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Antimicrobial resistance in the environment of an Australian veterinary referral hospital

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Abstract

The fight against antimicrobial resistance (AMR) is a crucial element of the “One Health” concept. The presence of AMR in veterinary health care facilities presents a significant risk to animal and human health. The spread of AMR can be facilitated in such settings by the frequent interactions between animals, humans and their shared environment. This environment is an important reservoir that can be involved in the transmission of antimicrobial-resistant bacteria between animals and humans. Practice-specific environmental surveillance is likely to aid immediate identification of AMR risks and assist in implementation of efficient infection control strategies. Application of novel molecular methods to explore environmental resistomes can provide valuable information but requires protocols that are reliable and practical for routine use in clinical settings. Exploration of environmental resistomes and genetic characterisation of antimicrobial-resistant environmental bacteria in clinical settings can provide useful insights into the local epidemiology of AMR in veterinary practices. It is also likely that investigations in one clinical setting may guide the development of better control measures in other settings.

This study identified potential infectious risks associated with antimicrobial resistance in a large veterinary teaching hospital and improved the infection control practices in the setting. This was achieved using metagenomic sequencing, whole genome sequencing and *de novo* hybrid genome assembly methods.

First, the applicability of the rapid and portable Oxford Nanopore MinION DNA sequencing technology to exploration of clinically relevant environmental resistomes was evaluated. The workflow developed was then used to identify, quantify and assess the potential clinical risks associated with antimicrobial resistance genes (ARGs) from different environmental sites of the veterinary hospital. ARGs detected in the veterinary hospital environment could

potentially confer resistance to antimicrobials commonly used in companion animal therapy, including aminoglycosides, beta-lactams, cephalosporins, fluoroquinolones, sulphonamides, macrolides and tetracyclines. Phenotypic methods confirmed the expression of some genotypic resistance patterns indicating their activity in the veterinary hospital environment. ARGs constituting a potential clinical risk, specifically ARGs located in the genome sequences of pathogenic bacterial species or on mobile genetic elements, were found to be shared between interconnected environmental sites and possible circulation pathways of these genes within the hospital were investigated. The relative abundances and the extent of co-localisation of the high-risk ARGs were higher in waste collection points than in the immediate environment of the animals. Therefore, the waste collection points in the premises were identified as amplifying reservoirs for clinically important ARGs. The biosecurity practices of the veterinary hospital were improved based on the findings of this work. This exemplifies the applicability of rapid DNA sequencing to efficient exploration of environmental resistomes in veterinary hospitals. Furthermore, knowledge about environmental resistomes was proven to be useful in implementing evidence-based infection control measures.

Secondly, repeated isolates of extended-spectrum-beta-lactamase (ESBL) producing organisms from the same environmental site of the veterinary hospital were phenotypically and genotypically characterised. The genomes were compared and their phenotypic resistance to antimicrobials, their resistance determinants (ARGs), and the genetic context of these resistance determinants and their capacity for horizontal transfer were evaluated. A strain of multiple drug-resistant *Enterobacter hormaechei* was detected in the hand washing sink of the veterinary hospital's intensive care unit (ICU) for approximately one month. This organism carried a large IncH12 conjugative plasmid with novel genetic rearrangements that resulted in two multidrug resistance loci containing genes conferring resistance to nine

classes of antimicrobials. The antimicrobial resistance gene clusters were associated with class 1 integrons and *IS26*, *IS903* and *ISCR* transposable elements. The ESBL resistance gene *blaSHV-12*, acquired fluoroquinolone resistance gene *qnrB2* and colistin resistance gene *mcr-9.1* were co-located in the same multidrug resistance locus, which was flanked by two *IS903* elements. This genetic structure is particularly concerning because of its putative ability to co-transfer multiple resistance determinants into other animal pathogens in the hospital environment. Thorough disinfection of the ICU eventually resulted in the elimination of the organism from the hand washing sink. However, the persistence of such organisms in the environment can lead to further accumulation and spread of AMR, necessitating targeted routine environmental surveillance.

Overall, findings of the work presented in this thesis contributed to the progress of antimicrobial stewardship and the mitigation of potential AMR risks in one large veterinary hospital, and identified sites of risk that should be examined in other veterinary hospitals.

Declaration

The experiments in this thesis constitute work carried out by me in the Faculty of Veterinary and Agricultural Sciences in the University of Melbourne unless otherwise stated. Due acknowledgement has been made in the text to all other material used. The thesis is less than 50,000 words in length, exclusive of tables, figures, bibliography and appendices, and complies with the stipulations set out for the degree of Master of Philosophy by the University of Melbourne.

Kanishka Kamathewatta

March 2021

Preface

All experimental work, data analysis and writing carried out for this Master of Philosophy was undertaken by Kanishka Kamathewatta, aside from the following cases. Ribosomal multilocus sequence typing, phylogenetic analysis and minimum inhibitory concentration testing were performed by supervisory author Dr. Marc Marenda. The conjugation experiment was performed by collaborating author Ms. Fannana Rafa.

Peer reviewed papers

Chapter 2 was published in the journal PLOS ONE on 30/05/2019. The citation and contributions of each author are listed below. Kanishka Kamathewatta contributed greater than 50% of the work.

Kamathewatta, K.I., Bushell, R.N., Young, N.D., Stevenson, M.A., Billman-Jacobe, H., Browning, G.F. and Marenda, M.S., 2019. Exploration of antibiotic resistance risks in a veterinary teaching hospital with Oxford Nanopore long read sequencing. PLOS ONE 14(5): e0217600. https://doi.org/10.1371/journal.pone.0217600

KIK developed the experimental design, collected and processed samples, conducted experiments and analyses, and wrote the manuscript. RNB provided resources and reviewed the manuscript. NDY conducted analyses and reviewed the manuscript. MAS conducted analyses and reviewed the manuscript. HBJ developed the experimental design and reviewed the manuscript. GFB developed the experimental design and reviewed the manuscript. MSM conceptualised and developed the experimental design, revised and reviewed the manuscript.

Chapter 3 was published in the journal Antimicrobial Resistance and Infection Control on 21/10/2020. The citation and contributions of each author are listed below. Kanishka Kamathewatta contributed greater than 50% of the work.

Kamathewatta, K.I., Bushell, R.N., Rafa, F., Browning, G.F., Billman-Jacobe, H., and Marenda M.S., 2020. Colonization of a hand washing sink in a veterinary hospital by an *Enterobacter hormaechei* strain carrying multiple resistances to high importance antimicrobials. Antimicrobial Resistance and Infection Control 9:163. https://doi.org/10.1186/s13756-020-00828-0

KIK developed the experimental design, conducted experiments and analyses, and wrote the manuscript. RNB processed samples, isolated bacteria and reviewed the manuscript. FR conducted conjugation experiment and reviewed the manuscript. GFB developed the experimental design and reviewed the manuscript. HBJ developed the experimental design and reviewed the manuscript. MSM conceptualised and developed the experimental design, conducted analyses, revised and reviewed the manuscript.

Conference presentations

Kamathewatta, K.I., Bushell, R.N., Young, N.D., Stevenson, M.A., Billman-Jacobe, H., Browning, G.F. and Marenda, M.S., 2018. Resistomes and associated risks in environmental sites of a veterinary teaching hospital intensive care unit. Australian Veterinary Antimicrobial Stewardship Conference, Sunshine Coast, Qeensland, Australia, 11- 13 November. Oral presentation.

Kamathewatta, K.I., Bushell, R.N., Young, N.D., Stevenson, M.A., Billman-Jacobe, H., Browning, G.F. and Marenda, M.S., 2018. Evaluation of Oxford Nanopore long read sequencing to explore antimicrobial resistance risks in a veterinary teaching hospital. MedVetPATHOGENS, 5th Prato Conference on Animal Bacterial Pathogens, Prato, Italy, 8-11 October. Oral and poster presentations.

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The dream to pursue a research higher degree at the University of Melbourne would not have become a reality without the financial assistance from the Melbourne Research Scholarship

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I would like to thank all my school and university teachers, particularly Dr. Rasika Jinadasa, for instilling the passion for microbiology in me, providing the opportunity to work in the field and for encouraging me to pursue a research higher degree.

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Table of Contents

Abstract II
Declaration V
Preface VI
Acknowledgements VIII
Abbreviations XI
List of Figures XIII
List of Tables XIV
Chapter 1: Literature Review 2
1. Introduction 2
2. An overview of antimicrobial resistance 2
3. AMR in hospital environments 16
4. Methods for exploring AMR in microbial ecosystems 29
5. Research aims 36
6. References 37

Chapter 2: Exploration of antibiotic resistance risks in a veterinary teaching hospital with Oxford Nanopore long read sequencing 61
1. Introduction 61
2. Publication 62
3. Conclusion 84
4. Appendix 85

Chapter 3: Colonization of a hand washing sink in a veterinary hospital by an *Enterobacter hormaechei* strain carrying multiple resistances to high importance antimicrobials 93
1. Introduction 93
2. Publication 93
3. Appendix 110

Chapter 4: General Discussion 118
References 125

Abbreviations

AMR	antimicrobial resistance
ANI	average nucleotide identity
ARG	antimicrobial resistance gene
BPW	buffered peptone water
CAT	chloramphenicol acetyltransferase
ECOFF	epidemiological cut-off
ESBL	Extended-spectrum-beta-lactamase
FFPE	formalin-fixed paraffin-embedded
HGT	horizontal gene transfer
ICC	infection control committee
ICE	integrative conjugative element
ICO	infection control officer
ICU	intensive care unit
IR	inverted repeat
IS	insertion sequence
LT	laundry trolley
MB	mop bucket
MDR	multiple drug resistance
MGE	mobile genetic element
MH	Mueller Hinton
MIC	minimum inhibitory concentration
MLST	multi-locus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSP	Methicillin-resistant *Staphylococcus pseudintermedius*
NGS	next generation sequencing
OC	office corridor
ONT	Oxford Nanopore Technologies
oriT	origin of transfer
OUT	operational taxonomic unit

PBP	penicillin binding protein
Pc	promoter
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
qPCR	quantitative polymerase chain reaction
rMLST	ribosomal multi-locus sequence typing
SNP	single nucleotide polymorphism
SOP	standard operation procedures
SSI	skin and soft tissue infection
T4CP	type IV coupling protein
T4SS	type IV secretion system
Tn	transposon
TSB	tryptic soy broth
UTI	urinary tract infection
WHO	World Health Organization
XDR	extensively drug resistant

List of Figures

Chapter 2

Fig 1. Comparison of WaferGen ARG $2^{-\Delta CT}$ values with MinION ARG counts (hits) with reference to the 16S rRNA gene **P70**

Fig 2. Abundance of bacterial reads carrying ARGs relative to their taxonomic origin in the ICU cages (ICU), Laundry Trolley (LT), Mop Bucket (MB) and Office Corridor (OC) **P71**

Fig 3. Venn diagrams showing the distribution of shared ARGs in the ICU cages (ICU), Laundry Trolley (LT), Mop Bucket (MB) and the Office Corridor (OC) samples **P76**

Fig 4. Relative abundances of high-risk genes shared between hospital sites **P76**

Fig 5. Microbial composition at family level in ICU cages (ICU), Laundry Trolley (LT), Mop Bucket (MB) and Office Corridor (OC) samples **P77**

Fig 6. Co-occurrence of ARGs and MGEs in nanopore reads (top) and Illumina contigs (bottom) **P78**

Chapter 3

Fig 1. Phylogenetic analysis of 136 *Enterobacter* sp. genomes from RefSeq and the two sink isolates CM18-216 and CM18-242-2 **P99**

Fig 2. Genetic map of the plasmid pCM18-216 **P101**

Fig 3. Schematic representation of the genetic context of the *IS26* transposable elements in pCM18-216 and comparison with related plasmids pEC-IMPQ (NC_012556.1) and pIMP4- SEM1 (KX810825.1) **P103**

Fig 4. Comparative analysis of pCM18-216 against 269 complete plasmid sequences from *Enterobacteriaceae* **P104**

Fig 5. Systematic comparative alignments of pCM18-216 and *qnrB2*-carrying IncH12 plasmids from *Enterobacteriaceae* **P106**

List of Tables

Chapter 1

Table 1. Examples of widespread dissemination of AMR genes on plasmids **P12**

Chapter 2

Table 1. Input DNA amounts versus sequencing yields **P68**

Table 2. Percentages of reads assigned at different taxonomic levels with four classification methods **P69**

Table 3. Antimicrobial resistance genes associated with mobile genetic elements in the veterinary hospital **P72**

Table 4. Comparison of phenotypic and genotypic detection of antimicrobial resistance in the veterinary hospital **P75**

Table 5. Sequences carrying multiple antimicrobial resistance genes in the veterinary hospital **P77**

Chapter 3

Table 1. Nucleotide differences identified between the chromosomes of isolates CM18-216 and CM18-242-2 **P97**

Table 2. Nucleotide differences identified between the plasmids pCM18-216 and pCM18-242- 2 **P98**

Table 3. ANI analysis of CM18-216, *E. cloacae* and *E. hormaechei* type strains **P99**

Table 4. Antimicrobial resistance genes in *Enterobacter* CM18-216 **P100**

Table 5. MIC of *Enterobacter* sink isolates, DH5 alpha transconjugant (TG) and parental recipient strain used in mating experiments **P102**

Table 6. IncH12 plasmids carrying *qnrB2* selected for comparative analysis **P105**

Chapter 1: Literature Review

1. Introduction

Antimicrobial resistance (AMR) is a global health concern because it limits therapeutic options for life-threatening infections. It is a complex problem because of the involvement of multiple resistance mechanisms, continuous bacterial evolution and the capacity for spread of resistance genes between different bacteria. The early detection of AMR in veterinary health-care settings is important to prevent its spread across animal patients, humans and the environment. Environmental sites in these facilities can act as reservoirs of resistance, and environmental monitoring for the presence of resistant bacteria and their resistance determinants provides useful insights that can improve infection control and prevention strategies. The first section of this review provides an overview of different resistance mechanisms and the genetic basis of AMR. The second part reviews the presence of antimicrobial-resistant nosocomial pathogens and antimicrobial resistance determinants in hospital environments, with a veterinary focus. Finally, the literature on different methods used to explore AMR in complex microbial eco- systems is analysed to identify an efficient methodology for the study of environmental resistomes in veterinary hospitals.

2. An overview of antimicrobial resistance

2.1. Definitions of antimicrobial resistance

AMR can be defined differently depending on the circumstances that are being considered [1]. Generally, a strain is classified as resistant if it grows in the presence of higher concentrations of the drug than phylogenetically related strains [2]. A strain is defined as clinically resistant when it survives antimicrobial therapy [2]. Clinical breakpoints are derived based on the pharmacokinetics of an antimicrobial in target animal species and the distribution of minimum inhibitory concentrations (MIC) in the pathogen of interest. This definition is

useful when determining the most effective antimicrobials to use to treat an animal (including humans). It is only applicable for animal-associated bacteria and for antimicrobial compounds that have established MIC breakpoints [1]. An organism is defined as having a reduced susceptibility when its MIC value for an antimicrobial is higher than the epidemiological cut-off value for resistance (ECOFFs) in that species [1]. ECOFF breakpoints are determined by plotting the MIC values of a large number of isolates of a species and correspond to the upper MIC limit of the wild-type population [1]. Use of ECOFF-based definition is not restricted to the analysis of specific microorganisms and antimicrobials as is the case with clinical resistance. It also has the advantage of defining low level resistances that may be overlooked by using the clinical MIC breakpoints. However, most environmental bacteria cannot be analysed using this definition because of the lack of appropriate databases for comparisons [1]. An alternative operational definition that has been proposed is gene-centric and based on comparison of a strain that has acquired a mutation or an exogenous resistance gene with the parental strain from which it was derived. Under this definition a strain is considered resistant when its MIC for a specific antimicrobial drug is higher than that of its parental strain [1]. Although this definition cannot be used to determine the susceptibility of a naturally occurring isolate, it is suitable for studies that look at the genetic determinants of AMR.

2.2. Mechanisms of AMR in bacteria

The inhibition of bacterial growth by antimicrobials can depend on several steps, the penetration of the drug into the bacterial cell, the chemical activation of the drug (in the case of pro-antibiotics) and the accumulation of the drug in an active form inside the bacterial cell to the concentration required for binding to and acting on the target. Bacteria can acquire a capacity to counteract any one of these steps, or, in the case of intrinsic resistance, may have a naturally occurring barrier at any one of these steps [3-5].

2.2.1. Inactivation of antimicrobials

Some bacteria can produce enzymes that either hydrolyse the antimicrobial or modify it into an inactive derivative. For example, the aminoglycosides, beta-lactams and chloramphenicol are susceptible to enzymatic inactivation. Chloramphenicol acetyltransferases (CAT), and aminoglycoside adenylases and phosphotransferases, bind to and modify radicals on the antimicrobials rendering them inactive, while beta-lactamases hydrolyse the active drugs into inactive molecules [3, 6].

2.2.2. Antimicrobial target modification

Blocking the capacity of an antimicrobial molecule to bind to its target protects the bacterial cell from the effects of the antimicrobial. This resistance mechanism is known as target modification and is classified into four types [3, 5, 7]: (1) mutations in the genes coding the targets that change the affinity of the targets to antimicrobials, preventing the antimicrobial binding (for example, mutations in *gyrA* and *parC* genes lead to amino acid substitutions reducing the affinity of fluoroquinolones for the products of these genes) [8]; (2) target replacement, as is seen in some beta-lactam resistances, where chimeric penicillin binding proteins (for example, altered PBP1a, PBP2b, PBP2x and PBP2a, which are encoded by mosaic *pbp* genes, in *Streptococcus pneumoniae*) that have a low affinity for beta-lactams replace the original PBPs [9]; (3) enzymatic changes to the target of an antimicrobial (for example, the enzyme 16S rRNA methylase methylates ribosomal RNA, thereby preventing aminoglycoside binding to ribosomes, resulting in higher levels of resistance) [10]; and (4) production of different proteins that protect the target from the antimicrobial (for example, the protein QnrA protects the bacterial topoisomerase by binding to it and thus destabilizing the complex between the topoisomerase, cleaved DNA and a quinolone. This leads to the release of the quinolone, religation of the DNA and regeneration of active topoisomerase,

resulting in resistance) [3, 5, 7].

2.2.3. Reduction of intracellular antimicrobial concentrations

A common antimicrobial resistance mechanism relies on reducing the intracellular concentration of the antimicrobial, thus preventing or interfering with the interaction between the drug and its target. This is achieved by cellular permeability barriers and/or active efflux pumps [3, 11].

The outer membrane of Gram-negative bacteria is a low-permeability barrier to antimicrobial drugs. The lipopolysaccharide molecules in the outer membrane form strong lateral interactions, resulting in low membrane fluidity [11-13]. This results in slow passive diffusion of hydrophobic antimicrobials, such as aminoglycosides, macrolides, rifamycins, novobiocin, fusidic acid and cationic peptides [13]. The porins that traverse the outer membrane are responsible for intake of small hydrophilic antimicrobials, such as beta-lactams. Some bacteria develop resistance to these antimicrobials through loss of porins [14]. The replacement of porins that permit transport of larger molecules with porins that only permit transport of smaller molecules can also reduce the intake of antimicrobials into bacterial cells [15]. Mutations can also result in functional changes that reduce the permeability of porins to antimicrobials [13].

Active drug efflux systems are responsible for expulsion of cytoplasmic solutes, including antimicrobials, to the extracellular environment. Efflux pumps are usually protein complexes that traverse the bacterial cell membrane. Some of these systems can export more than one compound and therefore can confer multiple drug resistance. Bmr, EmrB, MvrC, QacA, QacE and Smr are a few examples of multidrug efflux systems. The EmrB system in *E. coli* can export carbonyl cyanide, m-chlorophenylhydrazone, phenylmercuric acetate, nalidixic acid and thiolactomycin [3, 11]. Tetracycline resistance can also be conferred by efflux pumps. Tetracycline efflux genes, such as *tetA*, encode membrane-associated proteins that export

tetracycline from the cell and reduce its intracellular concentration [16].

2.2.4. Sequestering of antimicrobials

Some bacteria produce antimicrobial interceptors that can isolate or conceal the antimicrobial molecules [3]. The antimicrobials then fail to reach their targets due to this sequestration. Some interceptor molecules structurally resemble antimicrobial targets. The antimicrobials bind the structural analogues and are thereby prevented from binding to their targets [17]. However, other interceptors, which can be proteins, polysaccharides or DNA molecules, can bind to and sequester antimicrobials without relying on a structural similarity to the drug target. Cationic peptide antimicrobials, such as polymyxin B, interact with anionic bacterial surface polysaccharides to reach bacterial cells. Some bacteria, such as some encapsulated strains of *Klebsiella pneumoniae*, can release their anionic outer polysaccharide capsule upon exposure to peptide antimicrobials. This cationic capsule then binds with the anionic peptide antimicrobials and prevents them from reaching the bacterial surface [18]. Negatively charged DNA molecules can sequester positively charged antimicrobial molecules through electrostatic interactions [17]. Antimicrobial sequestration is also a significant mechanism contributing to antimicrobial resistance in biofilms [17].

2.2.5. Persistence

Apart from employing various mechanisms to resist antimicrobials, bacteria can also engage a phenomenon known as ‘persistence’ to evade the effects of antimicrobial drugs. A subpopulation of bacteria can differentiate into physiologically dormant persister cells under environmental stress conditions. These persister cells can tolerate antimicrobial treatment because they are metabolically inactive [19]. These cells revert to their infectious state after surviving antimicrobial therapy, resulting in recurrent infection. Examples include survival of uropathogenic *E. coli* after exposure to multiple antimicrobial agents [20] and biofilm-

associated infections [21].

2.3. Emergence, evolution and genetic basis of AMR

2.3.1. Naturally-occurring antimicrobial compounds and resistance

AMR is ancient and widespread in nature [22]. Microorganisms naturally exist in complex communities, where they compete for resources. Species that produce compounds that inhibit the growth or survival of neighbouring microorganisms have a competitive advantage. Naturally-occurring antimicrobial compounds were among the first antimicrobial drugs used to treat infections in humans. Penicillin was discovered by Alexander Fleming in 1928 and was not used medically until 1941 [3]. However, the identification of the enzyme penicillinase in 1940 [23], even before the therapeutic use of penicillin, demonstrates that there was already a reservoir of resistance determinants in natural microbial populations [24].

Intrinsic AMR had been observed in bacteria long before the use of antimicrobials for therapy and therefore the selection pressure imposed by it [25, 26]. Most bacteria are innately resistant to one or more antimicrobial agents and this type of resistance is mediated by natural structural [27] or functional [28-30] systems or determinants [31-35] that are always present in bacterial cells. Intrinsic resistance is clinically relevant because it limits the spectrum of activity of antimicrobials. However, the presence of innate resistance or its determinants in complex microbial eco-systems is less significant as they are not readily transmissible between bacteria [1].

2.3.2. Selection pressure imposed by therapeutic use of antimicrobials

The medical use of antimicrobials further exacerbated the prevalence of AMR. This was initially highlighted by the early use of streptomycin, an antibiotic synthesised by the soil bacterium, *Streptomyces griseus*. This drug was discovered in 1943 and was first used medically in 1944, but its use in monotherapy was rapidly limited by the development of

resistance during treatment of patients [36]. Antimicrobial substances can contaminate the environment as residues from therapeutic use. Inappropriate or overuse of antimicrobials can further increase environmental contamination [37]. This creates a selection pressure for AMR in the environment. This can select for naturally resistant populations of bacteria or for chromosomal mutations that result in an AMR phenotype, or for a more resistant phenotype [37]. The selection pressure imposed by one antimicrobial agent can also select for resistance to other antimicrobials with similar modes of actions, a phenomenon known as cross-resistance [38]. Therefore, resistance to a specific antimicrobial drug can emerge even in the absence of the use of that specific antimicrobial.

2.3.3. Acquired resistance

The development of AMR in intrinsically susceptible bacteria is known as acquired AMR. The selection pressure imposed by an antimicrobial can favour: (1) accumulation of chromosomal mutations that reduce susceptibility to the drug and/or (2) the acquisition and retention of foreign genetic material containing antimicrobial resistance genes (ARGs) [39]. Resistance acquired through mutations is vertically transferred to daughter cells, creating resistant sub-populations, whereas resistance traits acquired by acquisition of foreign genetic material can be transferred both horizontally and vertically.

The mobilisation of chromosomal ARGs into transferrable plasmids or non-transferrable integrons facilitates the emergence and evolution of AMR in bacterial populations. Integrons accumulate ARGs and potentially move to transferrable mobile genetic elements (MGEs) that carry ARGs between different bacteria [40]. This horizontal gene transfer (HGT) plays a crucial role in the emergence and evolution of AMR by disseminating ARGs beyond specific bacterial clones. HGT events are particularly likely between closely related bacteria that inhabit the environments exposed to the selective pressure of antimicrobials, metal residues and biocides [40].

Acquired resistance is significant both clinically and ecologically. The ARGs associated with MGEs in complex microbial eco-systems have much greater potential for dissemination, and thus are much more likely to be a risk to public health [1].

2.3.3.1. Mechanisms of horizontal gene transfer

Transformation, transduction and/or conjugation can be involved in HGT events in bacteria. The uptake of exogenous DNA in the environment surrounding bacteria is known as transformation [41]. If the exogenous DNA contains ARGs, the initially susceptible recipients will be transformed into a resistant phenotype. Transduction refers to the introduction of foreign DNA into a bacterial cell by a bacteriophage. The bacteriophage acts as a vehicle to transfer genetic material, which may contain ARGs from a donor bacterial cell, to a recipient cell [42]. Conjugation requires close contact between two bacterial cells. A sex pilus is formed between the donor and recipient cells resulting in a mating pair, enabling DNA to be transferred from the donor to the recipient. The transferred DNA can contain ARGs [43].

2.3.3.2. Mobile genetic elements

1 - Plasmids

Plasmids are small, circular or linear, extrachromosomal DNA molecules capable of independent replication [44]. Previously susceptible bacteria can become resistant to a number of antimicrobials by acquiring ARG-carrying plasmids through conjugation or transformation [41, 45]. Self-transmissible plasmids contain genes encoding an origin of transfer (oriT), a relaxase protein, a type IV coupling protein (T4CP) and a type IV secretion system (T4SS), which are required for conjugation [44]. Many ARGs conferring resistance to clinically important antimicrobials, including aminoglycosides [46, 47], carbapenems [48, 49], chloramphenicol [47], colistin [50-53], extended-spectrum beta lactams [54-56], quinolones [57], sulphonamides [47], tetracyclines [46] and trimethoprim [48], can be located on self-

transmissible plasmids, enhancing their dissemination throughout bacterial populations.

2 - Integrative Conjugative Elements (ICEs)

ICEs are located on host chromosomes and have a modular structure, with three core modules. These essential modules contain genes encoding integration/excision, conjugation and regulation of the ICEs. Apart from the essential modules, ICEs can also contain genes encoding phenotypic characteristics, such as AMR. The ICEs are excised from the donor cell chromosome and circularised, before being replicated and transferred to a recipient cell by conjugation and then integrating into the recipient chromosome [58]. Horizontal transfer of ARGs in ICEs has been observed in multiple bacterial species, including *Haemophilus influenzae* [59], *Vibrio cholerae* [60], *Pasteurella multocida* [61] and *Shewanella* species [62, 63].

3 - Insertion sequences (IS) and transposons (Tn)

These are transposable elements that are involved in mobilising ARGs within bacterial genomes. IS elements are short DNA sequences composed of a transposase gene flanked by inverted repeats (IR) and do not contain any other genes [64, 65]. The Tns are longer DNA sequences containing functional genes, such as ARGs, together with transposase genes [65]. A composite transposon is a common Tn type defined as "a region bounded by two copies of the same or related IS that can move as a single unit" [64]. The IS elements enhance and disseminate AMR by forming composite Tns carrying ARGs, mobilising adjacent DNA regions containing ARGs, and by promoting the expression of captured or adjacent ARGs [66, 67]. *IS26* [68-70], *IS257* [71], *ISEcp1* [72, 73], *ISApl1* [74] and *ISCR*[75] are frequently associated with ARGs and promote their dissemination. A second type of Tn involved in mobilisation of ARGs is known as a unit transposon. Typical examples are the members of the *Tn3* family, which contain a transposase gene, a resolvase gene and passenger gene(s), which may be ARGs, with the unit flanked by IR elements [64]. The *blaTEM* resistance genes

that confer resistance to beta-lactam antibiotics have been commonly identified within *Tn3* Tns [76]. Transposition is the movement of transposable elements from one genomic location to another and may be conservative and replicative. In conservative transposition the IS is removed from the donor site and then inserted into the recipient site, whereas in replicative transposition the element is copied from the donor site before insertion into the recipient site [64].

4 - Integrons

The basic structure of integrons consists of *intI* genes, *attI* recombination sites and promoters (Pc). These elements are able to incorporate gene cassettes through recombination. Gene cassettes are small circular elements containing single genes, mostly ARGs, and an *attC* recombination site. The *intI* gene encodes a site-specific tyrosine recombinase and this enzyme catalyzes the recombination between the *attI* and *attC* sites. This recombination inserts the gene cassette into the integron. Insertion of multiple cassettes results in an integron containing an array of ARGs. The expression of the inserted ARGs is regulated by the Pc promoter of the integron. Integrons are classified based on the type of *intI* gene they contain, with class 1 integrons considered to be the most important carriers of ARGs [64]. Gene cassettes commonly found inserted into class 1 integrons include metallo beta-lactamases (*VIM* and *IMP* type), extended-spectrum beta-lactamases (ESBLs), *OXA*-like enzymes, *aacA4*/*aac(6')-Ib* and *dfrA17*/*aadA5* [77].

2.3.3.3. Significance of MGE-mediated AMR acquisition

MGEs have contributed to the expansion of the host range of ARGs into additional bacterial strains and species, and thus to the ecological range of ARGs, into additional animal species and new geographical locations (Table 1). Two factors further aggravate the problem: (1) the involvement of drug-resistant nosocomial pathogens, and (2) the horizontal transfer of MGEs

carrying ARGs conferring resistance to high-importance antimicrobials.

There is both direct and indirect evidence for horizontal dissemination of ARGs through MGEs. Detailed comparative genomic analyses, combined with epidemiological data [78-81] and *in-vitro* experiments [82-85], directly confirm lateral spread of ARGs via MGEs, while the occurrence of MGEs with a high level of sequence similarity in different species or strains of bacteria without clear epidemiological links [86-90] and metagenomic studies revealing a high correlation between the presence of ARGs and MGEs [91, 92] provide indirect evidence for the spread of ARGs by MGEs. Taken together, it is clear that MGEs play a key role in the dissemination of AMR.

