Title: A genomic epidemiology study of multidrug-resistant *Escherichia coli, Klebsiella pneumoniae* and *Acinetobacter baumannii* in two intensive care units in Hanoi, Vietnam

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Key Words

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Abstract (298 words)

Background

Vietnam has high rates of antimicrobial resistance (AMR) but limited genomic surveillance, impeding our ability to assess transmission dynamics. This study aimed to use whole genome sequencing (WGS) to examine the transmission of key AMR pathogens in two intensive care units in Hanoi, Vietnam.

Methods

A prospective surveillance study of all adults admitted to two intensive care units (ICUs) at the National Hospital for Tropical Diseases (NHTD) and Bach Mai Hospital (BMH) was conducted between June 2017 and January 2018. Clinical and environmental samples were cultured on selective media, characterised using MALDI TOF MS, and Illumina sequenced. Phylogenies based on the *de novo* assemblies (SPAdes) were constructed using Mafft (PARsnp), Gubbins and RAxML. Resistance genes were detected using Abricate against the NCBI database.

Findings

3,153 *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* isolates from 369 patients were analysed. Phylogenetic analysis revealed predominant lineages within *A. baumannii* (global clone [GC]2, sequence types [ST]2, ST571) and *K. pneumoniae* (ST15, ST16, ST656, ST11, ST147) isolates. Colonisation was most common with *E. coli* (88.9%) followed by *K. pneumoniae* (62.4%). 91% of *E. coli* carried a *bla*CTX-M variant, while 81% of *K. pneumoniae* isolates carried *bla*NDM (54%) and/or *bla*KPC (45%). Transmission analysis using single nucleotide polymorphisms (SNPs) identified 167 clusters involving 251 (68%) patients, in some cases involving patients from both ICUs. There were no significant differences between the lineages or AMR genes recovered between the two ICUs.

Interpretation

This study represents the largest prospective surveillance study of key AMR pathogens in Vietnamese ICUs. Clusters of closely related isolates in patients across both ICUs suggests recent transmission prior to ICU admission in other healthcare settings or in the community.

Funding

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Research in context (269):

Evidence before this study:

Globally, antimicrobial resistance (AMR) is projected to cause 10 million deaths annually by 2050. While 90% of these deaths are expected to occur in African and Asian low- and middle-income countries (LMIC), attributing morbidity and mortality is difficult without the availability of comprehensive AMR data in these settings. Whilst efforts have been made to improve AMR surveillance in these settings, this is often hampered by limited infrastructure, training and financial resources.

Added value of this study:

This is the largest prospective surveillance study of three key AMR pathogens (*E. coli, K. pneumoniae* and *A. baumannii*) conducted in critical care settings in Vietnam. All patients were colonised or infected with one or more extended spectrum beta-lactamase (ESBL) producing and/or carbapenem-resistant organism. Colonisation with more than one organism was very common, with resistant *E. coli* predominantly isolated from stool. A small number of predominant lineages were identified for *K. pneumoniae* and *A. baumannii*, while the *E. coli* isolates were highly genetically diverse. A large number of genomic clusters were identified within the two ICUs, some of which spanned both ICUs. There were no significant differences between lineages or AMR genes between the two ICUs.

Implications of all the available evidence:

This study found high rates of colonisation and infection with three key AMR pathogens in adults admitted to two Vietnamese ICUs. Whilst transmission was common within ICUs the finding of similar lineages and AMR genes in both ICUs suggests that dissemination of AMR occurs prior to ICU admission, from either referral sites or in community settings prior to hospital admission. Strategies to tackle AMR in Vietnam will need to account for this by extending surveillance beyond ICU to hospital and community settings.

Introduction

Low- and middle-income countries (LMICs) have reported widespread antimicrobial resistance (AMR) in healthcare, community and agricultural settings. In South-East Asia, dense human populations, intensive animal farming, unrestricted access to antibiotics and limited laboratory infrastructure have all contributed to the rapid expansion of AMR (*1, 2*).

Much of this burden comes as a result of excessive use of antimicrobials in human and animal populations. In Vietnam, antimicrobial usage has been estimated to be two times higher in humans, and 1.5 times higher in animals, as compared to the European Union (*3*). Despite legal restrictions in Vietnam, antibiotics are often dispensed without prescriptions in the community (*4*). Broad-spectrum antibiotics are also commonly administered in healthcare settings to mitigate the effects of limited capacity for microbiological testing and infection control (*4, 5*). Detection of both resistant bacteria and antimicrobials have been recorded in the environment (*6, 7*), hospital waste (*8*) and food sources (*9, 10*).

Extensive AMR has led to increased pressure on hospitals and is particularly problematic in critical care settings. A study conducted in Thailand between 2008 and 2012 found that almost 80% of nosocomial infections were caused by resistant bacteria, accounting for roughly 100,000 AMR cases and 50,000 deaths annually (*11*). *Klebsiella pneumoniae* is considered a dire threat because of high rates of AMR and virulence (*12*) and widespread resistance to last-line treatments, such as carbapenems and polymyxins (*13*). *Acinetobacter baumannii* is a leading cause of ventilator-associated pneumonia (VAP) in critically ill patients, and is commonly treated with colistin due to widespread carbapenem resistance (*14, 15*).

Whilst AMR surveillance based on phenotypic antimicrobial susceptibility testing in Vietnam has improved in recent years, the infrastructure required for systematic genomic surveillance remains to be established. This is particularly important to determine circulating lineages and elucidate potential transmission events, as other methods do not provide the same level of resolution (*16*). Over the past decade, various studies have demonstrated the utility of WGS in characterising AMR, transmission routes, and dominant lineages (*17-19*). LMICs however remain relatively understudied, with few studies conducted in Vietnamese hospitals (*20-23*). With increasing globalisation, understanding the dynamics of circulating lineages and

136 evolving AMR in these regions is necessary to address both local and global efforts to detect,
137 monitor and manage resistant bacterial infections.
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139 In order to address this knowledge gap, we conducted a prospective genomic surveillance
140 study of key AMR pathogens in two hospitals in Vietnam. We targeted intensive care unit
141 (ICU) patients as we hypothesised that these would be most likely to have been treated with
142 antibiotics and to harbour AMR pathogens. Furthermore, we focussed our analysis on the
143 three most commonly isolated species (*Escherichia coli*, *K. pneumoniae*, and *A. baumannii*)
144 that were extended-spectrum beta-lactamase (ESBL) producers and/or carbapenem-resistant.
145 An additional in-depth analysis focussed primarily on a subset of the *K. pneumoniae* isolates
146 was also performed in a separate project (Pham et al., personal communication).
147
148 **Methods**
149 *Study design, setting and participants*
150 This prospective observational cohort study was conducted in two hospitals, the National
151 Hospital for Tropical Diseases (NHTD) and Bach Mai Hospital (BMH) in Hanoi, Vietnam,
152 between June 2017 to January 2018. All patients (aged 18 years or older) admitted to the
153 adult ICUs of the two hospitals were eligible for inclusion in the study. NHTD is a specialist
154 hospital for infectious and tropical diseases with a 22-bedded ICU which receives up to 400
155 patients per year. BMH is a large tertiary referral hospital, with a 45-bedded ICU that
156 receives up to 1,200 patients per year. Both hospitals are located in the same area of Hanoi
157 but operate independently of each other and do not share laboratory facilities, equipment or
158 staff. Patients are not commonly transferred between the two hospitals.
159
160 *Study procedures*
161 Screening specimens were collected from ICU patients on admission, on discharge and
162 weekly during their ICU stay. Specimens included stool/rectal swabs, urine, skin/wound
163 swabs and sputum/tracheal aspirates. Environmental samples were collected using flocked
164 swabs (from door handles, bed rails, medical equipment and patient tables) on a monthly
165 basis. Clinical data related to the ICU admission were collected from the medical records and
166 entered into a case record form and then into an electronic database. Laboratory data were
167 collected and recorded in an electronic database.
168
169 *Laboratory methods and sequencing*

