Nanopore metagenomic sequencing to investigate nosocomial transmission of human metapneumovirus from a unique genetic group among haematology patients in the United Kingdom

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SUMMARY

Background: Human metapneumovirus (HMPV) infection causes a spectrum of respiratory tract disease, and may be a significant pathogen in the context of immunocompromise. Here, we report direct-from-sample metagenomic sequencing of HMPV using Oxford Nanopore Technology.

Methods: We applied this sequencing approach to 25 respiratory samples that had been submitted to a clinical diagnostic laboratory in a UK teaching hospital. These samples represented 13 patients under the care of a haematology unit over a 20-day period in Spring 2019 (two sampled twice), and ten other patients elsewhere in the hospital between 2017–2019.

Results: We generated HMPV reads from 20/25 samples (sensitivity 80% compared to routine diagnostic testing) and retrieved complete HMPV genomes from 15/20 of these. Consensus sequences from Nanopore data were identical to those generated by Illumina, and represented HMPV genomes from two distinct sublineages, A2b and B2. Sequences from ten haematology patients formed a unique genetic group in the A2b sublineage, not previously reported in the UK. Among these, eight HMPV genomes formed a cluster (differing by ≤3 SNPs), likely to reflect nosocomial transmission, while two others were more distantly related and may represent independent introductions to the haematology unit.

Conclusion: Nanopore metagenomic sequencing can be used to diagnose HMPV infection, although more work is required to optimise sensitivity. Improvements in the use of metagenomic sequencing, particularly for respiratory viruses, could contribute to antimicrobial stewardship. Generation of full genome sequences can be used to support or rule out nosocomial transmission, and contribute to improving infection prevention and control practices.

Introduction

Human metapneumovirus (HMPV) is a 13 kb, single-stranded, negative-sense RNA virus of the *Paramyxoviridae* family.[1] Since its first description in 2001, HMPV has been recognized as an important cause of respiratory diseases, ranging from mild upper respiratory tract infections to severe bronchiolitis and pneumonia,[2–4] especially in young children and immunocompromised patients, such as those with hematologic malignancies, allogeneic stem cell transplant recipients, and lung transplant recipients.[5,6] However, studies of HMPV transmission and genetic diversity have been limited, with only ~150 full length HMPV genomes published to date.[7] Two major genetic lineages (A and B) and five sublineages (A1, A2a, A2b, B1, and B2) of HMPV have been described.[8]

The Nanopore sequencing platform (Oxford Nanopore Technology, ONT) can generate real-time sequencing data and has the long-term potential to become a point-of-care test for infectious diseases in clinical settings,[9] thus contributing to clinical management decisions including antimicrobial prescribing and stewardship, and informing infection control practice. The sequence data generated can provide insights into the molecular epidemiology and evolutionary genomics of infectious diseases, and also help to identify nosocomial transmission of infection. Nanopore sequencing has already been successfully applied to investigate outbreaks caused by emerging pathogens, including Lassa fever in Nigeria[10] and Ebola in West Africa.[11]

Here we describe Nanopore metagenomic sequencing of HMPV genomes from our tertiary referral teaching hospital in the United Kingdom. We generate sequence data from an outbreak of respiratory infection due to HMPV on the haematology ward in Spring 2019. We also report HMPV genomes from an additional ten respiratory samples from immunocompromised patients in the same hospital between 2017 and 2019 to provide a broader representation of sequence diversity in our patient population. Our objectives were to investigate the hypothesis that there was nosocomial transmission of HMPV among patients under the care of the haematology unit, and to elucidate the potential genetic variants of HMPV in this setting.

Methods

Sample collection and selection

Throat swab samples were collected by healthcare workers based on clinical indications. Samples were collected in viral transport medium and sent to the routine clinical diagnostic laboratory. They were tested for respiratory viruses based on the clinical standard operating protocol using either the BioFire® FilmArray® Respiratory Panel 2 assay (BioFire Diagnostics, Salt Lake City, UT, USA) ($n = 24$), or by Xpert Xpress Flu/RSV assay (Cepheid, Sunnyvale, CA, USA) ($n = 1$) (Table 1), then frozen at −80C for retrospective Nanopore sequencing.