The horizontal transfer of ARGs between bacteria is mainly mediated by conjugative plasmids and ICEs, while integrons and transposons play a role in intra-genome mobility and accumulation of ARGs. Plasmid-mediated HGT events are important as they can spread resistance determinants from commensal/environmental microorganisms to pathogenic bacteria [91, 93], or *vice versa* [78, 85]. This results in infections that are more difficult to treat or reservoirs of resistant organisms in close proximity to humans and animals, respectively. The evidence for local, regional and global dissemination of plasmids carrying ARGs between bacteria in food, the environment, animals and humans underscores the extent of this problem (Table 1).

Table 1. Examples of widespread dissemination of AMR genes on plasmids

Resistance transferred	**Plasmid type**	**Isolates sharing the plasmid**	**Host/s or Origin**	**Location**	**Reference[a]**
Colistin (*mcr-1.1*)	IncI2	Different *E. coli* strains	Human, dog, chicken	Ecuadorian household	[80]
ESBL (*blaCTX-M-1*) BL (*blaCMY-2*)	IncK	Different *E. coli* strains	Broilers, humans working and living on broiler farms	The Netherlands	[94]

ESBL (*blaCTX-M-1*)	IncI1	Different *E. coli* strains	Turkey	Canadian turkey farms	[95]
ESBL (*blaCTX-M-15*)	IncFII	Different *Klebsiella pneumoniae* strains	Healthy and hospitalised children	Tanzania	[86]
Carbapenem (*blaKPC*)	IncHI2/HI2A	Different *E. coli* strains	In-patients	Hospital in Manchester, England	[96]
Carbapenem (*blaKPC*)	IncN	Different *E. coli* strains	Global surveillance schemes	Different cities and countries across four continents	[87]
Carbapenem (*blaKPC-2*)	IncU/IncX5	*Klebsiella quasipneumoniae* *Serratia marcescens*	In-patients Hospital environment	Hospital in Virginia, USA	[97]
Carbapenem (*blaKPC-2*)	repA_1_pKPC-2	*Citrobacter freundii* *E. coli* *K. pneumoniae*	In-patients	Two hospitals in the USA	[98]
Carbapenem (*blaNDM-1*)	IncR/IncFIB/IncFII	*K. pneumoniae* *Klebsiella michiganensis* Different *Enterobacter* species *S. marcescens* *C. freundii*	In-patients	Ten different private hospitals in Durban, South Africa	[99]
Carbapenem (*blaNDM-1*) Tigecycline (*tet(X)*)	Untypeable plasmid (pCMG3-2-1)	*Acinetobacter lwoffii* *Acinetobacter indicus* *Acinetobacter schindleri*	Goose Duck Soil Sewage	Three geographic areas in China	[100]
Vancomycin (*vanA* cluster)	Linear plasmid (pELF2)	*Enterococcus faecium* *Enterococcus raffinosus* *Enterococcus casseliflavus*	In-patients	Hospital in Japan	[101]

BL, beta-lactamase; ESBL, extended-spectrum beta-lactamase

[a]The Web of Science (all databases) was searched using the keywords ‘horizontal’ AND ‘dissemination OR

spread OR transfer OR transmission' AND 'antimicrobial OR antibiotic' AND 'resistance genes' AND 'plasmids'. Records of meetings, abstracts and reviews were excluded. The records were further refined with six keywords ('veterinary OR animal', 'food', 'environment', 'global', 'regional', 'local') and the manuscripts identified were then screened manually. The two most recent publications from each of the six categories are summarised in the table to illustrate the widespread nature of plasmid-mediated horizontal dissemination of AMR.

Plasmids carrying genes encoding carbapenemases and ESBLs have been transferred between diverse strains, species and genera of the family *Enterobacteriaceae*, resulting in outbreaks of nosocomial infections caused by different bacteria, all of which have carried the same MGE [78, 79, 86, 102-108]. Similarly, MGEs have been involved in the spread of vancomycin resistance between different outbreak strains of enterococci [101, 109-111], and polyclonal spread of resistance to colistin, a last-resort antimicrobial used to treat unresponsive infections, in *Klebsiella pneumoniae* in human hospitals has caused considerable alarm [112]. Investigations that focus only on single bacterial strains are unable to demonstrate the magnitude of such outbreaks [79]. More extensive multi-species surveillance systems that include plasmid and resistance tracing are needed. However, some outbreak strains can silently carry and transfer resistance genotypes on conjugative plasmids, with phenotypic resistance only expressed after exposure to the cognate antimicrobials [111]. These silent HGT events further complicate outbreak investigations. The complex nature of MGE-mediated nosocomial outbreaks can be unraveled using whole genome sequencing, but this is not always practical, particularly during ongoing outbreaks that require rapid investigation so that control measures can be implemented. However, complete sequences of plasmids shared by bacteria responsible for outbreaks have been used to develop polymerase chain reaction (PCR) assays for rapid detection of such plasmids [105]. The fast turnaround times offered by novel sequencing technologies have also been useful in molecular investigations of plasmid-related outbreaks [79].

Control of disease outbreaks caused by bacteria carrying MGEs can also be difficult [78, 103]. This is particularly the case when a plasmid or MGE with a broad host range is involved, as a variety of ecological niches could harbour the resistance genes. In some cases, intensive attention to eliminating the risks imposed by environmental niches, such as improved environmental hygiene, containment of cross-transmission, extensive cleaning of plumbing, and/or removing and replacing sinks have still not been effective in the control of outbreaks caused by the organisms carrying the same plasmid [102, 103, 112]. However, a stringent multifaceted outbreak control strategy was able to successfully contain an outbreak caused by carbapenemase-producing *K. pneumoniae* strains [79]. This strategy involved improved hand hygiene, cohorting and isolation of patients, rectal screening of patients, environmental cleaning and education of staff, patients and visitors. However, implementation of some measures, such as construction of additional hand washing stations, assigning dedicated staff and equipment for separate areas of the hospital, increasing the frequency of patient screening and hydrogen peroxide vaporisation of hospital units [79], require additional resources and can therefore be expensive to implement. Furthermore, some changes to patient care, such as isolating patients awaiting screening results and increasing the frequency of rectal screening [79], could have implications for patient welfare. Prevention of outbreaks, rather than controlling them when they arise, would be a better approach, and this necessitates identification of potential sources of resistant organisms, including environmental reservoirs [102, 103, 106, 113].

Bacteria that have acquired resistance to at least one antimicrobial agent in each of three or more classes of antimicrobial drugs are known as multiple drug resistant (MDR) organisms [114]. Integrons and transposons are particularly important in the accumulation of multiple ARGs and in the generation of novel combinations and arrangements of these ARGs [115]. The multiple ARG regions that result can then be integrated into plasmids or ICEs, which

promote horizontal dissemination between organisms [60, 63, 116, 117]. The MDR phenotype results in co-selection, as selection pressure imposed by one antimicrobial also selects for the other resistance genes carried on the MGE [38]. Exposure to disinfectants or heavy metals can also co-select for resistance to multiple antimicrobials if ARGs are co-located with genes conferring resistance to these compounds [118, 119]. Co-selection is an important clinical consideration as ARGs conferring resistance to last-resort antimicrobials can become widespread, even in the absence of frequent use, if they are co-located with other ARGs conferring resistance to regularly used antimicrobials.

In summary, MGEs play an important role in dissemination of antimicrobial resistance, in outbreaks caused by drug-resistant nosocomial bacteria and in the generation of MDR organisms. Therefore, an understanding of the association between ARGs and MGEs is crucial to assessing the clinical risk attributable to resistance determinants, particularly when they are detected in environmental reservoirs.

3. AMR in hospital environments

Baquero and colleagues [120] have highlighted the importance of natural and man-made environmental systems in the evolution of antimicrobial resistance. High levels of biological connectivity, high rates of generation of variation and the presence of selection pressures in some environments facilitate the generation of mutations and recombination between the members of the microbial populations residing in these environments. Such a system has been described as a genetic reactor. Four genetic reactors have been identified as crucial niches for the evolution and spread of antimicrobial resistance: (1) human and animal microbiota; (2) settings housing susceptible individuals; (3) waste water; and (4) soil and water [120]. All these reactors are known to harbour large numbers of bacterial species, explaining the high biological connectivity and the generation of variation. Furthermore, constant exposure to antimicrobials

imposes the selection pressure required for the development and spread of antimicrobial resistance.

A high selection pressure is imposed in hospital environments because of the continuous administration of antimicrobials for therapy and for prophylaxis. This high selection pressure can lead to the development of AMR in pathogenic, commensal and environmental bacteria in hospitals. The environment can then act as an AMR reservoir and facilitate the transfer of drug-resistant organisms to susceptible individuals, resulting in nosocomial infections.

3.1. Antimicrobial-resistant nosocomial pathogens in the environment of veterinary hospitals

The World Health Organization (WHO) has published a list of twelve antibiotic-resistant pathogenic bacteria that are global targets for discovery and development of new antibiotics [121]. These targets were selected based on a number of criteria, including the burden they impose in healthcare settings. Healthcare-associated infections are frequently caused by the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) and antibiotic-resistant members of this group are categorized as critical or high priority organisms in the WHO priority list [121]. Drug-resistant ESKAPE organisms are also of importance in animal health because they are frequently involved in nosocomial infections in veterinary hospitals [122]. Another common pathogen implicated in veterinary nosocomial infections is multiple drug-resistant *Escherichia coli*. Although the epidemiology of microorganisms responsible for nosocomial infections in small animal practice is not well established, staphylococci appear to be the most important ESKAPE pathogens, in veterinary medicine followed by the *Enterobacteriaceae,* enterococci, *Acinetobacter* species and *Pseudomonas* species [122]. These organisms are often multidrug-resistant and often carry ARGs conferring resistance to high-importance antimicrobials, such as the carbapenems and vancomycin,

leaving no therapeutic options for control of infections caused by them [123]. The AMR risks associated with the above pathogens are exacerbated by the frequent involvement of MGEs in their drug resistance. An understanding of their occurrence in hospital environments can provide useful insights that can improve hospital infection control procedures and reduce the incidence of nosocomial infections.

3.1.1. *Enterococcus* species

Enterococcus faecium is a commensal organism found in the intestinal tracts of humans and other animals. It has emerged as a significant health-care-associated pathogen, over the last three decades, with the acquisition of multiple ARGs, including *vanA*, *vanB* and *vanM*, conferring intermediate to high level resistance to vancomycin [124]. The clinical diseases caused by *E. faecium* in small animals include urinary tract infections (UTIs) [125-129], surgical site infections (SSIs) [130] and wound infections [126]. Isolates frequently display MDR phenotypes, with or without vancomycin resistance, and in some cases they are similar to the strains in the hospital-adapted clonal complex infecting humans [125, 126, 128]. This suggested that there may be a reservoir of hospital-adapted *E. faecium* strains in companion animals that may cause infection in humans.

E. faecium survives well in the environment and contamination of surfaces and medical equipment in human hospitals has been associated with a significant risk of infection with vancomycin-resistant enterococci [131-134]. A similar, or potentially higher, level of risk could be expected in veterinary hospital environments as a result of faecal carriage of MDR enterococci by companion animals [135, 136] and the relative frequency of faecal contamination of the veterinary hospital environment. Several studies have shown that identical MDR *Enterococcus* spp. can be shared by veterinary patients, animal owners, veterinary staff, resident animals and the veterinary hospital environment [137-139],

indicating that cross-transmission can occur.

3.1.2. *Staphylococcus* species

The occurrence of methicillin-resistant *Staphylococcus* (MRS) species in the environment of veterinary hospitals has been more commonly investigated than that of the other ESKAPE pathogens, probably because of the frequency of infection with MRS, and the severity of the disease they cause, in companion animals. *Staphylococcus pseudintermedius* is a commensal organism of skin and mucosal membranes of small animals and *S. aureus* is known to colonize healthy dogs and cats [140]. They are opportunistic pathogens and acquisition of the *mecA* gene renders them resistant to the isoxazolyl penicillins, such as methicillin. Staphylococci that have acquired the *mecA* gene are commonly MDR. Methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant *S. pseudintermedius* (MRSP) commonly cause infections of the skin and mucosal membranes of small animals, including pyoderma, abscesses, wound infections, otitis and conjunctivitis [141-147]. In one study, the prognosis for the canine MRSA infections was good, in contrast to the common belief that these infections are untreatable [148]. However, the majority of the isolates in this study were from superficial infections, so this finding may not be applicable to more invasive infections, such as respiratory tract infections [149, 150], UTIs [143, 149, 151] and septicaemia [149, 152], caused by MRSA and MRSP in small animals.

MRSA and/or MRSP have been identified in veterinary patients, staff and their environments. In North America, MRSA have been detected on carts, doors, examination tables and floors, and in the air, of veterinary hospitals [153-156]. They have been shown to persistently colonize the environment [153]. The environment of a Swedish veterinary hospital was contaminated with strains of MRSP and MRSA and MRSP isolates with similar pulsed field gel electrophoresis (PFGE) patterns were obtained from canine patients, indicating potential

transmission between patients and the environment [157]. The same MRSA and MRSP strains have been detected in staff and their immediate environment in a German veterinary hospital [158]. MRS species have also been found to be common in the veterinary hospital environment in Korea [139] and MRS isolates from humans and animals in animal hospitals in Malaysia and Korea have also been found to be closely related [159, 160].

In contrast, surveillance studies conducted in Australia and Africa did not detect any MRS species in veterinary hospital environments [161, 162]. However, the Australian study isolated MRSA from veterinary staff and MRSP from patients and the authors suggested that the small sample size, the lack of longitudinal sampling, the restricted range of sites and equipment sampled, and good infection control procedures in the veterinary facilities surveyed could have contributed to the failure to detect MRS isolates in the environment [161]. These studies from different parts of the world indicate that environmental contamination and cross-transmission of MRS species is a common problem in veterinary hospitals and that control measures are needed to reduce the impact of these organisms and prevent their spread.

3.1.3. Klebsiella pneumoniae

Hospital-acquired UTIs, pneumonia, septicaemias and soft tissue infections caused by *K. pneumoniae* are often difficult to treat because the strains responsible are resistant to multiple antimicrobials. *K. pneumoniae* can acquire multiple resistance genes, including those encoding carbapenemases and ESBLs, conferring resistance to the carbapenems and third generation cephalosporins. In small animal medicine ESBL and carbapenemase producing *K. pneumoniae* have been found to be responsible for nosocomial infections, mainly of the skin and soft tissues, as well as UTIs [163-169]. Drug-resistant *K. pneumoniae* can cause post-surgical wound infections in dogs and cats and these can be fatal [169]. Some veterinary nosocomial strains appear to be unique to small animals [165, 167], suggesting independent evolution of

nosocomial lineages in animals. Others belong to the major high-risk clones detected in humans [163, 164, 166, 168, 169], suggesting the presence of a common source of infection shared by both small animals and humans. One such common source of infection could be the shared environmental surfaces in veterinary hospitals.

Contamination of human hospital environmental surfaces, medical equipment, waste and waste water with MDR *K. pneumoniae* has been detected [170-175]. Though veterinary hospital environments have not been studied as commonly as those of human hospitals, ESBL-producing *K. pneumoniae* have been detected in these environments and in patients and clonal spread between them has been observed [176].

3.1.4. *Acinetobacter* species

Multiple drug resistant *A. baumannii* is a leading cause of serious nosocomial infections in humans and carbapenem-resistant strains are the highest ranked pathogens in the WHO priority list [121]. *A. baumannii* can result in life-threatening systemic infections (sepsis, pneumonia and UTIs) and local infections that are difficult to treat (wounds, abscesses, cellulitis) in dogs and cats [177, 178]. Outbreaks of hospital-aquired infections with *A. baumannii* with high mortality rates have been reported in the intensive care units of veterinary hospitals [179, 180], highlighting the clinical significance of this organism in veterinary medicine. The outbreak strains are often resistant to many antimicrobials, and may only be susceptible to amikacin, ticarcillin, imipenem and colistin [179, 180]. Although early treatment with effective antimicrobials has resulted in a better prognosis [179], the need for use of last-resort antimicrobials complicates treatment, as they are expensive and are generally reserved for human medicine.

Prolonged hospitalisation and the use of invasive devices have been identified as predisposing factors for nosocomial infections caused by *A. baumannii* [179, 180], underlining the

importance of the hospital environment and equipment as potential sources of infection. *A. baumannii* can persist in the environment under harsh conditions because of its ability to survive desiccation, nutritional deprivation and exposure to many antimicrobials [181, 182]. This is further enhanced by its capacity to form biofilms [183]. Carbapenem-resistant *A. baumannii* are more commonly detected in samples from human hospital environments than in clinical specimens and some isolates from these two sources have been found to be closely related [184-186], suggesting that an environmental reservoir is likely to be the primary source for human infection. Carbapenem-resistant *A. baumannii* can survive on fabrics for more than a week [187], and has been detected in the air, and on furniture, medical devices and gloves of health care workers in intensive care units [186], potentially facilitating infection of patients. The presence and role of MDR *A. baumannii* in veterinary clinical environments is less well studied, but a few reports have suggested the presence of an environmental reservoir in veterinary hospitals [179, 180, 188]. Such reservoirs can serve as a source of this important pathogen, not only for patients but also for veterinary health care workers and animal owners.

3.1.5. *Pseudomonas* species

Multiple or extensively drug resistant (XDR) *P. aeruginosa* is a significant nosocomial pathogen in both human and veterinary medicine. While *P. aeruginosa* is intrinsically resistant to many antimicrobials, it is also capable of acquiring new resistance traits. Antimicrobial therapy, medical devices, patient and hospital environment factors have been identified as risk factors for colonization or infection with XDR *P. aeruginosa* [189]. *P. aeruginosa* is mainly associated with canine skin and soft tissue infections, including otitis externa [190], pyoderma [191], wound infections [191], abscesses [192], surgical site infections [193] and corneal ulcers [194]. It has also been reported as a cause of UTIs [195] and sepsis [196] in small animals. *P. aeruginosa* is clinically relevant because it is frequently the most resistant aetiological agent of canine and feline infections [190, 197-199].

It can survive in hospital environments for long periods of time [200], possibly in part because of its ability to form biofilms, as common hospital-grade biocides are not effective in eliminating biofilms [201]. As a result, prolonged environmental contamination can occur, facilitating opportunities for transmission. XDR *P. aeruginosa* strains commonly colonize moist environmental sites in hospitals, such as sinks, drains, plumbing systems, water and waste water, and a number of investigations have linked this persistence to nosocomial infections or outbreaks in human hospitals [202-207]. The genetic similarities of environmental *P. aeruginosa* and the strains colonizing hospitalised patients in human burns wards have suggested contamination of patients' wounds as a result of poor infection control [208, 209]. Contaminated intravenous catheters [196] and household water [210] have been identified as sources of *P. aeruginosa* infections in dogs. *P. aeruginosa* is the Gram-negative organism most commonly isolated from inanimate surfaces in both human [211, 212] and veterinary [139, 193] hospitals, indicating the importance of environmental reservoirs in the epidemiology of this important nosocomial pathogen.

3.1.6. *Enterobacter* species

Enterobacter species are gut commensals and are ubiquitous in the environment. However, they can also act as opportunistic pathogens. *Enterobacter aerogenes* and members of the *E. cloacae* complex are commonly associated with nosocomial infections. The *E. cloacae* complex is comprised of seven species, of which *Enterobacter cloacae* and *Enterobacter hormaechei* are the species most frequently associated with infections in humans [213]. The species in the *E. cloacae* complex share high levels of phenotypic and genotypic similarity, making their accurate identification difficult without whole genome sequencing [213]. *Enterobacter* species are intrinsically resistant to many beta-lactams because of their expression of AmpC beta-lactamase and many strains have acquired resistance to high-importance or last resort antimicrobials and have MDR phenotypes [213]. *Enterobacter*

species are known to cause UTIs, skin and soft tissue infections, respiratory tract infections and sometimes sepsis [214-219] in small animals. Pneumonia, sepsis, peritonitis and deep surgical site infections caused by this organism have resulted deaths in small animals [215]. Some isolates harboured both ESBL genes and plasmid-mediated quinolone resistance genes, which resulted in resistance to both third generation cephalosporins and fluoroquinolones [214, 217, 219]. This demonstrates the clinical significance of this pathogen, as these are the high importance antimicrobials that are most commonly used in companion animal practice [220]. *Enterobacter* species have also been found to be responsible for hospital acquired urinary tract [216] and surgical site [215] infections in small animals. The source of the organisms responsible in these cases could be the veterinary hospital environment and/or contaminated medical devices, such as urinary catheters.

Drug-resistant strains have been detected in various moist and dry environmental sites in human hospitals [173, 203, 212, 221-225] and are often associated with outbreaks of bacteraemia in neonates [226-229]. ESBL-producing Gram-negative bacteria, including *Enterobacter* species, are more commonly found in handwashing basins than other environmental sites in hospitals [230]. Nosocomial outbreaks have been directly linked with the colonization of moist environmental sites, including sinks and drains, by multiple drug and disinfectant-resistant *Enterobacter* species in human hospitals [231, 232]. One such outbreak in Australia was caused by a diverse set of carbapenem-resistant bacteria harbouring a class 1 integron with a *blaIMP-4* gene cassette that was carried by a conjugative plasmid. The majority of the environmental *E. cloacae* isolates carried this MGE, which harboured multiple ARGs, including an imipenem resistance gene. Clonal dissemination of some strains between patients and the hospital environment, and horizontal dissemination of the MGE containing *blaIMP-4* into diverse bacterial species circulating in the hospital environment, resulted in a prolonged outbreak [232]. This example highlights the complex nature of outbreaks caused

by *Enterobacter* species and the importance of the hospital environment as a reservoir of resistance. Regular monitoring of high risk environmental sites such as sinks and drains in both human and veterinary hospitals would enhance our understanding of the extent of AMR and the dynamics of its spread in organisms in these settings.

3.1.7. Escherichia coli

This organism is a well-known cause of nosocomial infections and is often associated with plasmid-mediated ESBLs and fluoroquinolone resistances [233]. Extraintestinal infections, most commonly UTIs, caused by multiple drug-resistant *E. coli* have been reported in Australian dogs [218, 234, 235]. Acquisition and rectal carriage of these organisms in dogs have been linked to an increased duration of hospitalization, antimicrobial therapy, and predisposing disease conditions [218, 236]. Some evidence suggests contamination of the veterinary hospital environment results from the rectal carriage of multiple drug resistant *E. coli* by dogs [234]. This contaminated environment may then play a role in wide-spread dissemination within veterinary hospitals. Environmental isolates have been shown to carry ESBL and plasmid-mediated fluoroquinolone resistance genes [237]. The dissemination of these organisms and the genes they carry is clinically significant as they confer resistance to high-importance antimicrobials used in veterinary medicine. Furthermore, some strains are shared between companion animals and humans, indicating interspecies spread [234, 238]. Another public health implication is the role of companion animals as drug-resistant *E. coli* reservoirs. In particular, *E. coli* carrying the plasmid-associated *qnrB* gene appear to be more common in companion animals than humans [238], suggesting that they are a source of this important drug-resistant pathogen.

Thus, a number of studies have shown that the ESKAPE and other important nosocomial organisms can persist in the environment in veterinary clinical settings for long periods of time because of repeated introductions by animals (in the case of the commensal species)

and/or their ability to survive harsh environmental conditions. This persistence results in an environmental reservoir that can facilitate transmission of these nosocomial pathogens between the environment, animals, animal owners and veterinary staff. Furthermore, the extended presence of these organisms in the environment can facilitate genetic exchange, recombination and genomic re-arrangements that may promote the evolution and emergence of novel nosocomial strains. Therefore, the environmental persistence of the above pathogens can be considered a significant risk factor for infections associated with veterinary healthcare.

3.2. Value of next-generation sequencing strategies in understanding molecular epidemiology of resistant nosocomial pathogens

Detailed genetic characterization of antimicrobial-resistant nosocomial pathogens provides useful insights into the evolution, transfer and persistence of these organisms in the environment of clinical settings. This knowledge is crucial to enhancing our understanding of the local and global molecular epidemiology of AMR and to mitigating the spread of resistant pathogens, and ultimately reducing risks they pose [239]. The rapid advance of third generation sequencing technologies have made comprehensive analyses of bacterial genomes possible. Hybrid genome assembly, which combines the advantages of short and long read sequencing technologies to generate accurate and complete sequences of genomes and plasmids, is particularly useful when studying the molecular epidemiology of AMR. Several recent studies have effectively combined Illumina and PacBio sequencing technologies to completely assemble the genomes and plasmids of carbapenemase producing bacteria. In-depth genetic comparisons of these assemblies have provided evidence of nosocomial transmission of carbapenemase producing bacteria from hospital environments to patients [113, 240], of horizontal transfer of plasmids carrying carbapenem resistance genes between different bacterial species or strains [102, 241], of the diversity and distinctive features of plasmids carrying carbapenem resistance genes circulating in hospitals [242], and of novel plasmids

harbouring carbapenem resistance genes [102] and their genetic context [240]. Nanopore sequencing, a convenient and rapid long read sequencing technology, has also been used in studies employing hybrid assembly techniques to resolve the genetic characteristics of drug-resistant organisms [243, 244].

3.3.Antimicrobial resistance genes in the environment of veterinary hospitals

The collection of all genes that could contribute to a phenotype of antimicrobial resistance is known as resistome [245]. Exploration of resistomes allows determination of the diversity and abundance of resistance genes present in genetic reactors. Attribution of these resistance determinants to their bacterial host and/or studies of their genetic context can provide further useful insights into the prevalence of AMR in environmental systems.

Human hospital environmental resistomes have not been investigated routinely (and veterinary hospital resistomes have not been investigated at all) because of the technical complexity of conducting such investigations, but the limited studies that have been performed have detected a high abundance of ARGs on hospital surfaces [246] or in air-conditioning systems [247]. Most other studies have focused on the abundance of ARGs in hospital effluent. The effluent from Cambridge University Hospitals was found to carry ARGs in high abundances and metatranscriptomic studies detected overexpression of beta-lactam resistance genes, which was attributed to the high rates of use of beta-lactam antimicrobials in the facility [248]. Similarly, analysis of waste water from a Spanish hospital revealed high copy numbers of the *blaTEM*, *qnrS*, *ermB*, *sul1* and *tetW* resistance genes [249]. In addition to the high prevalence of ARGs, integrons have been identified at high frequencies in the hospital waste water, suggesting a risk of accumulation of multiple ARGs by organisms in this environment and dissemination of them into the natural environment [250]. Hospital plumbing systems and waste water have also been identified as reservoirs of bacterial

plasmids carrying carbapenem resistance genes [113] and bacteriophages conferring antimicrobial resistance [251].

In summary, environmental sites in human hospitals, particularly drainage and plumbing systems, have been identified as important reservoirs of clinically important antimicrobial resistance genes, mobile genetic elements and nosocomial pathogens. Therefore, environmental sites in veterinary hospitals could also harbour antimicrobial resistance determinants and represent a risk to patients. The importance of this issue in human hospitals suggests that much more investigation is needed of the environmental resistomes in veterinary hospitals.

Veterinary hospital settings can favour the spread of nosocomial infections because of the close interactions between patients, hospital staff, animal owners and the hospital environment. A survey performed to characterize the biosecurity and infection control practices at veterinary hospitals found that 82% of practices had experienced outbreaks of infections caused by nosocomial pathogens within a 5-year period [252]. This highlights the importance of paying attention to biosecurity and infection control procedures to mitigate the risk of nosocomial infections. Other studies have pointed out the need for better strategies to promote active participation of veterinary health care workers in infection control practices [253, 254]. More surveillance for pathogens specific to the veterinary practice environment is needed to provide an enhanced evidence base for infection control programmes [255]. Active surveillance can also provide insights to aid the development of more targeted surveillance programmes focused on the areas of greatest risk [255].

Environmental monitoring is an important component in active and targeted surveillance programmes [122]. Most environmental surveillance programmes target specific nosocomial pathogens and their antimicrobial resistances. However, other bacteria inhabiting the veterinary hospital environment can also harbour ARGs that confer resistance to clinically important

antimicrobials and these can also be a cause for concern, particularly if they are associated with MGEs. These organisms can transfer their resistance determinants to nosocomial pathogens that inhabit in the same environmental site [232, 256, 257]. While monitoring ARGs in nosocomial pathogens is important, other significant resistance determinants in the hospital environment may be overlooked by focusing only on nosocomial pathogens [257]. One reason for such a focus is the time, labour and financial constraints on routine surveillance of a broad range of environmental organisms. Studying the environmental resistomes in veterinary clinical settings could be a useful alternative to traditional environmental surveillance.

4. Methods for exploring AMR in microbial ecosystems

An understanding of the nature, presence, diversity and abundance of antimicrobial-resistant organisms and their resistance determinants in complex microbial ecosystems requires development of methodologies appropriate for the environment to be investigated. Currently, a number of culture-based and molecular methods are in use and all these techniques have their own advantages and disadvantages. A combination of methods can yield more comprehensive detail about resistomes and resistant organisms [258].

4.1. Culture-based and conventional end-point PCR methods

The culture-based approach relies on isolation and characterisation of specific antimicrobial-resistant bacteria from the environment by inoculating selective media. This method is laborious and often introduces biases because some environmental bacteria do not grow on routine laboratory media [259]. Despite these disadvantages, this method is still useful for correlating resistant phenotypes with their corresponding host species. Furthermore, if this approach is combined with conventional PCR techniques, the ARGs responsible for phenotypic resistance can be determined.

PCR assays can detect the presence of ARGs at high levels of sensitivity and is more rapid than

culture-based techniques. However, conventional PCR is not quantitative and does not provide information about the bacterial host of each ARG unless it is combined with culture-based methods. It is also challenging to extract high quality DNA devoid of PCR inhibitors, which can result in false negative results. False positives can also result from non-specific amplification. Careful optimization of PCR assays is essential to ensuring specificity and sensitivity and can be a laborious process if a large number of resistance genes are to be targeted [259].

