Effects of control interventions on *Clostridium difficile* infection in England: an observational study


SG, EJCG, APJ, MHW, TEAP, ASW, DWC are full professors.

*Corresponding author, tel: +44 1865 220870; fax: +44 1865 764192; email: kate.dingle@ndcls.ox.ac.uk

A complete list of investigators in the Modernising Medical Microbiology Informatics Group is provided in the Acknowledgements.


Department of Infectious Disease Epidemiology, Imperial College, London, UK and NIHR

Imperial Health Protection Research Unit on Healthcare Associated Infection and Antimicrobial Resistance (X.D.)

Department of Zoology, Oxford University, UK (R.M.H.)

Wellcome Trust Centre for Human Genetics, University of Oxford, UK (D.J.W.)


Microbiology Department, Oxford University Hospitals NHS Trust, Oxford, UK. (S.J.O.)


Leeds Teaching Hospitals NHS Trust, Leeds, UK. (P.H.)

Cubist Pharmaceuticals (2013-2014) (S.G.)

Tufts University School of Medicine, Boston, Massachusetts, USA (S.G.)

R M Alden Research Laboratory, Culver City CA (E.J.C.G., D.M.C.)


Royal Free London NHS Foundation Trust and Public Health England (S.H.)

Research in Context

Evidence before this study

England is almost unique in experiencing a marked, recent decline in the incidence of healthcare-associated *C. difficile* infections (CDI). Previous reports showed the decline of one epidemic genotype (PCR-ribotype 027), whereas other genotypes appeared to persist. These changes followed the implementation of a multifaceted national CDI control policy in 2007. However, the relative contributions made by the different interventions that were introduced simultaneously is unknown.

Added value of this study

This study is the first to investigate the contribution of specific public health interventions to the marked national decline in CDI. Our novel approach involved the integrated analysis of multiple, large, concurrent data sets concerning CDI incidence, antimicrobial prescribing, and, crucially, the whole genome sequences (WGS) of over 4000 human *C. difficile* isolates. Our key finding was that the post-interventions decline in CDI reflected the disappearance of fluoroquinolone-resistant isolates (predominantly from four, genetically distinct genotypes), while the incidence of CDI caused by fluoroquinolone-susceptible isolates (of many different genotypes) remained unchanged. WGS-based phylogenetic analyses of the entire *C. difficile* population, with one phylogeny constructed for each genotype, identified shorter, geographically clustered branches, specific to the fluoroquinolone-resistant regions. This is consistent with rapid nosocomial transmission preceding the disappearance of fluoroquinolone-resistant isolates. Among the susceptible isolates the numbers that were closely genetically related (and by inference transmitted, either directly or indirectly), did not change over time. This was despite the implementation of comprehensive infection prevention and control measures, which would have targeted fluoroquinolone-resistant and susceptible *C. difficile* equally. These data suggest that it was the restriction of
fluoroquinolone prescribing, above other interventions, (including cephalosporin restriction and infection control precautions), that appears to explain the decline in CDI incidence.

**Implications of all the available evidence**

This powerful population genetic and biostatistical analysis supports the restriction of fluoroquinolone prescribing as a cornerstone in the control of epidemic CDI in the UK and worldwide.
SUMMARY

Background

The control of Clostridium difficile infections (CDI) is an international clinical challenge. Uniquely, CDI incidence in England declined by ~80% after 2006, following implementation of national control policies; we investigated their role in this decline. This study tested two hypotheses. First, if CDI declines in England were driven by changes in use of particular antibiotics, then incidence of CDI caused by resistant isolates should decline faster than that caused by susceptible isolates across multiple genotypes (defined by multilocus sequence type (ST)). Second, if CDI declines we were driven by improvements in hospital infection control, then transmitted (secondary) cases should decline regardless of susceptibility.

Methods

Regional and national CDI incidence and antimicrobial prescribing data (1998-2014) were combined with whole genome sequences (WGS) from 4045 national and international C. difficile isolates. Genotype (multilocus sequence type) and fluoroquinolone susceptibility were determined from WGS. The incidence of CDI caused by fluoroquinolone-resistant and -susceptible isolates was estimated using negative-binomial regression, overall and per genotype. Selection and transmission were investigated using phylogenetic analyses.