Samples were selected for inclusion in this study based on two approaches:

(1) We focused on a clinical cluster of HMPV infection based on the results of routine diagnostic testing by our clinical microbiology laboratory, generating data for 13 patients (two patients sampled twice, 15 samples) on the haematology ward during a 20 day period in Spring 2019.

(2) In order to assess the HMPV clinical cluster in the wider context of sequence data representing the wider hospital setting, we also report on additional HMPV cases (ten patients, ten samples) generated from metagenomic sequencing of residual respiratory samples collected between December 2017 and April 2019. Among these, 9/10 had HMPV diagnosed by the BioFire® RP2 assay in the clinical diagnostic laboratory. The standard protocol in the microbiology laboratory is to perform this multiplex panel only on a selected subgroup of patients most at risk of severe disease (those with immunocompromise, patients under the care of infection and respiratory teams, and those on critical care units). The remaining sample was only tested for influenza/RSV by the diagnostic laboratory and HMPV was detected as a result of Nanopore metagenomic sequencing of the residual sample after the patient had been discharged from hospital as part of a methods development project.

Methods for sample preparation, Nanopore metagenomic sequencing, Illumina sequencing, and genomic analysis are described in supplementary material.

Haematology unit

The haematology unit in our hospital can accommodate 25 adult in-patients, in 15 individual en suite side-rooms and five two-bedded bays (all maintained at positive or neutral pressure). Rooms are arranged around three sides of a square, with entry points at each end.

Results

Recovery of complete HMPV genomes through Nanopore metagenomic sequencing

We undertook Nanopore sequencing of a total of 25 throat swabs collected from 23 patients. The median age of the patients was 54 years (range 1 - 87 years), and there were 17 (74%) males and six (26%) females. The samples represented two groups:

(i) Samples from patients under the care of the haematology unit (15 samples from 13 patients) who had been diagnosed with HMPV infection based on routine clinical diagnostic testing of throat swabs (Fig. 1; Suppl Table 1A). The clustering of these cases on a single unit over a 20-day period in Spring 2019 raised clinical concern about an outbreak.

(ii) Ten HMPV-positive samples that had been submitted to the clinical diagnostic laboratory from ten patients elsewhere in the hospital between December 2017 and April 2019 (Suppl Table 1B).

Nanopore metagenomic sequencing generated between 1.6×10^5 and 1.9×10^6 (mean 9.4×10^5) total reads per sample (Table 1). Using taxonomic classification and mapping (see Supplemenatry data for details), we identified HMPV reads in 20/25 samples (sensitivity 80%), ranging from 47 to 449,098 reads (Table 1). These 20 samples included 13 from the haematology unit (from 11 patients) and seven from patients elsewhere in the hospital. The remaining five samples had tested positive by BioFire® FilmArray® Respiratory Panel 2 assay (BioFire Diagnostics, Salt Lake City, UT, USA) in the clinical diagnostic laboratory, but no HMPV reads were generated by Nanopore sequencing. The highest proportion of HMPV reads was 63.0% of total reads (sample 19, collected on the paediatric ward) (Table 1B).

Mapping to a reference genome, we retrieved 15 complete HMPV sequences from 11/15 haematology samples and 4/10 samples from elsewhere in the hospital, at mean coverage depths between 100 and 24,000 (Table 1). Consensus sequences were generated via Nanopolish and filtering out of positions with <70% base concordance, as described in supplementary methods. Confirmatory Illumina sequencing of 16 samples showed similar genome coverage of HMPV consensus sequences compared to Nanopore sequencing (Suppl Table 2). From 13 samples with enough genome coverage, comparison showed no nucleotide difference between consensus sequences derived from these two methods.

