensuring all exposed surfaces were covered. The drain interior was swabbed using the same swab by access via the drain holes. The swab contents were streaked immediately onto a *Pseudomonas*-selective C-N agar plate using a streaking motion applied horizontally, vertically and diagonally on the surface and rotating the swab tip continuously. Plates were incubated aerobically at 37 °C for 48 h and read daily.

Confirmation of presumptive *P. aeruginosa* isolates

Presumptive *P. aeruginosa* colonies (based on colony morphology) were enumerated as c.f.u. Presumptive *P. aeruginosa* colonies were purified by harvesting a representative sample using a 10 ul loop and streaking onto Columbia Blood Agar (CBA) (Oxoid, Basingstoke, UK) and incubating at 37 °C for 24 h. Single colonies formed on CBA streak-plates were re-streaked in parallel onto Nutrient Agar (Oxoid, Basingstoke, UK) and Milk Cetrimide Agar (MCA; Oxoid, Basingstoke, UK) plates. An oxidase test was performed on colonies present on nutrient agar plates using test strips (BioConnections, UK). Casein hydrolysis (translucent zone around the colony) and fluorescence under 253–320 nm UV light (using a UV-illuminator viewing chamber) was observed for on colonies present on MCA plates.

Further confirmation of *P. aeruginosa* isolates using mass spectrometry

Isolates exhibiting growth on *Pseudomonas*-selective CN agar, presumptive plate morphology, oxidase-positive reaction, and casein-hydrolysis were considered to be *P. aeruginosa* [16]. Isolates exhibiting a positive oxidase reaction and/or hydrolysing casein on MCA plates were examined using MALDI-TOF mass spectrometry (MS) (Maldi-TOF Biotyper IVD system Bruker Daltronics) as final confirmation. Strains were transferred to cryopreservation beads (Microbank) and stored below −20 °C.

Antimicrobial susceptibility profiles of environmental *P. aeruginosa* isolates

Antimicrobial susceptibility tests (ASTs) were performed on 50% of shower and drain isolates of *P. aeruginosa* with the sample population determined by random allocation using a computer-aided generator. Susceptibility profiles of environmental *P. aeruginosa* isolated were determined by disc-diffusion assays [17] against12 antibiotics (Oxoid): amikacin (30 µg), gentamicin (10 µg), tobramycin (10 µg), aztreonam (30 µg), meropenem (10 µg), imipenem (10 µg), ceftazidime (10 µg), cefepime (30 µg), ciprofloxacin (5 µg), piperacillin (30 µg), piperacillin/tazobactam (36 µg), and ticarcillin/clavulanic acid (Biorad, 75/10 µg).

Overnight cultures of the test *P. aeruginosa* strains were prepared on CBA at 37 °C from which a colony was harvested using a cotton-tipped swab pre-moistened with sterile PBS (Oxoid, UK) before transferring to glass tubes containing 3 ml PBS and re-suspending (vortex-mixing). Further dilutions were performed to adjust the turbidity to 0.5 McFarland standard solution. Resulting suspensions were streak-transferred to prepare a lawn on the surface of a Muller–Hinton (MH) agar plate (90 mm diameter; 4 mm depth: Oxoid, UK) and the target antibiotic disc arrays. MH plates were incubated aerobically at 35 °C for 18+/-2 h prior to reading. Susceptibility patterns were calculated by measuring the diameter of inhibition zones around each antibiotic disc against the corresponding EUCAST breakpoints for *P. aeruginosa* (version 11.0) [18] and the outcome assigned as susceptible (S), intermediate (I) or resistant (R).

Standard remediation procedure during the study

Management of remedial actions was undertaken by the hospital estates department and any testing of the waters (routine and retest samples) performed by the hospital water testing services.

The frequency of sampling was based on a risk assessment by the local Water Safety Group and deemed appropriate for sampling every 6 months at the start of the study. On identifying a contaminated outlet on an augmented care ward, cleaning, flushing, chemical disinfection with peracetic acid disinfectant (0.1%) and retesting were performed as recommended [8]. Colonized showers were withdrawn from use but flushed daily and sampled every 2 weeks until cleared. Patients and staff were instructed to avoid placing shower handles onto the wall mount but to suspend the shower vertically to allow the excess water to drain under gravity.

TMV remediation (replacement) procedure

If outlets remained positive for *P. aeruginosa* following hose change, contamination of TMV or contiguous plumbing was assumed. Where subsequent remedial actions failed, replacement of the thermostatic mixing valve (TMV) was considered. Replacement TMVs were chlorinated prior to fitting and, when installed, the external surfaces of the housing sprayed with a chlorine disinfectant. The TMV unit was then flushed for 2 min. Water samples were taken for microbiological analysis for *P. aeruginosa* a week before and 72 h after TMV replacement.

Enhanced remediation procedures

Where standard remediation measures failed to eradicate *P. aeruginosa* contamination despite several attempts, the Water Safety Group agreed to test enhanced measures in three shower rooms from each ward (i.e. six in total). Injection points were installed to allow disinfectant concentrate (50% hydrogen peroxide and 0.05% silver ions) dosing of the cold and hot water supplies.

To determine whether the *P. aeruginosa* was introduced to the building from an external source, water was sampled and tested from the incoming mains supply and from the basement level of the hospital building. Water samples were also collected for testing from shower rooms adjacent to the test shower rooms.

A control shower room was allocated on a separate floor located in the mid-point between ward A and B. This shower was installed with a pristine TMV and showerhead and hose units and closed off to patient use. The shower was not subjected to daily cleaning but was flushed daily (10 min cycles with a fully open valve).

Extraordinary remediation procedure

Where standard and enhanced remediation of shower outlets contaminated with *P. aeruginosa* failed, extraordinary remediation measures were adopted. In this case the TMV and all copper pipework supplying the affected room was replaced and fully disinfected (systemic chemical flushing with 0.1% peracetic acid). These procedures were undertaken after the survey period.

Ethics

Ethical approval was sought and deemed not required since the enhanced monitoring programmes was not an intervention or product trial and the setting was operating as per national guidelines.

Statistical analyses

Occurrence of *P. aeruginosa* at a given location (ward) and time point (week since shower hose change) during the monitoring was treated as a binary outcome (present/absent) and analysed using logistic multilevel (mixed) regression and the association with occurrence described as odds ratio (OR) and confidence interval (CI). Differences in occurrence were assessed as changes over time and time in categories. The former considered time as a continuous variable throughout the monitoring to examine occurrence of *P. aeruginosa*. The latter considered occurrence between the three time periods separated by shower hose changes. Statistical significance in the regression analysis was taken as P-value was<0.05.

RESULTS

Fourteen sampling sessions were conducted between 21 November 2018 and 6 June 2019, resulting in 280 shower water samples and 280 surface swabs taken from drains. Seven out of the ten showers on Ward A (non-augmented care unit) were dedicated to bed bays where the showers were shared between four patients, while the remaining three showers belonged to SIRs. All ten showers on Ward B (augmented care unit) belonged to single isolation rooms.

Shower water pressures were measured in both wards in weeks 1–3. In Ward A, the mean pressure was 2.71±0.71 bar (min/max: 2–3.9; n=8). However, in Ward B, water pressures were lower with a mean 0.83±0.05 bar (min/max: 0.8–0.9; n=6). Here, the flow rate was too low to use in conjunction with a POU filter.