Findings

National fluoroquinolone and cephalosporin prescribing correlated highly with CDI incidence (cross-correlations>0.88), in contrast to total antibiotic prescribing (cross-correlations<0.59). Regionally, CDI decline was driven by elimination of fluoroquinolone-resistant isolates (~67% of Oxfordshire cases in September 2006, ~3% in February 2013; annual incidence rate ratio: 0.52, (95%CI 0.48,0.56), versus fluoroquinolone-susceptible isolates: 1.02, (0.97,1.08)). CDI caused by fluoroquinolone-resistant isolates declined in four distinct genotypes (p<0.01). The regions of phylogenies containing fluoroquinolone-resistant isolates
were short-branched and geographically-structured, consistent with selection and rapid
transmission. The importance of fluoroquinolone restriction over infection control was
demonstrated by significant declines in inferred secondary (transmitted) cases caused by
fluoroquinolone-resistant isolates with or without hospital contact (p<0.0001), versus no
change in either group of cases caused by fluoroquinolone-susceptible isolates (p>0.2).

**Interpretation**

Restricting fluoroquinolone prescribing appears to explain the decline in CDI incidence,
above other measures, in Oxfordshire and Leeds, England. Antimicrobial stewardship should
be a central component of CDI control programs.

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INTRODUCTION

Clostridium difficile infection (CDI) remains a major clinical challenge worldwide.\textsuperscript{1,2} At least three antimicrobial classes are considered high-risk CDI triggers,\textsuperscript{3} including most cephalosporins, to which C. difficile is inherently resistant\textsuperscript{4} and clindamycin, to which genotypes causing early outbreaks had acquired resistance.\textsuperscript{5,6,7} Recent global dispersion of ‘hypervirulent’ NAP1/PCR-ribotype-027 CDI revealed an association between fluoroquinolone resistance and epidemic spread.\textsuperscript{8,9} Accordingly, restricting clindamycin or fluoroquinolone use has been employed, with other measures, to control localised CDI outbreaks.\textsuperscript{7,10,11}

Most CDI cases are temporally associated with healthcare,\textsuperscript{2} reflecting a combination of healthcare-associated acquisition, and healthcare-related triggers including antibiotics. Three UK studies using highly discriminatory whole genome sequencing (WGS),\textsuperscript{12,13,14} and a US study using alternative high-resolution typing,\textsuperscript{15} found as few as one-third of CDIs involved recent acquisition from an active case, leaving the source for two-thirds unexplained.

Contrasting with other countries,\textsuperscript{1,2} CDI incidence in England declined markedly over the last decade,\textsuperscript{16} following the introduction of national CDI prevention and management policies from June 2007.\textsuperscript{17,18} These included recommendations to avoid clindamycin and cephalosporins, minimise fluoroquinolone, carbapenem and aminopenicillin use, and improve infection prevention and control activities (Supplementary Methods).\textsuperscript{17} We investigated the impact of these interventions on C. difficile evolution, selection and transmission, to inform future CDI control policies for this global challenge.
METHODS

This study tested two hypotheses. First, if CDI declines in England were driven by changes in use of particular antibiotics, then incidence of CDI caused by resistant isolates should decline faster than that caused by susceptible isolates across multiple genotypes (defined by multilocus sequence type (ST)). Second, if CDI declines were driven by improvements in hospital infection control, then transmitted (secondary) cases should decline regardless of susceptibility.

To confirm that national policies affected antibiotic prescribing and CDI incidence, we first compared national antimicrobial prescribing data for hospitals and the community (obtained respectively from IMS Health, (Danbury, CT) and the Health & Social Care Information Centre (Supplementary Methods)) with national CDI incidence i.e. cases per English population per year (data from Public Health England).

The primary study dataset comprised WGS from clinical C. difficile isolates cultured from consecutive toxin enzyme immunoassay (EIA)-positive stool samples from symptomatic patients submitted to the Oxford University Hospitals NHS Trust between September 12, 2006-August 19, 2013 (n=2,021) (Supplementary Methods; Table S1). The hospital conducts all C. difficile testing in Oxfordshire, serving general practices, community hospitals and other providers, so incidence is per Oxfordshire population (~600,000) per year. This culture-positive CDI incidence was compared to Oxfordshire’s nationally-submitted EIA-positive incidence (incorporating changes in mandatory reporting requirements in 2008) to confirm representativeness of WGS. The latter was compared to English CDI incidence to assess generalisability.
Generalisability of Oxfordshire data were also assessed using comparable information from Leeds Teaching Hospitals NHS Trust, UK. This comprised WGS for consecutive clinical, toxin-positive (cytotoxin assay) isolates from symptomatic patients (August 2 2010 to May 1 2013; n=1,020) (Table S1), Leeds regional CDI incidence data (nationally-submitted) and ribotype prevalence, and antibiotic prescribing data.

Additional genetic context was provided by further regional and international C. difficile WGS (May 9, 2006-July 12, 2013) for isolates from: toxin-EIA-negative clinical samples of symptomatic Oxfordshire patients (n=395); toxin-positive samples representing two clinical trials of fidaxomicin in North America and Europe (n=803), and from healthy Oxfordshire infants (non-clinical, n=200) (Table S1).