Membrane filtration and subsequent culture of the membrane on plate count agar with or without antimicrobials have been used to enumerate resistant bacteria and calculate the proportion of bacteria that are resistant when levels of bacterial contamination of water are low, for example in river water samples. The culture medium is supplemented with different concentrations of various antimicrobials in order to select for bacteria resistant to different antimicrobials. The resistant isolates are then further characterised using a disc diffusion assay to determine their levels of resistance to a panel of drugs [260]. For samples with high bacterial loads, such as outflows from wastewater treatment plants, resistant bacteria can be recovered directly on selective Luria-Bertani agar plates containing antimicrobials, and cycloheximide (at 75 μg/ml) to prevent the growth of fungi. Plasmid DNA can be extracted from the resistant bacteria that are isolated and this can be screened for resistance genes using PCR assays [261]. To presumptively identify the bacterial species present in the sample and assess their resistance at the same time, viable plate counts can be performed using the spread plate method on multiple Chromocult agar plates, each containing a different antimicrobial at its breakpoint concentration. This method has been used to investigate the effect of fertilization with manure on the abundance of resistant coliform bacteria in soils and vegetables [262]. Selective and differential media supplemented with antimicrobials have been used to select for more specific resistant organisms, for example MacConkey agar has been used to detect *E. coli* and m-

Enterococcus agar to detect *Enterococcus* spp. [263, 264].

4.2. qPCR assays and qPCR arrays

The qPCR approach is comparatively faster than culture-based methods and can yield information not only about the presence, but also about the abundance, of antimicrobial resistance genes, albeit only for a restricted number of pre-defined gene targets [258]. A real-time qPCR assay has been employed to measure the amounts of tetracycline resistance genes in wastewater from cattle feedlots in parallel with phenotypic plate assays [265]. Comparison of the two methods demonstrated that the qPCR-based assay was more reliable in identifying the tetracycline resistance genes across the entire bacterial community in the waste water. However, it was not possible to detect the bacterial host of the genes without using the phenotypic methods [265].

Novel qPCR arrays can allow a large number of ARGs to be detected simultaneously. This overcomes the throughput limitations of conventional qPCRs. The Wafergen Bio-systems SmartChip Real-Time PCR can quantify more than 200 resistance genes and mobile genetic elements, while the Antibiotic Resistance Genes Microbial DNA qPCR Array by Qiagen can detect 97 different resistance genes. However, it is difficult to simultaneously optimise individual qPCR reactions and the detection limits are higher because of the small reaction volumes [259].

High capacity qPCR arrays have been employed in environmental resistome studies, including studies assessing the prevalence of ARGs in Chinese pig farms [266], in urban park soils [267], in effluents from waste water treatment plants [268], and in surface water [269]. DNA extraction is a crucial step and can be achieved by lysis of the bacterial cells using grinding, freeze-thawing and heating with SDS, together with high-salt treatment, to extract DNA with minimal shearing, followed by low-melting point agarose gel purification and phenol

extraction [266, 267, 270], or by using commercial kits such as the Fast DNA soil kit [268] or the Qiagen Blood and Tissue kit [269]. The Applied Biosystems OpenArray platform has been used to target 313 ARGs, together with the 16S rRNA gene, in DNA extracted from 36 manure and compost samples obtained from various pig farms in China [266]. ARG profiling was performed on soil samples from Chinese public parks irrigated with reclaimed water using the Wafergen Bio-systems SmartChip Real-Time PCR platform and 295 primer sets targeting 285 ARGs, 9 transposase genes and one 16S rRNA gene [267]. The capacity to customise the Wafergen qPCR array allowed another group to use slightly different sets of primers, targeting 282 ARGs, 4 integron associated genes, 9 transposases and the 16S rRNA gene, to measure the effect of chlorination on the resistome of waste water treatment plant effluents [268]. Further advances resulted in platforms capable of simultaneously performing qPCR arrays and generating amplicon libraries for sequencing. One such system is the Fluidigm Access Array, which has been used to identify clusters of ARGs that were enriched and remained associated with each other on pig farms [271].

4.3. Functional metagenomics

Functional metagenomic studies provide a better understanding of the functionality of different ARGs. This approach has been described as "a powerful experimental approach for studying gene function, starting from the extracted DNA of mixed microbial populations" [272]. The DNA is fragmented and small inserts are cloned into plasmid or fosmid vectors. These vectors are then introduced into host bacteria, such as *E. coli*, and the transformants are plated on selective media containing different antimicrobials. This allows the selection of antimicrobial-resistant clones. The vector DNA is then extracted and the inserts are sequenced. The sequences are analysed to identify the resistance genes of interest. Even though the method is labour-intensive and time-consuming, it does enable identification of novel resistance genes [258, 272].

ARGs in dairy cow manure have been explored using a functional metagenomics approach. Large DNA fragments from cow manure were extracted using the phenol-chloroform method, ligated into the pCC2Fos vector and fosmid libraries were prepared. Small DNA fragments from the cow manure were prepared using a commercial DNA miniprep kit and ligated into pCF430 to create small insert libraries. *E. coli* DH10β cells were transformed with the ligation products and plasmid DNAs extracted from antimicrobial-resistant clones were sequenced using the PacBio platform [273]. A similar functional metagenomics workflow has been used to profile the gut resistome of an intensive care unit patient with Oxford Nanopore MinION sequencing used to sequence the DNA from the antimicrobial-resistant clones. The bioinformatic pipeline poreFUME was developed to analyse the resulting sequences [274]. The PacBio and Nanopore sequencing platforms discussed here are useful not only for functional metagenomics, but also for metagenomics. The extra-long sequence reads produced by these technologies are particularly useful in resistome studies, as outlined later in this review.

4.4. Metagenomics

Metagenomic sequencing methods have been widely applied in environmental resistome studies. Metagenomics involves "direct genetic analysis of genomes contained within an environmental sample" [275]. The genetic material in complex microbial samples is extracted and subjected to high throughput sequencing. Next generation sequencing methods developed and commercialised by Illumina, 454/Roche, Pacific Biosciences and Oxford Nanopore are useful in metagenomics. The data obtained from sequencing are then assembled and annotated or searched directly for resistance genes. Specific antimicrobial resistance gene databases and bioinformatic tools have been developed for this purpose [276-278].

A major advantage of using metagenomics to study resistomes is that it does not restrict the analysis to a selection of pre-defined ARGs (as do PCR-based methods) or phenotypes (as do

culture-based methods). Instead, the entire pool of ARGs present in the complex microbial sample is examined [259]. Metagenomic studies also provide quantitative insights into the abundances of resistance genes [279, 280]. Careful selection of sequencing technologies and assembly pipelines can allow information on the genetic context of the ARGs and their genetic origins to be determined [281], knowledge that is important in determining the risks associated with the ARGs. However, this method is not capable of predicting the functionality of the ARGs [259], and combination of metagenomics with culture methods can help in overcoming this drawback. The results obtained using metagenomic approaches are largely dependent upon the ARG databases being used [282]. This bias can be reduced by combining the available resistance gene databases into a single non-redundant database. Another drawback of metagenomic studies is the high cost associated with next generation sequencing technologies [259]. Nevertheless, with recent advances and growing demand, some technologies seem to be becoming more affordable.

The 454/Roche technology yields read lengths of 600 to 800 bp, longer than the Illumina technology. It is possible to analyse up to 12 samples in a single run and nanograms of DNA are sufficient to prepare single-end libraries. However, the method can produce artificial replicate sequences, resulting in errors in gene abundance calculations [283], and it can also be subject to insertion or deletion errors [275]. In contrast, Illumina sequencing yields shorter read lengths, up to 300 bp, which can lead to failures in functional annotation [284]. Another disadvantage of the Illumina technology over that of 454/Roche is the longer run time. Despite these drawbacks, Illumina technology has low error rates, requires low amounts of starting material and allows greater sample multiplexing per lane. PacBio and Nanopore sequencing can generate greater read lengths than Illumina or 454/Roche sequencing, facilitating assembly and annotation [275], but the reads are more error-prone and require different bioinformatic tools for data processing.

The majority of the currently available metagenomic resistome studies were performed with Illumina sequencing and commercially available DNA extraction kits have been used to extract whole DNA from samples of human faeces [285], dairy cow faeces [286], polluted lake water [280] and waste water [279]. Paired-end sequence reads of 100 to 101 bp obtained with the Illumina HiSeq platform were directly annotated without assembly either by searching against ARG databases or by automated functional profiling using the MG-RAST pipeline [279, 280, 286]. While these studies provided valuable insights into the diversity and abundance of ARGs in their respective environments, the short lengths of the Illumina reads limited prediction of the origin of the ARGs and detection of multiple drug resistance in specific hosts. Differences were found in the ARG profiles obtained with the same dataset, derived from the wastewater of a non-hospital medical care facility, when the analyses were performed using two different approaches and databases [279]. These differences could be partly a result of insufficient coverage of reference genes by the short query sequence reads.

Assembly of Illumina short reads has been employed to overcome these drawbacks. The assembled contigs longer than 500 bp have been analysed to detect ARGs, their genomic context and the host of the ARGs in animal faeces, human gut microbiota and wastewater treatment plant influents. Around 43% of the ARG-carrying contigs from the human gut samples were classified as contigs carrying multiple ARGs and were also associated with metal resistance genes, transposases, integrases and virulence factors. ARG-carrying contigs were also able to be assigned to bacterial hosts [281]. Similarly, metagenomic assembly helped in assigning the ARGs to taxa found in human gut microbiomes in another study [285]. Although metagenomic assembly methods provide more information on ARGs, they are not quantitative and do not allow abundance calculations [275].

Application of long read sequencing methods, such as the PacBio and Nanopore technologies, in metagenomic and resistome studies would permit simultaneous quantitative and qualitative

studies of ARGs. The extra-long sequence reads produced by Oxford Nanopore Technologies (ONT), ranging from 10 to 50 kbp and sometimes up to 100 kbp [287], could be used directly without assembly to detect and quantify ARGs in their taxonomic and genomic contexts. Another advantage of ONT sequencing is the rapid and real-time generation of data [287] [288]. This is particularly important in pathogen or resistance surveillance studies, where sample to result time should be minimal [288, 289]. However, the accuracy of this sequencing technology is only 90-95% [290], significantly less than that of Illumina sequencing. Assembly-free targeted gene detection studies would be minimally affected by this lower accuracy, given that appropriate bioinformatic pipelines can be used. An online computational resource known as NanoARG was launched in 2019 and this tool can be used to detect, quantify and study ARGs in their genetic context using ONT long sequence reads [291]. A combination of ONT and Illumina technologies could be used in studies that require greater detail, such as detection of mutations in ARGs or novel resistance genes.

5. Research aims

Despite the importance of veterinary hospital environments as a source of AMR of clinical importance in humans and animals, exploration of antimicrobial resistances in these settings is mostly limited to studies focusing on specific nosocomial pathogens, such as methicillin-resistant *Staphylococcus* species. The overall abundance and diversity of environmental ARGs and resistant bacteria have not been described. The genetic characterization of nosocomial pathogens allows to enhance our understanding of the types, patterns and transmissibility of AMR these organisms possess. Improved understanding of veterinary hospital environmental resistomes is likely to assist in derivation or improvement of practice-specific environmental surveillance and infection control programmes to mitigate the risk of spread of antimicrobial-resistant nosocomial pathogens.

Of the currently available methods, metagenomic sequencing using the ONT long read

technology appears to be the best option for studying the resistomes present in veterinary clinical environments. Both quantitative and qualitative data can be obtained rapidly and in real-time for a reasonable cost using this technology. Moreover, the genomic context of the ARGs can be studied directly and this knowledge is extremely useful for determining the clinical risks associated with different ARGs. In spite of these benefits this method has not been applied previously for this purpose.

The work presented in this thesis aimed:

- To establish a reproducible protocol to explore the resistomes associated with veterinary hospital environments and to attribute the antibiotic resistance genes that were detected to their corresponding bacterial hosts or genetic origin using Nanopore sequencing. Environmental samples obtained from different sites in a veterinary hospital were tested using this protocol to evaluate the significance of differences in ARG profiles in relation to their clinical risk, their co-occurrence in their bacterial host and the potential for transmission between microorganisms, with the aim of improving infection control procedures in this facility.
- To phenotypically and genotypically characterise an ESBL-producing, MDR bacterial isolate recovered from the environment of a veterinary hospital. The persistence of this organism in the environment, the important ARGs it carried, their genetic context and the potential for horizontal dissemination of them were evaluated.

6. References

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Chapter 2: Exploration of antibiotic resistance risks in a veterinary teaching hospital with Oxford Nanopore long read sequencing

1. Introduction

Environmental surveillance is a crucial element in effective infection control and prevention programmes in hospitals. Most environmental surveillance programmes rely on culture-dependent methods, but the information derived from such methods is limited as they can only target few organisms and resistance types because they require considerable resources and time. Furthermore, the potential for dissemination of the ARGs cannot be inferred using culture-based methods. Molecular techniques particularly long read sequencing based methods, can be used to overcome these limitations. This chapter describes a feasible metagenomic sequencing based workflow (diagram 1) that can be used to profile ARGs, determine their bacterial hosts, and assess their association with MGEs and co-location with other ARGs in the environmental samples using ONT MinION technology.

The publication included in this chapter (section 2.2) describes the validation of this workflow and its application to explore environmental resistomes in an Australian veterinary referral hospital. The ARG profiles and their relative abundances obtained with this workflow were confirmed using a high-throughput qPCR array. The taxonomic classifications were compared with results from 16S rRNA (Illumina) sequencing and metagenomic assembly (Illumina). Assembled Illumina contigs were used to validate the genetic contexts of the ARGs obtained with the ONT long read sequence data. Culture-enriched metagenomic DNA samples originating from four environmental sites of the veterinary hospital were then examined for the presence, abundance and clinical importance of ARGs using the validated workflow. The knowledge derived from this work was used to

improve infection control and prevention strategies in the veterinary hospital.

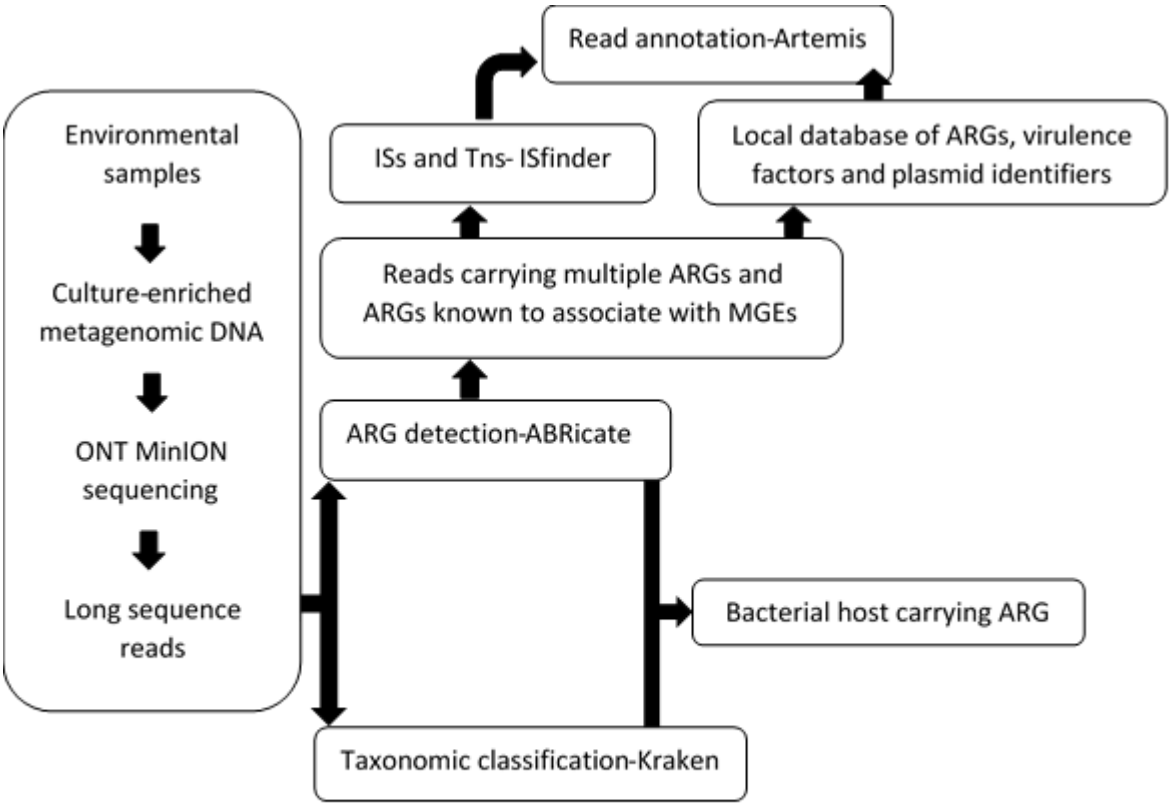

Diagram 1. ONT MinION workflow

2. Publication

PLOS ONE

RESEARCH ARTICLE

Exploration of antibiotic resistance risks in a veterinary teaching hospital with Oxford Nanopore long read sequencing

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Abstract

The Oxford Nanopore MinION DNA sequencing device can produce large amounts of long sequences, typically several kilobases, within a few hours. This long read capacity was exploited to detect antimicrobial resistance genes (ARGs) in a large veterinary teaching hospital environment, and to assess their taxonomic origin, genetic organisation and association with mobilisation markers concurrently. Samples were collected on eight occasions between November 2016 and May 2017 (inclusive) in a longitudinal study. Nanopore sequencing was performed on total DNA extracted from the samples after a minimal enrichment step in broth. Many ARGs present in the veterinary hospital environment could potentially confer resistance to antimicrobials widely used in treating infections of companion animals, including aminoglycosides, extended-spectrum beta-lactams, sulphonamides, macrolides, and tetracyclines. High-risk ARGs, defined here as single or multiple ARGs associated with pathogenic bacterial species or with mobile genetic elements, were shared between the intensive care unit (ICU) patient cages, a dedicated laundry trolley and a floor cleaning mop-bucket. By contrast, a floor surface from an office corridor without animal contact and located outside the veterinary hospital did not contain such high-risk ARGs. Relative abundances of high-risk ARGs and co-localisation of these genes on the same sequence read were higher in the laundry trolley and mop bucket samples, compared to the ICU cages, suggesting that amplification of ARGs is likely to occur in the collection points for hospital waste. These findings have prompted the implementation of targeted intervention measures in the veterinary hospital to mitigate the risks of transferring clinically important ARGs between sites and to improve biosecurity practices in the facility.

Introduction

In bacterial populations, the resistome is defined as "the collection of all genes that could contribute to a phenotype of antibiotic resistance" [1]. In human hospitals, monitoring of patient and environmental microbiomes has revealed complex patterns of surface colonisation and pervasive exchanges of resistance genes [2, 3]. While animal-associated microbiomes and resistomes have been studied in livestock flora [4, 5] as well as in farm environments and effluents [6–9], relatively few investigations have been carried out in veterinary hospitals. Large veterinary teaching hospitals accommodate a transient population of animals with various resident flora, infectious status, and previous exposure histories to antibiotics. This unique environment could act as a mixing reservoir for antimicrobial resistance genes (ARGs) to and from various sources including humans and animals. Most veterinary teaching hospitals have active and passive surveillance programs to monitor infectious risks and antimicrobial resistance trends associated with their patients [10], but the presence and diversity of ARGs in the hospital environment is often not explored. Therefore, we sought to determine the presence of ARGs in such environmental systems and to map their associations with their bacterial hosts or mobile genetic elements (MGEs).

Surveillance studies for ARGs have benefited from the rapid advances in next generation sequencing (NGS) technologies, which overcome the composition bias or limited detection capacity commonly associated with culture or PCR based methods [11, 12]. The Illumina NGS technology is cost effective and has low error rates, but its shorter read length makes the assembly and analysis of contiguous genomic sequence regions containing several, co-localised ARGs and/or MGEs challenging [13]. In contrast, long reads produced by the Oxford Nanopore Technologies MinION portable sequencer [14] readily permit the discovery of co-localised ARGs and their flanking genomic nucleotide sequences. This additional information is critical for correctly assigning reads to their taxonomic classification node and to provide insights into the bacterial host of the ARGs that are detected. The MinION sequencing technology has been employed previously to profile the gut resistome of a human patient, by functional metagenomics analysis of a plasmid expression library [15]. However, nanopore sequencing has not been applied to resistome analysis directly in veterinary environmental samples.

Here, a reproducible protocol was established to explore the resistomes associated with a veterinary hospital environment and to attribute the detected ARGs to their corresponding bacterial hosts or genetic origin using nanopore sequencing. The taxonomic classifications and quantitative detection of ARGs from MinION data were compared to reference methods, i.e. 16S rRNA analysis, WaferGen qPCR arrays for ARGs, Illumina sequencing and bacteriological culture surveys, to confirm the nanopore sequencing results. Finally, four MinION long read datasets obtained from different parts of the veterinary hospital environment were analysed to evaluate the significance of ARG profiles in relation to their clinical risk, co-occurrence in their bacterial host and potential for transmission between microorganisms, with the aim of improving infection control procedures in veterinary facilities.

Materials and methods

Sampling

This longitudinal study was carried out in the U-Vet Werribee Animal Hospital of The University of Melbourne between November 2016 and May 2017 (inclusive). Sampling was conducted across four environmental sources. The inner surfaces of patient cages in the intensive care unit (ICU cages) and the inner surfaces of a plastic laundry trolley (LT) were sampled on

eight independent occasions. The trolley, which is used to collect soiled bedding from the ICU cages but is kept outside the ICU room, was swabbed on the same days as the ICU cages. The liquid contents in a mop bucket (MB) used to clean the ICU floor were collected on eleven independent occasions. The floor of an office corridor (OC) with controlled access, no animal contact and limited foot traffic by the veterinary hospital staff was sampled once. A sterile gauze swab was moistened with buffered peptone water (BPW, Oxoid) and used to wipe approximately 1 m^2 from the hard surfaces (ICU cages, LT, OC). On each occasion an average of 4 (range 2 to 5) occupied ICU cages were swabbed and the swabs were pooled in a single sterile container. Pooled ICU cage swabs and single swabs from the LT and OC were transferred to the laboratory within 5 to 10 minutes of collection and processed immediately. The swabs were mixed with 100 mL BPW. The liquid contents (50 mL) from the MB were collected using sterile syringes, transferred into sterile containers and 75 ml of BPW was added. Aliquots of the suspensions were removed for culture (see below) and 20 mL aliquots were incubated at 30˚C for 16 hours without shaking to reach an OD_{600nm} of 0.8.

Bacterial cultures and phenotypic testing for antibiotic resistance

Aliquots (100 μL) from sample suspensions were ten-fold diluted with BPW and plated on Mueller Hinton (MH) agar without antibiotics, to estimate the total counts of viable bacteria. Undiluted sample suspensions (100 μL) were plated on MH agar containing ampicillin 50 μg/mL, enrofloxacin 2 μg/mL and gentamicin 15 μg/mL to select for multi-drug resistant organisms (MDR), and on Brilliance ESBL agar (Oxoid, UK) to select for extended spectrum beta lactamase (ESBL) producing organisms. The antibiotic concentrations used in MH agar plates were chosen empirically, based on routine antimicrobial susceptibility testing results observed in our diagnostic laboratory and the EUCAST database of MIC distributions in common pathogenic bacteria, available from the organisation website (http://www.eucast.org/). The plates were incubated for 24 to 48 hours at 37˚C.

DNA extraction

Cells from 20 mL of BPW cultures were collected by centrifugation at 1700 × *g* for 30 minutes at 4˚C on Allegra X-22R centrifuge with SX4250 swing bucket rotor (Beckman Coulter, USA). Genomic DNA was extracted from the cell pellets with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). After resuspension, one half of the cells were processed with the manufacturer's recommended protocol for Gram positive bacteria while the other half were processed with the protocol for Gram negative bacteria. The DNA concentration was measured by Qubit fluorometer (Invitrogen, USA) and the quality was determined by microspectrophotometry (NanoDrop ND-1000, NanoDrop technologies, Wilmington, DE). For each sampling occasion, equal volumes of Gram positive and Gram negative bacterial DNA extracts were mixed and cleaned by 1× SPRI beads (AMPureX, Beckman Coulter, CA, USA).

Library preparation, sequencing and read processing

The MinION sequencing libraries were prepared with the 1D genomic DNA sequencing kit SQK-LSK108 (Oxford Nanopore Technologies (ONT), UK). Briefly, 1–4 μg DNA was sheared into 8 kb fragments using g-tubes (Covaris, Brighton, UK) at 2539 × *g* for 1 minute, nicks were repaired with the Formalin-Fixed Paraffin-Embedded (FFPE) enzyme mix (New England BioLabs, USA), followed by end repair, dA-tailing and adaptor ligation, with or without barcoding. The sequencing was done in a portable MinION sequencing device, with R9.4 or R9.5 flow cells (ONT, UK). Raw reads were basecalled into fastq or fast5 files with the program Albacore version 2.1.7 (ONT, UK) unless specified otherwise. The fast5 files were converted to

fastq format using poretools version 0.6.0 [16]. Sequences were filtered with the program Fast5-to-Fastq [17] to select reads with a Phred quality score of ≥8 and read length of ≥250 bases. Filtered reads were then converted into fasta format using the program Fastaq [18]. A detailed explanation of the sequencing runs schedule is given in the S2 Table.

The Illumina sequencing libraries were prepared according to the TruSeq DNA v1.0 protocol. First, the DNA ends were repaired followed by A-tailing, index and adaptor ligation. Then the ligated libraries were enriched by PCR. Finally, the indexed and enriched libraries were pooled before sequencing in a NextSeq benchtop sequencer to obtain paired-end reads of 150 bp (Walter and Eliza Hall Institute of Medical Research, Victoria, Australia). The program Trim Galore version 0.4.4 [19] was used to remove the adaptors from the reads and to filter out the reads having a Phred quality score of <20. The adaptor trimmed and quality filtered fastq reads were assembled using the MEGAHIT version 1.1.4 [20].

Evaluation of microbial community composition

The Kraken taxonomic classifier version 1 [21] was used with the MiniKraken 2014 database [22] which contained complete bacterial, archaeal and viral genomes from RefSeq. Kraken outputs were then converted to show full taxonomic lineages using the script kraken-translate [21]. The program Centrifuge [23] was used with its own indexed bacterial and archaeal database. After taxonomic classification, reads with Centrifuge quality scores of less than 300 and less than 50 bp match lengths [23] were filtered out. Full taxonomic lineage for the Centrifuge output (NCBI taxonomic IDs) were obtained from the NCBI taxonomy website [24] and reports were combined using R version 3.4.0 [25]. Rarefaction curves were computed using the Kraken and Centrifuge outputs and using the contributed 'vegan' package version 2.4–5 [26] in R.

The 16S rRNA sequencing and diversity profiling was performed by the Australian Genome Research Facility (AGRF, Australia). The V3 and V4 hypervariable regions of 16S rRNA gene were PCR amplified using established universal forward 341F (`CCTAYGGGRBGCASCAG`) and reverse 806R (`GGACTACNNGGGTATCTAAT`) primers [27, 28]. The V1 and V3 hypervariable regions were amplified using established universal forward 27F (`AGAGTTTGATCMTGGCTCAG`) and reverse 519R (`GWATTACCGCGGCKGCTG`) primers [29]. The Illumina MiSeq platform was used with Illumina's Nextera XT Index Kit and Paired End Sequencing Chemistry. The sequence reads were analysed as described previously [30]. Briefly, overlapping paired reads were merged by aligning forward and reverse reads using USEARCH 8.1 [31] and seqtk toolkit was used to trim the primer sequences from the read-ends [32]. Then merged reads were filtered for length >400 bp and quality of <1 expected error [33], followed by clustering into operational taxonomic units (OTUs) [34] using USEARCH 8.1 [31]. The QIIME 1.9.1 pipeline was used to assign the representative sequences from each OTU into relevant taxa [35]. The taxonomy assignment script "assign_taxonomy.py" from QIIME was used with the Greengenes database [36] version 13_5.

Detection and quantification of antimicrobial resistance genes (ARGs)

ARGs were identified within MinION long read fasta sequences and Illumina contigs using ABRicate [37] and the following databases: (i) in-built antimicrobial resistance gene database, Resfinder [38], which contain 2228 ARG sequences; (ii) simplified/non-redundant subset of Resfinder ARGs containing 646 sequences; and (iii) a custom database of 16S rRNA sequences used to normalise the ARG counts. The non-redundant Resfinder ARG subset was constructed by clustering the Resfinder ARG database sequences with >90% nucleotide identity using the package CD-HIT-EST in program CD-HIT [39]. The custom-made 16S rRNA database

(rdpBac) was derived from 11,988 16S rRNA gene sequences downloaded from the Ribosomal Database Project [40], release 11. The BLAST hits having <70% coverage and <80% identity with the gene sequences from ABRicate databases were excluded from the analysis.

DNA samples were further analysed with a high capacity WaferGen SmartChip Real-time qPCR array (WaferGen Biosystems, Fremont, CA, USA). In this array, a total of 296 validated primer sets were used, including 285 primers targeting ARGs, 10 primers targeting MGEs and 1 primer targeting 16S rRNA gene [41, 42]. The relative abundances of ARGs and MGEs were calculated relative to the 16S rRNA gene using the $2^{-\Delta CT}$ method [43].

Taxonomic classification of individual long reads containing ARGs

For each sample, the standard kraken-translate output file, which contains the sequence read ID and the assigned taxonomic lineage was merged with each ABRicate ARG output file using the sequence read ID as the key in R version 3.4.0 [25]. The combined output was used to attribute ARGs to their corresponding bacterial hosts that were detected in the veterinary hospital environmental samples. Bubble plots were constructed with the package ggplot2 version 3.1.0 in RStudio version 1.0.143. Reads carrying ARGs known to occur in MGEs and reads carrying multiple ARGs were analysed using the BLASTN function in ISfinder server using a cut-off e-value of $1e^{-5}$ [44]. Nucleotide BLAST version 2.2.31+ was used to search a local BLAST nucleotide database of 8675 sequences, made by combining all antibiotic resistance genes, virulence factors and plasmids sequences provided by the program ABRicate [37]. The software Artemis [45] was used to annotate the reads based on the ISfinder and BLASTN outputs.

Statistical comparison of WaferGen and MinION results

The primersearch function in Jemboss 1.5 [46] was used to search WaferGen primer pairs with matches in the DNA sequences in Resfinder database. For each ARG having a representative sequence in ResFinder and a cognate PCR primer pair in the WaferGen array, the gene abundance was first normalised against the 16S rRNA gene abundance values calculated from the long reads analysis and the WaferGen results. Then, the differences between each of the normalised gene abundances and the 16S rRNA gene abundance were compared using Spearman's rank correlation coefficient in R version 3.4.0.