Shower water and drain contamination

Initially 30% (n=10) of the showers in Ward A and 50% (n=10) in Ward B were contaminated with *P. aeruginosa*. By the final sampling week, 50% (n=10) shower outlets and 90% (n=10) shower outlets respectively were contaminated. Change of hose and head cleared *P. aeruginosa* colonization temporarily in showers 3, 5, 7 and 10. Showers 2 and 8 were free of colonization throughout (Table 1). The other 14 showers were quickly re-contaminated. For all showers, a section of pipe connecting the TMV to outlet could not be accessed for sampling.

Between weeks 17–19, TMV remediation was applied to a random selection of showers in which the TMV was thought to be contaminated, i.e. the TMVs were replaced (three from each ward), and the pipework disinfected (hydrogen peroxide 50%, silver ions 0.05%).

Of the six showers sampled, with one exception, changing the TMV and disinfection of the housing was ineffective in clearing contamination. Neither hydrogen peroxide nor peracetic acid injected into the pipe between TMV and outlet eradicated *P. aeruginosa*. In the rooms adjacent to the ones where TMV was changed, there was no effect on the isolation of *P. aeruginosa* from showers. Sampling of supply water in the basement of the building did not show any growth of *P. aeruginosa*. A single shower was closed to both patient and staff access and was flushed but not cleaned for 3 months. No contamination with *P. aeruginosa* was observed at repeated monthly sampling.

P. aeruginosa was isolated from 25% (n=20) of drains initially and 50% (n=20) at the end of monitoring. Unlike shower heads, drains showed growth of multiple bacterial species. *P. aeruginosa* in the drain did not necessarily coincide with contamination of shower water (Table 1). *P. aeruginosa* colonized both shower water and the corresponding drain in 30% (n=260) samples, but only the drain or shower water on 4% (n=260) and 31% (n=260) of occasions, respectively. On 34% (n=260) occasions, *P. aeruginosa* was not found. Presence of *P. aeruginosa* in either shower or drain correlated with occurrence in corresponding drain or shower (chi-square: P<0.001).

MICROBIOLOGICAL SCREENING OF TAPS

All taps in the study rooms on Wards A and B remained clear of *P. aeruginosa* contamination, in hospital laboratory tests before and after the monitoring period for showers.

Associations between ward type and exposure (time)

(a) Occurrence of contamination over time

Logistic multi-level regression modelling demonstrated a significant association between duration of use of the shower in the clinical setting and occurrence of *P. aeruginosa* in shower waters (P=0.004) and drains (P=0.03), irrespective of hose/head changes (Figs 1 and 2). For each week elapsed, the odds for colonization/contamination with *P. aeruginosa* in the non-augmented care setting increased by 19% in shower water (OR=1.19; CI=1.09–1.31, P<0.001) and 18% in the drains (OR=1.18; CI=1.09–1.30, P<0.001). Odds for *P. aeruginosa* colonization/contamination over time remained unchanged in the augmented care wards for showers (OR=0.95; CI=0.84–1.07, P=0.42) and drains (OR=1.04; CI=0.98–1.11, P=0.23).

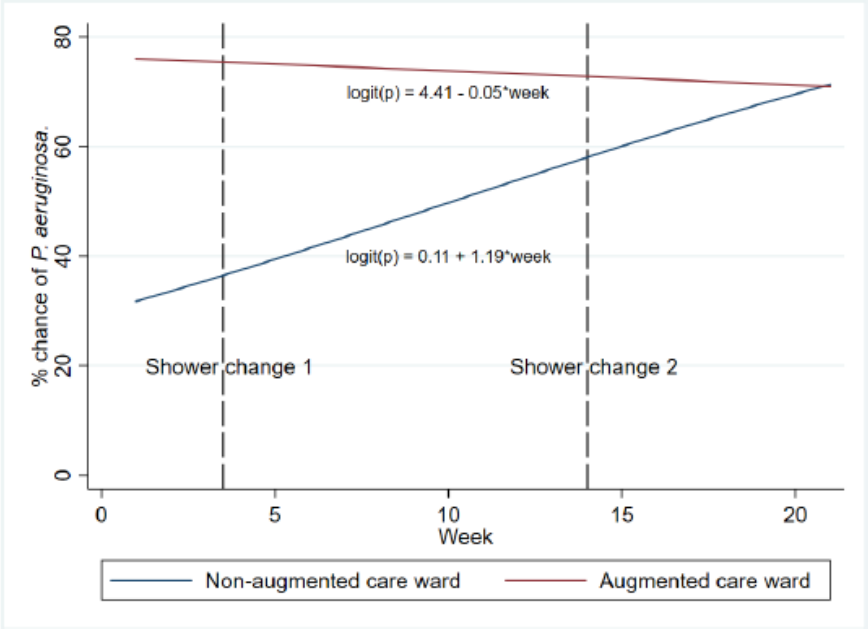

Fig. 1. Percentage chance of *P. aeruginosa* contamination of shower waters with time (weeks) in augmented and non-augmented care settings. *Y*-axis represents the occurrence ratio as a function of duration of exposure (*X*-axis). No changes were made to drains. Shower head-hose units were replaced with unused antimicrobial silver-impregnated types between weeks 3–4 and 13–15.

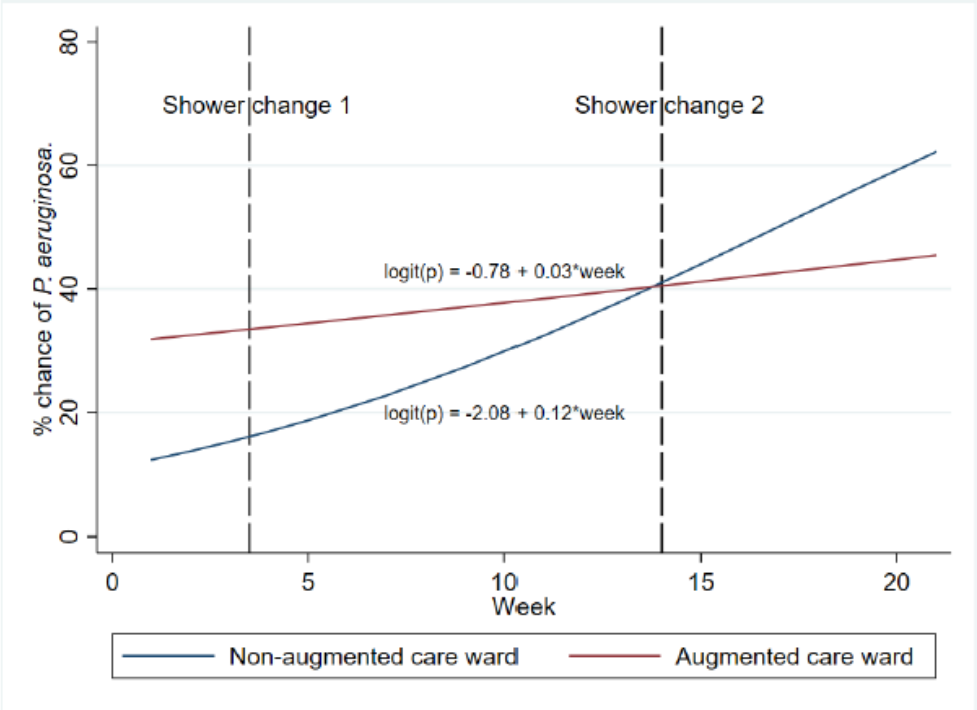

Fig. 2. Percentage chance of *P. aeruginosa* contamination of shower drain surfaces with time (weeks) in augmented and non-augmented care settings. *Y*-axis represents the occurrence ratio as a function of duration of exposure (*X*-axis). Shower head-hose units were replaced with unused antimicrobial silver-impregnated types between weeks 3–4 and 13–15.