**Genome Sequencing and Multilocus Sequence Type Identification**
Genomes were sequenced using Illumina technology. Velvet *de novo* assemblies and reference-based assemblies were generated, the latter mapped to C. difficile 630 (GenBank AM180355.1) (Supplementary Methods; reads submitted to NCBI, BioProjectID PRJNA304087, accession numbers Table S1). The sequences of loci defining C. difficile STs were identified and extracted using BIGSdb; STs were assigned using http://pubmlst.org/cdifficile/. The notation ST1(027) indicates, for example, Sequence-Type-1 (PCR-ribotype-027).

**WGS-derived Fluoroquinolone Susceptibility**
Isolates were designated fluoroquinolone-susceptible or -resistant based on specific non-synonymous substitutions within the quinolone resistance-determining region of gyrA/B genes extracted from WGS. gyrA C(245)T [T(82)I] and gyrB G(1276)A [D(426)N]
confer high-level fluoroquinolone resistance in C. difficile and other species.\textsuperscript{16,17} 

Susceptibility predictions were validated phenotypically for 387 fidaxomicin trial isolates\textsuperscript{19,20} (n=191 Canada, n=196 USA), using agar dilution (moxifloxacin minimum inhibitory concentration, (MIC)) (Figure S1, Supplementary Material).

\textbf{Statistical Analysis}

Univariable comparisons between English antimicrobial prescribing and CDI incidence were made using bivariate cross-correlations (Supplementary Methods). Genotype(ST)-specific incidence rates for CDI caused by toxin EIA-positive, culture-positive isolates were calculated using negative binomial regression accounting for missing data by probability weights (Supplementary Methods). For genotypes with >10% fluoroquinolone-resistant isolates, rates were calculated separately for fluoroquinolone-susceptible and fluoroquinolone-resistant isolates. Rates were also calculated separately for cases that could plausibly have arisen from secondary spread (transmission) inferred by close genetic relationships to prior cases (≤2 single nucleotide variants (SNVs) from the original case\textsuperscript{12}), and also separately for fluoroquinolone-susceptible and fluoroquinolone-resistant isolates.

Phylogenetic trees were constructed for each ST (or several closely related STs), using maximum likelihood, then corrected for recombination using ClonalFrameML version 1.0-6.\textsuperscript{24} Trees were time-scaled and made directly comparable post-1990 (Supplementary Methods). In each tree, the Evolutionary Distinctiveness (ED) score of each genome was calculated;\textsuperscript{25} low ED scores indicate closely related genomes, whereas high scores indicate their relative absence (Supplementary Methods).

\textbf{Role of the Funding Source}
The study sponsor had no role in study design, data collection, data analysis, data interpretation, or report writing. The corresponding author had full access to all study data and had final responsibility for the decision to submit for publication.
RESULTS

CDI Incidence and Antibiotic Prescribing

CDI incidence in England increased from 1998-2006 (p<0.0001) then declined rapidly to 2013 (p<0.0001) (Figure 1A). Declines occurred while total antibiotic prescribing was increasing (by 4.4%/year in the community (p<0.0001, 2006-2013) but only 0.5%/year in hospitals (p=0.053, 2006-2012)) (Figure 1B). Between 2005-2012 (when data were complete for England), the cross-correlations (CC) between English CDI incidence and total English antibiotic prescribing were -0.57 (95% CI -0.67,-.0.41), -0.59 (-0.68,-.0.44) and 0.29 (-0.19,0.60) (for hospital+community, community, and hospital prescribing respectively, optimum CC using a 1-year lag, Table S2, Supplementary Methods). During the same period, the strongest univariable associations between English CDI incidence and individual antimicrobials were with cephalosporins (CC=0.97 (0.82,0.98), 0.94 (0.68,0.97) and 0.97 (0.81,0.99) respectively (optimum 0-year lag)) and fluoroquinolones (CC=1.00 (0.84,1.00), 0.88 (0.48,0.95) and 0.93 (0.66,0.97) respectively (optimum 0-year lag)) (Figure S2A, Table S2), although hospital fluoroquinolone prescribing began to decline slightly earlier than community prescribing (p<0.0001 from 2005-2009, vs in the community p<0.0001 from 2007-2012; Figure 1A). Other antibiotics were more weakly associated (Figure S2A-F, Table S2).