Investigation of an outbreak demonstrating both nosocomial transmission and introduction of independent infections

A minimum spanning tree revealed that our HMPV samples could be divided as follows (Fig. 2):

• A major cluster (denoted the transmission cluster), containing eight sequences that were tightly grouped together and differed by ≤3 SNPs at consensus level, all from patients under the care of the haematology unit during a 20 day period in Spring 2019. These comprised samples from six inpatients and two outpatients who had been recently discharged from the ward. Within this transmission cluster, five sequences

Table 1

Summary of Nanopore sequencing data derived from 25 throat swabs positive for human metapneumovirus (HMPV) collected from a UK hospital cohort. Samples were taken from (A) patients who were part of a putative outbreak on the haematology unit; (B) patients attending other hospital units.

A: 15 samples collected from 13 patients on the haematology ward during an HMPV outbreak

Sample ID	Total reads generated	HMPV reads number (% of total reads)	HMPV genome coverage (mean mapping depth)	HMPV genetic lineage
7	702,001	160	10.4% (8)	A2b
8	1023,191	28,408 (2.8%)	complete (1622)	A2b
9	565,070	0	–	–
10*	999,084	317,401 (31.8%)	complete (17,120)	A2b
11	1429,921	181,286 (12.7%)	complete (9210)	A2b
12	1562,059	166,393 (10.7%)	complete (8473)	A2b
13	1920,010	60,575 (3.2%)	complete (3330)	A2b
14+	649,320	7181 (1.1%)	complete (390)	A2b
16	813,881	3003 (0.4%)	complete (176)	A2b
17	1261,728	0	–	–
18	530,863	18,591 (3.5%)	complete (1208)	A2b
20	1201,226	16,369 (1.4%)	complete (846)	A2b
21+	1100,477	612 (0.1%)	76.5% (34)	A2b
22*	537,013	15,386 (2.9%)	complete (953)	A2b
24	1257,193	2786 (0.2%)	complete (109)	A2b

B: Samples ($n = 10$) collected from other hospital in-patients

Sample ID	Total reads generated	HMPV reads number (% of total reads)	HMP genome coverage (mean mapping depth)	HMPV genetic lineage
1	168,811	46,425 (27.5%)	complete (5609)	A2b
2	234,208	6840 (2.9%)	complete (295)	B2
3	856,814	65	0 (2)	–
4	478,220	0	–	–
5	1291,696	0	–	–
6	785,255	47	0 (2)	–
15	1100,905	0	–	–
19	713,104	449,098 (63.0%)	complete (24,195)	B2
23	1131,790	246	60.9% (12)	B2
25	1418,948	42,514 (3.0%)	complete (2438)	A2b

Two pairs of samples collected from the same patient at different time points are marked with * or + sign. Sample IDs for those were also Illumina sequenced are underlined.
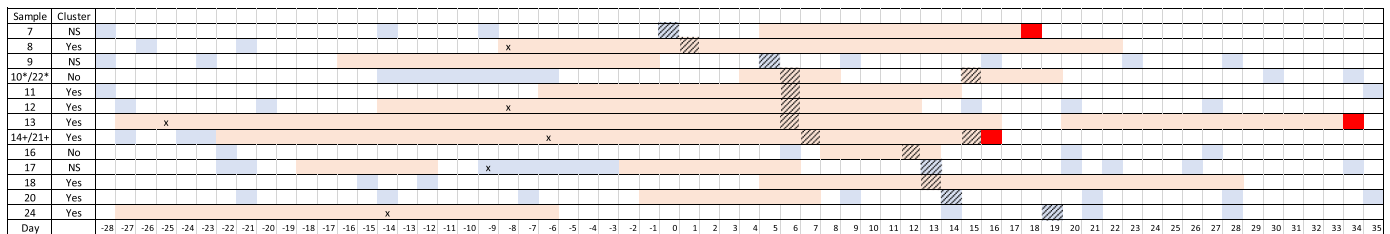