(b) Occurrence of colonization between hose/head changes

The frequency of contamination of shower water and drains with *P. aeruginosa* and odds of occurrence between hose changes in augmented and non-augmented care wards is shown in Table 2. Relative to the period before the first hose change, the likelihood of shower water and drains in the general setting (Ward A) becoming colonized with *P. aeruginosa* increased over time ($P<0.05$), with the highest density of colonized showers/drains after the second head/hose change. Colonization of showers and drains in augmented care (Ward B) persisted at high frequency regardless of the head/hose change.

ANTIMICROBIAL SUSCEPTIBILITY PROFILES

Of 274 positive *P. aeruginosa* samples, 117 were isolated from Ward A (69 shower head water; 48 shower drain) and 157 were isolated from Ward B (105 shower water and 52 shower drain). ASTs were performed on a 50% sample (Ward A shower water $n=35$; Ward A shower drain $n=24$; Ward B shower water $n=53$; Ward B shower drain $n=26$).

In the non-augmented care setting, cefepime resistance in *P. aeruginosa* from shower water declined after the first and second hose changes ($P<0.05$) relative to the baseline isolates (see Table S1, available in the online version of this article) with no changes observed in drain isolates (see Table S2). No other resistance profiles changed significantly in relation to interventions on this ward.

Approximately 50% of the sample population of *P. aeruginosa* isolated from shower waters in the augmented care wards were resistant to imipenem at the outset (see Table S3). Frequency of imipenem-resistance declined to 12 and 31% after the first and second intervention, respectively ($P<0.05$). No other significant differences were observed for the other antibiotics.

The final analyses compared between time periods for data from drains in Ward B. The results are summarized in Table S4. Drains in the augmented care wards harboured *P. aeruginosa* with resistance to aztreonam in 75% of occasions during the pre-intervention phase that declined to 20 and 0% after subsequent interventions (first and second intervention, respectively).

Table 2. Frequency of contamination and likelihood (odds) of colonization of new shower waters and drain surfaces with *P. aeruginosa* for each week elapsed in non-augmented care versus augmented care wards when showerhead and hose units are standardised and replaced every 3 months relative to a pre-change (control) phase (week 1–3)

Sample site	Clinical setting (Ward)	*P. aeruginosa* contamination rate *n/N* (%) and odds ratio (95% CI)			*P*-value
		Control phase week 1–3 (pre- shower head/hose change/control)	Phase 1 week 4–13 (first shower head/hose change)	Phase 2 week 15–21 (second shower head/hose change)	
Shower water	Non-augmented care (A)	12/30 (40%) 1.00	25/60 (42%) 1.14 (0.33, 3.91)	27/40 (68%) 11.4 (2.35, 55.1)	0.003
	Augmented care (B)	23/30 (77%)	44/60 (73%)	29/40 (73%)	0.73
		1.00	0.52 (0.08, 3.62)	0.45 (0.05, 3.66)	
	Non-augmented care (A)	4/30 (13%)	14/60 (23%)	23/43 (58%)	<0.001
		1.00	2.31 (0.61, 8.83)	18.2 (4.22, 78.6)	
Drain surfaces	Augmented care (B)	7/30 (23%)	24/60 (40%)	18/40 (45%)	0.09
		1.00	2.84 (0.91, 8.88)	3.79 (1.11, 12.9)	

Similarly, ciprofloxacin resistance in drain-derived *P. aeruginosa* declined after hose changes relative to baseline ($P<0.05$). Statistically significant differences between the three time periods were observed for ciprofloxacin, resistant strains were most frequently seen at the start of the study.

DISCUSSION

This study showed the extraordinary difficulty in eradicating *P. aeruginosa* bioburden in healthcare shower waters; the greater the number and duration of outlets being affected the more difficult control became. This hospital setting included areas with low water pressure that made use of POU filters impractical, despite the presence of vulnerable patients. Our study showed that routine 6-monthly sampling of augmented care wards was insufficient to prevent vulnerable patients being exposed to a risk of infection. Patients could be exposed for several months to *P. aeruginosa* when using a shower. As a result of the monitoring, all showers were later removed together with adjacent plumbing, and new showers were installed that could be more easily disinfected along with the wall plumbing. Follow-up routine sampling and microbiological analysis of the waters demonstrated *P. aeruginosa* contamination of the shower waters was reduced but recontamination of the hose/head still occurred during use (data not presented).

In other studies, timely identification of *P. aeruginosa* contamination required sampling schedules more frequent than those based on local risk assessment [8, 11, 15], even when there is no evidence of contamination of the mains water supply. However, the cost of sampling all showers in augmented care every 2 weeks would be difficult to justify in the long term without proving that transmission from shower to patient was occurring and that methods used to eradicate it were effective. Nonetheless, safeguarding vulnerable patient groups requires monitoring of shower water at intervals sufficient to identify clinical risks and implement effective remedial interventions in a timely fashion.

In 20% (n=20) of showers, replacement of the head and hose temporarily resolved water contamination, suggesting there was no colonization of the wall plumbing or TMV. In the non-augmented care ward the number of colonized outlets temporarily fell from 40% (n=10) to 20% (n=10) when the shower hoses were replaced. However, TMV and plumbing were progressively colonized during the investigation, probably from hose contamination by the patients, which rendered hose/head replacement ineffective. Thermostatic mixer valves were behind wall panels and not easily accessible for ad hoc disinfection, and the housing of the TMV could not be readily replaced. In this hospital, replacement of the TMV was ineffective likely due to colonization of inaccessible pipework and TMV housing. Water supplies in the hospital had between 80–365 milligrams per litre calcium carbonate (Thames Water) [19]. Biofilm formation was likely on surfaces exposed to hard water as limescale deposits were common.

Only 40% of showers (n=20) were free of *P. aeruginosa* colonization at the start of the study. Two showers remained free of contamination throughout the study. These rooms were not adjacent, did not share drains and were not designated for different types of patients than the rest of the ward. Once the shower hose, head or drain were colonized with *P. aeruginosa*, the organism persisted for extended periods and at high bacterial loads, particularly in augmented care wards where water pressure was low. Although POU filters were effective in sequestering *P. aeruginosa*, poor water pressure prevented their continued use in the

augmented care ward. In the non-augmented care setting, shower and drain colonization increased by 18% for each additional week despite the use of antimicrobial silver-impregnated shower materials. Although sampling for *P. aeruginosa* contamination in healthcare waters was not routine in Ward A (non-augmented care), there had been a recent cluster of *P. aeruginosa* wound infection and monitoring might have been beneficial.

HTM guidelines suggest that materials such as EPDM may be prone to colonization by *P. aeruginosa* on surfaces such as the inside of flexible lined hoses [11]. However, in this study, changing all hoses from EPDM to PVC materials did not affect the proportion of showers becoming contaminated/colonized [12].

Eradication of *P. aeruginosa* biofilms require antimicrobial-silver concentrations 10–100 times greater than that required for planktonic cells, with doses of ~5–10 ug ml^{-1} typically needed to demonstrate significant reductions [20]. In our study, the antimicrobial-shower head-hose units were commercial products. To achieve microbial reductions an antimicrobial material would require an adequate concentration of silver water-hose interface rather than in the thickness of the material. The concentration of silver in these shower units was not known. The absence of reduction in microbial contamination suggests the colonization was greater than the level against which silver would be effective or activity was inhibited by biofilm.