Results and discussion

Larger amounts of input DNA result in higher sequencing yields

The surface swabs collected throughout the veterinary hospital typically contained 10^4 to 10^5 total bacteria, based on colony counts on MH plates without antibiotics. As these samples did not provide sufficient biomass to extract the recommended amount of DNA for a standard MinION library preparation (at least 1 μg), the bacterial population was amplified by an incubation step for 16 hours at 30˚C in BPW. While this step is expected to reduce the taxonomic diversity of the initial sample, it was deemed that bacteria usually associated with major infectious risks in veterinary hospitals (e.g. *Enterobacteriaceae*, *Staphylococcus* sp.) are also the most likely to be recovered by this enrichment process and are therefore the most relevant organisms to consider in this study.

Different strategies for library preparation were tested to optimise the yield of sequenced long reads per run, using DNA extracts from the ICU cages and LT in 3 and 2 independent sequencing reactions, respectively. Increasing the amount of DNA in the pre-sequencing mix well above the recommended minimum of ~50–200 ng (up to ~900 ng) resulted in a proportional expansion of the total sequence outputs in Gbp over a comparable time frame of 16

Table 1. Input DNA amounts versus sequencing yields.

Sequencing library	DNA amount used for preparing library (μg)	DNA amount loaded on flow cell (ng)	Flow cell chemistry	Run time (hours)	Cumulative yield (gigabases)[a]	Cumulative yield (number of reads)[a]
ICU1	0.7	174	R 9.4	16	0.87	175,000
ICU2	1.2	322	R 9.5	27	1.45	275,000
ICU3	4	630	R 9.5	48	2.75	600,000
LT1	1.2	316	R 9.4	16	1.20	175,000
LT2	4.9	910	R 9.5	16	4.15	750,000

[a] values recorded after 16 hours of sequencing

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hours (Table 1). Thus, high DNA input, along with the advantages of nanopore sequencing libraries such as reduced preparation time and the longer read lengths [47] should allow to capture as many as possible microbial genomic data from the sample, a desirable aim in this type of study [48].

Nanopore sequencing is a reliable method to explore environmental microbiota

To validate the taxonomic assignments obtained with MinION sequencing, the microbial compositions of two aggregates of 19 ICU cage swabs and 4 LT swabs, were inferred from MinION long read data using the Kraken or Centrifuge classifiers and compared to sequence analysis results of 16S rRNA regions V1-V3 and V3-V4 from the same DNA extracts.

In the ICU cages and LT, all four methods (MinION-Kraken, MinION-Centrifuge, 16S/V1-V3, 16S/V3-V4) detected a similar composition of taxa at the phylum, class, and order levels (Table 2). At the family level, the four methods were concordant except for reads assigned to the family *Enterococcaceae* in the ICU cages, which composed approximately 21% of the reads according the MinION-Kraken, MinION-Centrifuge and 16S/V3-V4 but only 2% with the 16S/V1-V3 analysis. At the genus level, the MinION-Kraken and MinION-Centrifuge assigned taxonomic classification to 83% and 94% of the ICU cages reads, respectively. In contrast, 16S/V1-V3 and 16S/V3-V4 sequencing of the ICU cages sample successfully classified only 22% and 37% of the reads, respectively; a similar pattern was observed for the LT samples (Table 2). These results confirmed previous studies suggesting that long read nanopore sequencing provides better resolution at lower taxonomic levels compared to amplicon-based, short-read 16S rRNA sequencing [49–52]. Moreover, there was a lower agreement between 16S/V3-V4 and 16S/V1-V3 rRNA sequencing than between long read classifier tools, possibly due to known amplification biases between the two conserved 16S rRNA primer sets [53]. These results were confirmed by comparing the MinION long reads directly obtained from the ICU cages and LT against the MEGAHIT contigs assembled from Illumina reads using the same DNA preparations. The Kraken taxonomic assignments for both datasets were in agreement, further demonstrating the reliability of the MinION-Kraken approach.

A prior knowledge of the microbial community composition can improve 16S rRNA experimental design [54] but this information is not always available. Moreover, in a veterinary hospital environment, microbial communities are expected to represent several interconnected microbial eco-systems. As an example, the patient cages in ICU may contain microorganisms coming from animal skin, saliva, urine and faeces together with soil and other environmental microorganisms. These organisms contaminate bedding which in turn can contaminate the laundry trolley, and the mops used to clean the floor of the room. This makes nanopore sequencing an attractive approach to track ARGs and infectious risks in veterinary hospitals.

Table 2. Percentages of reads assigned at different taxonomic levels with four classification methods.

Taxa	% of reads assigned a taxonomic classification[a]							
	ICU cages				Laundry trolley			
	16S/ V1-V3	16S/ V3-V4	MinION/ Kraken	MinION/ Centrifuge	16S/ V1-V3	16S/ V3-V4	MinION/ Kraken	MinION/ Centrifuge
Phylum								
Proteobacteria	69	53	60	62	73	60	71	70
Firmicutes	31	47	40	38	27	40	29	30
Class								
Bacilli	27	42	39	37	18	30	26	27
Clostridia	5	5	1	1	9	10	3	3
Gammaproteobacteria	69	53	59	62	72	60	70	70
Order								
Bacillales	13	22	17	17	17	29	23	25
Clostridiales	5	5	1	1	9	10	3	3
Enterobacteriales	57	44	50	51	71	59	68	68
Lactobacillales	14	20	22	20	1	2	3	2
Pseudomonadales	11	9	9	10	1	1	2	2
Family								
Bacillaceae	9	16	14	14	14	22	22	24
Clostridiaceae	3	3	1	1	4	5	3	3
Enterobacteriaceae	57	44	50	47	71	59	68	67
Enterococcaceae	2	20	22	20	1	2	3	2
Peptostreptococcaceae	2	2	0	0	5	4	0	0
Planococcaceae	2	5	0	0	3	6	0	0
Pseudomonadaceae	11	9	9	10	1	1	1	2
Staphylococcaceae	1	1	3	3	0	0	1	1
Unassigned	12	0	1	0	0	0	1	0
Genus								
Assigned	22	37	83	94	10	39	92	99
Unassigned	78	63	17	6	90	61	8	1

[a] data for <1% assignments with all four methods in both samples are not shown in the table

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Detection and quantification of ARGs by MinION long read sequencing is confirmed by targeted qPCR assays

To assess the capacity of long read data to accurately detect the most abundant ARGs within complex environments, the DNA extracts from an aggregate of 19 ICU cages and 4 LT swabs (see S2 Table for details) were analysed with MinION and WaferGen technologies. The MinION sequence reads were searched for ARGs by the program ABRicate with the database Resfinder containing 2228 sequences. Read statistics for the MinION datasets are reported in S3 Table. Separately, the same DNAs were amplified in a WaferGen qPCR array with 296 gene-specific primer pairs. Only the sequence targets represented in both methods were compared.

Respectively, 93% and 97% of the ARGs detected in ICU cages and LT samples by MinION sequencing were also found with the WaferGen qPCR array. The normalised abundances of ARGs relative to the 16S rRNA gene were highly correlated between the two methods, with a Spearman's rank correlation coefficient of 0.67 for the ICU cages and 0.76 for the LT (Fig 1). The WaferGen qPCR array detected respectively 66 and 60 ARGs with a Ct value of 27 in the

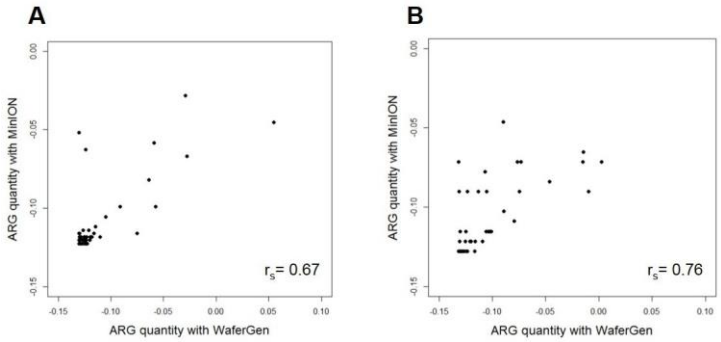

Fig 1. Comparison of WaferGen ARG $2^{-\Delta CT}$ values with MinION ARG counts (hits) with reference to the 16S rRNA gene. (A) ICU cages (B) Laundry Trolley. X axis: normalised differences in $2^{-\Delta CT}$ values of ARGs when compared to 16S rRNA $2^{-\Delta CT}$ value obtained with WaferGen; Y axis: normalised differences in counts of ARGs when compared to the 16S rRNA count obtained with program ABRicate using MinION sequence data.

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ICU cages and LT samples. Out of those, respectively 39 and 28 genes were also found in the MinION sequence datasets. The ARGs that were not found with the MinION sequencing but were detected by the WaferGen qPCR array were present in the sample at a very low relative abundance (0.08% or below) in the population, suggesting that qPCR methods were more sensitive for detecting low abundance ARG targets.

However, respectively 37/107 (35%) and 26/59 (44%) ARGs detected in the ICU cages and LT by MinION sequencing were not included in the WaferGen array. Some of these genes, such as *blaOXA*, confer resistance to clinically important beta lactam antibiotics and are known to be present on MGE as well [55]. Although different primer pairs could be included, the array capacity is currently limited to a maximum of 384 reactions, underlining the advantage of using a metagenomic approach to detect ARGs in complex samples. While nanopore sequencing has a low accuracy (typically 80–90%), it is expected that the relatively random distribution of errors within reads should not adversely impact the identification of most ARGs by ABRicate, which is based on BLASTN alignments and can tolerate some degree of sequence mismatches. This hypothesis was supported by examining alignments between individual nanopore reads that putatively contained ARGs and corresponding sequences from the resfinder database. Although a number of mismatches, attributable to sequencing errors, affected the overlapping region of the published ARG and the long read fragment, the alignement scores clearly indicated the presence of an ARG in the read.

A majority of environmental ARGs can be linked to a bacterial genus or a mobile genetic element

A recently developed ranking scheme [56] has proposed that ARGs found in a clinically relevant pathogen or in a MGE should be classified as high-risk ARGs, while resistances known to be intrinsic for a particular bacterial species will be considered only as markers for the presence of those bacteria. All reads containing ARGs were searched for information on their taxonomic or genetic origin in the adjacent sequences to evaluate the risks associated with ARGs in the veterinary hospital environment. By using this approach, it is possible to detect the bacterial host of the ARGs present on chromosomal sequences which are long enough to assign a taxonomic origin to the read. Although this method does not warrant the precise taxonomic identification of the bacterial hosts of plasmid sequences, it could point to a group of prokaryotes as the presumptive host for the element, and its potential for dissemination in bacterial populations.

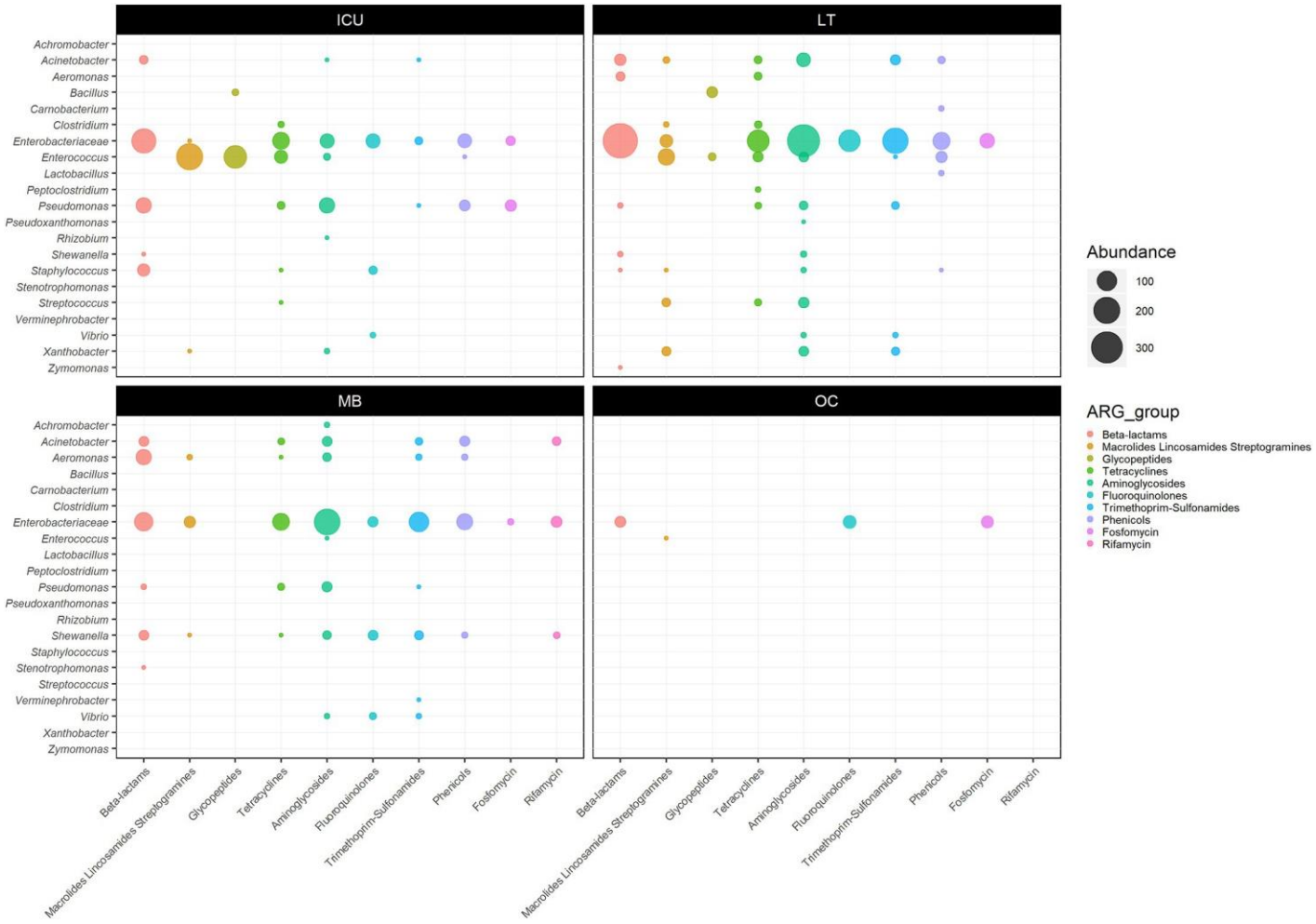

Fig 2. Abundance of bacterial reads carrying ARGs relative to their taxonomic origin in the ICU cages (ICU), Laundry Trolley (LT), Mop Bucket (MB) and Office Corridor (OC).

https://doi.org/10.1371/journal.pone.0217600.g002

Overall, 77% (1001/1299), 65% (1688/2592), 38% (813/2166) and 99% (80/81) of the ARGs detected respectively in the ICU cages, LT, MB and OC samples were also classified into a bacterial genus or family by the program Kraken. The lower rate of taxonomic assignment in MB sample might be due to the shorter read lengths observed in that particular sequencing library (i.e. mean read length of ~2 kb as opposed to ~5 kb in the other libraries). Many ARG-carrying taxons identified in the veterinary hospital fell into groups commonly found in animal flora, e.g. *Enterobacteriaceae*, *Enterococccus* and *Staphylococcus* (Fig 2 and S1 Table). The taxonomic classifications assigned by the Kraken and Centrifuge at the genus level were mostly consistent, except for some ARG-containing reads that were assigned by the two programs to different genera of *Enterobacteriaceae*. To ensure accuracy, these reads were reported at the family level only.

Respectively 23%, 67% and 65% of the ARGs-containing reads from the ICU cages, LT and MB samples also carried nucleotide sequences matching transposons, transposases and/or insertion sequences. Furthermore, the majority of aminoglycoside, tetracycline, trimethoprim or sulphonamide resistance genes were associated to MGEs (Table 3). Respectively 32%, 53% and 24% of beta lactam resistance genes detected in the ICU, LT and MB samples were

Table 3. Antimicrobial resistance genes associated with mobile genetic elements in the veterinary hospital.

Gene	n (N)[a] in Sample[b]		
	ICU	LT	MB
AACs[c]	**4 (4)**	**76 (77)**	**49 (70)**
aac(3)-IIa	-	19 (20)	28 (30)
aac(3)-IVa	-	4 (4)	-
aac(6')-aph(2'')	-	14 (14)	-
aac(6')-IIc	4 (4)	38 (38)	1 (1)
aac(3)-VIa	-	1 (1)	-
aac(6')-Ic	-	-	20 (39)
ANTs[c]	**5 (5)**	**71 (71)**	**19 (19)**
aadA1	-	24 (24)	18 (18)
aadA2	2 (2)	32 (32)	1 (1)
aadB	-	3 (3)	-
ant(6)-Ia	3 (3)	12 (12)	-
APHs[c]	**66 (71)**	**278 (278)**	**159 (159)**
aph(3')-Ia	4 (4)	77 (77)	4 (4)
aph(3')-IIa	24 (25)	-	-
aph(3')-III	2 (2)	12 (12)	-
aph(4)-Ia	-	6 (6)	-
aph(6)-Ic	24 (28)	-	-
aph(3')-VIa	-	2 (2)	-
strA	6 (6)	85 (85)	136 (136)
strB	6 (6)	96 (96)	19 (19)
ESBLs[c]	**84 (84)**	**208 (221)**	**20 (42)**
blaSHV	1 (1)	48 (61)	-
blaTEM	82 (82)	157 (157)	20 (20)
blaCARB	-	1 (1)	-
imiS	-	-	0 (22)
blaCTX-M-101	-	2 (2)	-
blaCTX-M-110	1 (1)	-	-
CATs[c]	**3 (3)**	**80 (82)**	**63 (85)**
cat(pC194)	-	14 (14)	-
cat(pC233)	1 (1)	12 (14)	-
catA1	1 (1)	8 (8)	-
catA2	1 (1)	37 (37)	14 (34)
catB8	-	9 (9)	46 (47)
catB3	-	-	3 (4)
DFRs[c]	**3 (3)**	**59 (59)**	**5 (5)**
dfrA1	-	5 (5)	-
dfrA12	-	12 (12)	-
dfrA18	-	15 (15)	-
dfrA30	3 (3)	11 (11)	-
dfrA32	-	2 (2)	-
dfrA7	-	10 (10)	-
dfrA10	-	1 (1)	-
dfrA13	-	1 (1)	-
dfrA15	-	2 (2)	-
dfrA14	-	-	5 (5)

(*Continued*)

Table 3. (Continued)

Gene	n (N)[a] in Sample[b]		
	ICU	**LT**	**MB**
MLSs[c]	**3 (3)**	**62 (64)**	**14 (14)**
ere(A)	2 (2)	29 (29)	1 (1)
mph(A)	-	6 (6)	12 (12)
mph(E)	-	1 (2)	-
erm(Q)	-	1 (1)	-
erm(B)	1 (1)	22 (23)	-
msr(E)	-	2 (2)	1 (1)
lnu(B)	-	1 (1)	-
QNRs[c]	**2 (17)**	**35 (36)**	**13 (20)**
QnrB10	0 (5)	20 (21)	-
QnrB12	0 (10)	11 (11)	-
QnrS1	-	4 (4)	-
QnrVC3	2 (2)	-	13 (20)
SULs[c]	**6 (6)**	**162 (163)**	**109 (118)**
sul1	2 (2)	134 (134)	29 (29)
sul2	4 (4)	28 (29)	80 (89)
TETs[c]	**54 (68)**	**108 (124)**	**46 (48)**
tet(A)	21 (21)	10 (10)	17 (17)
tet(B)	9 (9)	49 (49)	26 (28)
tet(C)	-	3 (3)	1 (1)
tet(D)	1 (1)	29 (33)	1 (1)
tet(E)	-	5 (7)	1 (1)
tet(L)	0 (1)	7 (10)	-
tet(M)	0 (4)	4 (11)	-
tet(O)	23 (32)	-	-
tet(X)	-	1 (1)	-
ARR[c]	**-**	**-**	**33 (34)**
ARR-3	-	-	33 (34)
Total	**228 (262)**	**1139 (1175)**	**530 (614)**

[a] Read counts: n = number of sequences showing significant similarities with the sequences of known MGEs; N = number of total sequences assigned to a particular ARG by ABRicate

[b] Sample: ICU: Intensive Care Unit; LT: Laundry Trolley; MB: Mop Bucket. ISFinder e-value: 1×10^{-5}

[c] Presence confirmed with Illumina sequencing

AACs: aminoglycoside acetyltransferases; ANTs: aminoglycoside nucleotidyltransferases; APHs: aminoglycoside phosphotransferases; ESBLs: extended spectrum beta-lactamases; CATs: chloramphenicol acetyltransferases; DFRs: dihydrofolate reductases; MLSs: macrolides, lincosamides, and streptogramins resistance genes; QNRs: quinolone resistance genes; SULs: sulfonamide resistance genes; TETs: tetracycline resistance genes; ARR: rifampin ADP-ribosyltransferase

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classified as Extended Spectrum Beta Lactamases (ESBLs). Again, most of these ESBL genes were also located next to MGEs, with the exception of the carbapenem resistance gene *imiS*, which belongs to Ambler Class B metallo-beta-lactamases [57], found in the MB sample.

In addition to high-risk ARGs, intrinsic resistance genes were also detected in all four samples. For instance in the ICU cages sample, 178 sequences classified into *Enterococcus faecalis* carried the *lsa* gene [58]. Similarly, 11 sequences matching the *msrC* gene were detected in

reads classified into *Enterococcus faecium* and 146 vancomycin resistance genes were carried by the *Enterococcus casseliflavus* intrinsic gene cluster *vanC, vanRC, vanSC, vanTC and vanXYC* [58]. Moreover, the *blaOXA-50* which occur naturally in *Pseudomonas aeruginosa* [59, 60] and *blaLEN* genes which are present on the chromosome of the *Klebsiella pneumoniae* isolates and lack the ability to develop into ESBL genes [61, 62] were identified.

Finally, none of the clinically relevant ARGs found in the veterinary hospital were detected in the OC. In this sample, 13 *oqxA* and 18 *oqxB* genes carried by *Klebsiella pneumoniae* were found, confirming that this species is a reservoir for *oqxAB* [63, 64]. The *fosA* resistance gene in *Klebsiella* and *Enterobacter* species [65] was also detected. These ARGs were located on chromosomal sequences and were not associated with MGEs. By contrast, the Illumina/MEGAHIT contigs which carried ARGs were often too short to provide the same level of insight on their taxonomic or genetic origin.

These results must take into account that using nanopore sequencing alone could underestimate the diversity of functional ARGs in the sample. Given the error rates of this method, it might not be possible to differentiate highly similar ARGs derived from a common ancestor. To address this issue, a thorough analysis of metagenomic sequence assemblies that combine long nanopore reads and short accurate Illumina reads is required.

Phenotypic resistance patterns of bacteria recovered from the veterinary hospital environment support MinION sequencing results

Bacteriological cultures of environmental samples from the veterinary teaching hospital confirmed the presence of bacteria displaying multiple drug resistance (MDR) to ampicillin, enrofloxacin and gentamicin, as well as ESBL producing phenotypes, which were predicted by the MinION sequencing data from the same samples (Table 4). As an example, a MDR and ESBL producing *Klebsiella* species was isolated from the LT sample in which the *Klebsiella*-associated ARGs *qnrB*, *aadA* and *blaSHV* sequences were detected. In the MB sample, oxidase positive Gram negative rods with a MDR phenotype were grown on selective agar plates, in accordance with the finding of sequencing reads classified as *Pseudomonas*, *Aeromonas* and *Shewanella* species carrying enrofloxacin, gentamicin and ampicillin ARGs. Finally, *Staphylococcus* species resistant to enrofloxacin and ampicillin were detected in the ICU cages sample by culture; MinION results also indicated the presence of *Staphylococcus* species carrying *norA* and *blaZ* genes. While the complete bacteriological identification of all species cultured from these samples was beyond the scope of this study, these preliminary observations broadly support the use of nanopore sequencing data as a means for monitoring bacterial resistance profiles in a veterinary hospital environment.

Laundry Trolley captures and amplifies the majority of ARGs present in the ICU cages

Saturation trends of the OTU rarefaction curves indicated that all datasets had sufficient sequencing depth to capture the diversity of the microbial population present in each sample (S1 Fig), allowing the comparative analysis of ARG compositions between samples. The ICU cages, LT, MB and OC samples were estimated to contain 77, 101, 51 and 8 categories of ARGs, respectively. The ABRicate analysis of both MinION long reads and assembled Illumina contigs resulted in the same profile of major ARG categories (Table 3). This confirms the fact that higher error rates in MinION sequence data did not adversely affect the accurate assignment of ARGs. The ICU cages shared 66% of their ARGs with the LT, but only 25% with the MB and 6% with the OC (Fig 3A). Furthermore, the ICU cages shared 77% of high-risk ARGs, as defined by Martinez et al. [56], with the LT, 41% with the MB, and none with the OC sample

Table 4. Comparison of phenotypic and genotypic detection of antimicrobial resistance in the veterinary hospital.

Sample	Conventional bacteriology		MinION sequencing	
	Organism	**Resistance phenotype**	**ARG (ABRicate)**	**Genus (Kraken)**
ICU	*Enterococcus*	MDR	*(aph(3')-III; ant(6)-Ia)*[a]	*Enterococcus*
	Staphylococcus	MDR	*blaZ*	*Staphylococcus*
			norA	
LT	*Klebsiella*	MDR ESBL	*(QnrB; aadA; blaSHV)*[a]	*Klebsiella*
			(blaCTX-M; QnrS)[a]	
			(blaTEM; aac(3)-IIa)[a]	
			(strA; strB; aph(3')-Ia; blaSHV; aadA)[a]	
	Enterobacteriaceae	MDR ESBL	*(aadA; aac(3)-VIa; QnrB; blaTEM; blaOXA; aadA)*[a]	*Salmonella*
			(blaTEM; strA; strB; aph(3')-Ia)[a]	
			(strB; strA; QnrB)[a]	
			(QnrB; blaDHA)[a]	*Escherichia*
			(aph(3')-Ia; strA; strB)[a]	
			blaTEM	
			(blaTEM; strB; strA)[a]	*Citrobacter*
			blaCMY	
			QnrB	
			(blaCMY; strA; strB)[a]	*Enterobacter*
			QnrB	
	Enterococcus	MDR	*(aph(3')-III; ant(6)-Ia, aac(6')-aph(2''))*[a]	*Enterococcus*
MB	Oxidase positive Gram negative rods	MDR	*(strA; strB)*[a]	*Pseudomonas*
			aac(3)-IIa	
			blaTEM	
			(aac(3)-IIa; QnrVC3)[a]	*Shewanella*
			(blaOXA; aadA)[a]	
			(blaTEM; aac(3)-IIa)[a]	*Aeromonas*
			imiS	
			(strA; strB)[a]	
			blaOXA	
			blaMOX	
			blaFOX	
	KESC group	ESBL	*blaTEM*	*Klebsiella* *Enterobacter* *Serratia*

[a] multiple ARGs on the same sequence read. MDR; multidrug resistance to Ampicillin = > 50 μg/ml: Enrofloxacin = > 2 μg/ml: Gentamicin = > 15 μg/ml. ESBL; extended spectrum beta lactam resistance. KESC; *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter*. ICU: Intensive Care Unit; LT: Laundry Trolley; MB: Mop Bucket

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(Fig 3B). The high-risk ARGs shared between the ICU and other sites included aminoglycoside, sulphonamide, trimethoprim, macrolide, chloramphenicol, and tetracycline resistance genes, as well as ESBLs. The relative abundance of ARGs was highest in the MB sample, with 0.89 copies per total sequence mega base, followed by LT (0.54), ICU cages (0.22) and OC (0.10). High-risk ARGs followed a similar pattern, with 0.83, 0.45 and 0.09 copies per total sequence mega base in the MB, LT and ICU cages samples, respectively. Moreover, higher relative abundances in the LT and MB samples compared to the ICU cages were observed for almost every category of ARG (Fig 4).

It is difficult to ascertain the impact of the culture enrichment step on the initial population collected, so the conclusions derived from our comparative analysis of relative abundances of

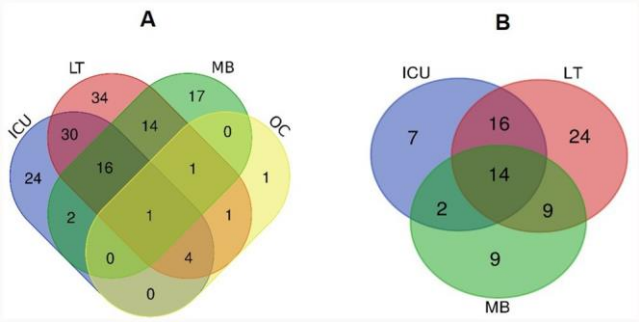

Fig 3. Venn diagrams showing the distribution of shared ARGs in the ICU cages (ICU), Laundry Trolley (LT), Mop Bucket (MB) and the Office Corridor (OC) samples. (A) Total ARG profiles; (B) High-risk ARG profiles (according to the ranking scheme from Martinez et al.).

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ARGs between samples must remain preliminary. However, it is interesting to note that bacterial compositions were similar between the ICU cages and the LT samples, whereas a different taxonomic diversity was observed in MB (Fig 5). These trends could be caused by recurrent contamination events followed by enrichment of resistant bacteria in favourable environmental conditions, and/or by the maintenance of different established microbial populations across the veterinary hospital. Therefore, a possible interpretation of our results is that high-risk ARGs, present at low levels in the immediate environment of the patients (cages), are amplified with their bacterial hosts in the LT, while selection pressures in the MB might create and maintain quite different populations and ARG profiles. To explore these questions, it would be useful to develop reliable culture-free DNA extraction protocols, with sufficient yields to undergo nanopore sequencing, directly from the environmental samples. Some low input DNA library kits and alternative sequence amplification strategies [66] offer promising solutions to address this problem and will be implemented in future experiments.