All patient and environmental specimens were cultured on selective media (CHROMagar™ ESBL, CHROMagar™ mSuperCARBA™, CHROMagar™ VRE, CHROMagar, France). Single colony picks of target organisms (*Escherichia coli*, *Acinetobacter baumannii* and *Klebsiella pneumoniae*) were selected and identified using MALDI-TOF MS (Bruker Diagnostics, Bremen, Germany) and stored at -80 °C. Stored isolates were shipped in two batches to the University of Cambridge, United Kingdom, where they were sub-cultured, re-identified using MALDI-TOF MS, and underwent antimicrobial susceptibility testing (Vitek-2, BioMérieux, Marcy L'Étoile, France). Isolate DNA was extracted using QIACube and the QIAamp 96 DNA QIACube HT kit (Qiagen, Hilden, Germany) prior to shipping to the Wellcome Sanger Institute for sequencing. DNA was sequenced in two batches on an Illumina HiSeq X10 machine (Illumina Inc., San Diego (CA), USA).

Read quality control

Raw Illumina reads were checked for quality using fastQC (v0.11.8) (*24*) and MultiQC (v 1.0.dev0) (*25*). Raw Illumina reads were also checked for contamination using Kraken2 (v2.0.7-beta) (*26*) and Bracken (v2.5) (*27*).

Assembly

Illumina reads were *de novo* assembled using SPAdes (v3.13.1) (*28*) and checked for quality using Quast (v5.0.2) (*29*) and CheckM (v1.0.18) (*30*). Further filtering and quality control methods for all assemblies is provided in the Supplementary Methods.

Phylogenetic construction

Prior to phylogenetic construction, reads were mapped to filtered assemblies and sites that had <90% consensus compared to the reference allele were masked. Masked assemblies were then aligned using PARsnp (v1.2) under default settings (*31*). Core multi-alignments produced using PARsnp were filtered for recombination using Gubbins (v.2.3.5) (*32*). Phylogenies were then constructed using RAxML (GTR-GAMMA model) (v8.2.12) (*33*) as implemented through Gubbins. *E. coli* phylogroups were determined using ClermonTyping (*34*). Trees were visualised using iTol v5.6.2 (*35*). Details on global reference selection are provided in the Supplementary Methods.

Antibiotic resistance gene detection

Resistance genes and plasmid replicons were detected from draft assemblies using Abricate (v1.0.1) (*36*) and the NCBI database (for resistance genes) (*37*)**.** Genes were considered present if there was 90% coverage at 90% nucleotide identity (*38*).

Multi-locus sequence typing (MLST)

Sequence types were determined using mlst (v2.19.0) (https://github.com/tseemann/mlst) and the associated species scheme (specifically the Pasteur scheme for *A. baumannii*) (*39-41*).

Transmission cluster analysis

Transmission clusters were constructed using Transcluster (using the makeSNPClusters method, which ignores time of sampling and uses a pure SNP-distance cut-off) (*42*) using single nucleotide polymorphisms (SNPs) determined after recombination filtering using Gubbins. Transmission cut-offs were evaluated based on intra- and inter-patient SNP diversity within each species phylogeny (Supplementary Figure 1).

Results

Samples included in the study

Between June 2017 to January 2018, a total of 3,367 isolates were cultured, comprising *Escherichia coli* (n=765), *Klebsiella pneumoniae* (n=1,372) and *Acinetobacter baumannii* (n=1,230). Thirty-one isolates were excluded from the analysis because of poor assembly quality. A further 150 isolates were excluded because of suspected inter-species contamination, and 33 isolates were excluded because of suspected intra-species (strain-level) contamination (Supplementary Figure 2). Thus 3,153 isolates (93.6%), comprising 2,901 isolates from 369 patients and 252 environmental isolates, passed quality filtering and were included in the final analyses.

Clinical data

Of the 3,153 isolates, 1,042 (33%) were collected from BMH, while 2,111 (67%) were collected from NHTD (Table 1). Both hospitals recruited a similar number of patients and the average age was 53-55 years (BMH median age 55, NHTD median age 57.5). The average length of stay (LOS) in BMH was 7 days (median 6 days), and 21 days (median 16 days) at NHTD (Supplementary Figure 3). Patient outcomes are summarised in Table 1.

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240 **Table 1: Summary of isolates and patients included in the study**

Variable	Bach Mai Hospital	National Hospital of Tropical Diseases
Isolates:		
Total	1042	2111
Clinical	993 (95%)	1898 (90%)
Environmental	49 (5%)	213 (10%)
Patients:		
Total	182	187
Male	104 (57%)	114 (61%)
Female	71 (39%)	50 (27%)
Gender not recorded	7 (4%)	23 (12%)
Age:		
Male	53 years (range 16-85)	55.2 years (range 5-92)
Female	53.4 years (range 19-91)	55.7 years (range 10-90)
Age not recorded	n=5 (3%)	n=19 (10%)
Length of stay:		
	7.5 days (range 0 – 35)	21 days (range 1 – 75)
Stay not recorded	n=4 (2.2%)	n=7 (3.7%)
Outcome at discharge from ICU		
Stable, discharged home	10 (6%)	38 (20%)
Improved, transferred to another ward	117 (64%)	83 (44%)
Deteriorated, transferred to another ward	3 (2%)	7 (4%)
Discharged home to die	44 (24%)	43 (23%)
Died in hospital	4 (2%)	9 (5%)
Not recorded	4 (2%)	7 (4%)

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All of the 369 patients were colonised or infected with one or more of the three species: *E. coli*, *A. baumannii* and *K. pneumoniae*: 146 patients (40%, 55 at BMH and 91 at NHTD) were colonised or infected with all three species; 133 patients (36%, 66 at BMH and 67 at NHTD) were colonised or infected with two of the three species; and 90 patients (24%, 61 at MNH and 29 at NHTD) only had one species detected.