Fig. 1. The timeline of 13 patients under the care of the haematology team during an HMPV outbreak in a UK tertiary referral teaching hospital. Each patient is represented by one row (13 patients, 15 samples). Timeline is indicated in days along the bottom of the plot, with day 0 representing the time of collection of the first positive HMPV samples, and the total time interval of 64 days selected to show the time course from the first admission to the last discharge/death. Timing of attendance or admissions on the haematology ward and haematology outpatient department are marked in orange; other non-haematology wards and outpatient departments are in blue. Cross hatching box indicates collection of a throat swab that was tested positive for HMPV in the clinical diagnostic laboratory, and black cross indicates a throat swab that tested negative for HMPV. Red box denotes death of a patient. Second column indicates whether the HMPV sequence was identified as part of the transmission cluster based on genetic sequence (Table 1A). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were identical; the remaining three sequences differed from these by one, one, and three SNPs. These five SNPs were in the P ($n = 1$), F ($n = 1$), and L ($n = 2$) genes, and the intergenic region between the M and F genes ($n = 1$).

- Two identical sequences which differed from the transmission cluster by 46 SNPs, and were collected from the same patient nine days apart on the haematology ward (samples 10 and 22).
- A further three A2b sublineage sequences, one from a haematology patient and two from elsewhere in the hospital, that were separated by >80 SNPs from both the haematology clusters and each other (Figs. 2 and 3).
- Two B2 sublineage sequences, one from paediatrics and one from the adult renal/transplant unit, that were separated from the haematology clusters by >2000 SNPs.

These results suggest that the eight HMPV sequences within the transmission cluster were associated with nosocomial transmission on the haematology ward during a 20 day period. However, a

further two HMPV-positive patients on the haematology unit had sequences outside the transmission cluster, suggesting that these may have been acquired independently despite arising within the same time period and in the same hospital ward.

We reviewed the locations of inpatients within the haematology unit, seeking to determine likely routes of HMPV infection. In total, HMPV-positive patients occupied 11 different bed locations at different times during the outbreak. These included both side rooms and two-bedded bays, and were spread evenly around the ward. We were therefore unable to draw any specific conclusions about patient-to-patient transmission, or spread by fomites, shared facilities or ward staff.

Characterisation of HMPV phylogeny shows a predominance of A2b sequences

Phylogenetic analysis showed that 13 of the 15 full length HMPV genomes recovered from 10 haematology patients and two other patients clustered within the A2b sublineage (Fig. 3). These
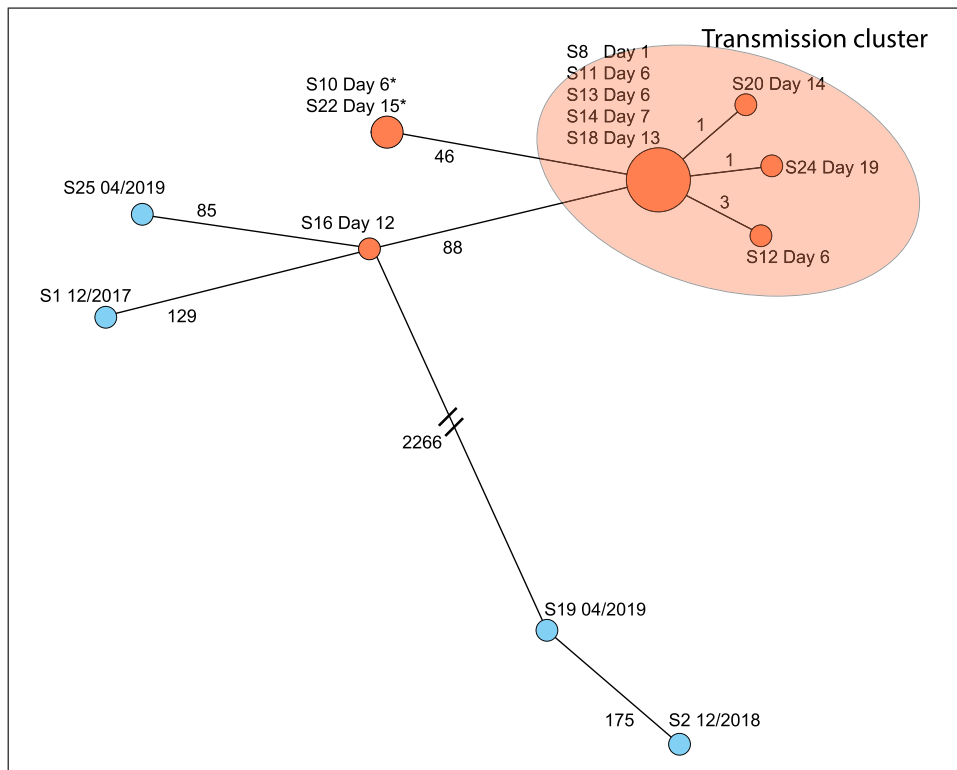
Fig. 2. Minimum spanning tree of full length HMPV sequences recovered from throat swabs collected at a UK teaching hospital. The tree was built on the basis of single nucleotide variant (SNP) distances between consensus sequences generated from Nanopore data. Sequences recovered from haematology patients during an HMPV outbreak are marked in orange (actual dates not shown to protect patient anonymity), and samples from non-haematology settings are marked in blue. SNP distance between each pair of sequence is denoted by number adjacent to the branch. A pair of samples collected from the same patient at different time points are marked with an asterisk. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sequences were closely related to strains from the United States and China, forming a unique genetic group supported by a boot-strap value of 100%. The remaining two full length sequences, from elsewhere in the hospital, clustered within the B2 sublineage.