Similar to English CDI incidence, Oxfordshire rates also decreased from 2007 (when isolate-level fluoroquinolone-susceptibility could be determined) (p<0.0001) (Figure 2A). Fluoroquinolone prescribing in Oxfordshire hospitals declined from a peak in 2005 until 2010 (p<0.0001), when usage began to increase again (p<0.0001 from 2010-2013). Hospital cephalosporin and fluoroquinolone prescribing were also positively associated with CDI incidence (CC=0.73 (0.15,0.86) and 0.62 (-0.09,0.81), Table S2), but associations were
estimated much less precisely given the much smaller population (~1% of England). Positive associations were also observed between CDI decline and decline in extended spectrum penicillins (CC=0.84 (0.24,0.90) and beta-lactamase resistant penicillins (CC=0.67 (-0.04,0.81), Figure S9. Community prescribing data was not available.

**Predicting *C. difficile* Fluoroquinolone Susceptibility from WGS**

Paired fluoroquinolone susceptibility phenotype and gyrA/B DNA sequences were assessed for 387 isolates representing 53 STs. Phenotype and WGS were 98.7% concordant (Figure S1; sensitivity 97.8%, specificity 99.5%); only 1/185 isolates predicted resistant by WGS22,23 lacked an elevated MIC. Conversely, only 4/202 isolates lacking resistance-associated substitutions22,23 had raised MICs (16 mg/L). gyrA/B sequence therefore reliably predicts fluoroquinolone resistance phenotype.

**Regional CDI Incidence and Fluoroquinolone Susceptibility**

The decrease in Oxfordshire cases was due solely to a decline in CDI caused by fluoroquinolone-resistant isolates (estimated at ~67% of all Oxfordshire CDI September 2006, falling to ~3% February 2013, annual incidence rate ratio (aIRR)=0.52 (95% CI 0.48-0.56) p<0.0001 (Figure 2B)). The majority (62%) of fluoroquinolone-resistant isolates were from genotype ST1(027), but the decline persisted even when excluding ST1(027) and pooling the remaining fluoroquinolone-resistant isolates together, (aIRR=0.73 (0.66-0.81), p<0.0001 for all non-ST1; aIRR=0.66 (0.59-0.75), p<0.0001 for all non-ST1 with >10% resistant isolates, Figures 3, S3D). Considering genotypes containing >10% resistant isolates separately, CDI caused by fluoroquinolone-resistant isolates declined significantly for four genotypes from three distinct chromosomal backgrounds26: clade 1 ST42(106) (p=0.00076),
ST3(001) (p=0.0054); clade 2 ST1(027) (p<0.0001) and clade 4 ST37(017) (p=0.0027), Figures 3, 4A-B, S3A-C).

Notably, the incidence of CDI caused by fluoroquinolone-susceptible isolates remained unchanged (aIRR=1.02 (95% CI 0.97-1.08) p=0.45, Figure 2B, heterogeneity p<0.0001 vs fluoroquinolone-resistant), and actually increased in three of the five genotypes with >10% but <99% resistant isolates (Figure 3, 4B, S3A-C). More limited data for Leeds, representing a geographically independent region, were broadly similar (aIRR=0.55 (0.49-0.61) p<0.0001 pooling predominantly fluoroquinolone-resistant ribotypes, aIRR=1.03 (1.01-1.05) p=0.0031 pooling fluoroquinolone-susceptible ribotypes, Figure S4, Table S2), as were national ribotyping data, supporting generalisability.

**Phylogenetic evidence for fluoroquinolone-driven *C. difficile* transmission**

Nineteen phylogenies (Figures 4D-F, S5A-D), were constructed representing the 22 most common *C. difficile* genotypes in Oxfordshire and Leeds. The phylogeny of each genotype containing >10% fluoroquinolone-resistant isolates (Figure 4D,E, S5A) indicated rapid, geographically structured clonal expansion(s) associated with resistance. This observation was reproduced internationally among parts of the phylogenies representing Calgary, Canada (Figure 4D,E) and among isolates from three cities in Northern Italy; Modena, Turin and Arsizio (Figure S5A). It was supported by significantly lower ED scores for resistant versus susceptible areas of phylogenies containing both fluoroquinolone-resistant and fluoroquinolone-susceptible isolates (e.g. ST3 p<0.0001 Figure 4E, ST37 p<0.0001, Figure S5A). By contrast, the phylogenies of genotypes consisting primarily of susceptible isolates (Figure 4F, S5A-D) were geographically unstructured and had longer branches. This was also seen internationally among susceptible isolates from Calgary and Montreal, Canada (Figure...
Among fluoroquinolone-susceptible genotypes, the ED scores (and, by inference, transmission) did not differ significantly between Oxfordshire and Leeds clinical isolates (p>0.1) (Figure S5).