Both *E. coli* and *K. pneumoniae* were isolated primarily from stool / rectal swabs (627/721 [87.0%] and 822/1316 [62.5%], respectively). *K. pneumoniae* was also isolated from other sites including sputum (325/1316 [24.7%]), urine samples (63/1316 [4.8%]) and skin swabs (17/1316 [1.3%]). In contrast, *A. baumannii* isolates were mostly isolated from sputum (621/1116 [55.6%]), followed by stool / rectal swabs (247/1116 [22.1%]), urine (49/1116 [4.4%]) and skin swabs (36/1116 [3.2%]). *A. baumannii* also accounted for the highest number of environmental isolates (161/1116, [14.4%]), compared to 6.5% (85/1316) for *K. pneumoniae* and 2.2% (16/721) for *E. coli.*

Whole genome sequencing reveals predominant circulating lineages

Phylogenetic trees for each species were constructed to explore lineage diversity within the dataset. The *E. coli* isolates were found to be genomically diverse, with isolates spread over eight phylogroups and 80 sequence types (STs) (Figure 1). The most prevalent ST was ST648 (phylogroup A; 11.8%), followed by ST410 (phylogroup C; 9.7%), ST617 (phylogroup A; 9.2%), ST131 (phylogroup B2; 7.9%) and ST1193 (phylogroup B2; 7.4%). Overall, 33 of the 80 STs only had one representative isolate in this dataset.

In contrast, the *K. pneumoniae* and *A. baumannii* isolates appeared to be centred around specific dominant lineages. More than 80% of the *K. pneumoniae* isolates were from one of five STs, including ST15 (n=34%), ST16 (n=20%), ST656 (n=12%), ST11 (n=11%) and ST147 (n=7%). The majority of *A. baumannii* were global clone (GC)2 (n=832, 74.6%) (*43*) and mainly belonged to ST2 (n=48%) and ST571 (n=24%) (based on the Pasteur scheme). There did not appear to be any relationship between STs and hospitals, with all of the major ST lineages detected in both ICUs.

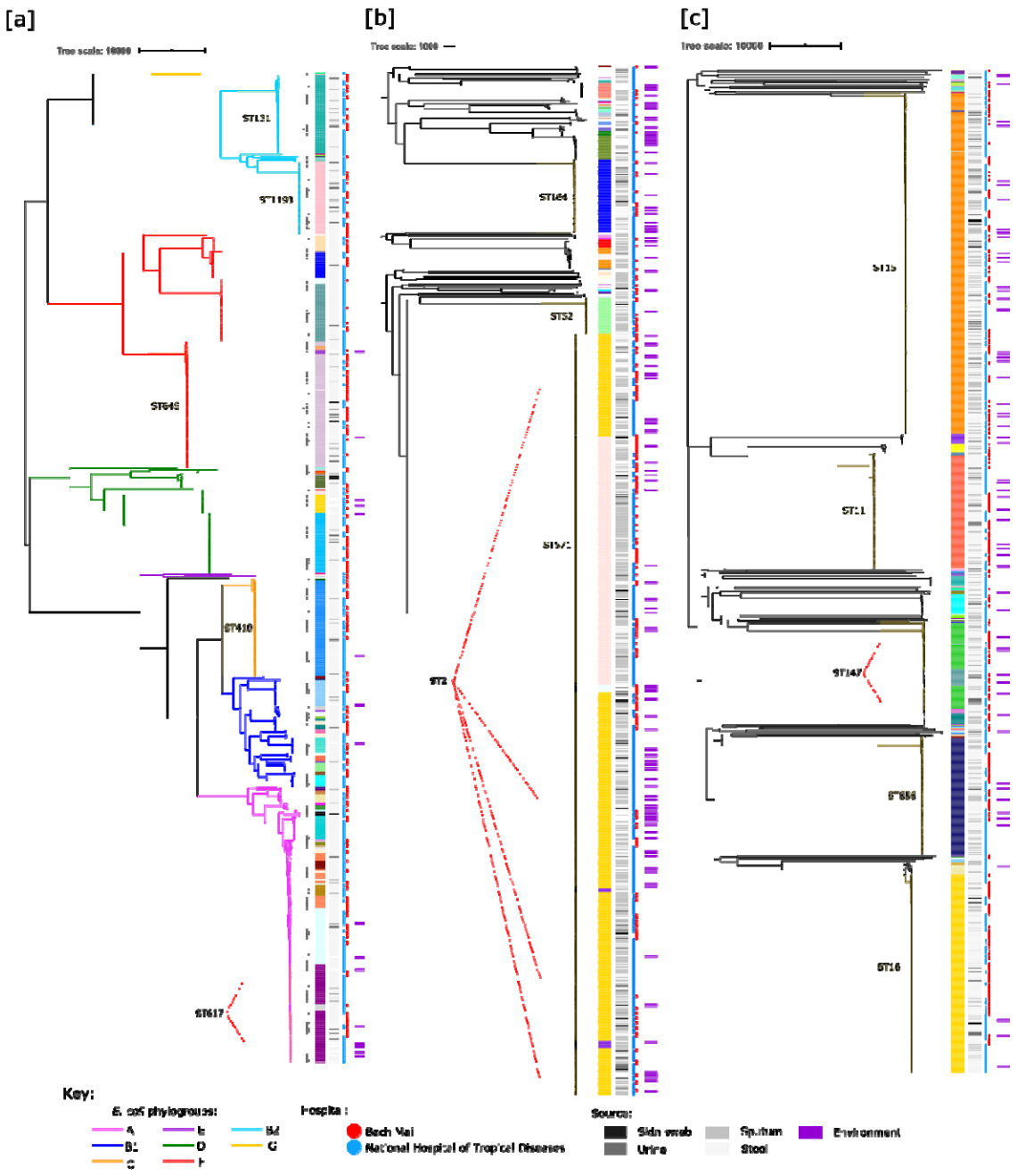

Figure 1: Whole genome phylogenies for [A] *E. coli,* [B] *A. baumannii,* and [C] *K. pneumoniae:* recombination-filtered core-SNP trees with mid-point root. Tree metadata includes (from left to right column beside trees): MLST, source and hospital. Outermost purple bars indicate environmental isolates. Branches corresponding to *E. coli* phylogroups are coloured accordingly. Main STs are highlighted in the image using the pale-yellow boxes.

In order to gain broader insight into the lineages, we selected globally representative strains to contextualise our dataset. Addition of these global representatives into the *E. coli* phylogeny showed that most isolates belonged to a globally diverse set of STs that were not unique to Vietnam, but found across parts of North America, Europe and Asia (Supplementary Figure 4). Similarly, several of the major *K. pneumoniae* lineages were represented globally, particularly ST147, ST11 (mainly from China and the USA) and ST15 (mainly Asian countries) (Supplementary Figure 5). However, it was also clear that local expansion was prominent, particularly among the ST656, ST16 and ST15 lineages. For *A. baumannii*, we focused primarily on GC2 isolates (Supplementary Figure 6). There was very little representation of global strains similar to lineages within our dataset, and those that were available consisted mainly of strains from other parts of Asia.