There were no differences in cleaning frequency or bed occupancy between the wards and both bays and single rooms were almost fully occupied throughout the period of study. Under HTM 04–01 guidelines, routine monitoring for *P. aeruginosa* is not required in non-augmented care wards but showers were shared between patients and transmission was a possibility. Drains were a reservoir of *P. aeruginosa,* and these drains were open. Contamination of water outlets from drains in baths has been demonstrated [21]. Shower heads can be contaminated from drains through contaminated droplets/bioaerosols generated during ablutions and/or handling of the shower head with contaminated hands.

The risk of contamination of showers was greater than taps, possibly due to the larger volume of stagnant water in a shower between TMV and outlet. Contaminating organisms in a shower hose have a large available luminal surface area on which biofilm plaques can develop. Biofilms have been reported to attach more firmly to surfaces where there is a high shear stress [22, 23] but in this study contamination at the outset was more common in the ward where water pressure was low. The cleaning protocols would not have removed biofilm from the inside surfaces of the shower and external decontamination relied on mechanical scrubbing to remove biofilm. Application of disinfectant alone would not be adequate to eradicate *P aeruginosa* within biofilm [24]. Although shower hose and head were replaced periodically, biofilm in the plumbing and TMV was not affected. Hence a more radical approach to biofilm removal was needed, such as local thermal disinfection [25].

Showers and taps represent a significant reservoir of *P. aeruginosa* for patients vulnerable to developing bacteraemia. The presence of indistinguishable genotypes of *P. aeruginosa* in water outlets and patients has been described, although the direction of transmission is often unclear [2, 26, 27]. Patients with mucositis, intravascular catheters or foot wounds may be susceptible to potentially invasive contamination from the environment. In a study of outlets in 23 augmented care units over 16 weeks [28], between 0.9 and 16% of outlets demonstrated colonization. Whole-genome sequencing suggested a single genotype persisted within an outlet, possibly related to contamination in manufacture. Judging by epidemiological links in time and place, indistinguishable isolates suggested acquisition from the environment in 5% of patients. In another study, taps in ten ICUs were repeatedly sampled and isolates typed by pulsed-field electrophoresis [29]. More infections appeared to be transmitted between patients than from the outlets to patients. A tap water source of organisms detected on patient screening was implicated in 17% of patient acquisitions. Strains persisted in taps a median of 5 weeks (or longer in electronic taps). However, non-augmented care areas were not sampled in either study. In 141 isolates taken from showers in a burns unit, whole-genome sequencing showed clustering of isolates by room and outlet and three patients had identical genotypes to their environment [30]. A thermostatic mixer valve was shown to be a source of water contamination.

Among the antibiotic susceptibility tests against 12 antibiotics, there were few statistically significant differences over the period of study and some resistance was to antibiotics not used in the wards, such as aztreonam. Resistance to aztreonam has been reported to be persistent, even in the absence of any selective pressure [31]. Since there were few significant differences in antibiograms of strains isolated in this study, it was not possible to deduce any links in transmission of isolates between rooms and time-points. Consequently, epidemiological links would require molecular analyses, such as whole-genome sequencing.

After the end of the surveillance study, full replacement of pipework (extraordinary remediation), TMV and shower head/hose unit followed by systemic disinfection was undertaken and subsequent sampling demonstrated eradication of *P. aeruginosa* colonization.

There were limitations to this investigation. The distribution of single and shared showers and water pressures within the building may not be generalizable to other hospitals. The time and frequency of shower use and the volume of water used per episode was not known. The presence of a length of pipe in the wall between TMV and outlet and fixed TMV housing was inaccessible for disinfection and compromised control of *P. aeruginosa* and made changing the TMV ineffective. Dead legs (blind-ended or unused pipes) or a very low level of contamination in supply water could not be excluded as a source.

CONCLUSIONS

Microbiological monitoring of shower waters for *P. aeruginosa* based on local risk assessment may be insufficient to capture changes in colonization-status and should be reviewed to avoid repeated patient exposure. Colonization can be transient and affect both drain and shower hose/head. Once established in plumbing, shower hose replacement, installation of antimicrobial shower materials (heads and hose) and repeated local disinfection may be ineffective in reducing colonisation. POU filters in showers carry a significant cost and are not practicable when water pressures are low. In this hospital setting, more effective means of preventing and removing *P. aeruginosa* colonization were required, for example, surface disinfection before every use, as well as easily replaced wall plumbing complemented by more frequent environmental monitoring. Healthcare shower waters in the non-augmented care setting are an unrecognised reservoir of *P. aeruginosa* and a risk for acquisition/carriage by patients, especially where shower facilities are shared.

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Conflicts of interest
The author(s) declare that there are no conflicts of interest.