The patients kept in ICU often require antimicrobial treatments, and the residues of these drugs are likely to accumulate in the cage environment via urine, faeces and other biological

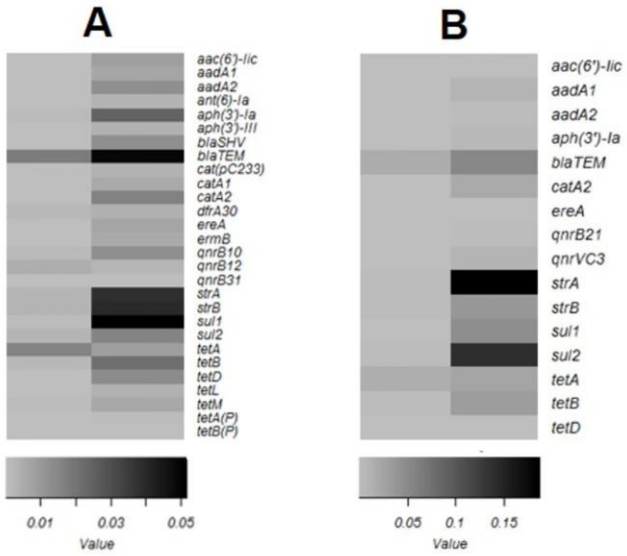

Fig 4. Relative abundances of high-risk genes shared between hospital sites. Comparative abundance between (A) ICU cages and Laundry Trolley (B) ICU cages and Mop Bucket. Grey scale indicates ARG counts per mega base pairs.

https://doi.org/10.1371/journal.pone.0217600.g004

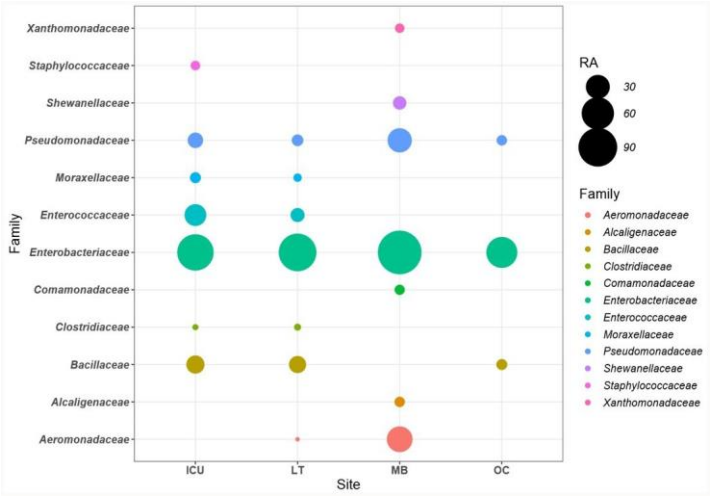

Fig 5. Microbial composition at family level in ICU cages (ICU), Laundry Trolley (LT), Mop Bucket (MB) and Office Corridor (OC) samples. Only the families with relative abundance of >1 copy per mega base pair are represented.

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materials from the patient. This would add to the selective pressure already applied by the therapeutic course on the flora of the animal, and further increase the risk of developing antimicrobial resistance. The problem could become particularly serious if the laundry trolleys and the mop buckets are less frequently cleaned and disinfected compared to the ICU cages. Veterinary hospital waste represents a major source for re-introduction of resistance and should be monitored regularly. As a practical outcome of this study, the infection control procedures of the veterinary teaching hospital were revised, to enforce the systematic rinsing and drying of buckets and regular disinfection of mop heads and laundry trolleys. Long read sequencing is currently applied to other sections and sites of the veterinary hospital, such as drains, sinks and consultation tables in order to further identify critical control points and better inform future infection control plans.

Multiple drug resistances are revealed in long reads from veterinary hospital environments

Significant proportions of ARG-containing reads carried multiple antibiotic resistances in the LT and MB samples, and to a lesser extent in the ICU cages (Table 5). The majority of these reads also carried sequences related to MGEs. For instance, in LT and MB an *aac(3)-II* gene was located close to an *IS6* transposase on the same read. In ICU cages and LT, *blaTEM* and

Table 5. Sequences carrying multiple antimicrobial resistance genes in the veterinary hospital.

Category	Percentage %		
	ICU	LT	MB
SDR	87.16	69.39	78.92
MDR2	6.09	14.70	17.60
MDR3	2.34	8.96	2.58
MDR≥4	2.06	6.10	0.78
Total MDR	**10.50**	**29.76**	**20.96**

SDR: single drug resistance; MDR2: multiple drug resistance due to two different ARGs; MDR3: multiple drug resistance due to three different ARGs; MDR≥4: multiple drug resistance due to four or more different ARGs. ICU: Intensive Care Unit; LT: Laundry Trolley; MB: Mop Bucket

https://doi.org/10.1371/journal.pone.0217600.t005

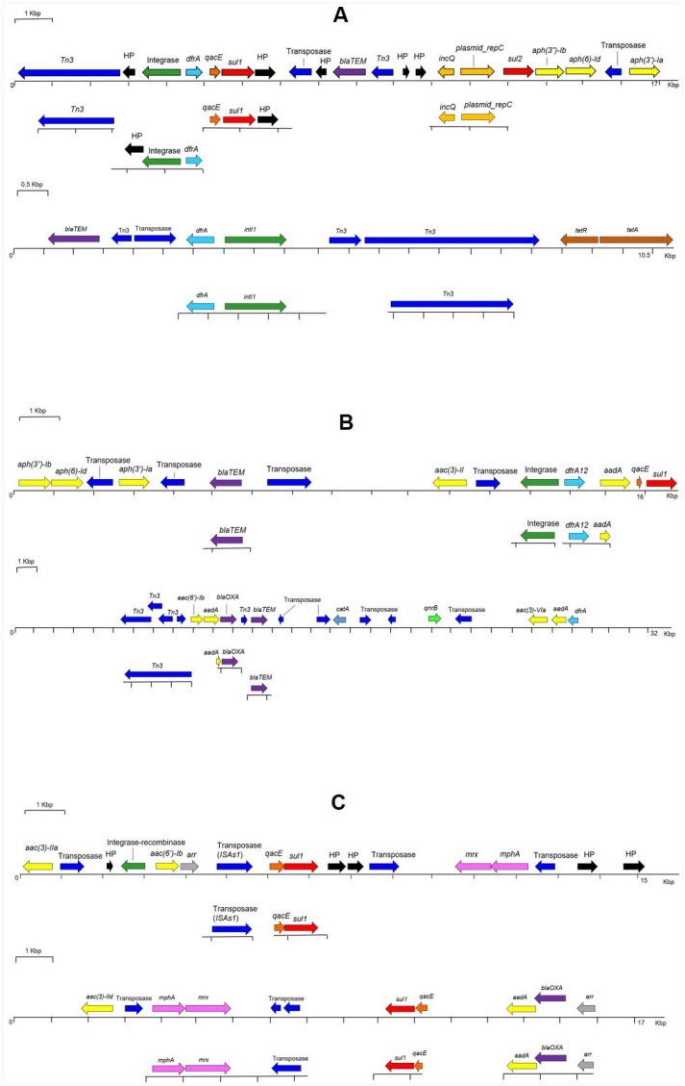

Fig 6. Co-occurrence of ARGs and MGEs in nanopore reads (top) and illumina contigs (bottom). Gene organisation of representative sequences from (A) ICU cages (B) Laundry Trolley and (C) Mop Bucket. Resistance genes, light blue: trimethoprim; red: sulphonamides; purple: beta-lactams; yellow: aminoglycosides; grey: rifamycin; pink: macrolides; blue: chloramphenicol; brown: tetracycline; luminous green: fluoroquinolone; dark orange: quaternary ammonium compounds. Mobile Genetic Elements, dark blue: transposases; green: integrases; light orange: plasmid-associated. HP: Hypothetical Proteins.

https://doi.org/10.1371/journal.pone.0217600.g006

aph(3')-Ia genes were present adjacent to the *IS6* or *Tn3* family transposase genes, and a *sul1*, *qacE* and *dfrA* gene cluster was found close to an integrase gene, suggesting the presence of an integron. In MB, *qacE* and *sul1* genes were found adjoining *ISAs1* transposase. The *ISAs1* family transposases were previously described for disrupting a gene cassette in class I integron [67] and *sul1/qacE* are well known components of class I integrons [68]. The mapping of the assembled and annotated Illumina contigs to the annotated MinION long reads further confirmed the arrangement of these genes. The length of Illumina contigs were limited and therefore less descriptive when compared to the long reads. This makes the MinION long read sequencing, a much favourable approach to monitor the potential spread of multiple drug resistance in the environment. Some examples of such reads are given in Fig 6A, 6B and 6C. The presence of

clinically important ARGs adjacent to MGEs is of particular concern as it reflects the potential risk of disseminating multiple drug resistance within a veterinary clinical environment. While the accumulation of ARGs on individual reads was relatively low in the ICU cages sample, their higher proportion in the LT suggests that exchanges and recombination of ARG occurs in waste environment.

Conclusions

Nanopore sequencing is a convenient and portable method for routine monitoring of environmental risks associated with infectious agents and antimicrobial resistance in veterinary hospitals. Our findings identified possible transfers of ARGs between interconnected environmental sites and identified waste collection points as significant amplifying reservoirs for clinically important ARGs. This work has led to improving biosecurity practices in the investigated premises and demonstrated the usefulness of rapid DNA sequencing to implement evidence-based operational measures for infection control in veterinary facilities.

Supporting information

S1 Table. Bacterial read counts carrying ARGs in the ICU cages (ICU), Laundry Trolley (LT), Mop Bucket (MB) and Office Corridor (OC).
(DOC)

S2 Table. Detailed information on independent MinION sequencing runs.
(DOCX)

S3 Table. Sequence read statistics after filtering for the quality.
(DOCX)

S1 Fig. Rarefaction curves for number of species detected in samples ICU cages, LT, MB and OC.
(DOC)

S1 File. Ct values obtained with WaferGen SmartChip Real-time qPCR array.
(XLSX)

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3. Conclusion

The workflow described here enabled accurate identification and quantification of ARGs and ARG-carrying organisms in veterinary clinical environments. It was superior to traditional culture-based methods as it could target a large number of organisms and resistance determinants at the same time and predict the potential for horizontal dissemination of the ARGs. The workflow was cost effective because it could generate results rapidly with a relatively low requirement for resources. Overall, it could be incorporated into routine environmental surveillance programmes of veterinary hospitals as an efficient molecular tool to identify potential antimicrobial resistance risks.

The measurements of the relative abundances of ARGs, frequency of MDRs, ARG associations with MGEs, and ARG-carrying bacterial hosts allowed identification of environmental sites with higher AMR risks. The ARG-carrying organisms appeared to be transferred from the ICU cages to the laundry trolley through soiled bedding and enriched within the laundry trolley, possibly due to the favourable environmental conditions in it. The mop bucket maintained a different population of bacteria that carried ARGs in high abundances, probably because of the selective effect of disinfectants in the contents of the bucket. Exchange and recombination events could also have occurred in the waste collection points (laundry trolley and mop bucket) resulting in a high frequency of MDR genotypes. The findings were immediately applied to the prevention of infections in the veterinary hospital that was studied, resulting in an improvement in its biosecurity practices, with a particular focus on eliminating the repeated use of the same disinfection solution for mopping and on more frequent disinfection of mops and laundry trolleys.

4. Appendix

Supplementary Tables and Figures

Table S1. Bacterial read counts carrying ARGs in the ICU cages (ICU), laundry trolley (LT), mop bucket (MB) and office corridor (OC)

Taxon[a]	Site	Resistance genes[b]									
		BL	**MLS**	**GP**	**Tet**	**AG**	**FQ**	**TS**	**Phe**	**Fos**	**Rif**
Enterococcus	ICU	-	200	146	34	5	-	-	1	-	-
	LT	-	67	7	17	14	-	1	23	-	-
	MB	-	-	-	-	1	-	-	-	-	-
	OC	-	1	-	-	-	-	-	-	-	-
Pseudomonas	ICU	55	-	-	7	56	-	1	20	23	-
	LT	2	-	-	4	9	-	7	-	-	-
	MB	2	-	-	5	17	-	1	-	-	-
Enterobacteriaceae	ICU	170	1	-	68	43	44	7	41	13	-
	LT	378	32	-	132	321	128	188	74	49	-
	MB	88	22	-	71	193	17	99	63	3	20
	OC	20	-	-	-	-	32	-	-	27	-
Staphylococcus	ICU	30	-	-	1	-	8	-	-	-	-
	LT	1	1	-	-	2	-	-	1	-	-
Acinetobacter	ICU	10	-	-	-	1	-	1	-	-	-
	LT	25	4	-	7	42	-	17	6	-	-
	MB	15	-	-	4	15	-	6	16	-	10
Xanthobacter	ICU	-	1	-	-	2	-	-	-	-	-
	LT	-	11	-	-	15	-	8	-	-	-
Streptococcus	ICU	-	-	-	1	-	-	-	-	-	-
	LT	-	9	-	5	19	-	-	-	-	-
Bacillus	ICU	-	-	4	-	-	-	-	-	-	-
	LT	-	-	20	-	-	-	-	-	-	-
Aeromonas	LT	11	-	-	7	-	-	-	-	-	-
	MB	56	2	-	1	9	-	3	3	-	-
Shewanella	ICU	1	-	-	-	-	-	-	-	-	-
	LT	2	-	-	-	3	-	-	-	-	-
	MB	14	1	-	1	9	15	11	3	-	4
Clostridium	ICU	-	-	-	3	-	-	-	-	-	-
	LT	-	2	-	6	-	-	-	-	-	-
Vibrio	ICU	-	-	-	-	-	2	-	-	-	-
	LT	-	-	-	-	2	-	2	-	-	-
	MB	-	-	-	-	2	5	2	-	-	-
Rhizobium	ICU	-	-	-	-	1	-	-	-	-	-
Carnobacterium	LT	-	-	-	-	-	-	-	2	-	-
Lactobacillus	LT	-	-	-	-	-	-	-	2	-	-
Peptoclostridium	LT	-	-	-	2	-	-	-	-	-	-
Zymomonas	LT	1	-	-	-	-	-	-	-	-	-
Pseudoxanthomonas	LT	-	-	-	-	1	-	-	-	-	-
Achromobacter	MB	-	-	-	-	2	-	-	-	-	-
Stenotrophomonas	MB	1	-	-	-	-	-	-	-	-	-
Verminephrobacter	MB	-	-	-	-	-	-	1	-	-	-

[a] Taxonomic classification determined by Kraken.

[b] Antimicrobial: BL, beta-lactam; MLS, macrolide/lincosamide/streptogramin; GP, glycopeptide; Tet, tetracycline; AG, aminoglycoside; FQ, fluroquinolone/quinolone; TS, trimethoprim/sulphonamide; Phe, phenicol; Fos, Fosfomycin; Rif, rifamycin.

Table S2. Detailed information on independent MinION sequencing runs

Dataset	Sample	Date of collection	Number of swabs	DNA pools	Barcodes	MinION sequencing run
ICU1[a]	IC1	14.11.2016	5	P1	NA	1
	IC2	28.11.2016	5			(22.03.2017)
	IC3	12.12.2016	5			
	IC4	13.02.2017	4			
ICU2[a]	IC1	14.11.2016	5	P1	NA	2
	IC2	28.11.2016	5			(01.08.2017)
	IC3	12.12.2016	5			
	IC4	13.02.2017	4			
ICU3[a]	IC1	14.11.2016	5	P1	BC1	3
	IC2	28.11.2016	5			(25.08.2017)
	IC3	12.12.2016	5	P2	BC2	
	IC4	13.02.2017	4			
	IC5	27.02.2017	4	P3	BC3	
	IC6	27.03.2017	3			
	IC7	01.05.2017	2	P4	BC4	
	IC8	15.05.2017	4			
LT1[b]	L1	14.11.2016	1	P1	NA	4
	L2	28.11.2016	1			(09.05.2017)
	L3	12.12.2016	1			
	L4	13.02.2017	1			
LT2[b]	L1	14.11.2016	1	P1	BC1	5
	L2	28.11.2016	1			(24.01.2018)
	L3	12.12.2016	1	P2	BC2	
	L4	13.02.2017	1			
	L5	27.02.2017	1	P3	BC3	
	L6	27.03.2017	1			
	L7	01.05.2017	1	P4	BC4	
	L8	15.05.2017	1			
MB[c]	M1	12.12.2016	NA	P1	BC1	6
	M2	13.02.2017	NA	P2	BC2	(13.12.2017)
	M3	27.02.2017	NA			
	M4	17.03.2017	NA	P3	BC3	
	M5	20.03.2017	NA			
	M6	21.03.2017	NA			
	M7	23.03.2017	NA			
	M8	27.03.2017	NA			
	M9	28.03.2017	NA	P4	BC4	
	M10	04.04.2017	NA			
	M11	12.04.2017	NA			
OC[d]	OC1	28.05.2017	1	P1	NA	7
						(05.06.2017)

[a] The sequence reads of these runs were combined to form the final dataset for the ICU cages
[b] The sequence reads of these runs were combined to form the final dataset for the laundry trolley
[c] The dataset for mop bucket
[d] The dataset for office corridor
NA = not applicable

Table S3. Sequence read statistics after filtering for quality[a]

Dataset	Size (Gbp)	Mean length (Kbp)	N50 (Kbp)
ICU cages	6	5	10
LT	5	6	10
MB	2	2	4
OC	1	7	11

[a] Reads shorter than 250 bp and reads with a phred quality score less than 8 were filtered out

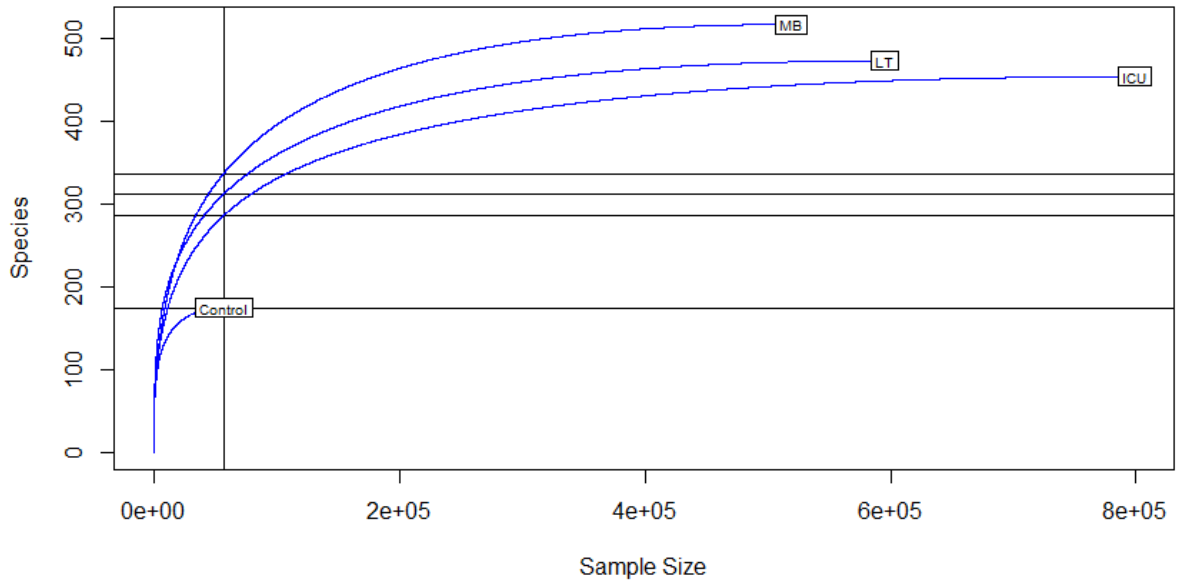

Figure S1. Rarefaction curves for numbers of species detected in samples from the ICU cages, LT, MB and OC (Control)

Table S4. List of ARGs detected and their relative abundances

Gene	ICU	LT	MB	OC
aac(3)-IIa_1	0	0.006671	0.038303	0
aac(3)-IVa_1	0	0.001042	0	0
aac(3)-VIa_1	0	0.000208	0	0
aac(6')-aph(2'')_1	0	0.003335	0	0
aac(6')-Ic_1	0	0	0.025124	0
aac(6')-IIa_1	0	0	0.000412	0
aac(6')-IIc_1	0.000666	0.008547	0.000412	0
aadA1_1	0.000167	0.006671	0.010708	0
aadA2_2	0.0005	0.012508	0.003295	0
aadB_1	0	0.005629	0	0
ampH_1	0	0	0.002883	0
ant(6)-Ia_1	0.0005	0.002918	0	0
aph(3')-Ia_1	0.001332	0.024599	0.007414	0
aph(3')-IIa_1	0.006162	0	0	0
aph(3')-IIb_1	0.004663	0	0	0
aph(3')-III_1	0.000333	0.003544	0	0
aph(3')-VIa_1	0	0.000417	0	0
aph(4)-Ia_1	0	0.001668	0	0
aph(6)-Ic_1	0.005662	0	0	0
ARR-3_4	0	0	0.031301	0
blaACC-2_1	0.000167	0	0	0
blaACC-3_1	0.000167	0	0	0
blaACT-10_1	0	0.000417	0.002059	0.0025543
blaACT-14_1	0.008659	0.007922	0	0.00894
blaACT-9_1	0	0.011257	0	0
blaADC-25_1	0.000999	0.000834	0	0
blaCARB-10_1	0	0.000417	0	0
blaCFE-1_1	0	0.000417	0	0
blaCMY-18_1	0.003997	0.005629	0.002471	0
blaCMY-98_1	0.006994	0	0	0
blaCTX-M-101_1	0	0.000417	0	0
blaCTX-M-110_1	0.000333	0	0	0
blaDHA-1_1	0	0.010215	0.013591	0
blaFOX-10_1	0	0	0.004942	0
blaHERA-1_1	0.000167	0	0	0
blaL1_3	0	0	0.000412	0
blaLEN16_1	0	0	0	0.0140485
blaMOX-5_1	0	0.001042	0.000412	0
blaOXA-1_1	0	0	0.004942	0
blaOXA-100_1	0.0005	0.000834	0	0
blaOXA-101_1	0	0	0.007825	0
blaOXA-134_1	0	0.000417	0	0
blaOXA-146_1	0	0.000417	0	0

blaOXA-162_1	0	0	0.004119	0
blaOXA-164_1	0	0.000208	0	0
blaOXA-211_1	0	0.00271	0.000824	0
blaOXA-213_1	0.000167	0	0	0
blaOXA-50_1	0.00433	0	0	0
blaOXA-504_1	0	0.002293	0.00659	0
blaOXA-9_2	0	0.000417	0	0
blaOXY-1-1_1	0.0005	0.001042	0.000412	0
blaPAO_1	0.004496	0	0	0
blaPLA-3A_1	0	0.001668	0.000824	0
blaSHV-100_1	0.000167	0.012716	0	0
blaSRT-1_1	0	0	0.016063	0
blaTEM-101_1	0.017818	0.048989	0.052718	0
blaZ_32	0.005495	0.000208	0	0
cat(pC194)_1	0	0.003752	0	0
cat(pC233)_1	0.000167	0.003335	0	0
cat_1	0.006661	0	0	0
catA1_1	0.000167	0.00542	0	0
catA2_1	0.000333	0.01626	0.019357	0
catB3_1	0	0	0.001647	0
catB7_1	0.003497	0	0	0
catB8_1	0	0.002918	0.023064	0
cmx_1	0	0	0.000412	0
dfrA1_1	0	0.001251	0.000412	0
dfrA10_1	0	0.000625	0	0
dfrA12_1	0	0.004169	0	0
dfrA13_1	0	0.000417	0	0
dfrA14_1	0	0	0.00659	0
dfrA15_1	0	0.000417	0	0
dfrA18_1	0	0.010632	0	0
dfrA30_1	0.001832	0.004169	0	0
dfrA32_1	0	0.000417	0	0
dfrA7_1	0	0.002502	0	0
dfrG_1	0	0.000208	0	0
ere(A)_2	0.000333	0.007296	0.000412	0
erm(B)_18	0.000167	0.005629	0	0
erm(C)_1	0	0.000208	0	0
erm(Q)_1	0	0.000417	0	0
floR_1	0	0.000417	0	0
fosA_1	0.003997	0	0	0
fosA_10	0	0.00271	0	0.0153257
fosA_15	0.002165	0.006879	0.001236	0.0204342
fosA_2	0	0.000834	0	0
imiS_1	0	0	0.009061	0
lnu(B)_1	0	0.000208	0	0
lsa(A)_1	0.032806	0.003335	0	0.0012771

mph(A)_1	0	0.004795	0.037891	0
mph(A)_2	0	0.004378	0.033361	0
mph(E)_3	0	0.000417	0.001647	0
msr(C)_1	0.001832	0.00813	0	0
msr(E)_4	0	0.000417	0.001647	0
norA_1	0.001665	0	0	0
oqxA_1	0.000999	0.003752	0	0.0166028
oqxB_1	0.00383	0.015218	0	0.0242656
QnrB10_1	0.001665	0.012925	0	0
QnrB12_2	0.004663	0.00271	0	0
QnrB21_1	0.000333	0	0.002471	0
QnrB31_1	0.000167	0.000208	0	0
qnrE_1	0	0	0.001236	0
QnrS1_1	0	0.000834	0	0
QnrS2_1	0	0	0.014003	0
QnrVC3_1	0.000333	0	0.010708	0
str_1	0	0.000208	0	0
strA_1	0.002998	0.038149	0.186985	0
strB_1	0.003164	0.039817	0.037891	0
sul1_2	0.001166	0.051491	0.0486	0
sul2_7	0.002998	0.015635	0.141269	0
tet(39)_1	0	0.001251	0	0
tet(41)_1	0	0	0.015651	0
tet(A)_2	0.015654	0.008547	0.025124	0
tet(B)_3	0.002165	0.021472	0.032125	0
tet(C)_2	0	0.000625	0.000412	0
tet(D)_1	0.000333	0.013759	0.000412	0
tet(E)_3	0	0.001459	0.000412	0
tet(H)_1	0	0.000208	0	0
tet(J)_1	0.00766	0.003335	0	0
tet(L)_2	0.000167	0.003544	0	0
tet(M)_1	0.000833	0.006254	0	0
tet(O)_1	0.006328	0	0	0
tet(U)_1	0	0.000208	0	0
tet(X)_1	0	0.000208	0	0
tetA(P)_1	0.000333	0.001042	0	0
tetB(P)_3	0.000333	0.000625	0	0
VanC_1	0.0005	0	0	0
VanC_3	0.005828	0.000208	0	0
VanH-Pt_5	0.000167	0	0	0
VanPt_5	0.000167	0	0	0
VanR-A_1	0.000333	0.000625	0	0
VanR-C_1	0.0005	0.000208	0	0
VanR-C_3	0.005662	0.000417	0	0
VanS-C_1	0.000666	0.000208	0	0
VanS-C_3	0.002998	0.000208	0	0

VanS-Pt_5	0.0005	0.001668	0	0
VanT-C_1	0.0005	0	0	0
VanT-C_2	0.00383	0.000208	0	0
VanW-Pt_2	0.000167	0	0	0
VanX-A_1	0.000167	0	0	0
VanXY-C_1	0.0005	0	0	0
VanXY-C_3	0.006661	0.000417	0	0
VanY-F_1	0.000167	0	0	0
VanY-Pt_5	0.000167	0.001251	0	0
VanZ-F_1	0.000167	0.000625	0	0

Note, this table was not included in the published paper

Chapter 3: Colonization of a hand washing sink in a veterinary hospital by an *Enterobacter hormaechei* strain carrying multiple resistances to high importance antimicrobials

1. Introduction

Implementation of routine environmental surveillance programmes allows early identification of drug-resistant organisms in hospitals. The application of molecular techniques (as described in Chapter 2) to support these programmes further enables prediction of clinical risks associated with resistance determinants and their potential for dissemination. However, insights into their molecular epidemiology, including their environmental persistence, genetic re-arrangements and recombination events, can only be achieved through detailed genetic characterisation of those organisms. This knowledge is valuable in understanding the nature and evolution of important multidrug-resistant nosocomial pathogens.

This publication (Section 3.2) describes an investigation of repeated isolations of multidrug-resistant, ESBL producing bacteria from the hand washing sink in the ICU of the veterinary hospital. Phenotypic and molecular characterisation of the isolates, comparative genome analysis and conjugation experiments were employed for this purpose.

2. Publication

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Antimicrobial Resistance and Infection Control

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Colonization of a hand washing sink in a veterinary hospital by an *Enterobacter hormaechei* strain carrying multiple resistances to high importance antimicrobials

Kanishka Kamathewatta[1], Rhys Bushell[1], Fannana Rafa[2], Glenn Browning[2], Helen Billman-Jacobe[2] and Marc Marenda[1*]

Abstract

Background: Hospital intensive care units (ICUs) are known reservoirs of multidrug resistant nosocomial bacteria. Targeted environmental monitoring of these organisms in health care facilities can strengthen infection control procedures. A routine surveillance of extended spectrum beta-lactamase (ESBL) producers in a large Australian veterinary teaching hospital detected the opportunistic pathogen *Enterobacter hormaechei* in a hand washing sink of the ICU. The organism persisted for several weeks, despite two disinfection attempts. Four isolates were characterized in this study.

Methods: Brilliance-ESBL selective plates were inoculated from environmental swabs collected throughout the hospital. Presumptive identification was done by conventional biochemistry. Genomes of multidrug resistant *Enterobacter* were entirely sequenced with Illumina and Nanopore platforms. Phylogenetic markers, mobile genetic elements and antimicrobial resistance genes were identified in silico. Antibiograms of isolates and transconjugants were established with Sensititre microdilution plates.

Results: The isolates possessed a chromosomal Tn7-associated silver/copper resistance locus and a large IncH12 conjugative plasmid encoding resistance against tellurium, arsenic, mercury and nine classes of antimicrobials. Clusters of antimicrobial resistance genes were associated with class 1 integrons and IS26, IS903 and ISCR transposable elements. The *bla*SHV-12, *qnrB*2 and *mcr*-9.1 genes, respectively conferring resistance to cephalosporins, quinolones and colistin, were present in a locus flanked by two IS903 copies. ESBL production and enrofloxacin resistance were confirmed phenotypically. The isolates appeared susceptible to colistin, possibly reflecting the inducible nature of *mcr*-9.1.

Conclusions: The persistence of this strain in the veterinary hospital represented a risk of further accumulation and dissemination of antimicrobial resistance, prompting a thorough disinfection of the ICU. The organism was not recovered from subsequent environmental swabs, and nosocomial *Enterobacter* infections were not observed in the hospital during that period. This study shows that targeted routine environmental surveillance programs to track organisms

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with major resistance phenotypes, coupled with disinfection procedures and follow-up microbiological cultures are useful to control these risks in sensitive areas of large veterinary hospitals.