Closer inspection of the global representatives found several strains in each species that were closely related (<5 core SNPs) to isolates in our dataset (Supplementary Table 1). Most of these global representatives were also isolated in Asian countries. The exception was *E. coli*, where two closely related global representatives were from the United Kingdom and Australia (ST1193 and ST131, respectively). Agricultural isolates were also linked to these lineages, as the other two closely related *E. coli* representatives were originally isolated from poultry (biosample SAMEA104188722) and a farm worker in Vietnam (biosample SAMEA5277968).

High prevalence of antibiotic resistance genes among majority of isolates

Almost all isolates carried acquired resistance genes belonging to at least 3 antibiotic classes, with 90% of *E. coli*, 97% of *K. pneumoniae* and 41% *A. baumannii* carrying genes across 5 antibiotic classes (Figure 2). There were no discernible differences based on sample source or hospital, with the exception of *E. coli* detected in pus/skin swabs (n=6) which appeared on average to carry resistance to more antibiotic classes. *K. pneumoniae* isolates tended to fall into one of three “peaks” (Figure 2). This was due to lineage-specific carriage of acquired resistance genes, where ST15 isolates tended to carry resistance to more classes, compared to ST16 which often carried the least. The other three main lineages (ST656, ST11, ST147) fell between these two peaks. The exception was in the environmental samples, where only two peaks can be seen. This is likely due to very few ST16 isolates detected in the environment.

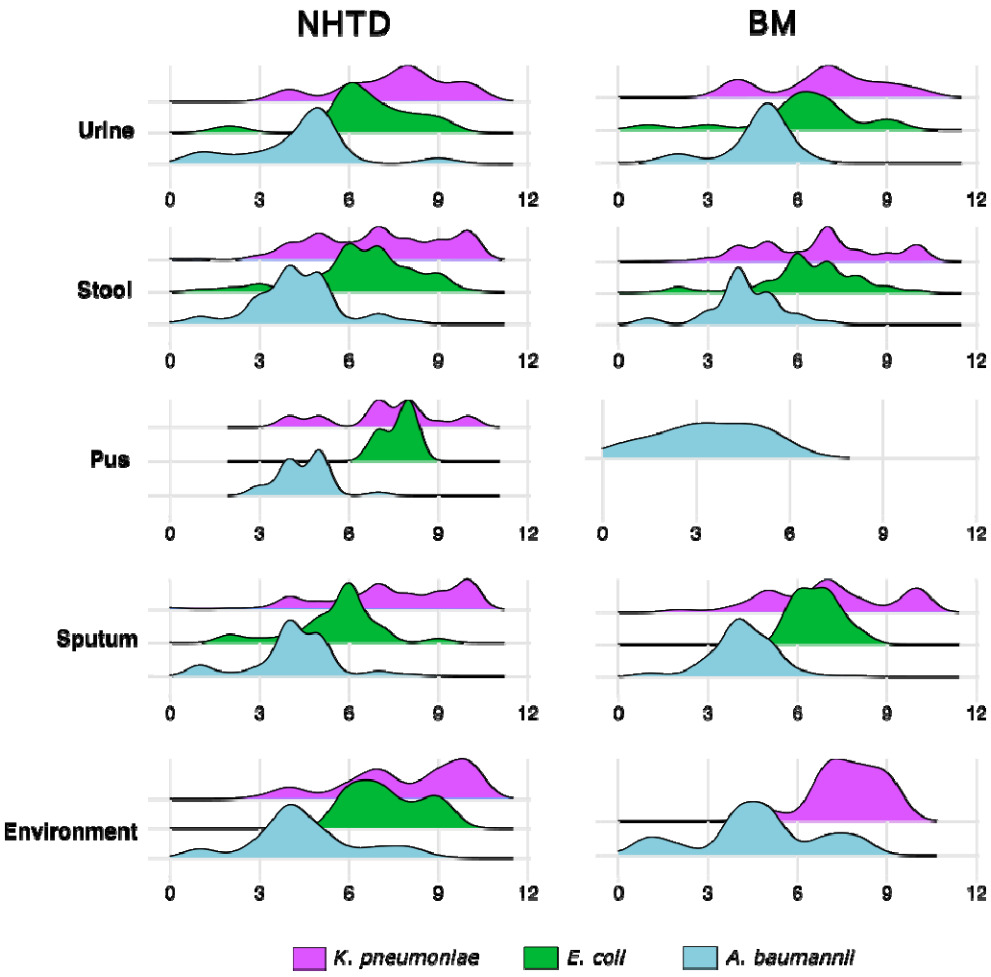

Figure 2: Summary of isolates and the number of antibiotic resistance classes separated by species, hospital and sample type.

Resistance to antibiotics classes varied across the *E. coli* phylogeny, reflective of the diversity of strains within the dataset (Supplementary Figure 7). *bla*CTX-M genes were found in most *E. coli*, with *bla*CTX-M-15 (36%), *bla*CTX-M-27 (30%) and *bla*CTX-M-55 (17%) the most prevalent (Table 2). *bla*KPC-2 (13%) and *bla*NDM-[1,4,5,7] (24%) were present sporadically across the phylogroups, suggesting independent acquisitions events. Only 4% (n=28) of isolates carried *mcr* genes conferring resistance to colistin. Again, these seemed to be independent acquisitions, with the exception of an ST206 cluster (phylogroup A; n=11) involving three patients from NHTD.

Conversely, MDR gene presence across the *K. pneumoniae* isolates appeared consistent with the main lineages, suggesting clonal expansion rather than diverse sampling of the species

(Supplementary figure 8). Similar to the *E. coli*, incidence of *bla*CTX-M-15 (37.5%) was high, but less so overall compared to blaKPC-2 (45%) and *bla*NDM (54%) (NDM-4 [27.9%], NDM-1 [24.7%] and NDM-5 [1.8%]) (Table 2).

Acquired AMR genes were overall less prevalent among the *A. baumannii* isolates. Similar to the *K. pneumoniae*, resistance to specific classes tended to be a feature of each distinct lineage, suggesting clonal expansion (Supplementary Figure 9). The carbapenemase gene *bla*OXA-23 was present in 83% of the dataset, with *bla*OXA-58 and *bla*OXA-72 present at much lower frequencies (5% and 0.2% respectively) (Table 2). The aminoglycoside resistance gene *armA* was also highly prevalent (76%).

Overall, 133 AMR genes were detected in BM and 154 were detected in NHTD. 49 genes were unique to either hospital (35 in NHTD, 14 in BM), but were only detected at a prevalence of less than 0.1%, suggesting sporadic cases. The remaining 129 genes were the same across both hospitals. The genes with the highest prevalence (at least 1%) were found to be almost identical in both hospitals, with the exception of *bla*NDM-4 (0.98%), *dfrA12* (0.92%), *rmtB1* (0.86%), *qnrB6* (0.74%) and *bla*OXA-181 (0.56%) which were below 1% prevalence in NHTD.