Estimation of most recent common ancestor

Time-scaled phylogenetic analysis suggested that the eight sequences within the transmission cluster shared a common ancestor originating in December 2018 (95% highest posterior density [HPD], August 2018 to March 2019). The substitution rate was estimated to be 0.63×10^{-3} substitutions per site per year (95% HPD, 0.36×10^{-3} to 0.91×10^{-3}; 8.36 substitutions per genome per year), comparable to previous estimates.[8]

Recovery of a complete HMPV genome from a sample not tested by the diagnostic laboratory

The earliest sample sequenced (sample 1) was from an adult patient with cystic fibrosis (CF). A throat swab was taken following presentation with an exacerbation of respiratory symptoms. This sample tested negative in the clinical diagnostic laboratory by Xpert Xpress Flu/RSV assay (Cepheid, Sunnyvale, CA, USA) for influenza A, influenza B, and respiratory syncytial virus, but was not tested for HMPV. We applied Nanopore metagenomic sequencing to the residual sample, from which 27.5% of the total reads were HMPV, allowing recovery of a complete HMPV genome (Table 1B). Sequencing did not generate reads likely to represent other significant pathogens from this sample.

Longitudinal sampling demonstrates waning of viral load in clinical samples

Two of the patients on the haematology unit were sampled twice, providing the opportunity to determine longitudinal changes in HMPV within the metagenome. One patient was represented by sample numbers 10 and 22, taken on day 6 and day 15 of the haematology outbreak (0 SNPs, Fig. 1). At the time of the index sample, HMPV accounted for 31.8% of total reads generated, with a mean mapping depth of >17,000 (Table 1A). Nine days later, HMPV reads had declined to account for only 2.9% of total reads, with a mean mapping depth of 953. Likewise HMPV reads in samples 14 and 21 from a single patient taken on days 7 and 15, declined from 1.1% to 0.1% of total reads, and from a mean mapping depth of 390 to 34 over an eight day interval (Table 1A). These cases illustrate the potential importance of the timing of sample collection, with steep declines in both proportion of reads and mapping depth over a period of a few days.