Additional phylogenies for three prevalent fluoroquinolone-susceptible genotypes revealed similar branch lengths irrespective of sampling region size (Figure S6A,B). Oxfordshire phylogenies (Figure S6B), containing genomes from toxin EIA-positive and -negative samples, plus genomes from healthy, asymptomatic, community infants, demonstrated a lack of structure by source, even within a single region. Interestingly, ED scores were generally lower for clinical toxin EIA-positive genomes compared with infant and EIA-negative genomes, especially in ST8(002) (p=0.0033) and ST2(014/020) (p=0.0014) (Figure S6A,B), consistent with greater transmission in the former.

Fluoroquinolone restriction and multiple enhanced infection control measures were introduced simultaneously in England in 2007. Therefore, we investigated the hypothesis that infection control, not antimicrobial stewardship, reduced CDI incidence by reducing transmission; e.g. fluoroquinolone-resistant isolates were simply more prevalent in hospitals where infection control efforts were concentrated. Secondary spread (transmission) was inferred when subsequent cases had closely genetically related isolates. The Oxfordshire incidence of inferred secondary cases was estimated separately for fluoroquinolone-resistance versus fluoroquinolone-susceptibility, and also for cases where hospital-based contact occurred between primary and secondary cases. There was strong evidence for declines in secondary CDI cases caused by fluoroquinolone-resistant isolates, both with (aIRR=0.21 (95% CI 0.13–0.34, p<0.0001)) and without (aIRR=0.45 (0.29-0.71), p<0.0001) hospital contact with a previous case. Declines occurred in secondary cases caused by
fluoroquinolone-resistant isolates from ST1(027) and non-ST1(027) genotypes (p≤0.012, Figures 5, S8). By contrast, there was no evidence of declines in secondary CDI cases caused by fluoroquinolone-susceptible isolates, either with (aIRR=0.87 (0.67–1.13), p=0.29), or without (aIRR=1.14 (0.92–1.42), p=0.23) hospital contact with a previous case, supporting the importance of fluoroquinolone restriction over infection control interventions.
Here, analysis of multiple WGS datasets demonstrates that reductions in CDI incidence caused by fluoroquinolone-resistant isolates (of multiple genotypes) plausibly has driven the CDI decline in Oxfordshire and Leeds, England from 2007. Declines occurred alongside significant reductions in fluoroquinolone use in hospitals and the community. Extensive WGS phylogenies show that acquisition of fluoroquinolone resistance preceded the emergence of multiple, prevalent genotypes (Figures 4, S5A); after fluoroquinolone prescribing was controlled, incidence declines were specific to CDI caused by fluoroquinolone-resistant isolates of these same genotypes (Figures 3, 4, S3B, S4). By contrast, the incidence of CDI from multiple fluoroquinolone-susceptible genotypes remained constant (Figures 3, 4C, S3C), unaffected by changes in fluoroquinolone use or other national policy measures, such as restricted cephalosporin prescribing and enhanced infection control interventions (irrespective of genotype) (Figures 5, S2A, Table S217). Critically, there was no evidence of a decline in plausibly nosocomially transmitted secondary cases caused by fluoroquinolone-susceptible C. difficile, which would be expected if improved infection control had made a major contribution to CDI declines, whereas secondary cases caused by fluoroquinolone-resistant C. difficile decreased markedly (Figure 5, S8).

The phylogenetically estimated date of fluoroquinolone resistance emergence preceded the clinical emergence of multiple problematic C. difficile genotypes of different phylogenetic clades26; not only ST1(027)9 but also ST42(106), ST3(001) and ST37(017) (Figures 4, S5A).28,29. The recent emergence of fluoroquinolone-resistant ST17(018) in Italy (Figure S5A) also followed high fluoroquinolone usage.30 Our greater sampling density9 revealed short branched, geographically structured phylogenies of fluoroquinolone-resistant C. difficile consistent with rapid spread within hospitals, and occasional transmission between them.
Inclusion of international isolates allowed us to demonstrate generalisability of our findings outside of the UK. Although fluoroquinolone-susceptible, limited ST8(002) and ST2(014/020) transmission plausibly occurred, as indicated by small, short branched clusters, and lower ED scores for clinical-toxin EIA-positive isolates versus infant/EIA-negative isolates (Figure S6A,B). However, the absence of large-scale geographic structure in the long branched phylogenies of all fluoroquinolone-susceptible genotypes (Figure S5B/C, S6) suggests that most were introduced independently into the clinical environment from alternative potential reservoirs.\(^ {31,32}\) Fluoroquinolone-susceptible *C. difficile* may therefore represent a population lacking large-scale adaptation to antimicrobial selection pressures of clinical environments.