In order to determine if certain time points throughout the study had different gene burdens (potentially indicative of mobile genetic element [MGE]-mediated transmission), we plotted gene presence versus date for genes equivalent to 1% prevalence in either hospital (Supplementary Figures 10). Overall, we found that both ICUs had a consistently high burden, making it difficult to distinguish significant gene fluctuations over time. In NHTD, we observed three genes that seemed to peak between November to December 2017. Examination of our dataset for isolates with these three genes (*bla*NDM-4, *bla*OXA-181 and *rmtB1*) revealed a subset of ST16 *K. pneumoniae* that carried all three genes as well as a single ST11 isolate from BM. We plotted the presence of these isolates over time, which mirrored the rise in prevalence in NHTD over the November to December period (Supplementary Figure 11). No *E. coli* nor *A. baumannii* isolates in this dataset carried all three genes.

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370 **Table 2: Summary of resistance genes found in the three species**

Resistance gene class	***Escherichia coli***	***Acinetobacter baumannii***	***Klebsiella pneumoniae***
Tetracycline	563 (78.1%)	701 (62.8%)	753 (57.2%)
Sulphonamide	649 (90%)	746 (66.8%)	969 (73.6%)
Fluoroquinolone	161 (22.3%)	19 (1.7%)	1187[3] (90.2%)
Colistin	28 (3.9%)	0 (0%)	10 (0.8%)
Fosfomycin	48 (6.7%)	5 (0.4%)	1316[3] (100%)
MLS	579 (80%)	816 (73.1%)	623 (47.3%)
Trimethoprim	621 (86.1%)	41 (3.7%)	982 (74.6%)
Phenicols	274 (38%)	176 (15.8%)	675 (51.3%)
Bleomycin	179 (24.8%)	36 (3.2%)	717 (54.5%)
β-lactamase	718 (99.6%)	1018 (91.2%)	1286 (97.7%)
Class C:			
EC	721[1]	2	0
ACT	0	1	0
CMY	209	1	2
DHA	26	2	24
Class A:			
LAP	17	0	142
CARB	0	63	0
PER	0	68	0
TEM	347	697	686
SHV	7	9	1292[3]
VEB	0	12	3
CTX	613	4	681
KPC	94	3	593
Class D:			
OXA	251	996[2]	611
Class B:			
IMP	0	6	1
NDM	173	35	716
Rifamycin	114 (15.8%)	63 (5.6%)	810 (61.6%)
Aminoglycoside	680 (94.3%)	1115 (99.9%)	1290 (98%)
Streptothricin	11 (1.53%)	8 (0.7%)	0 (0%)

371 [1] blaEC intrinsic in *E. coli*

372 [2] blaADC and blaOXA intrinsic in *A. baumannii* (except OXA-[1,10,23,58,72])

[3] fosA (fosfomycin), oqxAB (fluoroquinolone) and blaSHV intrinsic in *K. pneumoniae*

ESBL resistance genes are highlighted in grey and carbapenem resistance genes in yellow

Most patients carried several AMR strains

Over half of the patients in this study had multiple isolates of the same species during their stay (*E. coli*: 51%, *K. pneumoniae*: 57%, *A. baumannii*: 58%). Of these patients, 60-70% had different sequence types (ST) (*E. coli*: 67%, *K. pneumoniae*: 68%, *A. baumannii*: 64%) (Figure 3). For *E. coli* and *K. pneumoniae*, the majority of patients only had isolates detected in stool (80% and 54%, respectively). Conversely, most patients with *A. baumannii* were detected only in sputum (38%), or sputum and stool (32%).

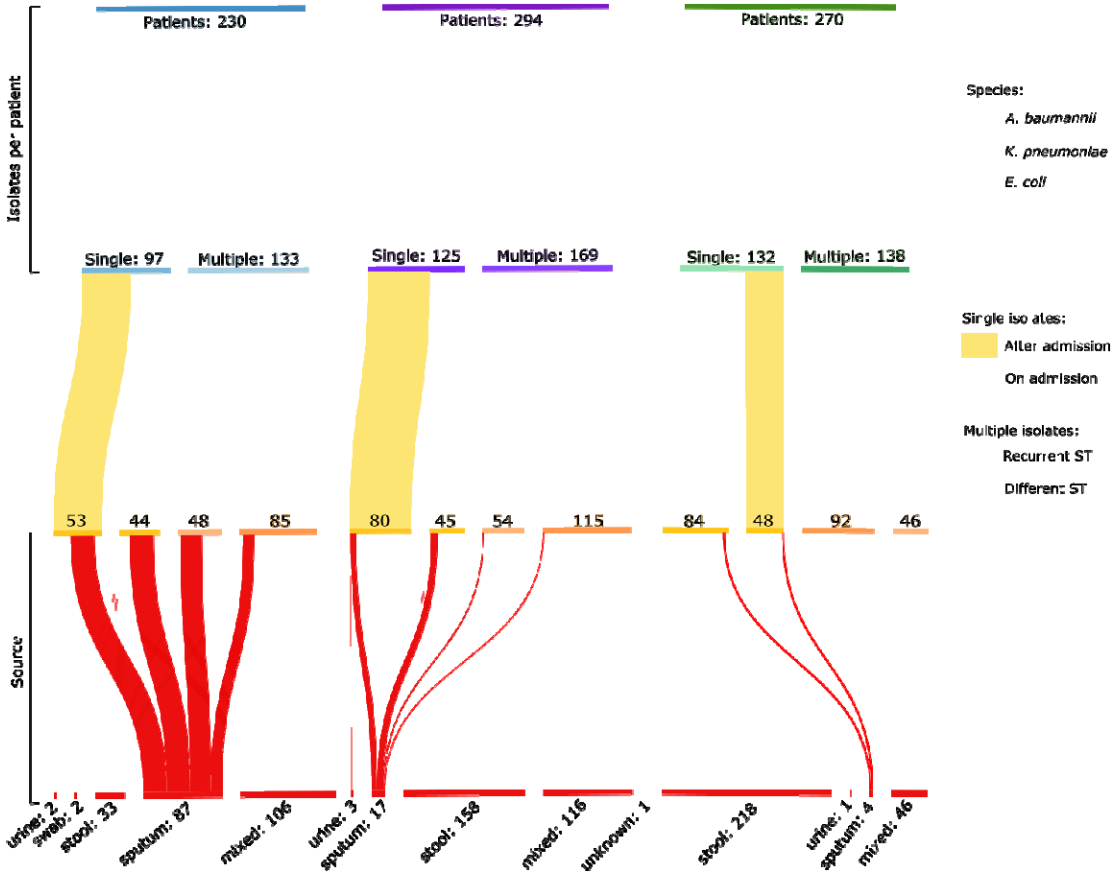

Figure 3: Overview of strain diversity, recurrence and source among study patients: "Patients" refers to the total number of patients in this study that had at least one isolate of that species. Within each species, we evaluated whether patients had (i) only a single isolate for that species, or (ii) multiple isolates. If only a single isolate, we determined whether it was collected on admission to the ICU or after. For multiple isolates, we determined if the patient's isolates were the same ST (recurrent) or a different ST. We finally looked at how

many patients had isolates from one site (urine, swab, stool or sputum) or mixed sites (any combination of sites).