Outcomes of HMPV infection

We considered whether we could determine the likely contribution of HMPV infection to morbidity and mortality in this patient group. However, all of the haematology patients from whom our samples derived had malignant disease, and 7/13 (54%) had positive blood cultures (with a diverse range of organisms represented) during the course of the same in-patient episode (shown in Fig. 1). Three deaths occurred, but all in the context of complex multi-system disease and confirmed or probable bacterial infection as well as HMPV. Deeper interrogation of patient records would have been unlikely to refine our understanding further, and was not appropriate on ethical grounds.
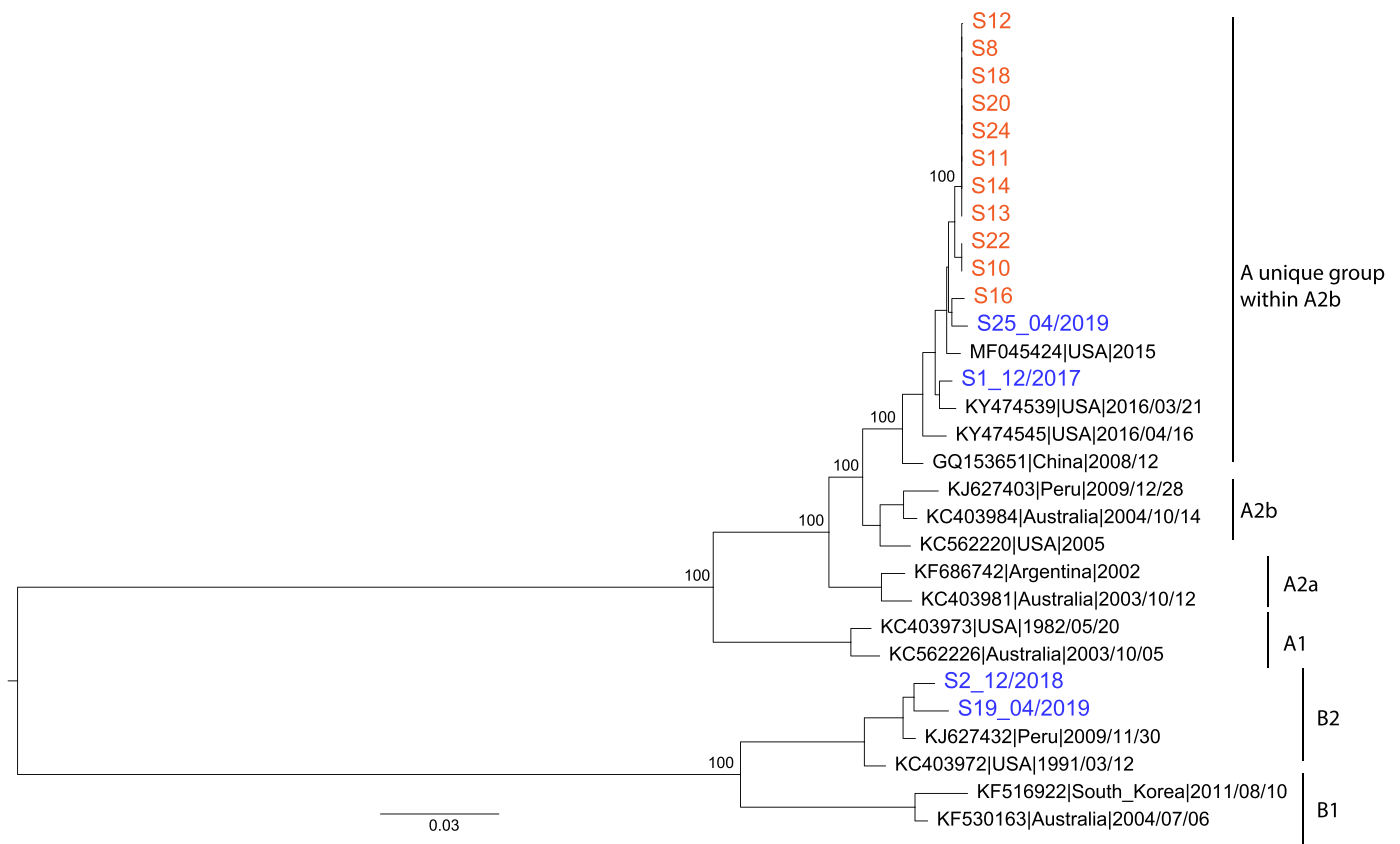
Fig. 3. Maximum-likelihood (ML) phylogenetic trees of HMPV sequences recovered from respiratory samples in a UK teaching hospital. HMPV sequences recovered from samples collected on the haematology ward/outpatients are marked in orange, and samples from other clinical settings in blue. Reference sequences (in black) are selected on the basis of the complete ML tree that includes all 154 published HMPV sequence as shown in Suppl Fig. 1. The five known genetic sublineages of HMPV, A1, A2a, A2b, B1, and B2, are indicated on the right of the tree. Numbers at the nodes indicate bootstrap support evaluated by 500 replicates, only bootstrap values >70 are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Nanopore metagenomic sequencing contributes to the identification and genomic characterisation of other organisms