Keywords: Veterinary hospital, ICU, Antimicrobial resistance, IncH12 plasmid, *Enterobacter hormaechei*

Background

Hospital acquired infections are a significant threat to human and animal health. Hospital environments are critical reservoirs for drug-resistant bacteria [1–3]. Investigations into nosocomial infections outbreaks caused by *Enterobacterales*, *Pseudomonas* and *Acinetobacter*, have revealed that contaminated hand washing sinks in intensive care units were an important source of these microorganisms [4–8]. Dissemination of bacteria from the hand washing sinks is droplet-mediated [9]. Mobile genetic elements such as plasmids, integrons, and transposons play a key role in maintaining and propagating antibiotic resistance genes (ARGs). Common plasmid sequences have been detected in different species of carbapenemase-producing bacte- ria that colonised both the patients and the plumbing of an intensive care unit (ICU) in a human hospital [10]. Good biosecurity practices and routine, targeted environmental surveillance are two important tools to prevent outbreaks of nosocomial infections caused by multidrug resistant opportunistic or obligate pathogens in hospital premises. The genomic analysis of the bacteria isolated through these surveillance programs provides useful information on the origin and potential spread of antibiotic resistance genes. This knowledge can be used to improve infection control procedures. The aim of this study was to exploit the findings of a surveillance program for multidrug resistant organisms in the environment of a teaching veterinary hospital. The genus *Enterobacter* represents a group of phylogenetically diverse opportunistic pathogens, often harboring multiple drug resistance genes and are involved in hospital-acquired infections [11]. Here, we report the repeated isolation of an extended spectrum beta lactamase (ESBL) producing, multidrug resistant *Enterobacter* sp. from the hand washing sink of a large veterinary hospital ICU, and its genotypic and phenotypic characterization. Antimicrobial resistance genes, integrons and transposons were identified in the isolates and their potential for mobility and horizontal transfer was investigated through comparative sequence analysis and conjugation experiments. A successful decontamination protocol was implemented in the hospital to eliminate the organism from the sink in response to these findings.

Methods

Bacterial isolates

Swabs from ICU sink and drain were submitted to the clinical microbiology laboratory of the Melbourne Veterinary School U-Vet hospital in Werribee, Victoria, Australia, as part of the routine environmental surveillance program. The swabs were placed in 100 ml of buffered peptone water (BPW) and incubated at 37 ºC for 24 h. ESBL screening plates (Oxoid) were inoculated with one loop of broth culture and incubated at 37 ºC for 24 h. Presumptively ESBL positive green or blue colonies were sub-cultured onto sheep blood agar and MacConkey agar (MicroMedia, Australia) plates, which were incubated at 37 ºC for 24 h. Phenotypic identifications were performed based on colony morphology, Gram staining characteristics, oxidase test and biochemical properties using the API rapidID 32E test (bioMerieux, Marcy-l'Étoile, France) and the Entero-Pluri test (Liofilchem) kits. ESBL production was confirmed with double disk diffusion synergy assays using cefotaxime, ceftazidime and amoxicillin-clavulanate [12]. Antimicrobial susceptibility testing was performed with the Calibrated Dichotomous Susceptibility method [13] and the broth microdilution method using Sensititre plates COMPGN1F and GNX2F (Thermo-Fischer) on a Aris2X machine according to the manufacturer's instructions.

DNA extraction

Single colonies from pure overnight cultures on sheep blood agar were inoculated into 10 ml of tryptic soy broth (TSB), which was incubated at 37 °C overnight. Cells from 1 ml of each TSB cultures were collected by centrifugation at 15,000×*g* for 2 min and genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol for Gram negative bacteria. The DNA concentration was measured using a Quantus fluorometer (Promega) and the quality was determined by microspectrophotometry (NanoDrop ND-1000, NanoDrop Technologies). The DNA extracts were cleaned using SPRI beads (AMPureX, Beckman Coulter).

Nanopore sequencing

The sequencing libraries were prepared according to the 1D native barcoding genomic DNA sequencing protocol with EXP-NBD103 and SQK-LSK108 kits (Oxford

Nanopore Technologies, Oxford, UK). At least 1 μg of DNA was processed by treatment with the Formalin-Fixed Paraffin-Embedded (FFPE) enzyme mix (New England Bio Labs, Ipswich, USA) and then end-repaired. Barcode-adaptor ligation was performed after dA-tailing. The sequencing was performed out in a MinION device with flow cell version FLO-MIN107 (Oxford Nanopore Technologies). The raw reads were basecalled into fastq files with Albacore version 2.2.7 (Oxford Nanopore Technologies). De-multiplexing and adaptor trimming was performed using Porechop version 0.2.3 (https://github.com/rrwick/Porechop), before filtering out 20% of the reads with the lowest quality, using the program Filtlong version 0.2.0 (https://github.com/rrwick/Filtlong).

Illumina sequencing

Illumina sequencing was performed at the Australian Genome Research Facility (AGRF, Melbourne, Victoria, Australia) using the Illumina HiSeq2500 platform, generating 125 bp long paired-end reads. The sequencing adaptors were removed and the reads with a Phred quality score of < 20 were filtered out using Trim Galore version 0.4.4 [14].

Genome assembly and analysis

Hybrid (short Illumina and long Nanopore reads) or long read-only de novo genomic assemblies were performed using Unicycler version 0.4.7 [15]. The identity of the genomes and their sequence type was determined using mlst (https://github.com/tseemann/mlst) within the PubMLST database [16]. The genomes and plasmids resulting from hybrid assemblies were annotated using the program Prokka version 1.14 [17] and the RAST annotation server [18]. Sequence visualization and plotting were performed with the Artemis program suite [19]. The annotations were manually curated using BLASTP to search the non-redundant protein database (NCBI). The antibiotic resistance genes were identified by searching the Prokka predicted open reading frames (ORFs) against the CARD protein database [20] with BLASTP. Transposons and integrons were predicted using ISfinder [21] and Integron Finder [22], respectively. The program IslandViewer [23] was used to visualise genomic islands. The origins of transfer regions on plasmids were identified using oriTfinder [24]. Multilocus Sequence Type (MLST) and Ribosomal Multilocus Sequence Typing (rMLST) analysis were performed on the pubMLST server [25, 26]. The program ABRicate [27] version 0.9.8 was used to detect antimicrobial genes with the databases ncbi and card, and incompatibility groups with the database plasmidfinder.

Comparative sequence analysis

Full genome and plasmid alignments were performed using Mauve aligner version 2.4.0 [28]. Detailed single nucleotide polymorphism (SNP) and gap analysis of genome and plasmid alignments were performed using Geneious version 11.1.2. The plasmid sequences were searched against the NCBI nucleotide database and the PLSDB plasmid database [29] using BLASTN. Comparative plasmid visualizations were performed using the genoPlotR [30] package in R version 3.4.0. and the CGView Comparison Tool [31]. Phylogenetic trees were built with MegaX software [32] from concatenated multiple alignments of housekeeping gene sequences produced with the program Muscle [33].

Mating

Broth mating experiments were performed as described before [34, 35]. Briefly, the recipient *E. coli* DH5α and the donor CM18-216 were inoculated into 1 mL of LB and grown respectively at 37 °C (recipient) and either 27 °C or 37 °C (donor) for 18–20 h without shaking. One volume of donor cultivated at either 27 °C or 37 °C was mixed with four volumes of recipient and incubated at the same temperature for 2 h. Subsequently, 100 μl of the conjugation mixtures were plated onto LB agar plates containing 16 μg/ml tetracycline and 16 μg/ml nalidixic acid, and incubated at 27 °C and 37 °C for 48 h and 24 h, respectively. Colonies were randomly picked and sub-cultured on Sheep Blood Agar and MacConkey Agar plates for phenotypic testing.

Results

Persistence of a multidrug resistant strain of *Enterobacter hormaechei* in a sink

Four ESBL producing *Enterobacter* sp. were isolated on selective media from environmental swabs collected in a veterinary teaching hospital ICU over a period of approximately one month. The first isolate, CM18-216, was obtained from a hand-washing sink as part of the hospital routine surveillance program. A second isolate, CM18-242-2, was obtained from a follow-up assessment of the tap handles and sink edges after a first disinfection attempt. Two more isolates, namely CM18-269-1 and CM18-269-2, were later recovered from the drain and the edge of the same sink, after a second disinfection attempt. All isolates had identical antimicrobial resistance profiles. The biochemical characterisation of isolates CM18- 216 and CM18-242-2 established with the rapid ID 32E strip (Bio-Merieux) resulted in the profile 46772514741, which the ApiWeb database reports as an excellent identification of an *Enterobacter cloacae* (%ID 99.9, T 0.97).

This identification was confirmed by an Entero-pluri test, giving the biocode 32261. However, an atypical result, Lactose negative, was indicated by the test. Moreover, the sink isolates did not ferment lactose on MacConkey plates.

The genomes of isolates CM18-216 and CM18-242-2 were completely sequenced, using Illumina and Oxford Nanopore platforms. A summary of sequence read statistics for both methods is provided in Additional file 1: Table S1. Isolate CM18-216 contained a 4,689,992 bp chromosome and a 288,096 bp plasmid; isolate CM18-242-2 contained a 4,689,986 bp chromosome and a 288,061 bp plasmid. The two isolates had nearly identical chromosome sequences, with only 44 single nucleotide differences and 6 small insertion/deletions which accounted for the 6 bp length difference (Table 1). These nucleotide differences were all in hypothetical proteins or in non-coding regions, except for one position within a 16S rRNA region. Several prophages and genomic islands were also identified on the chromosome (Additional file 2: Fig. S1). The sequence alignments of the large plasmids, hereafter named pCM18-216 and pCM18-242-2, revealed 16 nucleotide differences between the two sequences, as well as 6 gaps in pCM18-216 and 41 gaps in pCM18-242-2 (Table 2). These nucleotide differences were all clustered in a region of approximately 380 bp encoding an IS5 family transposase. The plasmids belonged to the incompatibility group IncHI2 and contained all the genes required for transfer and an oriT region, indicating that it was capable of conjugation.

The genomes of the two other isolates subsequently recovered from the ICU sink, CM18-269-1 and CM18-269-2, were sequenced with Nanopore reads only, and were assembled into 2 circular contigs corresponding to a chromosome and a plasmid. Multiple sequence alignments showed high levels of colinearity between all chromosomal contigs, suggesting that all four *Enterobacter* isolates recovered from the ICU sink over one month were related. However, while all four genomes carried a complete prophage of approximately 32 kb located 1160 kb from the chromosomal origin, the isolate CM18- 269-2 possessed a second prophage, which was absent from the 3 other genomes, 1800 kb apart (Additional file 3: Fig. S2).

The Sequence Type of CM18-216 was determined by the online pubMLST server as ST110, using the *Enterobacter cloacae* database. Ribosomal Multilocus Sequence Typing (rMLST) genome analysis identified CM18-216 and CM18-242-2 as *E. hormaechei*. Average Nucleotide Identity (ANI) analysis confirmed this result, indicating a higher proximity with *E. hormaechei* than with *E. cloacae* type strains (Table 3). Phylogenetic analysis of concatenated alignments of the house keeping genes *groL, gyrA*,

Table 1 Nucleotide differences identified between the chromosomes of isolates CM18-216 and CM18-242-2

Position		Nucleotide difference	Protein/region
CM18-216	CM18-242-2		
308741	308741	G/A	Hypothetical protein
308748	308747	C/T	Hypothetical protein
309078	309077	G/A	Non-coding region
309785	309784	T/A	Non-coding region
309787	309786	C/T	Non-coding region
424216	424215	T/A	Hypothetical protein
425369	425368	G/A	Non-coding region
426076	426075	T/A	Non-coding region
426078	426077	C/T	Non-coding region
426577	426576	C/T	Non-coding region
1201725	1201724	C/T	16S rRNA
1203869	1203868	T/A	Hypothetical protein
1204685	1204684	G/A	Hypothetical protein
1204692	1204690	C/T	Hypothetical protein
1205022	1205020	G/A	Non-coding region
1205729	1205727	T/A	Non-coding region
1205731	1205729	C/T	Non-coding region
3657012	3657010	G/A	Non-coding region
3657014	3657012	A/T	Non-coding region
3657721	3657719	C/T	Non-coding region
3658051	3658049	G/A	Hypothetical protein
3658058	3658055	C/T	Hypothetical protein
3658874	3658871	A/T	Hypothetical protein
4172066	4172063	G/A	Non-coding region
4172068	4172065	A/T	Non-coding region
4172775	4172772	C/T	Non-coding region
4173928	4173925	A/T	Hypothetical protein
4437299	4437296	G/A	Non-coding region
4437301	4437298	A/T	Non-coding region
4438008	4438005	C/T	Non-coding region
4438338	4438335	G/A	Hypothetical protein
4438345	4438341	C/T	Hypothetical protein
4439161	4439157	A/T	Hypothetical protein
4515406	4515402	G/A	Non-coding region
4515408	4515404	A/T	Non-coding region
4516445	4516441	G/A	Hypothetical protein
4516452	4516447	C/T	Hypothetical protein
4517268	4517263	A/T	Hypothetical protein
4640694	4640689	G/A	Non-coding region
4640696	4640691	A/T	Non-coding region
4641403	4641398	C/T	Non-coding region
4641733	4641728	G/A	Hypothetical protein
4641740	4641734	C/T	Hypothetical protein
4642556	4642550	A/T	Hypothetical protein
308742	308742	T/-	Hypothetical protein
1204686	1204685	T/-	Hypothetical protein

Table 1 (continued)

Position		Nucleotide difference	Protein/region
CM18-216	CM18-242-2		
3658054	3658052	A/-	Hypothetical protein
4438341	4438338	A/-	Hypothetical protein
4516448	4516444	A/-	Hypothetical protein
4641736	4641731	A/-	Hypothetical protein

gyrB, rpoB and *dnaA* from 142 *Enterobacter* sp. complete genomes from RefSeq (Additional file 1: Table S2) placed the two sink isolates on the same branch, amongst a cluster of *E. hormaechei* strains (Fig. 1). Of note, some entries identified as "*E. cloacae*" in the Ref_Seq database which were used to build the tree also fell into *E. hormaechei* clades. These genomes were individually analysed by rMLST, which re-classified them as *E. hormaechei*. The isolate CM18-216 was selected as representative of the *E. hormaechei* strain repeatedly found in the hospital ICU sink.

Resistance genes to clinically important antimicrobials and to heavy metals are clustered on the large IncH12 plasmid

Thirty-five ARGs were detected on *E. hormaechei* CM18-216 genome by the program ABRicate, with 18 on the chromosome and 16 on the IncH12 plasmid (Table 4). The clinically important ARGs were clustered within two loci on the plasmid (Fig. 2). These ARGs were identified as *bla*SHV-12, *qnrB2*, *mcr*-9.1, *bla*-TEM, *cat*II, *tetD*, *sul*1, *dfrA*19, *ereA*, *arr*, *aac*(3)-II, *aac*(6′)-IIc, *aph*(6)-Id, *aph*(3″)-Ib and *ant*(3″)-Ia.

Table 2 Nucleotide differences identified between the plasmids pCM18-216 and pCM18-242-2

Position		Nucleotide difference	Protein/region
pCM18-216	pCM18-242-2		
22872	22870	T/G	IS5 family transposase
22873	22871	T/C	IS5 family transposase
23000	22975	A/G	IS5 family transposase
23029	23004	A/G	IS5 family transposase
23080	23047	A/T	IS5 family transposase
23081	23048	C/G	IS5 family transposase
23111	23076	A/G	IS5 family transposase
23117	23082	A/C	IS5 family transposase
23166	23129	G/A	IS5 family transposase
23181	23144	G/A	IS5 family transposase
23183	23146	G/T	IS5 family transposase
23184	23150	C/A	IS5 family transposase
23185	23151	A/G	IS5 family transposase
23254	23219	T/C	IS5 family transposase
23255	23220	G/A	IS5 family transposase
218820	218785	G/A	IS5 family transposase
22869	22869	CT/-	IS5 family transposase
22889	22887	TTCCGA/------	IS5 family transposase
22924	22916	-/A	IS5 family transposase
22950	22943	T/-	IS5 family transposase
22968	22960	CGGATTAACCCGTTCCT/-	IS5 family transposase
23054	23029	TG/--	IS5 family transposase
23066	23039	GCGCTT/------	IS5 family transposase
23085	23052	A/-	IS5 family transposase
23093	23059	T/-	IS5 family transposase
23118	23083	-/T	IS5 family transposase
23157	23123	CAG/---	IS5 family transposase
23183	23146	---/ACC	IS5 family transposase
23242	23208	CC/--	IS5 family transposase
23253	23217	-/A	IS5 family transposase

Table 3 ANI analysis of CM18-216, *E. cloacae* and *E. hormaechei* type strains

ANIm value (aligned percentage)	CM18-216	*Enterobacter cloacae* ATCC 13047 [T]	*Enterobacter hormaechei* ATCC 49162 [T]
CM18-216	*	87.97 (78.21)	95.30 (83.74)
Enterobacter cloacae ATCC 13047 [T]	87.97 (70.88)	*	87.89 (69.12)
Enterobacter hormaechei ATCC 49162 [T]	95.31 (85.51)	87.89 (77.99)	*

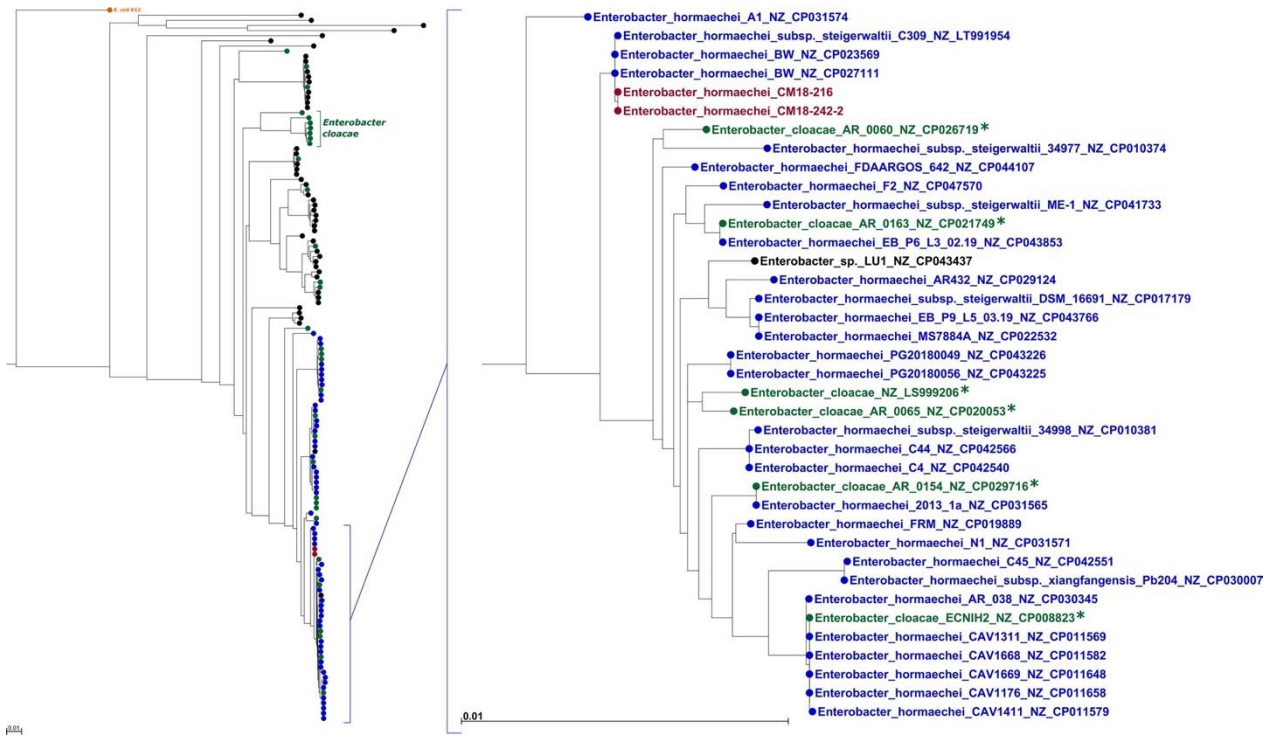

Fig. 1 Phylogenetic analysis of 136 *Enterobacter* sp. genomes from RefSeq and the two sink isolates CM18-216 and CM18-242-2. A maximum likelihood tree was built from concatenated alignments of *groL*, *gyrA*, *gyrB*, *rpoB* and *dnaA* genes, representing 12174 positions, using the General Time Reversible model with discrete gamma distribution G (5 categories, 0.5920) and invariable sites I (58.19%). The *E. coli* K12 MG1655 strain is used as an out-group (orange). The *E. hormaechei*, *E. cloacae*, other *Enterobacter* sp., and sink isolates are respectively indicated in blue, green, black and red. The sub-tree containing the sing isolates is presented on the right. The asterisks indicate *E. cloacae* genomes re-classified as *E. hormaechei* by rMLST analysis. Scale bars represent the number of substitutions per site

Phenotypic testing of the isolates broadly confirmed the resistance patterns predicted by genetic analysis. The isolates CM18-216 and CM18-242-2 possessed high Minimal Inhibitory Concentrations (MICs) values for penicillins, cephalosporins, monobactams, aminoglycosides, phenicols, trimethoprim-sulfonamides and tetracyclines (Table 5). Analysis of MICs for third generation cephalosporins and double-disk diffusion synergy assays confirmed the ESBL phenotype seen on selective plates during the primary isolation of the organism from environmental swabs. The MIC for enrofloxacin of both isolates was 1 ug/mL. The organism displayed a susceptible phenotype to colistin and polymyxin B in broth and agar diffusion tests.

Gene operons or clusters for tellurium, mercury and arsenic metal resistance were also detected on the plasmid (Fig. 2). The tellurium resistance gene cluster was located between nucleotide positions 76066 and 82286, and consisted of *terZ, terA, terB, terC, terD, terE* and *terF*. The components of the mercury resistance operon, *merE, merD, merA, merC, merP, merT* and *merR*, were located between nucleotide positions 101821 and 105561. The arsenic resistance operon contained *arsH, arsR, arsB* and *arsC* and was located

Table 4 Antimicrobial resistance genes in *Enterobacter* CM18-216

Gene	Start	End	Strand	% Cov	% Id	Resistance
Chromosome						
bacA	652522	653339	+	99.51	83.37	Peptide
emrB	1048287	1049816	–	99.42	84.25	Fluoroquinolone
emrR	1051153	1051683	–	100	84.18	Fluoroquinolone
oqxA	1315626	1316800	+	99.91	87.08	Phenicol Quinolone
oqxB	1316824	1319943	+	98.95	89.14	Phenicol Quinolone
acrD	1356278	1359376	–	99.52	81.41	Aminoglycoside
yojI	1537237	1538876	+	99.64	78.93	Peptide
baeR	1652616	1653326	–	98.34	82.98	Aminocoumarin Aminoglycoside
mdtC	1656139	1659216	–	99.94	82.44	Aminocoumarin
mdtB	1659217	1662339	–	99.9	80.23	Aminocoumarin
H-NS	2063229	2063642	–	100	85.51	Cephalosporin Fluoroquinolone Macrolide Penam Tetracycline
marA	2349683	2350058	+	97.92	84.84	Various
msbA	2956274	2958022	–	100	83.25	Nitroimidazole
acrA	3457822	3459015	+	100	87.94	Various
acrB	3459038	3462184	+	99.87	85.02	Various
fosA	3985418	3985843	–	100	96.01	Fosfomycin
CRP	4226794	4227426	+	100	87.99	Fluoroquinolone Macrolide Penam
cpxA	4567511	4568877	+	99.49	83.17	Aminocoumarin Aminoglycoside
Plasmid						
*bla*TEM-1	112126	112986	–	100.00	100.00	Beta-lactam
*cat*A2	122343	122984	+	100.00	100.00	Chloramphenicol
tet(D)	124596	125780	+	100.00	99.92	Tetracycline
*sul*1	128882	129721	–	100.00	100.00	Sulfonamide
ere(A)	130245	131304	–	86.31	99.44	Macrolide
arr	132166	132579	–	100.00	100.00	Rifamycin
aac(3)-II	132707	133516	–	100.00	100.00	Gentamicin
aac(6′)-IIc	135601	136182	–	100.00	100.00	Gentamicin Kanamycin Tobramycin
mcr-9.1	219313	220932	+	100.00	100.00	Colistin
aph(6)-Id	224139	224975	–	100.00	100.00	Streptomycin
aph(3″)-Ib	224975	225777	–	99.88	100.00	Streptomycin
*dfrA*19	227552	228121	–	100.00	100.00	Trimethoprim
*sul*1	230833	231672	–	100.00	100.00	Sulfonamide
*qnrB*2	232163	232807	+	100.00	100.00	Quinolone
*sul*1	236554	237393	–	100.00	100.00	Sulfonamide
*aadA*2	237898	238689	–	100.00	100.00	Streptomycin
*bla*SHV-12	242434	243294	+	100.00	100.00	Cephalosporin

% Cov.: percentage of coverage; % Id.: percentage of identity

between nucleotide positions 199610 and 201790. The operon was co-located with an ISNCY family transposase, to the left of *arsH*. In addition to these plasmid operons, two complete copper and silver resistance loci, *pcoABCDRSE* and *silESRCFBAP* were present on the chromosome, next to Tn7-like transposases, in a predicted genomic island located between nucleotide positions 4356976 and 4393429 (Additional file 2: Fig. S1).

The multidrug resistance plasmid pCM18-216 is conjugative

Mating between *E. hormaechei* CM18-216 and a laboratory strain of *E. coli* DH5α (lactose negative, nalidixic acid resistant) in broth at 27 °C for 2 h resulted in the appearance of tetracycline-resistant transconjugants, which were confirmed as the *E. coli* recipient by conventional biochemistry. Mating performed at the higher temperature of 37 °C did not result in transconjugants.

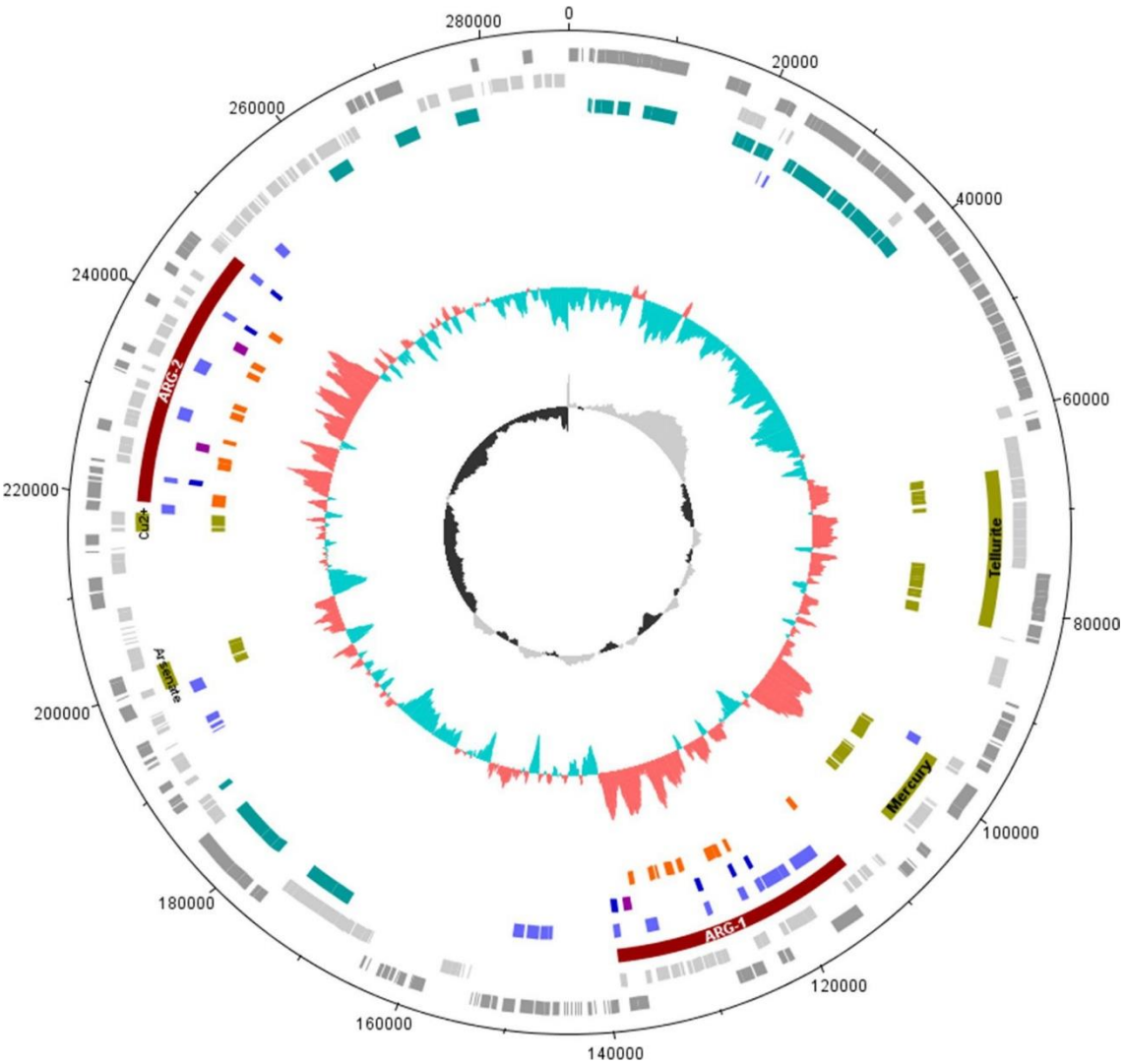

Fig. 2 Genetic map of the plasmid pCM18-216. From outer to inner circles: 1-nucleotide positions; 2,3-grey bars: CDSs; 4-teal: conjugation, dark red: ARG loci, green-brown metal/other resistance loci; 5-light blue: transposases CDSs; 6-dark blue: IS26 elements, purple: integron recombinase CDSs; 7-orange: ARGs, green-brown: other resistance genes; 8-GC% plot; 9-GC skew plot

The MICs of four randomly picked transconjugants were compared to the *E. hormaechei* and *E. coli* DH5α parents (Table 5). All transconjugants had MICs identical to the donor and higher than the recipient for ampicillin, chloramphenicol, gentamicin, tetracycline, and trimethoprim-sulfamethoxazole. Moreover, the transconjugants had MICs higher than DH5α albeit slightly lower compared to the donor, for amoxicillin/clavulanic acid, first and third generation cephalosporins (cefalexin, cefazolin, cefovecin, cefpodoxime, ceftazidime), and doxycycline. However, the transconjugants MICs for fluoroquinolones were similar to the unconjugated DH5α recipient, and lower than the *E. hormaechei* donor.