The CDI incidence decline following national restriction of high-risk antimicrobials is consistent with previously-successful small-scale interventions restricting high-risk antimicrobials as part of control packages.\(^ {7,10,11}\) However, our study demonstrated conclusively that Oxfordshire CDI declines were due to the parallel disappearance of fluoroquinolone-resistant isolates of multiple genotypes (Figures 2, 3) suggesting that any selective advantage specific to resistant isolates may be lost when the antimicrobial is withdrawn. In England, additional antimicrobials were also targeted for restriction.\(^ {17}\) However, only cephalosporin use also fell (Figures 2A, S2). Since all *C. difficile* is inherently resistant to most cephalosporins\(^ {4}\) their restriction cannot explain the fluoroquinolone-susceptibility specific declines in incidence observed. Similarly, if an ST1(027)-specific factor had led to its decline, there would be no reason for CDI caused by fluoroquinolone-resistant isolates of several other genotypes (ST42(106), ST3(001), ST37(017)) in two other *C. difficile* clades (1 and 3\(^ {26}\)) to decline concurrently (Figures 3, 5). While univariate cross-correlations between CDI decline and hospital-prescribed extended-spectrum penicillins
(mostly amoxicillin alone) and beta-lactamase resistant penicillins (mostly flucloxacillin alone) were stronger than for fluoroquinolones in Oxfordshire, the use of many antibiotics in these groups actually rose because they were instead used in combinations such as co-amoxiclav. Penicillins have generally been shown to have a lesser risk of provoking *C. difficile* than other classes of antibiotics,\textsuperscript{8,33} and when taking community prescribing into account, (which forms a larger proportion of overall antimicrobial use than hospital prescribing) the correlation between these penicillin groups and CDI incidence in England disappears. Unfortunately, community prescribing data were not available for Oxfordshire for comparison. Finally, the much smaller population size meant these univariate cross-correlations were estimated imprecisely compared with England as a whole. Our study therefore clarifies the issue of whether fluoroquinolone or cephalosporin restriction alone or in combination is key to CDI control.\textsuperscript{34,35,36} However, changes in dominant genotypes over time have been reported in a single centre in the absence of antimicrobial restriction policies.\textsuperscript{37} ST1(027)-outbreak control has also been achieved when total antimicrobial (not only fluoroquinolone) use was reduced,\textsuperscript{38} although this could still reflect predominantly the impact of fluoroquinolones.

Similar to cephalosporin restriction, enhanced infection control measures\textsuperscript{17} such as isolation, contact precautions, and enhanced environmental cleaning do not target specific *C. difficile* genotypes and should therefore reduce numbers of symptomatic patients infected with transmitted strains, irrespective of fluoroquinolone-susceptibility. Analysis of closely related *C. difficile* genomes from different patients, i.e. representing possible transmissions\textsuperscript{12} potentially preventable by infection control measures, demonstrated clearly that incidence only fell for secondary cases caused by fluoroquinolone-resistant *C. difficile*, irrespective of hospital contact with a previous closely genetically related case, with no change in secondary
cases caused by fluoroquinolone-susceptible isolates (Figure 5, S8). This is consistent with previous work\(^{38}\) finding no change in CDI incidence after infection control procedures were strengthened. This supports the greater importance of fluoroquinolone restriction in both hospitals and the community over enhanced infection control in recent reductions in English CDI incidence.

Antimicrobial stewardship targeted all patients in hospitals and the community,\(^{17}\) so clinically adapted resistant *C. difficile* may conceivably have been eliminated from asymptomatic carriers and cases. If fluoroquinolone-resistant *C. difficile* persisted in carriers, outbreak conditions should have returned rapidly once fluoroquinolone prescribing increased. This did not occur even after post-2010 increases in hospital fluoroquinolone prescribing in Oxford and Leeds (Figures 2A, S4). However, whereas pre-2007 fluoroquinolones were prescribed widely, including among the elderly, increases post-2010 do not necessarily equate to increased exposure of high-CDI-risk patients. Instead they may reflect new, specific indications such as neutropenic prophylaxis (e.g. Figure S7 for Leeds; equivalent data not available in Oxford), consistent with observations that fluoroquinolone use is not a risk factor under non-outbreak conditions\(^{39}\). The lack of rise in fluoroquinolone-resistant CDI nationally also supports their almost complete eradication from both symptomatic patients and asymptomatic carriers in England, consistent with regional (Oxfordshire) findings that by late 2011, fluoroquinolone-resistant isolates of the commonest incidence genotype (ST1(027)) had disappeared from asymptomatic colonization as well as infection.\(^{31}\)