From the Nanopore metagenomic sequencing data, we recovered one complete genome of rhinovirus A, and two complete genomes of human parainfluenza 3 (Suppl Table 3). We also identified reads from several other viral genomes, including human parainfluenza virus 1, influenza A virus, and influenza B virus; and four bacterial species (*Pseudomonas aeruginosa, Streptococcus pneumoniae, Moraxella catarrhalis*, and *Haemophilus influenzae)* (Suppl Table 3).

Discussion

Diagnosis and full genome sequencing of HMPV infection

In this study, the first to report full length HMPV genomes from the UK, we identified HMPV genomes originating from a unique genetic group within the A2b sublineage, which were associated with nosocomial transmission among haematology patients. This genetic group within the A2b sublineage was only recently identified from phylogenetic analysis of fusion and attachment genes,[12] and comprises sequences generated in East and Southeast Asia between 2006 and 2012 (including Malaysia, Vietnam, Cambodia, China, and Japan),[13] and in Croatia between 2011 and 2014.[14] A previous study of HMPV infections in children suggested that lineage A could be more pathogenic than lineage B.[15] However, we also observed HMPV genomes from two distinct sublineages, A2b and B2, from patients in different locations within the same hospi-

tal during the same time period, highlighting the potential under-ascertainment of this respiratory pathogen.[4] Although generation of consensus sequences for analysis inevitably masks some of the diversity present within quasispecies, our results are in keeping with previously estimated substitution rates for HMPV, and we demonstrate that the method is sufficient to distinguish between distinct viral genetic lineages.

HMPV outbreaks in haematology patients

An outbreak of HMPV A2a has been previously described in 15 patients with haematological malignancies in Germany, in which it was reported to have been associated with four deaths from pneumonia and multi-organ failure.[5] The virulence of HMPV from the unique A2b group, and the extent to which this virus contributes to morbidity or mortality in the haematology patients in our study, is uncertain. However, the recovery of the complete genome and the high proportion of HMPV reads identified from the metagenomic data suggest active infection that could have been completely or partly responsible for clinical syndromes.

In this small retrospective study, it is not possible for us to comment on causation of disease or attributable mortality, as our cohort represents a small group of complex patients with multisystem disease and profound immunocompromise, and we did not collect data about clinical syndromes in real time. Retrospective assessments of clinical records could potentially be biased. Furthermore, we did not collect clinical or laboratory parameters from other patients on the unit at the same time, so do not have denominator data with which to calculate attributable morbid-

ity or mortality. However, our results are a proof of principle in demonstrating the way in which Nanopore metagenomic sequencing could be applied in real time to help identify or monitor outbreaks of infection.

Implications for investigation of a clinical outbreak

Our study demonstrated the applicability of Nanopore metagenomic sequencing to generate complete consensus genomes with accuracy equivalent to Illumina, and provided high resolution investigation of a clinical cluster. Nosocomial transmission of HMPV has previously been reported based on sequencing of the partial genome.[5,16] However, whole genome sequencing showed higher sensitivity and specificity than partial genome sequencing for the identification of a transmission cluster, as exemplified by influenza.[17]

Potential pathways of transmission on the haematology ward include surface contamination of shared equipment, patient-to-patient spread, transmission within outpatient clinics, transmission by healthcare workers or other ward staff, and visitors coming from the community into the unit.[18] Phylogenetic reconstruction allowed us to define eight closely related cases that represent a transmission cluster, and also to differentiate a further two cases in which sequencing suggests independent introduction of infection. Therefore, more than one of these transmission pathways was likely responsible for the clinical outbreak. This inference can only be made on the basis of sequence data, as the standard diagnostic test does not allow differentiation of co-circulating strains and a point-source outbreak may have been the most likely conclusion based on routine clinical testing.