Antimicrobial resistance genes are associated with transposable elements

The two antibiotic resistance gene loci carried by plasmid pCM18-216 contained transposons and/or class 1 integrons putatively forming complex transposable elements.

Locus 1 was identified as an 18 kbp fragment consisting of two composite transposons and a class 1 integron fused together. The locus contained four IS26 copies, with the chloramphenicol resistance gene *cat*II between the first two, the tetracycline resistance gene *tet*D and its regulator *tet*R between the second and third, and a complete class 1 integron between third and fourth IS26 elements. The integron contained the aminoglycoside

Table 5 MIC of *Enterobacter* sink isolates, DH5 alpha transconjugant (TG) and parental recipient strain used in mating experiments

Antimicrobic	*E. hormachei* 18-216	*E. hormachei* 18-242-2	DH5 alpha_TG	DH5 alpha
Amikacin	≤4	≤4	≤4	≤4
Amoxicillin/Clavulanic Acid	>8	>8	=8	=4
Ampicillin	>8	>8	>8	=2
Aztreonam	>16	>16	>16	n/d
Cefalexin	>16	>16	=16	=4
Cefazolin	>32	>32	=32	=2
Cefepime	≤2	≤2	≤2	n/d
Cefotaxime	=8	=8	=2	n/d
Cefovecin	>8	>8	=8	=0.5
Cefpodoxime	>8	>8	=8	≤1
Ceftazidime	>16	>16	=16	≤4
Chloramphenicol	>32	>32	>32	≤2
Ciprofloxacin	≤0.25	≤0.25	≤0.25	n/d
Colistin	≤0.25	≤0.25	≤0.25	n/d
Doripenem	≤0.12	≤0.12	≤0.12	n/d
Doxycycline	>16	>16	=16	=0.5
Enrofloxacin	=1	=1	≤0.12	≤0.12
Ertapenem	≤0.25	≤0.25	≤0.25	n/d
Gentamicin	>8	>8	>8	≤0.25
Imipenem	≤1	≤1	≤1	≤1
Levofloxacin	≤1	≤1	≤1	n/d
Marbofloxacin	=0.5	=0.5	≤0.12	≤0.12
Meropenem	≤1	≤1	≤1	n/d
Minocycline	=16	=16	=8	n/d
Orbifloxacin	=4	=4	≤1	≤1
Piperacillin/tazobactam constant 4	≤8	≤8	≤8	≤8
Polymixin	≤0.25	≤0.25	≤0.25	n/d
Pradofloxacin	=0.5	=0.5	≤0.25	≤0.25
Tetracycline	>16	>16	>16	≤4
Ticarcillin/clavulanic acid constant 2	=32	=32	=32	n/d
Tigecycline	=0.5	=0.5	=0.5	n/d
Tobramycin	=8	>8	=2	n/d
Trimethoprim/sulfamethoxazole	>4	>4	>4	≤0.5

The values are compiled from Sensitre plates COMPGN1F and GNX2F. n/d: no data available for the organism

resistance gene *aac*(6′)-IIc upstream of the integrase gene, an IS1380 family transposase gene, and the aminoglycoside, rifampicin and erythromycin resistance genes *aac*(3)-II, *arr* and *ere*A, between the transposase and the 3′-CS of the integron. This structure appears to be the result of genetic re-arrangements involving IS26 family composite transposons conferring chloramphenicol and tetracycline resistance, together with a class 1 integron carrying the other resistance genes. This brought together 7 complete and 2 truncated ARGs that potentially could be mobilised in a single horizontal gene transfer event. Moreover, a beta-lactamase gene *bla*-TEM associated with a Tn3 transposon was located at the end of locus 1. These various components were also detected in plasmids with high levels of sequence similarity with pCM18-216, exemplified by pEC-IMPQ (NC_012556.1) carried by an *Enterobacter* isolated from a hospital environment in Taiwan, and pIMP4-SEM1 (KX810825.1) carried by a *Salmonella* isolated from a cat in Australia. However, the different genetic elements forming the pCM18-216 ARG locus 1 were located in separate regions in those replicons (Fig. 3a).

Locus 2 was a 26 kbp structure, also containing IS26 elements. The region is bordered by two IS903 copies and carries composite transposons and a complex class 1 integron containing two integrase genes, surrounding

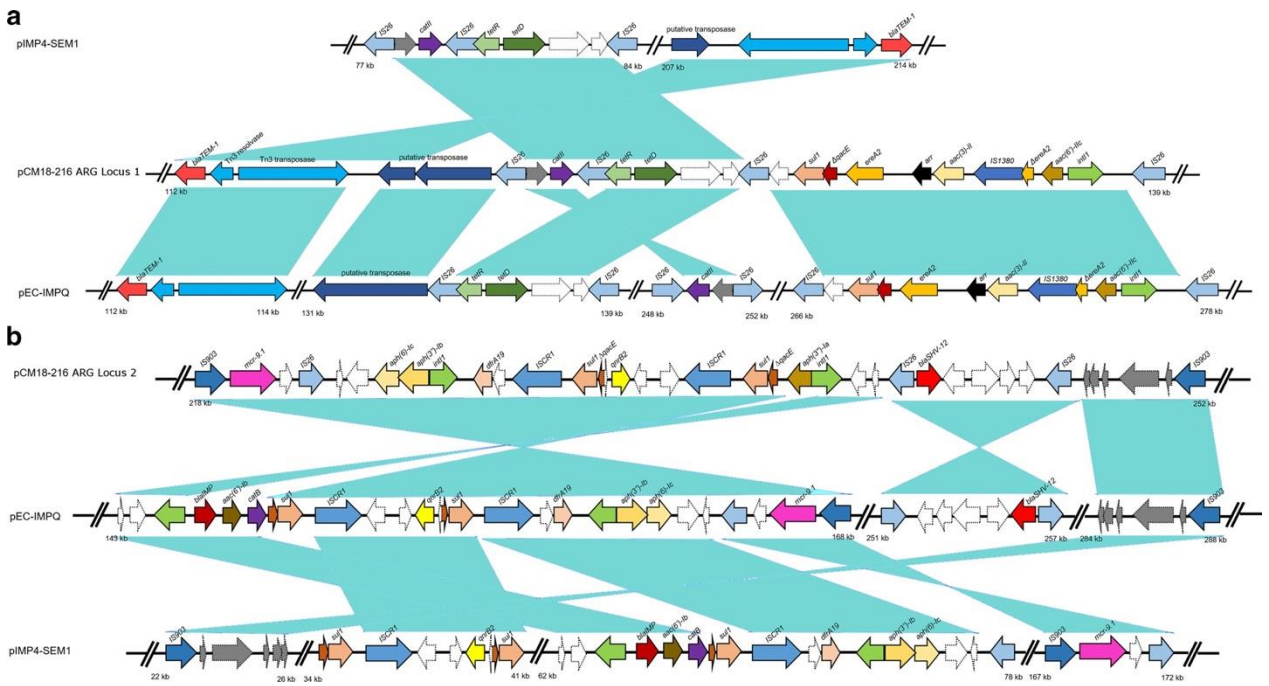

Fig. 3 Schematic representation of the genetic context of the IS26 transposable elements in pCM18-216 and comparison with related plasmids pEC-IMPQ (NC_012556.1) and pIMP4-SEM1 (KX810825.1). Resistance genes and mobile elements are represented by color-coded arrows. **a** ARG locus 1; **b** ARG locus 2

two "insertion sequence common region 1" elements (ISCR1, or IS91 family transposases). Eight ARGs were found in locus 2, including the ESBL *bla*SHV-12, fluoroquinolone resistance *qnrB2* and colistin resistance *mcr*9.1, which were respectively associated with copies of IS26, ISCR1 and IS903. As for locus 1, these structures were also found in pEC-IMPQ and pIMP4-SEM1 but were organized differently and carried a slightly larger repertoire of ARGs (Fig. 3b).

pCM18-216 shows similarities with a subset of large multidrug resistance plasmids from *Enterobacteriaceae*

Since the ARG loci-1 and -2 shared several genetic components with other multidrug resistance plasmids, the pCM18-216 sequence was compared to a set of 269 large plasmids of various incompatibility groups from *Enterobacteriaceae* (Additional file 1: Table S2). BLASTN DNA-DNA alignments showed that over 100 of these plasmids shared most of their sequence with pCM18-216 (Fig. 4a). However, BLASTP analysis of pCM18-216 CDS products indicated that some sequences were shared with only a smaller subset of replicons (Fig. 4b); for the most part these genes corresponded to ARG-carrying and mercury resistance loci of the plasmid (Fig. 4c, d). All 98 IncHI2 plasmids present in the dataset displayed overall sequence similarity with pCM18-216, but only 22 and 8 plasmids possessed a *bla*SHV-12 and *qnrB2* gene, respectively.

A comparative analysis of IncH12 plasmids carrying *qnrB2* and displaying high levels of similarity with pCM18-216 (Table 6) showed that they all shared a common backbone with a number of sequence re-arrangements and inversions (Additional file 4: Fig. S3). The pEC-IMPQ sequence was the most closely related to pCM18-216 with 99.94% sequence similarity, and carried an IS26-flanked composite transposon containing *bla*SHV-12 and a class 1 complex integron containing ISCR1 elements and *qnr*B2, but these components were located distantly on the replicon. Similarly, the plasmid p34977 from *Enterobacter hormaechei* subsp. *steigerwaltii* (CP_012170.1) possessed an IS26-*bla*SHV-12 transposon located 21 kbp away from the class 1 complex integron above described. By contrast, in pCM18-216 ARG locus-2, the IS26-*bla*SHV-12 transposon was immediately adjacent to the complex class 1 integron (Fig. 3b).

Systematic alignments of these plasmids with the CGView Comparison tool confirmed that ARGs-carrying regions are associated with most of the gene diversity within the subset (Fig. 5). While all plasmids except one carried an ESBL gene (*bla*SHV-12 or *bla*OXA1), only 3 plasmids (namely pEC-IMPQ, pIMP4-SEM1

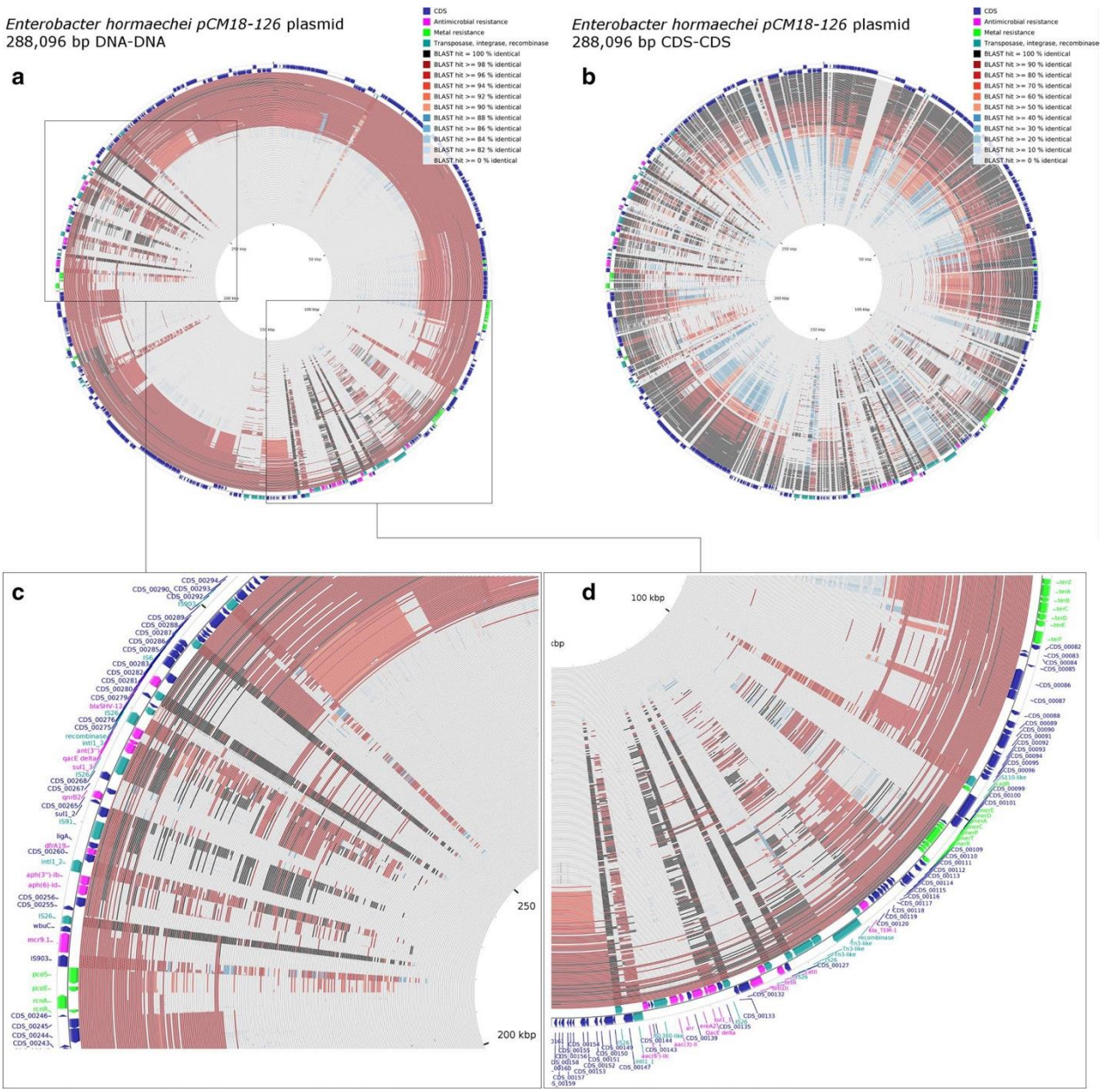

Fig. 4 Comparative analysis of pCM18-216 against 269 complete plasmid sequences from *Enterobacteriaceae*. Outer rim represents the pCM18-216 map; ARGs are indicated in pink, metal resistance genes in green, transposable elements and integrases in teal. Each inner rim represents an individual plasmid sequence. **a** DNA-DNA alignments; **b** CDS-CDS alignments; **c** close-up view of ARG locus2; **d** close-up view of ARG locus1

and pMS7884A) also encoded metallo beta lactamases (*bla*IMP-4 or *bla*IMP-8) conferring resistance to carbapenems.

Discussion

The veterinary hospital investigated in this study has been using a registered commercial disinfectant containing benzalkonium chloride and biguanide hydrochloride for regular decontamination procedures. This type of product is widely used in animal care premises as it is considered efficacious against common veterinary pathogens as well as being safe for pets and staff. For cleaning and disinfection of sinks, the Standard Operating Procedure (SOP) enforced in the hospital is performed in two steps. First, a detergent or scrubbing agent is used to remove most organic material, followed by a thorough

Table 6 IncH12 plasmids carrying *qnr*B2 selected for comparative analysis

Plasmid	NCBI ID	Assigned taxon	Host	Country	Year
pEC-IMPQ	NC_012556.1	Enterobacter cloacae	Human	Taiwan	2009
p34977-263	NZ_CP012170.1	*Enterobacter hormaechei* subspecies steigerwaltii	Human	USA	2015
p09-036813-1A_261	NZ_CP016526.1	Salmonella enterica subspecies enterica serovar Heidelberg	Horse	Canada	2016
pIMP4-SEM1	KX810825.1	Salmonella enterica subspecies enterica serovar Typhimurium	Cat	Australia	2016
pMS7884A	NZ_CP022533.1	*Enterobacter hormaechei*	Human	Australia	2017
Plasmid "unnamed-4"	NZ_CP029717.1	Enterobacter cloacae	-	USA	2018
pGMI14-002_1	NZ_CP028197.1	Salmonella enterica subspecies enterica serovar Concord	-	Czech Republic	2018

rinsing with water. Then, the disinfectant is applied liberally and allowed to dry, ensuring a minimum 10 min contact time, as per the manufacturer instructions. Hospital staff members are supervised and trained by the Hospital Infection Control Officer (ICO) to ensure compliance with the SOP. The repeated isolation of *Enterobacter* in the hospital ICU exemplifies the capacity of some microorganisms to persist in health care premises despite normal disinfection attempts. The plasmid-encoded efflux pump *qacE* delta1 may have played a role in conferring partial resistance against the quaternary ammonium compound present in the disinfectant. However we cannot rule out that the other factors, such as the presence of grooves or hard-to-reach parts in the sink structure, may have initially interfered with the correct application of the product. Here, the hospital ICO played a crucial role to ensure that proper decontamination protocols were followed, including the manufacturer's recommendations for dilution, temperature and contact time of the disinfectant. The *Enterobacter* strain was not detected from swabs collected after a third round of decontamination of the sink, suggesting that the correct measures were eventually applied with success. Benzalkonium chloride is still used in the veterinary hospital. Routine environmental surveillance of the premises has not indicated the presence of intractable infectious agents, when the disinfectant is applied correctly.

Although two biochemical identification kits classified the *Enterobacter* isolates as *E. cloacae*, the absence of lactose fermentation was atypical for this species [36], as 93% of *E. cloacae* strains but only 9% of *E. hormaechei* strains appear lactose positive on MacConkey plates after 48 h [37]. Within the *E. cloacae* complex, accurate species identification by MALDI-TOF can be difficult, prompting for DNA sequencing to resolve taxonomic ambiguities [38]. The various genome analysis methods used in our study (Ribosomal Multilocus Sequence Typing, Average Nucleotide Identity and pylogenetic tree construction) identified the sink isolates as *E. hormaechei*. These results illustrate the current limitations of identification kits and databases for the correct classification of species in the *E. cloacae* complex.

All chromosomal ARGs were components of multidrug efflux pumps, except for *bacA*, which confers resistance to bacitracin by target alteration [39], whereas the conjugative plasmid pCM18-216 encoded specific resistance mechanisms against important antimicrobials, such as fluoroquinolones and cephalosporins. Although no ECOFF value is currently available for *E. hormaechei* against enrofloxacin, the MIC of 1 ug/mL observed with this antimicrobial was well above the ECOFF value of 0.125 ug/mL reported by EUCAST for *E. coli*, suggesting the presence of an acquired (albeit modest) resistance to the drug, likely due to the *qnr*B2 gene. However, the *E. coli* transconjugants carrying pCM18-216 were susceptible to fluoroquinolones. The reason for this is unclear, but the impact of a *qnr*B2 resistance on therapeutic outcomes in animals infected by *E. hormaechei* or other nosocomial agents carrying pCM18-216 cannot be dismissed. The pCM18-216 carried the *mcr*-9.1 gene, encoding a newly described phosphoethanolamine transferase which can confer an inducible resistance to colistin upon exposure to sub-inhibitory concentrations of the drug [40]. The sink isolates appeared susceptible to colistin and polymyxin B based on conventional testing methods. Although preliminary attempts at inducing colistin resistance in CM18-216 and CM18-242–2 by sub-culturing the isolates in presence of the antibiotic in broth or solid media failed to demonstrate a reversible increase in MIC in our hands, this question deserves further scrutiny. In *E. coli*, the two component system encoded by *qseC* and *qseB* is proposed to regulate the expression of polymixin/colistin resistance [41]. These genes are localised next to *mcr*-9.1 and IS903 in some *E. coli* and *E. hormaechei* plasmids [40]. While *qseC* and *qseB* were not carried pCM18-216, homologous sequences were found on the isolate chromosome, between nt positions 691981 and 693986. It is unclear whether *E. hormaechei* CM18- 216 can display colistin resistance under certain inducing conditions, which remain to be defined, but as this

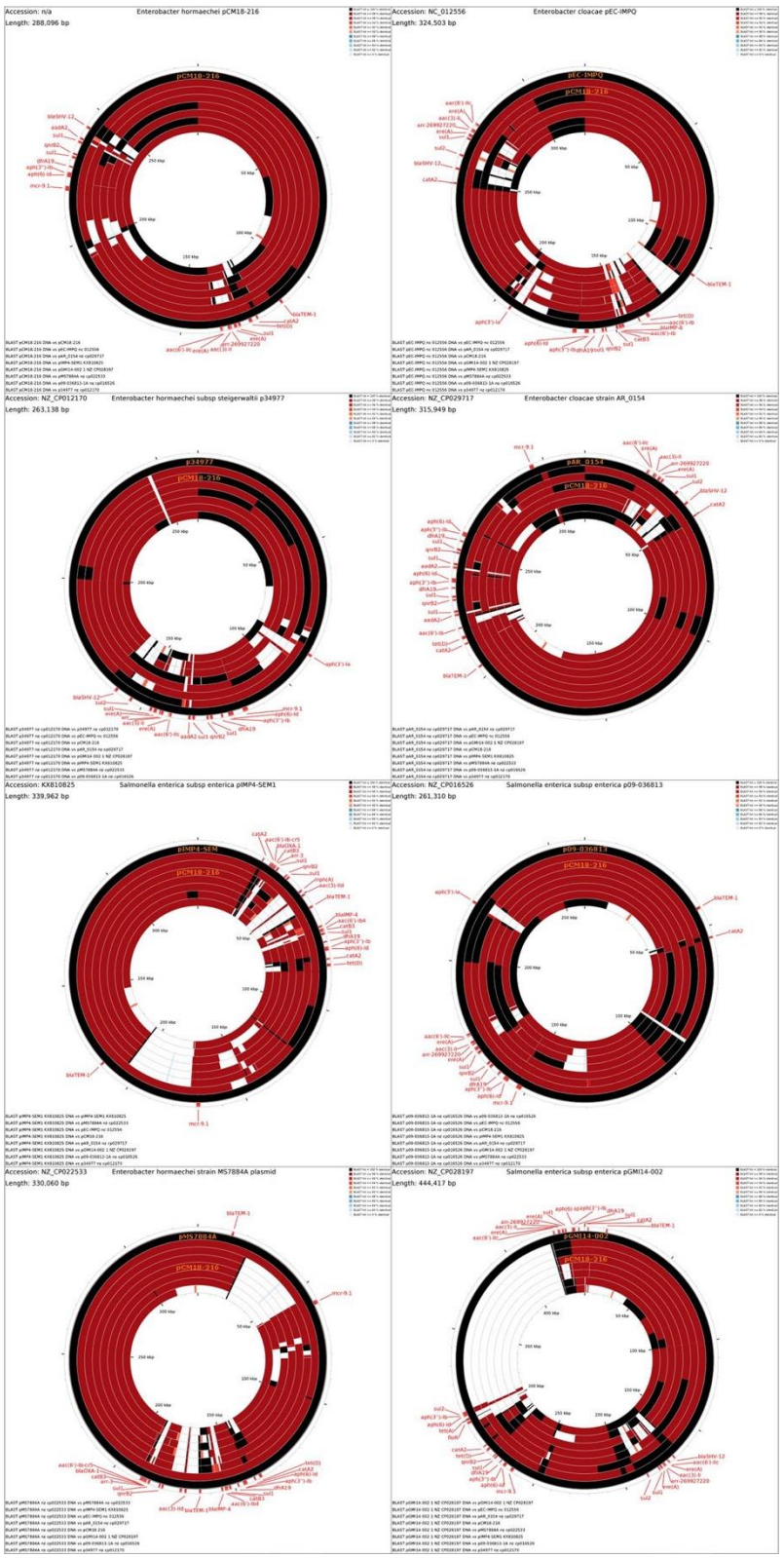

Fig. 5 Systematic comparative alignments of pCM18-216 and *qnrB2*-carrying incH12 plasmids from *Enterobacteriaceae* (see Table 6 for details). Each panel represents a query sequence plasmid (black outer circle) and the seven subject sequences (inner circles) arranged by decreasing order of similarity with the query. Positions of antimicrobial resistance genes in each query sequence are indicated in red

antimicrobial is a last resort, high importance drug for humans, the presence of an organism carrying *mcr*-9.1 in a veterinary ICU is concerning.

The co-selection of resistant organisms and propagation of resistance genes in veterinary hospital environments has been explored recently in our group, with a particular focus on the ICU [42]. The phenotypic characterization of metal resistances in the sink isolates was beyond the scope of this study, but it is worth noticing that pCM18-216 carried tellurium resistance gene clusters typically found in IncH12 plasmids [43] and heavy metal resistance genes organized similarly to other plasmids and transposons of Gram negative bacteria [44, 45]. The ESBL production was putatively attributed to the plasmidic gene *bla*SHV-12; the association of ESBL-encoding and metal resistance genes has been recently reported in *E. hormaechei* [46]. Topical preparations containing silver and fluoroquinolones are commercially available in Australia for the treatment of ear infections in companion animals, raising questions about the risks associated with the accumulation of heavy metals and antimicrobials residues in veterinary premises. The temperature requirements observed in mating experiments between CM18-216 and *E. coli* are also found in the conjugative transfer of IncHI plasmids, which occurs only within a 22–28 °C range [34, 47]. This suggests that pCM18-216 can transfer from *E. hormaechei* to other bacteria and disseminate heavy metal and multidrug resistances, including ESBLs, in the hospital normal environmental conditions.

The isolates also carried several mobile genetic elements. The presence of an additional chromosomal prophage in one of the four isolates indicates that the *Enterobacter* population colonizing the ICU sink may have acquired or rearranged mobile genetic elements over time. Several transposases and integrases were also found in the plasmid sequence, with important consequences for the physical organisation and potential co-transfer of ARGs. In Australia, ISCR1 have been described in IncL/M plasmids and IS26-associated class 1 integrons carrying *qnrB2* [48]. The ISCR1 elements are involved in rolling-circle transposition to form complex class 1 integrons [49]. IS26 mediated genetic re-arrangements are also well documented [50–52], particularly for their role in dissemination of antimicrobial resistance genes. The accumulation of antimicrobial resistance genes in genetic loci flanked by IS26 elements was more pronounced in pCM18-216 compared to other plasmids. No other sequence in the Genbank nucleotide database possessed a complete colinearity with the pCM18-216 full ARG locus-2, suggesting that this structure was created by intra-plasmidic sequence relocation. Because of the physical proximity of these ARGs and the presence of two bordering IS903 copies, the ARG locus-2 of pCM18-216 has the potential to facilitate the simultaneous horizontal gene transfer of the ESBL gene *bla*SHV-12 and the fluoroquinolone resistance gene *qnr*B2, along with other antimicrobial resistance genes, through a single transposition event. In Australia, *bla*IMP-4 genes have been associated with IncHI2 plasmids carried by *E. hormaechei* with various MLST profiles, but only two ST110 isolates [53]. These antimicrobials are considered of very high importance and their use in companion animals is not generally recommended (https://vetantibiotics.fvas.unimelb.edu.au/). Although the isolates CM18-216 and CM18-242 were susceptible to carbapenems and did not carry *bla*IMP sequences on their plasmids, the presence of the same mobile genetic elements found on *bla*IMP plasmids and pCM18-216 opens the question whether the organism is able to acquire such resistance. This underlines the importance of early detection of multidrug resistant organisms and decontamination to control the risks of dissemination of resistance within the hospital.

Conclusions

The presence in the veterinary hospital ICU of an ESBL, as well as fluoroquinolone and putative colistin resistance genes within an IS26 transposon in a conjugative plasmid for nearly one month underlines the risk of horizontal dissemination of ARGs into other bacterial species and nosocomial infections with reduced possibilities of treatment. This was concerning, even though the *Enterobacter* host was not phenotypically resistant to colistin and presented only intermediate MICs levels against ciprofloxacin. Repeated rounds of disinfection of the sink pipes were implemented until the organism could no longer be detected by environmental sampling. Based on these results, routine environmental surveillance programs incorporating the rapid detection of organisms capable of ESBL production and resistance to fluoroquinolones, colistin and carbapenems, should be considered in large veterinary hospitals.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13756-020-00828-0.

Additional file 1: Table S1. Sequencing read statistics after quality filtering. Table S2. Details on the genomes used to construct the phylogenetic tree.

Additional file 2: Figure S1. Chromosomal map of the E. hormaechei isolate CM18-216. From outer to inner circles: 1, nucleotide positions; 2 and 3, CDSs (grey); 4, tRNA (green); 5, predicted genomic islands and prophages (red); 6, pco/sil copper/silver resistance(brown-green), transposases

(purple), salmochelin synthesis and uptake (blue); 7, GC% plot; 8, GC skew plot. Inset: detailed map of the pco/sil resistance locus.

Additional file 3: Figure S2. Mauve alignment of the chromosome sequences of the four Enterobacter strains isolated from the ICU sink over approximately one month. Local blocks of colinearity are labelled with different colors. Predicted coding sequences are indicated underneath each genome. CM18-216 and CM18-242-2 (two top rows) were obtained from hybrid assemblies of Illumina and Nanopore reads; CM18-269-1 and CM18-269-2 (two bottom rows) were assembled from nanopore reads only. Position of a putative phage in CM18-269-2 is indicated by a red box.

Additional file 4: Figure S3. Alignment of pCM18-216 with IncH12 plasmids carrying qnrB2. Horizontal black lines indicate the lengths of the plasmid sequences. Dark blue horizontal bars on the top (forward strand) and the bottom (reverse strand) of the black lines indicate areas of sequence homology. Vertical bars connecting the horizontal lines show areas of sequence homology.

Abbreviations

ICU: Intensive care units; ESBL: Extended spectrum beta-lactamase; MIC: Minimum inhibitory concentration; ARG: Antimicrobial resistance gene.