References

1. **Loveday HP, Wilson JA, Kerr K, Pitchers R, Walker JT, *et al.*** Association between healthcare water systems and *Pseudomonas aeruginosa* infections: a rapid systematic review. *J Hosp Infect* 2014;86:7–15.
2. **Bédard E, Prévost M, Déziel E.** *Pseudomonas aeruginosa* in premise plumbing of large buildings. *Microbiologyopen* 2016;5:937–956.
3. **Recio R, Mancheño M, Viedma E, Villa J, Orellana MÁ, *et al.*** Predictors of mortality in bloodstream infections caused by *Pseudomonas aeruginosa* and impact of antimicrobial resistance and bacterial virulence. *Antimicrob Agents Chemother* 2020;64:e01759-19.
4. **Fingertips.** AMR local indicators. P. aeruginosa bacteraemia all counts and rates by acute trust - Public Health England; 2022. https://fingertips.phe.org.uk/profile/amr-local-indicators/data#page/3/gid/1938132910/pat/158/par/TE_trust/ati/118/are/RGT/iid/93399/age/1/sex/4/cid/4/tbm/1 [accessed 10 January 2022].
5. **Ortolano GA, McAlister MB, Angelbeck JA, Schaffer J, Russell RL, *et al.*** Hospital water point-of-use filtration: a complementary strategy to reduce the risk of nosocomial infection. *Am J Infect Control* 2005;33:S1–19.
6. **Wu W, Jin Y, Bai F, Jin S.** Pseudomonas aeruginosa. *J Mol Med* 2015;2:753–767.
7. **Exner M, Kramer A, Lajoie L, Gebel J, Engelhart S, *et al.*** Prevention and control of health care-associated waterborne infections in health care facilities. *Am J Infect Control* 2005;33:S26–40.
8. **Department of Health.** Health Technical Memorandum 04-01: Safe water in healthcare premises. Part C: *Pseudomonas aeruginosa* – advice for augmented care units. 2016.
9. **American National Standards Institute.** ASTM D5612-94 - Standard Guide for Quality Planning and Field Implementation of a Water Quality Measurement Program; 2008. https://webstore.ansi.org/Standards/ASTM/ASTMD5612942008 [accessed 17 January 2022].
10. **American National Standards Institute.** How Standards Support National Water Quality Month; 2015. https://www.ansi.org/news/standards-news/all-news/2015/08/how-standards-support-national-water-quality-month-11 [accessed 17 January 2022].
11. **Department of Health.** Health Technical Memorandum 04-01: Safe water in healthcare premises Part A: Design, installation and commissioning. 2016.
12. **Hutchins CF, Moore G, Webb J, Walker JT.** Investigating alternative materials to EPDM for automatic taps in the context of *Pseudomonas aeruginosa* and biofilm control. *J Hosp Infect* 2020;106:429–435.
13. **Blanc DS, Nahimana I, Petignat C, Wenger A, Bille J, *et al.*** Faucets as a reservoir of endemic *Pseudomonas aeruginosa* colonization/infections in intensive care units. *Intensive Care Med* 2004;30:1964–1968.
14. **Moore G, Walker J.** Presence and control of *Legionella pneumophila* and *Pseudomonas aeruginosa* biofilms in hospital water systems. *Biofilms Infect. Prev. Control A Healthc.* Handb., Elsevier Inc.; 2014, p. 311–37. https://doi.org/10.1016/B978-0-12-397043-5.00017-7.
15. **Department of Health.** Health Technical Memorandum 04-01: Safe water in healthcare premises Part B: Operational management. 2016.
16. **PHE.** UK Standards for Microbiology Investigations-standards-for-microbiology-investigations-smi-quality-and-consistency-in-clinical-laboratories PHE Publications gateway number: 2015013 UK Standards for Microbiology Investigations are produced in association with; 2015
17. **Hudzicki J.** Kirby-Bauer disk diffusion susceptibility test protocol author information. *ASM* 2012:1–13.
18. **European Committee on Antimicrobial Susceptibility Testing (EUCAST).** Breakpoint tables for interpretation of MICs and zone diameters Version 11.0; 2022. http://www.eucast.org
19. **Thames Water.** Thames Water Utilities Limited Water Quality Report. 2021., pp. 1–12. https://www.thameswater.co.uk/help/water-quality/check-your-water-quality#/search
20. **Bjarnsholt T, Kirketerp-Møller K, Kristiansen S, Phipps R, Nielsen AK, *et al.*** Silver against *Pseudomonas aeruginosa* biofilms. *APMIS* 2007;115:921–928.
21. **Berrouane YF, McNutt LA, Buschelman BJ, Rhomberg PR, Sanford MD, *et al.*** Outbreak of severe *Pseudomonas aeruginosa* infections caused by a contaminated drain in a whirlpool bathtub. *Clin Infect Dis* 2000;31:1331–1337.
22. **Stoodley P, Cargo R, Rupp CJ, Wilson S, Klapper I.** Biofilm material properties as related to shear-induced deformation and detachment phenomena. *J Ind Microbiol Biotechnol* 2002;29:361–367.
23. **Stoodley P, Jacobsen A, Dunsmore BC, Purevdorj B, Wilson S, *et al.*** The influence of fluid shear and AlCl3 on the material properties of *Pseudomonas aeruginosa* PAO1 and *Desulfovibrio* sp. EX265 biofilms. *Water Sci Technol* 2001;43:113–120.

24. **Buckingham-Meyer K, Goeres DM, Hamilton MA.** Comparative evaluation of biofilm disinfectant efficacy tests. *J Microbiol Methods* 2007;70:236–244.
25. **Yui S, Karia K, Ali S, Muzslay M, Wilson P.** Thermal disinfection at suboptimal temperature of *Pseudomonas aeruginosa* biofilm on copper pipe and shower hose materials. *J Hosp Infect* 2021;117:103–110.
26. **Cholley P, Thouverez M, Floret N, Bertrand X, Talon D.** The role of water fittings in intensive care rooms as reservoirs for the colonization of patients with *Pseudomonas aeruginosa*. *Intensive Care Med* 2008;34:1428–1433.
27. **Aumeran C, Paillard C, Robin F, Kanold J, Baud O, *et al.*** *Pseudomonas aeruginosa* and *Pseudomonas putida* outbreak associated with contaminated water outlets in an oncohaematology paediatric unit. *J Hosp Infect* 2007;65:47–53.
28. **Halstead FD, Quick J, Niebel M, Garvey M, Cumley N, *et al.*** *Pseudomonas aeruginosa* infection in augmented care: the molecular ecology and transmission dynamics in four large UK hospitals. *J Hosp Infect* 2021;111:162–168.
29. **Coppry M, Leroyer C, Saly M, Venier A-G, Slekovec C, *et al.*** Exogenous acquisition of *Pseudomonas aeruginosa* in intensive care units: a prospective multi-centre study (DYNAPYO study). *J Hosp Infect* 2020;104:40–45.
30. **Quick J, Cumley N, Wearn CM, Niebel M, Constantinidou C, *et al.*** Seeking the source of *Pseudomonas aeruginosa* infections in a recently opened hospital: an observational study using whole-genome sequencing. *BMJ Open* 2014;4:e006278.
31. **Jorth P, McLean K, Ratjen A, Secor PR, Bautista GE, *et al.*** Evolved aztreonam resistance is multifactorial and can produce hypervirulence in *Pseudomonas aeruginosa* *mBio* 2017;8:e00517-17.

E.2. Failure of a hollow-fibre shower filter device to prevent exposure of patients to *Pseudomonas aeruginosa*

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Failure of a hollow-fibre shower filter device to prevent exposure of patients to *Pseudomonas aeruginosa*

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SUMMARY

Background: *Pseudomonas aeruginosa* in hospital water is a risk for invasive infection. Point-of-use (POU) filters are used to reduce patient exposure to the organism, and hollow-fibre filters are becoming more popular. However, retrograde colonization of the filter mechanism may contaminate the effluent.

Aims: To assess the efficacy of POU filter head (polysulfone; hollow-fibre matrix) shower filters in preventing the exposure of high-risk patient groups to *P. aeruginosa*.

Methods: Pre-flush (opening the outlet and collecting the first 100 mL of water) samples were analysed to measure *P. aeruginosa* contamination from 25 shower outlets (~21% of all showers on the six wards), with and without a hollow-fibre filter. *P. aeruginosa* was measured in a subset of outlets harbouring *P. aeruginosa* (sampling period 19th August 2019 to 10th January 2020).

Findings: Water from all 25 showers was heavily colonized [>300 colony-forming units (cfu)/mL] with *P. aeruginosa* at the showerhead. *P. aeruginosa* was found in 32% (8/25) of post-filter shower water effluent samples with a geometric mean of $4x10^6$ cfu/mL (N=4) ($6.8x10^4$–$2x10^8$). Filters were sampled at 15–150 days of use (median 15 days), with 26% (6/23) of filter units becoming colonized before the expiry date.

Conclusion: POU filter showerhead units may not be effective in preventing exposure of vulnerable patients to *P. aeruginosa* in hospital water due to retrograde contamination (external contamination of the showerhead passed back to the filter cartridge itself) or failure of the hollow-fibre filter matrix. Reliance should not be placed on the use of hollow-fibre filters to protect patients from exposure to *P. aeruginosa* without repeated microbiological monitoring.

Introduction

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterial pathogen that causes hospital-acquired infection of surgical wounds, blood, the respiratory tract and the urinary tract, particularly in patients in haematology wards and intensive care units (ICUs) [1,2]. *P. aeruginosa* commonly colonizes hospital water systems, and has been associated with outbreaks of infection in vulnerable patients [3]. By analysing the relatedness of *P. aeruginosa* strains from patient infections and hospital water, studies have suggested that water systems, outlets and wet environments are implicated in serious

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infection in immunosuppressed patients [3,4]. The mode and direction of transfer between the patient and the clinical environment is difficult to demonstrate, but many studies have provided evidence of an association between *P. aeruginosa* infection and colonized taps and shower water [4,5]. As such, installation of filters on water outlets has been recommended when disinfection fails to eradicate the organism.