Evidence of extensive transmission between ICU patients

Temporal observation of the isolates found no obvious association of any time point with any ST to suggest an outbreak of a specific lineage. In order to investigate potential clusters within the ICUs at a higher resolution, we examined plausible short-term transmission events using single nucleotide polymorphisms (SNPs).

To identify closely related strains that could indicate recent transmission, we evaluated clusters based on SNP distances across the core genome of each species for this dataset. Given the short sampling period, none of the three major species were likely to acquire more than 1 SNP while in the hospital. As such, we looked at samples with genomic evidence of most recent transmission; zero SNP clusters. Clusters were defined when they involved >1 patient. Clusters involving a single patient and environmental samples were not included.

Most clusters were detected in *K. pneumoniae* and *A. baumannii* isolates, with 71 and 74 clusters representing 38% and 52% of total isolates for that species, respectively (Figure 4). *K. pneumoniae* had some of the largest clusters, ranging in size from 2 to 79 isolates, while *A. baumannii* clusters were smaller, between 2 to 33 isolates. Only 22 clusters were detected in *E. coli* and were generally small (median 3 isolates, range 2 to 9 isolates), representing only 13% of the *E. coli* dataset.

For all three species, the majority of clusters were detected between patients within a single ICU. Evaluating admission and discharge dates further confirmed patient overlap in these clusters (Figure 4, Supplementary Figures 12 to 17).

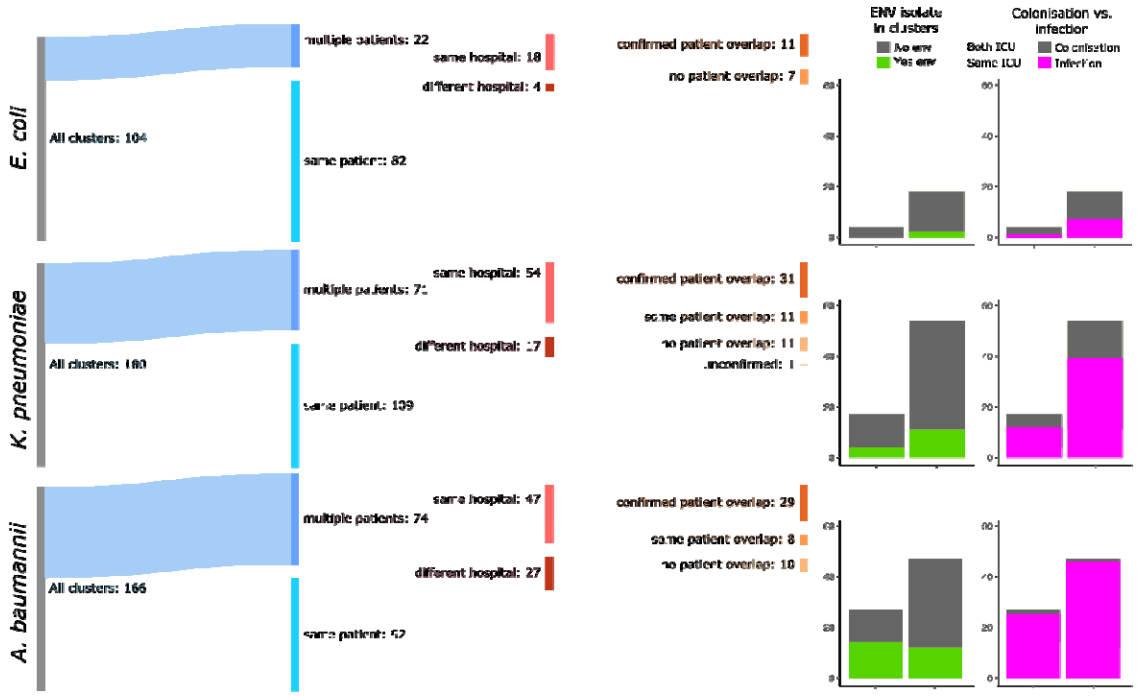

Figure 4: Summary of 0 SNP clusters in all species: Clusters were defined as (i) multiple patients: samples were derived from at least two different patients, or (ii) same patient: isolates were derived from the same patient, or only a single patient and the environment. Epidemiological evidence to support clusters was defined as (i) confirmed patient overlap: all patient ICU stays overlap with another in the same cluster, (ii) some patient overlap: at least 2 patient ICU stays overlap, and (iii) zero patient overlap between all patients in cluster. ENV isolate in clusters: clusters were counted if an environmental isolate was found in that cluster. Colonisation vs. infection: clusters were counted if they (a) had only isolates from stool (i.e. colonisation) or (b) had isolates from urine, swabs and/or sputum with or without isolates from stool (i.e. infection).

A. baumannii clusters were most often associated with environmental isolates (24/74 clusters, Figure 4). The largest *A. baumannii* cluster within the same hospital ICU involved 33 isolates from eight patients and eight environmental samples (Supplementary Figure 13; ST451, cluster number 13). Admissions for patients in this cluster overlapped with detection of the same strain in the environment, which was also detected in the hospital environment several months later. Only two *E. coli* clusters contained related environmental isolates. *K. pneumoniae* environmental isolates were more often found in within-hospital (ICU) clusters (n=11) compared to between-hospital clusters (n=4).

To broadly evaluate infection risk for each outbreak cluster, we determine whether clusters contained (i) stool sample / rectal isolates only, indicating colonisation or (ii) isolates from urine, skin swab or sputum samples, which could represent infection or colonisation at

multiple sites. The majority of *A. baumannii* clusters contained isolates from non-stool / rectal swab samples (Figure 4). Conversely, colonisation-only clusters were common for *E. coli*; the largest *E. coli* cluster involving both hospital ICUs contained only 5 isolates from four patients, which were all isolated from stool (Supplementary Figure 14; ST617, cluster number 37). *K. pneumoniae* clusters were a mix, with both colonisation-only clusters and infection clusters.