The genotypes ST1(027), ST42(106), ST3(001), and ST37(017), accounting for most fluoroquinolone-resistant isolates, represent three divergent *C. difficile* clades\(^{26}\), each with a genetically distinct, toxin-encoding Pathogenicity Locus.\(^{26}\) These genotypes could therefore
differ in virulence and/or transmissibility due to varying gene content. ST1(027), for example, is almost four times likelier than other genotypes to cause symptomatic infection over colonization\(^4\) (although this could reflect its fluoroquinolone-resistant phenotype in settings with high fluoroquinolone prescribing). It seems unlikely that other gene content should be completely confounded with fluoroquinolone resistance, particularly within the large clade \(^1\) containing ST42(106), ST3(001) and (Italian) ST17(018)). However, even if additional virulence factors are associated with ST1(027), the overall diversity of outbreak-associated genetic backgrounds in which fluoroquinolone resistance is found, suggests that this phenotype alone may unfortunately be sufficient to confer outbreak-potential.

A few sporadic fluoroquinolone-resistant isolates were identified in otherwise susceptible genotypes (Figure S5), suggesting that chance, combined with regional antibiotic prescribing policies, could trigger localised spread. ST11(078) was unusual, in that fluoroquinolone resistance occurred in 24/182 (13\%) of isolates, distributed throughout the phylogeny (Figure S5B). ST11(078) can be transmitted zoonotically,\(^3\) and the unstructured pattern of fluoroquinolone resistance within this phylogeny could reflect the sporadic emergence of resistance either during agricultural fluoroquinolone use, or following human colonisation and antibiotic exposure.

The main study limitation is being primarily based in one, albeit large (c.600,000 population) region, where 7 years of individual-isolate WGS enabled us to predict fluoroquinolone susceptibility. Leeds WGS were available for only 2.7 years, precluding a similar analysis to Figure 2 in another region. Different datasets from different sources were used for CDI incidence and antibiotic use because no one dataset was collected consistently across the entire period from a single source. Comparisons of CDI incidence and antibiotic use are
ecological, and therefore prone to unmeasured confounding. English hospital-level antibiotic
data are not available before 2013 (only subsequently\(^{41}\)), so we were unable to investigate
associations between fluoroquinolone use and CDI across Trusts in a broader ecological
analysis. However, our key characteristics, fluoroquinolone-susceptibility and genotype, were
unknown when the CDI occurred and were not inclusion/exclusion criteria. Therefore, the
phylogenetic analyses are representative of the genotypes circulating in the locations studied
when sampled.

In summary, fluoroquinolone resistance occurs in multiple genetically divergent \textit{C. difficile}
genotypes.\(^{26}\) The contrasting phylogenies of fluoroquinolone-resistant and fluoroquinolone-
susceptible \textit{C. difficile} likely reflect increased potential for healthcare-associated selection
and epidemic spread of fluoroquinolone-resistant bacteria. Thus the \textit{C. difficile} genotypes
causing infections at any given time and location, and the relative importance of different
transmission routes (nosocomial person-to-person versus multiple introductions) may be a
direct consequence of antimicrobial prescribing policies. The multifaceted approach to CDI
control adopted by England successfully curtailed transmission. WGS data suggest that
fluoroquinolone restriction plausibly played the most important role in this success.

Appropriate antimicrobial stewardship therefore is, and will likely remain, central to the on-
going control of CDI.
**FIGURE LEGENDS**

**Figure 1**

A. National CDI incidence and fluoroquinolone prescribing. Mandatory CDI incidence corresponds to all cases reported in over-2s (from 2004-2007 cases were only reported in over 65-s, and are upweighted to provide comparable estimates in over 2s, see Supplementary Methods). As mandatory reporting was only introduced in 2004, we have also included voluntary-reported CDI to give an indication of trends prior to that date.

B. National antibiotic prescribing overall. Dotted lines are estimates (see Supplementary Methods).

**Figure 2**

A. CDI incidence together with fluoroquinolone and cephalosporin prescribing for Oxfordshire. Mandatory CDI incidence corresponds to all cases reported in over-2s (from 2004-2007 cases were only reported in over 65-s, and are upweighted to provide comparable estimates in over 2s, see Supplementary Methods). Only toxin-positive culture-positive samples were used in the genotype-specific and phylogenetic analyses.

B. CDI incidence by fluoroquinolone susceptibility for Oxfordshire. IRR=Annual incidence rate ratio. *C. difficile* is inherently resistant to most cephalosporins.⁴

**Figure 3**

Oxfordshire *C. difficile* incidence trends by fluoroquinolone resistance and genotype.