Implications for infection prevention and control

As a result of this outbreak, in which we confirm eight cases of HMPV likely to have been nosocomially acquired, we have reviewed our infection prevention and control policies pertaining to respiratory virus infections. This includes recommendations for the management of patients, staff, visitors and the environment. Patients with confirmed or suspected viral respiratory tract infections should be nursed in negative or neutral pressure side-rooms with the door closed, extra vigilance is required around hand hygiene and cleaning of clinical areas and fomites, staff with symptoms of respiratory infections should take extra precautions and should be excluded from clinical duties if deemed at high risk of transmission, and visitors should not attend the ward if they have any symptoms of respiratory infection. Our full recommendations are available as a public document online (http://nssg.oxford-haematology.org.uk/bmt/clin-man/B-7-0-viral-respiratory-tract-infections.pdf).

Caveats and limitations

We adopted two different approaches to sample collection, firstly focusing on an HMPV cluster in a defined clinical location, and secondly using a small number of HMPV positive samples from other sources in the hospital. Due to the focused approach to clinical testing in our hospital, with comprehensive panels (BioFire® RP2) applied only to samples from individuals with significant immunocompromise, the sequences generated by this study do not represent the wider hospital or community populations. Moreover, we did not take a systematic approach to sequencing all HMPV-positive samples from the diagnostic laboratory, but selected a small number from similar periods to provide a genetic control group for comparison with cases in the haematology cluster.

Our data showed that Nanopore metagenomic sequencing is less sensitive than the current routine clinical diagnostic test, il-

lustrating that for diagnosis, Nanopore sequencing would currently not be the test of choice without further optimization. Such optimizations may include increasing the number of reads consistently generated per sample and implementation of a cost effective way to run each sample on individual flowcell. In previous optimization work, we have demonstrated that there is not a linear relationship between sample number and total read count, making it unlikely that multiplexing is a limiting factor on sample read yield,[19] but in future it is most likely that individual sample will be run on individual, smaller capacity flow cells, such as the Flongle device. We were unable to derive quantification of virus from the routine clinical diagnostic test (BioFire® RP2), which reports only a binary result, and therefore cannot draw any specific conclusions about the limits of detection of Nanopore sequencing. However, longitudinal sampling of two patients in this study indicate a quantitative or semi-quantitative output from the metagenomic data, as yield of HMPV sequences and coverage depth wanes over the time course of a clinical infection.

There are currently insufficient data to inform consistent practice in making the distinction between pathogens, commensal flora and environmental contaminants from metagenomic datasets. This is further complicated by differences between organisms, sample types and the clinical context in which a sample was collected. Recommendations are beginning to emerge,[20,21] but it remains the case that expert interpretation is required on a case-by-case basis based on attributes of the patient and sample, and characteristics of the specific sequencing data (including organism, genome coverage, read depth, and sample diversity). As more such data are generated and shared from within clinical practice, confidence in interpretation will increase.

Conclusions

The utility of metagenomic data is here exemplified by the generation of full length genome for investigating a clinical cluster, and by identification of potentially pathogenic viruses and bacteria simultaneously from individual samples. Our results show the potential for detection of coinfection, supporting clinical management decisions including antimicrobial stewardship, providing insights into the epidemiology and transmission of infection, and informing infection prevention and control interventions. Characterization of the microbiome of patients with complex underlying disease, both during periods of clinical stability and in the setting of lower respiratory tract infections, is valuable in informing clinical and infection control practices.

Declaration of Competing Interest

MC, RV and STP have previously received consumables free of charge from ONT, but not for the study presented here.

Acknowledgments

Ethical statement

These samples were surplus to diagnostic requirements and were sequenced without access to personal identifiers as part of a larger study with Research Ethics Committee approval (17/LO/1420). Samples from outbreak were sequenced as part of infection control activities. Anonymised clinical information pre-linked to laboratory identifiers was obtained through the Infections

in Oxfordshire Research Database which has Research Ethics Committee and Health Research Authority approval as a generic electronic research database (14/SC/1069, ECC5-017(a)/2009).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2020.02.003.

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