P. aeruginosa tends to become established in distal parts of a water system, such as sinks, taps and showers [3]. Showers are liable to develop *P. aeruginosa* biofilm due to the materials used, low flow rate and operating water temperature of 25–40 °C which favour the growth of this pathogen [6]. The aerosol droplets produced can be inhaled by patients or can contaminate intravenous line insertion sites and damaged mucous membranes, posing a risk, particularly to patients following chemotherapy.

In the UK, remedial actions to mitigate the risks posed by *P. aeruginosa* contamination in water systems are described in Health Technical Memorandum (HTM) 04–01 [7,8]. Where efforts to reduce the number of *P. aeruginosa* using mechanical (shearing by flushing water) and chemical (e.g. chlorine dioxide, silver/copper ion etc.) methods fail, a physical barrier approach, such as a point-of-use (POU) membrane filter unit, may be implemented if water pressure is adequate.

The two main types of POU filter units used on taps and shower outlets in the healthcare setting are membrane filters (disposable or reusable) and hollow-fibre filters. Depending on the manufacturer, standard membrane filter units comprise of a double layer membrane with a pore size of 0.1–0.2 μm which prevents the passage of *P. aeruginosa*, and a pre-filtration layer that retains larger particulates and organic matter [6]. Hollow-fibre filter units consist of a sealed chamber, and the incoming water must pass through 0.1-μm-diameter pores spanning the length of a matrix of hollow fibres before exiting the outlet. Standard membrane and hollow-fibre filter units operate as pass-through water filtration systems, and are prone to biofouling and bioscaling with organic debris and inorganic salts (e.g. calcium/magnesium carbonate). The ability of these filters to sequester *P. aeruginosa* effectively depends on the duration and frequency of use, as well as water quality. The efficiency of membrane POU water filters has been demonstrated, but some studies have reported that *P. aeruginosa* contamination can occur within the recommended term of use given by the manufacturer [9,10].

Hollow-fibre filters are gaining popularity as they allow greater flow of water, especially when water pressure is low [11,12]. The advantage of hollow-fibre filters against conventional flat membrane filters is the attainment of a large membrane surface within a limited volume as the membrane is in the form of hollow-fibre bundles [13]. Polysulfone and polyethylene are commonly used materials in hollow fibres, with average pore diameter ranging from 0.25 to 1.5 μm and from 0.5 to 2 μm, respectively [13,14]. Hollow fibres provide structural strength, and therefore increase the average life of a membrane. In addition, they increase water permeability due to their hydrophilic properties [11]. To determine whether these POU filters continue to prevent egress of *P. aeruginosa* during the manufacturer usage period, this study investigated the efficacy of 25 historically used polysulfone hollow-fibre shower filter units (medical shower filter; 0.1-μm pore size; polysulfone body; antimicrobial silver-impregnated; in-use lifecycle expiry of 92 days) in patient bathrooms in augmented and non-augmented care wards.

Methods

Clinical setting and selection criteria

Twenty-five patient bathrooms were selected at random from six wards with patients requiring augmented care (haematology, elderly care, adolescent haematology/oncology and infectious diseases) at a 700-bed multi-storey building teaching hospital in London, UK. Each ward was a single floor of the hospital building. The bathrooms selected were en-suite for single isolation rooms or those serving shared-occupancy bed bays (rooms with four to six beds). Apart from elderly care, cases of *P. aeruginosa* bacteraemia had occurred in all of the wards in the preceding 2 months. All of the bathrooms had a POU hollow-fibre filter integrated showerhead.

Shower water sample collection and assay by membrane concentration

Prior to sample collection, the showerheads were disinfected by wiping the entire outer surface with a sterile alcohol wipe (70% isopropyl alcohol) and allowed to air dry (~15 s).

The opening of a water sample collection bag (sterile grade) was placed over a showerhead and secured to capture a water sample. An incision was made aseptically to the bottom corner of the bag to create a second opening via which water could be channelled. The shower valve was opened and an aliquot of at least 100 mL of water was collected using the water collection bag into a sample container (pre-dosed with 1 mL of neutralizer solution; composition: 1 g/L sodium thiosulphate, 30 mL/L Tween 80 and 3 g/L lecithin in phosphate-buffered saline). The showerhead was then removed aseptically and placed on a pre-sterilized tray. A second 100 mL water sample was collected in the same manner into a second sample container. These two samples represented 'with/without POU filter' sample arrays, respectively. The showerhead was then re-attached, and the entire surfaces of the showerhead and hose were wiped with a sterile alcohol wipe prior to reinstating the shower. This process was repeated for 25 individual showers within the hospital. This represented 21% (25/119) of showers on the test wards. The sampling period was 19th August 2019 to 10th January 2020. Follow-up sampling was performed for two of the showers 24 days after the first water collection.

Water samples were transferred to a refrigerator (2–8 °C) within 2 h of collection and processed within 24 h. Shower samples (100±5 mL) were concentrated by vacuum filtration (max 65 kPa pressure) through a 47-mm nitrocellulose membrane of pore size 0.45 μm, followed by plating the membrane on to a Pseudomonas C–N agar plate. Plates were incubated aerobically at 37 °C for 48 h prior to counting the colonies. Water sampling and the subsequent procedures were performed in line with HTM guidelines recommended by NHS England [15].

Confirmation of P. aeruginosa isolates

Suspect colonies were distinguished by colony morphology (blue–green/green–yellow/red–brown) on selective agar

(Pseudomonas C–N), harvested for subculture on milk-cetrimide agar (MCA) and nutrient agar in parallel, and incubated at 37 °C for 24 h. Colonies growing on nutrient agar were tested for oxidase reaction, while hydrolysis on MCA was noted.

Isolates demonstrating oxidase-positive reactions and/or hydrolysis of casein on MCA plates were further confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis (Bruker Daltronics) in line with HTM guidelines. MALDI-TOF-MS analysis was performed as an additional confirmatory step [15].

Measurement of P. aeruginosa

The upper reading/counting limit of samples analysed using the membrane concentration assay technique was 300 colony-forming units (cfu)/100 mL.

A subset of four showers, selected at random, was assayed further by taking a 1-mL aliquot from the original sample and performing serial 1/10, 1/100 and 1/1000 dilutions before plating 100 µL on to Columbia blood agar from the neat, 1/10, 1/100 and 1/1000 arrays. Confirmation of *P. aeruginosa* was done as described previously.

Shower water pressure measurements

Water pressure measurements were performed with a pressure gauge (Bourdon Pressure Gauge 0–4 bar, RSRS Components, UK) on 74 showers from 10 wards. Showerheads were dismantled from the hose, and the screw thread of the pressure gauge was fitted directly to the end of the shower hose. The outlet was opened fully to allow maximum water and pressure values, recorded in bar units, once the gauge stabilized (~5 s). The pressure gauge was dismantled from the shower hose, and its end was disinfected by immersion into absolute ethanol (70% solution) for 2–3 s and then wiping the excess with an alcohol wipe. The showerhead was replaced on to the corresponding hose end, and further disinfected by wiping all external surfaces with a sterile alcohol wipe.

Validation of 70% ethanol sterilization protocol

The efficacy of the ethanol spray/wipe protocol for disinfection of the showerhead prior to sampling was validated in-house using representative shower types and a stainless steel control surface, inoculated with up to 10^6 cfu/cm^2 of *P. aeruginosa*. After spraying with 70% (v/v) ethanol solution and a manual wipe at 10 s, surfaces were sampled by a bead washing technique. Reductions of 6-log$_{10}$ were achieved (publication pending; data available upon request).