IRR=Annual incidence rate ratio. For genotypes with >10% resistant isolates (denoted FQR), rates were calculated separately for CDI caused by fluoroquinolone-susceptible and resistant isolates. To show that the difference in trends for resistant and susceptible isolates is not driven solely by the decline in ST1(027), rates were also calculated for all non-ST1(027) genotypes together, as well as for all genotypes with >10% resistant isolates (excluding ST1(027)) and for all genotypes with <=10% resistant isolates (FQS). Heterogeneity between
trends in CDI caused by fluoroquinolone-resistant vs fluoroquinolone-susceptible isolates:

All $p<0.0001$, Non-ST1 $p<0.0001$, Non-ST1 FQR $p<0.0001$, ST42 $p<0.0001$, ST37

$p=0.00015$, ST3 $p=0.0007$, ST35 $p=0.92$, ST11 $p=0.0053$.

**Figure 4**

Contrasting CDI incidence (Oxfordshire) and WGS phylogenies representing the fluoroquinolone-resistant genotype ST1(027), the mixed resistant and susceptible genotype ST3(001), and the almost entirely fluoroquinolone-susceptible genotype ST8(002).

**A.** CDI incidence by fluoroquinolone susceptibility for genotype ST1(027) in Oxfordshire.

IRR=Annual incidence rate ratio. Red bars indicate fluoroquinolone-resistant isolates, blue bars indicate fluoroquinolone-susceptible isolates, black bars indicate resistance not determined.

**B.** CDI incidence by fluoroquinolone susceptibility for genotype ST3(001) in Oxfordshire.

**C.** CDI incidence by fluoroquinolone susceptibility for genotype ST8(002) in Oxfordshire.

**D.** Time scaled phylogeny for ST1(027) generated using ClonalFrameML. Every third Oxfordshire isolate (by date) is shown. ED=Evolutionary Distinctiveness (ED),

R=fluoroquinolone-resistant, S=fluoroquinolone-susceptible. Phylogenies were scaled to be directly comparable post-1990; the grey shaded regions prior to 1990 represent the regions of the phylogenies that should not be compared, since they are not scaled identically.

Background colour indicates fluoroquinolone susceptibility; branch colour geographic location.

**E.** Time scaled phylogeny for the mixed fluoroquinolone resistant/susceptible genotype, ST3(001), generated using ClonalFrameML. Two fluoroquinolone-resistant areas of the phylogeny are indicated by red shading within the blue ‘susceptible’ region. Rapid clonal expansion after resistance emergence is supported by significantly lower ED scores for resistant versus susceptible areas.
Time scaled phylogeny for ST8(002) generated using ClonalFrameML. Every second Oxfordshire isolate (by date) is shown. Two fluoroquinolone-resistant isolates are indicated at the bottom of the panel.

**Figure 5**

The incidence of inferred secondary CDI cases in Oxfordshire, i.e. cases caused by *C. difficile* isolates that are genetically closely related (≤2 single nucleotide variants) to isolates recovered from a previous case, and therefore potentially transmitted. Incidence was calculated separately for inferred secondary cases caused by fluoroquinolone-resistant ST1(027), fluoroquinolone-resistant non-ST1(027) and fluoroquinolone-susceptible isolates, stratified by ‘with’ versus ‘without’ hospital-based contact. IRR=Annual incidence rate ratio.
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CONTRIBUTORS

KED, XD, TPQ, MHW, TEAP, ASW and DWC designed the study with input from DWE, NS, TG, RMH and DJW.

KED, DWE, NS, DG, AV, SJO, WNF, JF, KM and JM collected specimens from Oxfordshire and Leeds, cultured C. difficile and, or extracted chromosomal C. difficile DNA for WGS. SG, EJCG and DMC contributed the ‘Fidaxomicin clinical trial’ isolate collection.

EJCG and DMC performed fluoroquinolone susceptibility testing.

The Modernising Microbiology Informatics Group, JMF, TG and DHW optimised or performed the assembly of short DNA sequence reads.

KED derived genotype data from WGS and identified genomes for the construction of CFML dual-scaled phylogenies. All phylogenies were constructed by XD. KED combined phylogenetic and fluoroquinolone resistance genotype data.

PH, SH, MHW and DWC obtained antimicrobial prescribing data. RH and APJ obtained national incidence data for CDI.

Biostatistical analysis: TPQ, ASW and TEAP performed the analysis of Oxfordshire and national incidence and antimicrobial prescribing data. WNF, TPQ, ASW and MHW performed the analysis of Leeds incidence and antimicrobial prescribing data. DWE, TPQ, ASW and TEAP performed SNV analysis (Figure 5, S8).

KED, XD, TPQ, MHW, TEAP, ASW and DWC wrote the first draft of the article and all authors contributed to and had final approval of the Article.

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