In addition to suspected within-ICU transmission, we also detected a number of clusters involving patients from both hospital ICUs (Figure 4). The most pronounced example of this was a large ST15 *K. pneumoniae* cluster involving 79 isolates from 38 patients and 6 environmental samples (Supplementary Figure 18). Most were collected between July to September, with some late occurrences in October and November. All patients from NHTD between July to September had overlapping timelines, consistent with spread within the ICU (Supplementary figure 16; ST15, cluster number 15). Only one patient had no evidence of overlap (ND162) but did cluster with environmental isolates from the same timeframe, indicating a possible environmental source. Patients from BM appeared to have both patient overlap and consecutive acquisitions without patient overlap as time progressed. This suggests that patient transmission and also transmission via other routes (e.g. inadequate cleaning before the next patient, transmission via healthcare workers) may have been important factors in the spread of this strain.

The identification of closely related isolates between independently operating ICUs suggested that there may have been a common source located outside the ICU e.g admission to the same location prior to admission to ICU. To determine if certain lineages were more associated with acquisition within the ICU, we assessed diversity on arrival (i.e. the patients first sample) versus diversity within the ICU (all other samples). Based on ST alone, we found a slight increase in diversity in the ICU versus on arrival (Supplementary Figure 19). However, the unique STs recovered in either setting only represented a small portion of the isolates overall. All of the main lineages for each species were found on both admission and within the ICU (Supplementary Figure 19 and 20).

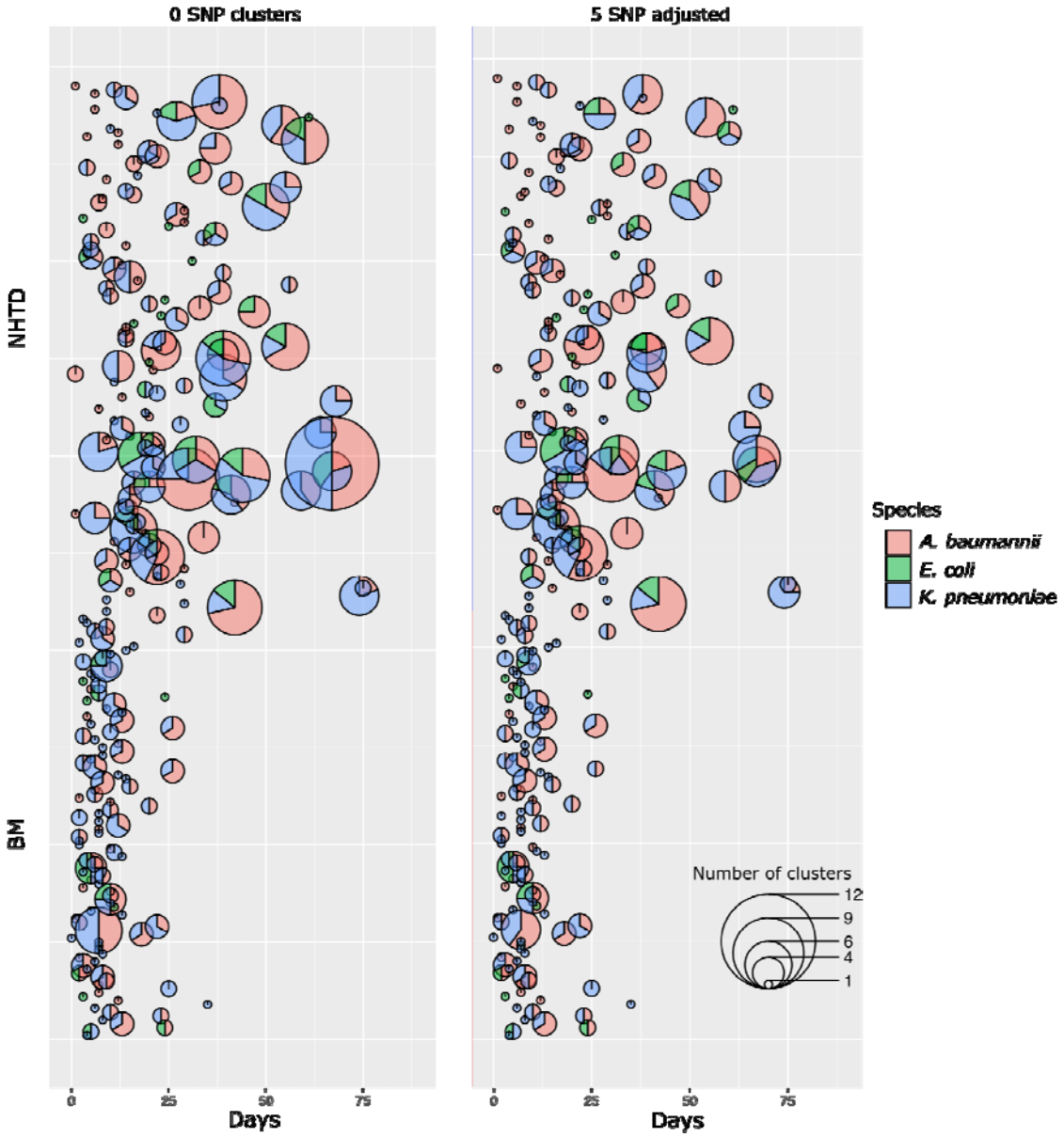

Figure 5: Scatterpie showing the number of clusters in patients across all species: y-axis represents patients from BM or NHTD involved in at least one 0 SNP cluster. X-axis represents length of stay for that patient; one pie is plotted per patient at the duration of their stay. Each circle represents 0 SNP clusters in a single patient. The size of the clusters corresponds to the number of clusters, while the colour relates to the species. The left plot shows all 0 SNP clusters, while the right plot shows clusters condensed at 5 SNPs.

Identification of multiple transmission clusters per patients

Overall, there were 251 patients (representing 68% of the cohort) involved in 167 clusters across the three species collected over the course of this study. 112 patients were only involved in a single cluster during their time in the ICU (Figure 5). However, the remaining 139 patients were involved in at least two clusters, with one patient involved in 12 clusters detected at 0 SNPs. For patients with at least two clusters, 20 had clusters from all three species, 94 had clusters from two species and 25 had only one species. Overall, we saw a general trend towards more clusters in a single patient as they spent more time in the ICU ward.

To determine if our clusters were potentially derived from a single original cluster predating their time in the ICU, we looked at SNP distances between clusters of the same ST (Supplementary Figure 21). At a threshold of five SNPs, several of the prominent STs within each species formed large clusters, including ST804 in *A. baumannii* and ST16 in *K. pneumoniae*. At this threshold, we found 29 clusters in the *A. baumannii* dataset (originally 74), 23 clusters in the *K. pneumoniae* (originally 71) and 19 clusters in *E. coli* (originally 22). By readjusting our analysis per patient using these clusters, we found that 123 patients had only a single cluster during their stay (Figure 5). 128 patients had 2 clusters, with the maximum number of clusters in a single patient being 7 (n=3 patients).