Statistical analysis

Chi-squared test with Yates' correction was performed to examine the difference between days of use of the showers that filtered the bacterial load effectively and the showers that failed to filter effectively.

Results

P. aeruginosa was found in the effluent from eight (32%) showers, despite the filter being in place (Table I). Six of those eight showerheads were found to have high bacterial counts (>300 cfu/100 mL). One filter (Shower #16) reduced the *P. aeruginosa* load in the effluent from >300 cfu to 8 cfu, while another (Shower #17) reduced the count to ~100 cfu. These eight showers had been in use for a mean of 60.87 (95% confidence interval 15.3–106) days. Shower #16 and Shower #17 were sampled on the 15th day of use. At the second sampling (39th day of use), these two showers showed *P. aeruginosa* 100 and >300 cfu/mL in the effluent, respectively, with the shower filter in place.

The remaining 18 showers filtered out the *P. aeruginosa* bioburden effectively, despite its presence at high numbers (i.e. >300 cfu/100 mL). The duration of use of the POU filters screened averaged 20.65 (standard deviation 12.57) days. There was no significant difference in the days of use between the showers that filtered the bacterial load effectively and the showers that failed to filter effectively (P=0.075).

P. aeruginosa was quantified in four of eight showers that had *P. aeruginosa* >300 cfu/100 mL with a filter showerhead in place. There was a geometric mean of 4x10^6 cfu/100 mL (6.8x10^4–2x10^8) (Table II).

In total, 74 shower water pressure measurements were taken from 10 floors of the hospital, with values averaging 2.94 bar (range 0.3–3.9). Pressure measurements of the four wards tested in this study were:

- Ward C: 10 shower water pressure measurements, mean 2.43 bar (range 2.3–2.8);
- Ward D: eight shower water pressure measurements, mean 1.8 bar (range 1.6–2.2);
- Ward E: eight shower water pressure measurements, mean 1.17 bar (range 1.1–1.25); and
- Ward F: six shower water pressure measurements, mean 0.83 bar (range 0.8–0.9).

Discussion

Exposure to shower water colonized with *P. aeruginosa* is a potential risk for the development of bacteraemia in immunosuppressed patients [3,4]. In this study setting, the use of hollow-fibre shower filters did not provide assurance of safety for patients in the shower environment. Although not necessarily due to a failure of the filter itself, external contamination and growth inside the showerhead had a similar effect, exposing some patients to high levels of organisms, with a risk of serious subsequent infection in immunosuppressed individuals. Without repeated monitoring, clinical teams may be unaware of the potential source of pseudomonas bacteraemia in vulnerable patients.

The hollow-fibre POU filter showerheads were *in situ* for 3 months before the sampling survey commenced; they replaced showers with non-filtration antimicrobial-impregnated showerhead/hose units.

The hollow-fibre technology was selected due to the high-capacity filtration via the 0.1-µm-diameter pores in the filter matrices, and the long shelf-life of 92 days (manufacturer communications). The POU filters were subjected to routine surveillance to assure efficacy against *P. aeruginosa* during the period of use.

Although the POU filters were effective in removing *P. aeruginosa* from the effluent in the majority of cases, the organism was found distal to the filter in one-third (8/25) of showers. While this study did not explore the sources of

Table I
Presence of *Pseudomonas aeruginosa* in the effluent of hospital shower water fitted with a point-of-use (POU) filter unit at various durations of use

POU shower filter details					Effluent water quality (presence of *P. aeruginosa*)[b]	
Shower ref. number	Ward ref.	Ward specialty	Location of corresponding shower (bay/SIR)	Age of filter (days in use)[a]	Without POU filter (cfu/100 mL)	With POU filter in place (cfu/100 mL)
1	Ward E	Haematology	SIR	15	>300	>300
2	Ward E	Haematology	SIR	15	>300	0
3	Ward E	Haematology	SIR	15	>300	0
4	Ward E	Haematology	SIR	15	>300	0
5	Ward E	Haematology	SIR	15	>300	0
6	Ward E	Haematology	SIR	15	>300	0
7	Ward E	Haematology	SIR	15	>300	0
8	Ward E	Haematology	SIR	15	>300	0
9	Ward F	Haematology	SIR	15	>300	0
10	Ward F	Haematology	SIR	15	>300	0
11	Ward F	Haematology	SIR	15	>300	0
12	Ward F	Haematology	SIR	15	>300	0
13	Ward F	Haematology	SIR	15	>300	0
14	Ward F	Haematology	SIR	15	>300	0
15	Ward F	Haematology	SIR	15	>300	0
16	Ward F	Haematology	SIR	15	>300	8
17	Ward F	Haematology	SIR	15	>300	100
18	Ward B	Elderly care	Bay	45	>300	>300
19	Ward C	Adolescent haematology/oncology	Bay	45	>300	>300
20	Ward C	Adolescent haematology/oncology	Bay	47	>300	0
21	Ward D	Oncology (adult)	Bay	47	>300	0
22	Ward A	Infectious diseases	Bay	47	>300	0
23	Ward F	Haematology	SIR	52	>300	>300
24	Ward C	Adolescent haematology/oncology	Bay	150	>300	>300
25	Ward C	Adolescent haematology/oncology	SIR	150	>300	>300

SIR, single isolation room; cfu, colony-forming units.
[a] Expiry date of POU filter units is 92 days from date of installation (manufacturer specifications). Numbers of *P. aeruginosa* present in shower water with and without a POU filter unit were determined by membrane concentration assay.
[b] Counts depicted as 0 cfu are below the detection limit (1 cfu).

contamination, the isolation of *P. aeruginosa* from filter-treated water was likely due to retrograde contamination from external reservoirs, or failure of the filter matrices in sequestering bacteria.

In this study, a high bacterial burden (>10^6 cfu/100 mL) in the pipework proximal to the filter may have overwhelmed the efficacy of the hollow-fibre filter matrix. However, a study using a 0.1-μm porous polyethylene hollow-fibre filter demonstrated >log 6 reduction when challenged with *Klebsiella terrigena* [16]. Retrograde contamination of taps, and even proximal piping, from drains despite POU filters has been reported [17].

POU filters are an alternative to chemical disinfection using chlorine dioxide, hydrogen peroxide or copper–silver

Table II
Quantification of *Pseudomonas aeruginosa* bioburden to determine water quality of the effluent from four showers

Shower description and details			Effluent water quality (presence of *P. aeruginosa*)
Shower ref. number	Ward reference	Ward specialty	cfu/100 mL without POU filter
16	Ward F	Haematology	$6.8x10^4$
17	Ward F	Haematology	$1.45x10^7$
23	Ward F	Haematology	$1.6x10^6$
25	Ward C	Adolescent haematology/oncology teenage cancer	$2.02x10^8$

cfu, colony-forming unit; POU, point of use.

ionization, and are effective when endemic potential pathogens cannot be eliminated [6]. In a surgical ICU, POU filters were associated with elimination of tap water contamination, and a reduction of pseudomonas colonization and infection in patients by 95% and 56%, respectively [9]. Use of 0.2-μm filters in wards in Japan removed all Gram-negative bacterial contamination in water for up to 2 months [6]. Studies in ICUs and bone marrow transplant units found that installation of filters reduced nosocomial pseudomonas infections [18,19].