Discussion

Here we present a large prospective surveillance study of three key AMR pathogens from two hospital ICUs in Vietnam. We used WGS to capture a high-resolution snapshot of the dominant circulating lineages over a six-month period. In this study we focused on *E. coli*, *K. pneumoniae*, and *A. baumannii*, as these were the most commonly isolated species from both ICUs. These three species have also been reported as highly prevalent in other Vietnamese studies (*44*), and are amongst the most clinically significant Gram-negative bacteria, having been designated as “critical” priority pathogens for research and development of new antibiotics by the World Health Organisation (*45*).

Phylogenetic analysis of all three species suggested local dominance of specific lineages for *K. pneumoniae* and *A. baumannii*. Comparison to global representatives found few closely linked strains, with an overall preference for local clustering. This was particularly evident

for *K. pneumoniae* ST15, ST656, ST11 and ST16 and many of the GC2 isolates for *A. baumannii*. One notable exception was from a study conducted in southern Vietnam (*46*), where a number of GC2 *A. baumannii* strains were found to be closely related to ST571 isolates from this study. Similar studies in other Vietnamese hospital settings have also identified *K. pneumoniae* isolates corresponding to ST15 (*23*), ST16 (*47*) and ST11 (*48*) with similar antimicrobial resistance profiles carrying *bla*KPC and *bla*NDM. This suggests that these lineages may not be restricted to referral hospitals in Hanoi but may potentially occur throughout Vietnam. In contrast, analysis of the study *E. coli* isolates and comparison with global references did not identify evidence for locally dominant lineages, instead showing large dispersal of global strains throughout the phylogeny.

Despite limiting our study samples to ESBL-producing and/or carbapenem-resistant isolates belonging to three species, we identified a large number of isolates with an average of 16.55 and 9.68 isolates/day from patients in BMH and NHTD, respectively. This is an exceedingly high number compared to other countries, such as the United Kingdom (UK), where a similar study only found 199 ESBL-producing Enterobacteriaceae over the course of one year (0.5 isolates/day) from three hospital sites between 2008 to 2009 (*49*). A point-prevalence survey conducted in a UK hospital in 2017 also identified no positive carbapenemase-producing Enterobacteriaceae (CPE) from 540 samples (*50*).

Nearly all of the isolates presented in this study were characterised as multi-drug resistant (MDR) on the basis of resistance to three or more antimicrobial drug classes (*51*). *K. pneumoniae* was found to be particularly resistant, with a number of isolates carrying genes to more than nine antibiotic classes. The only drug to which we did not observe extensive resistance was *mcr* gene-mediated colistin resistance, which has been reported in animal farming and agriculture in Vietnam (*52-55*). It is possible that the *mcr* gene has not yet become disseminated to more urban areas of Vietnam, despite the use of colistin in clinical settings. A limited number of isolates displayed mutational changes related to colistin resistance, such as interruption of the *mgrB* gene in *K. pneumoniae* (*56*). We did not further investigate the role of mutational colistin resistance as we did not have the capacity to corroborate genotypic resistance with phenotypic measurements in this study.

Colonisation was found to be a large reservoir for AMR, with the majority of *E. coli* and more than half of the *K. pneumoniae* isolated from stool samples, as has been documented

previously in Vietnamese hospitals (*57*). High community usage of antibiotics in Vietnam is likely to promote colonisation with AMR bacteria, as prior treatment with antibiotics is known to lead to colonisation (*58*). Colonisation itself has been identified as a risk factor for subsequent infection (*59-61*), and has been previously documented in ICU patients in southern Vietnam (*62*). Colonisation with AMR *E. coli* has also been identified as a risk for transferral of resistance to other colonising pathogens, such as *Shigella* (*63*).

Infection control is critically important for reducing the risk of hospital-acquired infections (HAIs) and mortality in ICUs. Here we found evidence for numerous recent transmission events involving multiple patients, where *A. baumannii* and *K. pneumoniae* were more often found in transmission clusters. *A. baumannii* is particularly problematic to control in hospital environments owing to its ability to resist desiccation and cleaning (*64*) and to survive for long periods of time on surfaces (*65, 66*). *K. pneumoniae* is also commonly responsible for outbreaks in healthcare settings globally (*67-69*), and requires immediate and appropriate intervention due to its propensity to be highly resistant and virulent (*70*). Detection and prevention of AMR transmission in LMICs is difficult for a number of reasons. These include limited capacity for microbiological testing, overcrowding of ICUs, inability to isolate ICU patients, and inadequate staff training or knowledge of infection control procedures (*71, 72*). The high level of AMR in this study would have made it difficult to discern specific clusters using phenotypic methods alone. WGS enables accurate investigation of transmission events but its use is limited by lack of infrastructure, expertise and high cost, particularly in resource-constrained settings.

In addition to evidence for recent transmission between patients on the same ICU, we also identified clusters involving patients from both hospital ICUs. This result was unexpected, as there was no direct transfer of patients between the two ICUs. The most likely explanation for these clusters is a source outside of the ICUs, such as other wards or other hospitals which may have referred patients to ICU. Another possibility is that AMR strains may have been acquired in the community, reflecting high rates antibiotic usage in the community and AMR detection in livestock and food. Based on the similarity between lineages and AMR across both ICUs, we suggest that transmission, particularly of the predominant *A. baumannii* and *K. pneumoniae* lineages, is likely already circulating outside of the ICUs, where it is then further propagated.

We acknowledge some limitations to our study. First, contamination of some of the isolates prior to WGS necessitated additional filtering steps for the majority of samples. Secondly, we did not extensively explore plasmid profiles amongst the samples because of the limitations of short read sequence data. Thirdly, although serial samples were collected and cultured, we selected single colony picks for sequencing. It is therefore possible that some diversity may have been lost at different timepoints during the study sampling period. Finally, this study focused on patient and environmental samples only. We were therefore unable to investigate potential transmission events involving hospital staff and/or visitors.

Nevertheless, we present the largest prospective surveillance study of multidrug-resistant *E. coli*, *A. baumannii* and *K. pneumoniae* in Vietnamese critical care patients to date. The extensive transmission and AMR detected within and between ICU wards suggests dominant circulating lineages of *A. baumannii* and *K. pneumoniae* existing both within hospitals, and potentially in community settings in Vietnam. Further work should be conducted to expand genomic surveillance in hospital and community settings to determine the levels of AMR and prominent lineages in order to inform AMR control strategies in Vietnam.

Data sharing

Genome sequence data have been deposited in the European Nucleotide Archive (ENA) under the Bioproject PRJEB29424. A list of the sample accession numbers is available in Supplementary Dataset 1. Isolate genome assemblies (heterogenous sites masked and unmasked) are available on Figshare under the following DOI: 10.6084/m9.figshare.13303253, 10.6084/m9.figshare.13302728.

Ethical statement

The study protocol was approved by the Scientific and Ethical Committees of the National Hospital for Tropical Diseases and Bach Mai Hospital and by the University of Cambridge Human Biology and Research Ethics Committee (reference: HBREC 2017.09). Written informed consent was obtained from the patient or from their relative prior to enrolment in the study.

Declaration of interests

All authors have no conflicts of interest to declare.

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