However, external contamination can affect the efficacy of POU filter devices, and represents an indefinite revenue commitment for replacements. In the present study, the hollow-fibre filters adopted had a specified lifespan of approximately 3 months. Nevertheless, 26% (6/23) of the POU filters became colonized before the expiry date of the device had elapsed. Two of the filters screened in this study were *in situ* beyond the expiry date, and were decommissioned from use immediately by the hospital estates and facilities management. Membrane filter devices are an alternative to hollow-fibre filter units, but contamination with *P. aeruginosa* has been demonstrated to occur within the recommended duration of use [9]. A study from France reported *P. aeruginosa* contamination 4 and 5 weeks after installation [10]. Although the contamination level may be low initially, *P. aeruginosa* can proliferate quickly, presenting a risk for cross-contamination. Polysulfone or polyethylene hollow-fibre filters have practical utility over standard membrane filters in low-pressure water systems, where water output would otherwise be severely attenuated [11,12]. However, they are susceptible to the same problems of external contamination within a few weeks of installation. A laboratory study of experimental contamination of pristine hollow-fibre filter devices (0.2-μm pore size) before placing on uncontaminated taps and showers found that hollow-fibre shower filters were effective in removing *P. aeruginosa* [11]. However, despite a recommended use time of 31 days, hollow-fibre tap filters showed early growth of *P. aeruginosa*, in one case from day 16. There was no back contamination after filters were removed.

The mains water supply of the hospital was screened at the incoming site to the hospital, and found to be free of *P. aeruginosa* (data upon request). In the present study, the water proximal to the filters harboured *P. aeruginosa* 10^6 cfu/100 mL. In cases where *P. aeruginosa* was isolated post-filtration, it could not be ascertained whether the contamination originated from retrograde contamination (e.g. aerosolized droplets from shower trays/drains), translocation through the filter matrix by high-pressure water flow, or as a consequence of perforation of the POU filter cartridge within the body of the showerhead. The pressure of water flow in the test building was below the upper tolerance (5 bar; manufacturer product specification) of the POU filter cartridge. Further exploratory and destructive analysis of the filter device, including microbiological and molecular characterization, is required. Low pressures present another risk because patients may then remove the showerheads and expose themselves to unfiltered shower water colonized by *P. aeruginosa*. Low shower pressures averaged 0.83 bar on Ward F; a haematology area occupied by immunosuppressed patients. In some cases, showerheads had already been removed by the patients when showers were inspected, despite warnings by nurses, ward sisters and wall posters not to do so.

An audit conducted after this study screened patients for rectal colonization between 24th January 2020 and 13th May 2020 (110 days). Six hundred and six samples (groin/rectal swabs) were collected from 155 patients. Four patients were *P. aeruginosa* negative in the first sample, but acquired *P. aeruginosa* during their stay (unpublished data).

Various devices are marketed on the premise of delaying retrograde biofilm formation, but efficacy in use against *Pseudomonas* spp. has not been demonstrated in peer-reviewed studies (e.g. copper inserts for tap outlets and silver-impregnated hoses). Although it is important to demonstrate the source of contamination, investigation of all possible routes of transmission is difficult. Hollow-fibre medical filter devices may be useful in preventing exposure of patients to *P. aeruginosa* from colonized shower water for short periods of use. However, application of POU shower filter units should be complemented with regular water testing, daily cleaning and internal disinfection of filtered water outlets in augmented care wards, especially when growth of *P aeruginosa* persists.

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Conflict of interest statement
None declared.

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References

[1] Ducel G, Fabry J, Nicolle L. Prevention of hospital-acquired infections. A practical guide. 2nd ed. Geneva: World Health Organization; 2002.
[2] Trautmann M, Lepper PM, Haller M. Ecology of *Pseudomonas aeruginosa* in the intensive care unit and the evolving role of water outlets as a reservoir of the organism. Am J Infect Control 2005;33(Suppl. 1):S41–9.
[3] Loveday HP, Wilson JA, Kerr K, Pitchers R, Walker JT, Browne J. Association between healthcare water systems and *Pseudomonas aeruginosa* infections: a rapid systematic review. J Hosp Infect 2014;86:7–15.
[4] Aumeran C, Paillard C, Robin F, Kanold J, Baud O, Bonnet R, et al. *Pseudomonas aeruginosa* and *Pseudomonas putida* outbreak associated with contaminated water outlets in an oncohaematology paediatric unit. J Hosp Infect 2007;65:47–53.
[5] Venier AG, Leroyer C, Slekovec C, Talon D, Bertrand X, Parer S, et al. Risk factors for *Pseudomonas aeruginosa* acquisition in intensive care units: a prospective multicentre study. J Hosp Infect 2014;88:103–8.
[6] Sasahara T, Ogawa M, Fujimura I, Ae R, Kosami K, Morisawa Y. Efficacy and effectiveness of showerheads attached with point-of-use (POU) filter capsules in preventing waterborne diseases in a Japanese hospital. Biocontrol Sci 2020;25:223–30.
[7] Department of Health. Part A: Design, installation and commissioning. In: Health Technical Memorandum 04-01: Safe water in healthcare premises. London: DoH; 2016.

[8] Department of Health. Part C: *Pseudomonas aeruginosa* – advice for augmented care units. In: Health Technical Memorandum 04-01: Safe water in healthcare premises. London: DoH; 2016.
[9] Trautmann M, Halder S, Hoegel J, Royer H, Haller M. Point-of-use water filtration reduces endemic *Pseudomonas aeruginosa* infections on a surgical intensive care unit. Am J Infect Control 2008;36:421–9.
[10] Florentin A, Lizon J, Asensio E, Forin J, Rivier A. Water and surface microbiologic quality of point-of-use water filters: a comparative study. Am J Infect Control 2016;44:1061–2.
[11] Totaro M, Valentini P, Casini B, Miccoli M, Costa AL, Baggiani A. Experimental comparison of point-of-use filters for drinking water ultrafiltration. J Hosp Infect 2017;96:172–6.
[12] Smith CM, Hill VR. Dead-end hollow-fiber ultrafiltration for recovery of diverse microbes from water. Appl Environ Microbiol 2009;75:5284–9.
[13] Schmittel A, Basagni M, Gaulle E, Keller T. Point-of-use water purifier with polysulfone hollow fibres. Vol. 1. US Pat Appl Publ; 2017. p. 1–15.
[14] Kamo J, Hirai T, Takahashi H, Kenji K. Porous polyethylene hollow fiber membrane of large pore diameter, production process thereof, and hydrophilized porous polyethylene hollow fiber membranes. 2017. p. 1–2.
[15] Department of Health. Part B: Operational management. In: Health Technical Memorandum 04-01: Safe water in healthcare premises. London: DoH; 2016.
[16] Hydreion L. Microbiological testing of the Sawyer 7/6B filter. Report No S05-03. 2005.
[17] Bédard E, Prévost M, Déziel E. *Pseudomonas aeruginosa* in premise plumbing of large buildings. Microbiologyopen 2016;5:937.
[18] Barna Z, Antmann K, Paszti J, Banfi R, Kadar M, Szax A, et al. Infection control by point-of-use water filtration in an intensive care unit – a Hungarian case study. J Water Health 2014;12:858–67.
[19] Cervia JS, Farber B, Armellino D, Klocke J, Bayer RL, McAlister M, et al. Point-of-use water filtration reduces healthcare-associated infections in bone marrow transplant recipients. Transpl Infect Dis 2010;12:238–41.