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Authors' contributions

Conceptualization, M.M.; methodology, M.M., H.B. and K.K.; validation, M.M., H.B. and G.B.; formal analysis, K.K., H.B. and M.M.; investigation, K.K. FR, MM and R.B.; resources, R.B.; data curation, K.K. and M.M.; writing–original draft preparation, K.K.; writing–review and editing, M.M., H.B. and G.B.; visualization, K.K., and M.M.; supervision, M.M., H.B. and G.B.; All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The datasets generated and analysed during the current study have been deposited in the Genbank repository under the following entries: BioProject PRJNA613546; BioSample SAMN14409014: CP05031 (chromosome CM18-216), CP050312 (plasmid pCM18-216); BioSample SAMN14449833: CP050506 (chromosome CM18-242-2), CP050507 (plasmid pCM18-242-2).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

3. Appendix

Supplementary Tables and Figures

Table S1. Sequencing read statistics after quality filtering

Isolate	Nanopore				Illumina	
	No. reads	Total sequence (Mbp)	Mean read length (kbp)	N50 (kbp)	No. reads	Read length (bp)
CM18-216	8,519	129	15	21	2,061,010	110-125
CM18-242-2	17,872	271	15	21	1,759,821	115-125
CM18_269_1	20,209	389	19	25	NA	NA
CM18_269_2	23,373	398	17	22	NA	NA

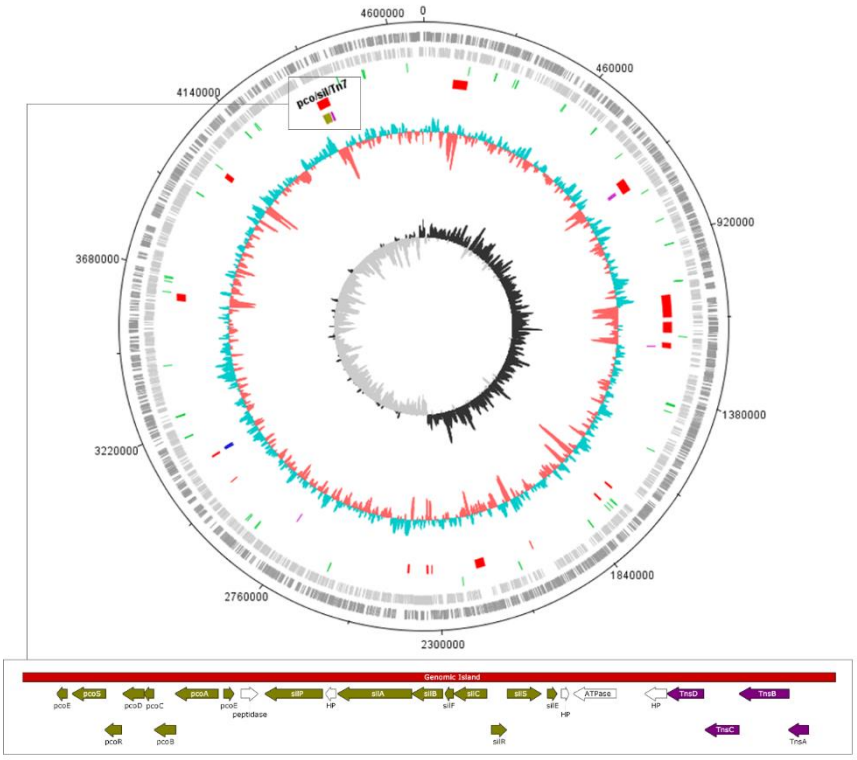

Figure S1. Chromosomal map of the *E. hormaechei* isolate CM18-216. From outer to inner circles: 1, nucleotide positions; 2 and 3, CDSs (grey); 4, tRNA (green); 5, predicted genomic islands and prophages (red); 6, pco/sil copper/silver resistance(brown-green), transposases (purple), salmochelin synthesis and uptake (blue); 7, GC% plot; 8, GC skew plot. Inset: detailed map of the pco/sil resistance

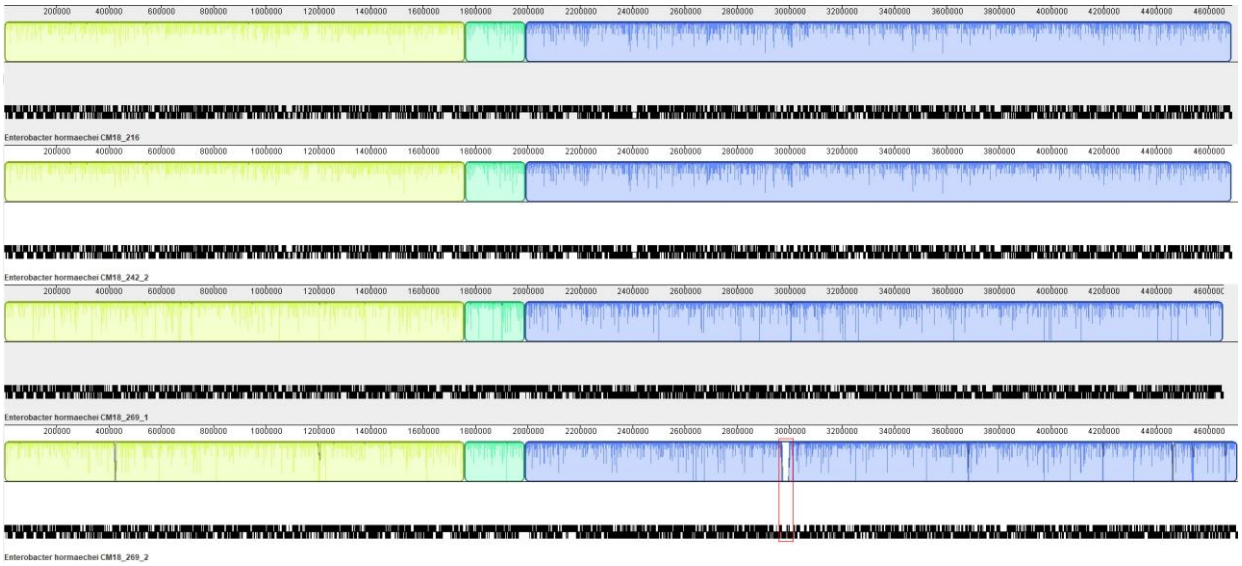

Figure S2. Mauve alignment of the chromosome sequences of the four *Enterobacter* strains isolated from the ICU sink over approximately one month. Local blocks of colinearity are labelled with different colors. Predicted coding sequences are indicated underneath each genome. CM18-216 and CM18-242-2 (two top rows) were obtained from hybrid assemblies of Illumina and Nanopore reads; CM18-269-1 and CM18-269-2 (two bottom rows) were assembled from nanopore reads only. Position of a putative phage in CM18-269-2 is indicated by a red box.

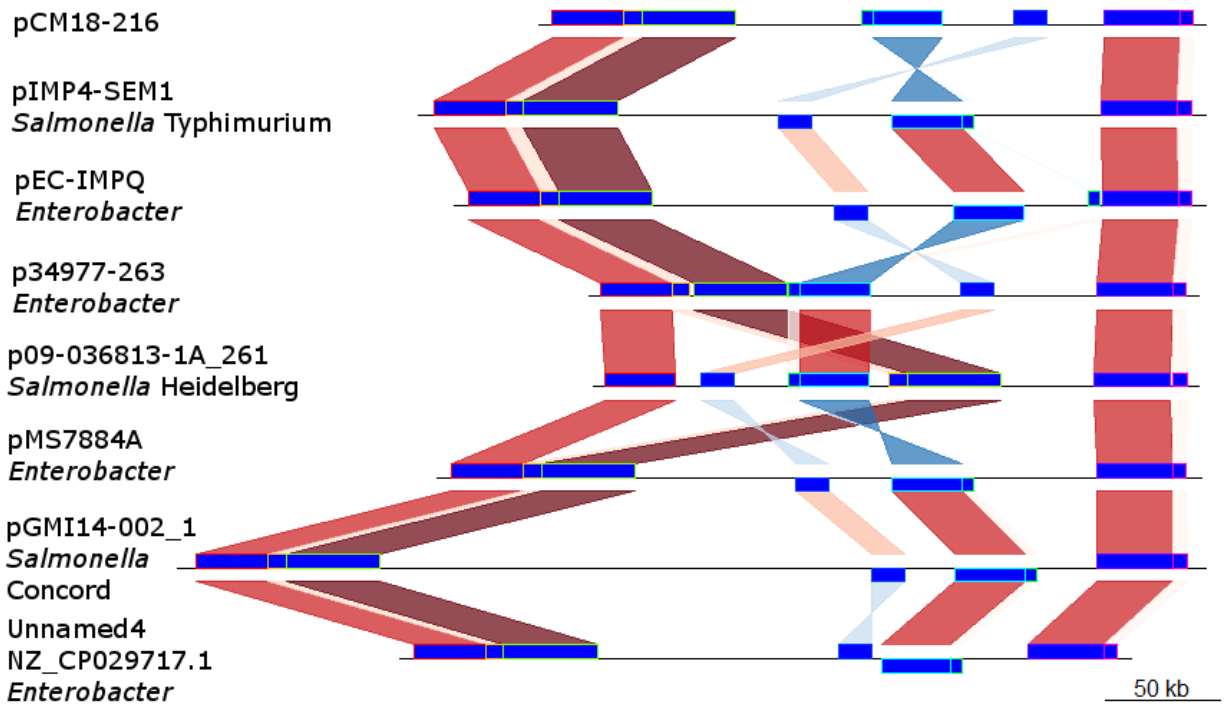

Figure S3. Alignment of pCM18-216 with IncH12 plasmids carrying *qnrB2*. Horizontal black lines indicate the lengths of the plasmid sequences. Dark blue horizontal bars on the top (forward strand) and the bottom (reverse strand) of the black lines indicate areas of sequence homology. Vertical bars connecting the horizontal lines show areas of sequence homology.

Table S2. Details on the genomes used to construct the phylogenetic tree.

Refseq Assembly	Species	Strain	Host	Source	Isolation Location	Isolation Date
GCF_000016325.1_ASM1632v1	*Enterobacter* sp.	638	n/a	n/a	n/a	n/a
GCF_000017665.1_ASM1766v1	*Cronobacter sakazakii*	ATCC_BAA-894	n/a	n/a	n/a	n/a
GCF_000025565.1_ASM2556v1	*Enterobacter cloacae* subsp. *cloacae*	ATCC_13047	n/a	n/a	n/a	n/a
GCF_000224675.1_ASM22467v1	*Enterobacter soli*	LF7a	n/a	n/a	n/a	n/a
GCF_000235765.1_ASM23576v3	*Enterobacter cloacae* subsp. *dissolvens*	SDM	n/a	n/a	n/a	n/a
GCF_000239975.1_ASM23997v1	*Enterobacter ludwigii*	EcWSU1	n/a	n/a	n/a	n/a
GCF_000286275.1_ASM28627v1	*Enterobacter kobei*	ENHKU01	n/a	n/a	n/a	n/a
GCF_000410515.1_ASM41051v1	*Enterobacter* sp.	R4-368	n/a	n/a	n/a	n/a
GCF_000512375.1_ASM51237v1	*Enterobacter ludwigii*	P101	n/a	n/a	n/a	n/a
GCF_000632395.1_ASM63239v1	*Enterobacter asburiae*	L1	n/a	lettuce	Malaysia	03-Apr-2013
GCF_000724505.1_ASM72450v1	*Enterobacter cloacae*	ECNIH2	n/a	sink drain	USA	2012
GCF_000750225.1_ASM75022v1	*Enterobacter hormaechei* subsp. *hoffmannii*	ECNIH3	Homo sapiens	tracheal aspirate	USA	2011
GCF_000750275.1_ASM75027v1	*Enterobacter hormaechei* subsp. *hoffmannii*	ECR091	Homo sapiens	urine	USA	2012
GCF_000770155.1_ASM77015v1	*Enterobacter cloacae*	GGT036	n/a	n/a	South Korea	n/a
GCF_000783675.2_ASM78367v2	*Enterobacter cloacae complex* sp.	FDAARGOS_77	Homo sapiens	Rectal Swab	USA	07-Sep-2013
GCF_000784865.1_ASM78486v1	*Enterobacter cloacae*	ECNIH4	n/a	sink drain	USA	2012
GCF_000784905.1_ASM78490v1	*Enterobacter cloacae*	ECNIH5	n/a	sink drain	USA	2011
GCF_000801755.2_ASM80175v2	*Enterobacter* sp.	E20	Oryza sativa	glyphosate polluted soil	China Zhejiang	Oct-2010
GCF_000807405.2_ASM80740v4	*Enterobacter hormaechei* subsp. *oharae*	34978	Homo sapiens	bodily fluid	USA	2011
GCF_000807415.2_ASM80741v4	*Enterobacter roggenkampii*	35734	Homo sapiens	Excreted bodily substance	USA	2010
GCF_000807425.2_ASM80742v4	*Enterobacter hormaechei* subsp. *steigerwaltii*	34998	Homo sapiens	bodily fluid	USA	2011
GCF_000814125.3_ASM81412v3	*Enterobacter hormaechei* subsp. *steigerwaltii*	34977	Homo sapiens	bodily fluid	USA	2009
GCF_000814205.1_ASM81420v1	*Enterobacter hormaechei* subsp. *hormaechei*	34983	Homo sapiens	bodily fluid	USA	2010
GCF_000814225.1_ASM81422v1	*Enterobacter hormaechei* subsp. *xiangfangensis*	34399	Homo sapiens	Excreted bodily substance	USA	2011
GCF_001022015.1_ASM102201v1	*Enterobacter hormaechei*	CAV1311	Homo sapiens	Urine Genitourinary	USA Virginia	2011-01
GCF_001022055.1_ASM102205v1	*Enterobacter hormaechei*	CAV1668	Homo sapiens	Perirectal	USA Virginia	2012-08
GCF_001022075.1_ASM102207v1	*Enterobacter hormaechei*	CAV1411	Homo sapiens	Respiratory	USA Virginia	2011-06
GCF_001022095.1_ASM102209v1	*Enterobacter asburiae*	CAV1043	Homo sapiens	n/a	USA Virginia	2008-03
GCF_001022255.1_ASM102225v1	*Enterobacter hormaechei*	CAV1669	Homo sapiens	Perirectal	USA Virginia	2012-08
GCF_001029645.1_ASM102964v1	*Enterobacter ludwigii*	UW5	n/a	soil	Canada Waterloo	29-Sep-1994
GCF_001521715.1_ASM152171v1	*Enterobacter asburiae*	ATCC_35953	Homo sapiens	physical	n/a	2014-12-01
GCF_001617645.1_ASM161764v1	*Enterobacter asburiae*	ENIPBJ-CG1	Homo sapiens	n/a	China Beijing	2014-07-03
GCF_001623605.1_ASM162360v1	*Enterobacter* sp.	ODB01	n/a	crude oil contaminated soil	China	2014-09-01
GCF_001719105.1_ASM171910v1	*Enterobacter* sp.	HK169	n/a	Tomato roots	South Korea Daejeon	2015
GCF_001729705.1_ASM172970v1	*Enterobacter hormaechei* subsp. *oharae*	DSM_16687	Homo sapiens	n/a	Germany	n/a
GCF_001729725.1_ASM172972v1	*Enterobacter hormaechei* subsp. *steigerwaltii*	DSM_16691	Homo sapiens	wound	Belgium	n/a
GCF_001729745.1_ASM172974v1	*Enterobacter hormaechei* subsp.	DSM_14563	Homo sapiens	n/a	Germany	n/a

	hoffmannii					
GCF_001729765.1_ASM172976v1	*Enterobacter kobei*	DSM_13645	Homo sapiens	blood	Japan	n/a
GCF_001729785.1_ASM172978v1	*Enterobacter hormaechei* subsp. *xiangfangensis*	LMG27195	n/a	Chinese traditional sourdough	China Heilongjiang province	n/a
GCF_001729805.1_ASM172980v1	*Enterobacter roggenkampii*	DSM_16690	Homo sapiens	n/a	Germany	n/a
GCF_001750725.1_ASM175072v1	*Enterobacter ludwigii*	EN-119	Homo sapiens	n/a	n/a	n/a
GCF_001874505.1_ASM187450v1	*Enterobacter hormaechei*	CAV1176	Homo sapiens	Perirectal	USA Virginia	2010-05
GCF_001888805.2_ASM188880v2	*Enterobacter* sp.	SA187	n/a	roots of desert plants	Saudi Arabia Jizan	01-Mar-2014
GCF_001922365.1_ASM192236v1	*Enterobacter cloacae*	AR_0002	n/a	n/a	n/a	n/a
GCF_001984825.2_ASM198482v2	*Enterobacter chengduensis*	WCHECl-C4	Homo sapiens	blood	China Chengdu Sichuan	2015-02-01
GCF_002007805.1_ASM200780v1	*Enterobacter roggenkampii*	R11	n/a	sewage water	China Shandong	2016-12-17
GCF_002025685.1_ASM202568v1	*Enterobacter ludwigii*	AA4	Zea mays	root	USA Boston	Dec-2012
GCF_002055735.1_ASM205573v1	*Enterobacter cloacae*	AR_0065	n/a	n/a	n/a	n/a
GCF_002192355.1_ASM219235v1	*Enterobacter cloacae*	AR_0163	n/a	n/a	n/a	n/a
GCF_002192395.1_ASM219239v1	*Enterobacter cloacae*	AR_0053	n/a	n/a	n/a	n/a
GCF_002197345.1_ASM219734v1	*Enterobacter cloacae*	A1137	Homo sapiens	blood	n/a	n/a
GCF_002201815.1_ASM220181v1	*Enterobacter cloacae*	AR_0050	n/a	n/a	n/a	n/a
GCF_002204775.1_ASM220477v1	*Enterobacter cloacae*	AR_0136	n/a	n/a	n/a	n/a
GCF_002208095.1_ASM220809v1	*Enterobacter cloacae complex* sp.	ECNIH7	n/a	n/a	USA	2014
GCF_002211685.1_ASM221168v1	*Enterobacter roggenkampii*	704SK10	n/a	wastewater	Switzerland Basel	Dec-2015
GCF_002237465.1_ASM223746v1	*Enterobacter hormaechei*	MS7884A	Homo sapiens	endotracheal tube	Australia	15-Jun-2015
GCF_002303275.1_ASM230327v1	*Enterobacter cloacae*	M12X01451	Homo sapiens	Stool	n/a	n/a
GCF_002787395.1_ASM278739v1	*Enterobacter* sp.	CRENT-193	Homo sapiens	wound	South Korea Seoul	2013
GCF_002850575.1_ASM285057v1	*Enterobacter cancerogenus*	CR-Eb1	Galleria mellonella	gut 3 4th instar larva	South Korea Daejeon	2015-09
GCF_002863825.1_ASM286382v1	*Enterobacter* sp.	Crenshaw	Rhizoctonia solani	brown patch in grass	USA Kansas	12-Dec-2016
GCF_002947755.1_ASM294775v1	*Enterobacter cloacae*	AR_0060	n/a	n/a	n/a	n/a
GCF_002954165.1_ASM295416v1	*Enterobacter cloacae*	AR_0072	n/a	n/a	n/a	n/a
GCF_002968455.1_ASM296845v1	*Enterobacter hormaechei* subsp. *hoffmannii*	AR_0365	n/a	n/a	n/a	n/a
GCF_002982195.1_ASM298219v1	*Enterobacter cloacae*	PIMB10EC27	Homo sapiens	urine	Viet Nam	2010
GCF_003010695.1_ASM301069v1	*Enterobacter cloacae complex* sp.	FDA-CDC-AR_0132	n/a	n/a	n/a	n/a
GCF_003031445.1_ASM303144v1	*Enterobacter cloacae*	109	Homo sapiens	trachael aspirate	USA Boston	2015
GCF_003031755.1_ASM303175v1	*Enterobacter cloacae*	174	Homo sapiens	blood	USA Boston	2015
GCF_003051945.2_ASM305194v2	*Enterobacter hormaechei*	SCEH020042	Homo sapiens	n/a	China Panzhihua Sichuan	2016-10-10
GCF_003053755.1_ASM305375v1	*Enterobacter cloacae*	AR_0093	n/a	n/a	n/a	n/a
GCF_003071645.1_ASM307164v1	*Enterobacter cloacae complex* sp.	FDA-CDC-AR_0164	n/a	n/a	n/a	n/a
GCF_003073995.1_ASM307399v1	*Enterobacter hormaechei*	AR432	n/a	n/a	n/a	n/a
GCF_003186415.1_ASM318641v1	*Enterobacter hormaechei*	234	Homo sapiens	wound	USA Boston	2016
GCF_003186565.1_ASM318656v1	*Enterobacter hormaechei*	388	n/a	n/a	USA Boston	2017
GCF_003204095.1_ASM320409v1	*Enterobacter cloacae*	AR_0154	n/a	n/a	n/a	n/a
GCF_003254805.1_ASM325480v1	*Enterobacter hormaechei* subsp. *xiangfangensis*	Pb204	n/a	acid mine decant and tailings from	South Africa West Rand Gauteng	Jan-2014
GCF_003264955.1_ASM326495v1	*Enterobacter hormaechei*	20710	Homo sapiens	sptum	China Shandong	2011-08
GCF_003288475.1_ASM328847v1	*Enterobacter hormaechei*	AR_038	n/a	n/a	n/a	n/a
GCF_003382725.1_ASM338272v1	*Enterobacter hormaechei* subsp.	OSUVMCKPC4-2	canine	n/a	USA Ohio Columbus	2016-07-27

	xiangfangensis					
GCF_003408555.1_ASM340855v1	*Enterobacter hormaechei*	2013_1a	Homo sapiens	Rectal Swab	n/a	2013
GCF_003408575.1_ASM340857v1	*Enterobacter hormaechei*	N1	Homo sapiens	Rectal Swab	n/a	2015
GCF_003408595.1_ASM340859v1	*Enterobacter hormaechei*	A1	Homo sapiens	Rectal Swab	n/a	2015
GCF_003428425.1_ASM342842v1	*Enterobacter hormaechei*	WCHEH020038	Homo sapiens	n/a	China Sichuan Chengdu	2016-11-02
GCF_003444755.1_ASM344475v1	*Enterobacter hormaechei*	FRM	n/a	high concentration of fluoride	China	2008-10-30
GCF_003586025.1_ASM358602v1	*Enterobacter hormaechei* subsp. *xiangfangensis*	OSUKPC4_L	Canis lupus familiaris	Bite Wound	USA Ohio Columbus	09-Sep-2016
GCF_003660125.1_ASM366012v1	*Enterobacter hormaechei*	C15117	n/a	Burns unit surveillance	Australia Sydney	2007
GCF_003665375.1_ASM366537v1	*Enterobacter kobei*	WCHEK045523	Homo sapiens	n/a	China Sichuan Chengdu	2017
GCF_003719615.1_ASM371961v1	*Enterobacter cloacae*	E3442	Penaeus vannamei	n/a	Netherlands	Mar-2017
GCF_003812145.1_ASM381214v1	*Enterobacter roggenkampii*	FDAARGOS_523	Homo sapiens	Rectal Swab	n/a	28-Dec-2015
GCF_003940765.1_ASM394076v1	*Enterobacter asburiae*	CAV1043	n/a	water	USA	18-Jun-2018
GCF_003964795.2_ASM396479v2	*Enterobacter hormaechei* subsp. *xiangfangensis*	WCHEX045001	Homo sapiens	blood	China Chengdu Sichuan	2018-01
GCF_003965345.2_ASM396534v2	*Enterobacter hormaechei*	WCHEH090011	Homo sapiens	n/a	China Chengdu Sichuan	2017
GCF_004006055.1_ASM400605v1	*Enterobacter* sp.	N18-03635	Homo sapiens	Rectal Swab	Canada	n/a
GCF_004118875.1_ASM411887v1	*Enterobacter hormaechei*	S11_16	Homo sapiens	n/a	United Kingdom	2016
GCF_004138605.1_ASM413860v1	*Enterobacter roggenkampii*	ECY546	Homo sapiens	n/a	China Wenzhou	2008-10-30
GCF_004151605.1_ASM415160v1	*Enterobacter cloacae*	EN3600	Homo sapiens	blood	China Anhui	May-2015
GCF_004193715.1_ASM419371v1	*Enterobacter cloacae*	CZ-1	n/a	paddy soil	China Zhenzhou	14-Jul-2016
GCF_004355165.1_ASM435516v1	*Enterobacter cloacae complex* sp.	N13-01531	Homo sapiens	n/a	Canada Alberta	2013
GCF_004684365.1_ASM468436v1	*Enterobacter roggenkampii*	BP10374	Homo sapiens	blood	India	2018
GCF_004804375.1_ASM480437v1	*Enterobacter bugandensis*	220	Homo sapiens	throat swab	Germany Hessen	15-Jan-2019
GCF_004804395.1_ASM480439v1	*Enterobacter bugandensis*	1367	Homo sapiens	blood	Germany North Rhine Westphalia	01-Jan-2011
GCF_005518115.1_ASM551811v1	*Enterobacter ludwigii*	JP6	Tobacco	rhizosphere soil	China Hunan Province	2011-05-01
GCF_005848825.1_ASM584882v1	*Enterobacter ludwigii*	JP9	n/a	Tobacco rhizosphere soil	China Hunan Province	2011-05-01
GCF_005890075.1_ASM589007v1	*Enterobacter ludwigii*	I42	n/a	Lycium barbarum rhizosphere soil	China Ningxia Hui Autonomous Region	2015-09-01
GCF_006228165.1_ASM622816v1	*Enterobacter cloacae*	NH77	Homo sapiens	n/a	Thailand Chiang Mai	Jan-2018
GCF_006385655.1_ASM638565v1	*Enterobacter hormaechei*	C126	n/a	urine	India	2016
GCF_006385915.1_ASM638591v1	*Enterobacter ludwigii*	I140	n/a	soil	China Ningxia Hui Autonomous Region	2011-05-01
GCF_007035645.1_ASM703564v1	*Enterobacter asburiae*	1808-013	Homo sapines	urine	Japan Osaka	2018-08
GCF_007035805.1_ASM703580v1	*Enterobacter asburiae*	17Nkhm-UP2	n/a	river water	Japan Osaka	2017-09
GCF_007035975.1_ASM703597v1	*Enterobacter* sp.	18A13	n/a	river water	Japan Osaka	2018-08
GCF_007556795.1_ASM755679v1	*Enterobacter hormaechei* subsp. *steigerwaltii*	ME-1	n/a	n/a	n/a	2018
GCF_008123985.1_ASM812398v1	*Enterobacter hormaechei*	PG20180056	n/a	mouse gut	n/a	18-May-2018
GCF_008124025.1_ASM812402v1	*Enterobacter hormaechei*	PG20180049	n/a	mouse gut	n/a	18-May-2018
GCF_008271405.1_ASM827140v1	*Enterobacter* sp.	LU1	goat	rumen content	Poland Lubelskie Voivodeship	2010-2011
GCF_008365235.1_ASM836523v1	*Enterobacter kobei*	EB_P8_L5_01.19	Homo sapiens	screening swab	United Kingdom London	2019-01
GCF_008505035.1_ASM850503v1	*Enterobacter hormaechei*	EB_P6_L3_02.19	Homo sapiens	rectal screen	United Kingdom London	2019-02-27
GCF_008693905.1_ASM869390v1	*Enterobacter hormaechei*	FDAARGOS_642	Homo sapiens	clinical isolate	USA KY	n/a
GCF_008931325.1_ASM893132v1	*Enterobacter hormaechei*	C44	Homo sapiens	clinical sample	Australia Sydney	15-Apr-2013
GCF_008931405.1_ASM893140v1	*Enterobacter hormaechei*	C15	Homo sapiens	clinical sample	Australia Sydney	23-Feb-2009
GCF_008931465.1_ASM893146v1	*Enterobacter* sp.	E76	n/a	Shower 3	Australia Sydney	07-May-2014
GCF_008931525.1_ASM893152v1	*Enterobacter hormaechei*	E5	n/a	Shower 3	Australia Sydney	13-Jun-2012

GCF_008931545.1_ASM893154v1	*Enterobacter kobei*	C16	Homo sapiens	clinical sample	Australia Sydney	12-Mar-2009
GCF_008931585.1_ASM893158v1	*Enterobacter hormaechei*	C4	Homo sapiens	clinical sample	Australia Sydney	28-Jun-2007
GCF_008931645.1_ASM893164v1	*Enterobacter hormaechei*	C45	Homo sapiens	clinical sample	Australia Sydney	27-Apr-2013
GCF_008931785.1_ASM893178v1	*Enterobacter hormaechei*	EB_P9_L5_03.19	Homo sapiens	Rectal Swab	United Kingdom London	2019-03
GCF_009036245.1_ASM903624v1	*Enterobacter cloacae*	SGAir0282	n/a	air	Singapore	14-May-2015
GCF_009176645.1_ASM917664v1	*Enterobacter oligotrophica*	CCA6	n/a	n/a	Japan Hiroshima Higashi Hiroshima	n/a
GCF_009184765.2_ASM918476v2	*Enterobacter roggenkampii*	WCHER090065	Homo sapiens	n/a	China Chengdu Sichuan	2016-08
GCF_009497055.1_ASM949705v1	*Enterobacter hormaechei*	AUH-ENM30	Homo sapiens	n/a	Lebanon	2016
GCF_009648915.1_ASM964891v1	*Enterobacter cancerogenus*	MiY-F	n/a	Cilantro	USA MI	2014-12-10
GCF_009707405.1_ASM970740v1	*Enterobacter cloacae*	CBG15936	Homo sapiens	sputum	China Guangzhou	29-Mar-2017
GCF_009728975.1_ASM972897v1	*Enterobacter hormaechei*	ECL69214	Homo sapiens	urine sample	China Guangzhou	04-Feb-2018
GCF_009738085.1_ASM973808v1	*Enterobacter hormaechei*	L51	Homo sapiens	feces	China Zhejiang	2016-04-08
GCF_009755685.1_ASM975568v1	*Enterobacter asburiae*	AEB30	n/a	ginger	USA Albany California	Jul-2015
GCF_009834325.1_ASM983432v1	*Enterobacter hormaechei* subsp. *hoffmannii*	MYJARB-EH1	Homo sapiens	n/a	USA	2018
GCF_009905155.1_ASM990515v1	*Enterobacter hormaechei*	F2	n/a	River	China Guangzhou Guangdong	2015-06-01
GCF_009930835.1_ASM993083v1	*Enterobacter hormaechei*	BW	Homo sapiens	Blood and wound	USA University of Michigan University Hospital	2013-08-24
GCF_009930935.1_ASM993093v1	*Enterobacter hormaechei*	BW	Homo sapiens	n/a	USA Ann Arbor Michigan	2014-05-30
GCF_900322715.1_C309	*Enterobacter hormaechei* subsp. *steigerwaltii*	C309	Homo sapiens	hospital	France	2015
GCF_900322725.1_C45	*Enterobacter cloacae complex* sp.	C45	Homo sapiens	hospital	France	2014
GCF_900324475.1_EB-247	*Enterobacter bugandensis*	EB247	Homo sapiens	blood	Tanzania	2010-01-05
GCF_900497145.1_ASM90049714v1	*Enterobacter cloacae*	EC-TO80	n/a	n/a	n/a	n/a
GCF_900635705.1_32407_E01	*Enterobacter cloacae*	NCTC11571	n/a	n/a	n/a	1800_2017

Chapter 4: General Discussion

Infection control strategies play a crucial role in mitigating nosocomial infections caused by drug-resistant pathogens in health care settings, and systematic surveillance is a key contributor to derivation of evidence-based infection control programmes [1, 2]. Appropriate surveillance can include monitoring patients for disease and/or monitoring the hospital environment for the presence of infectious agents [2, 3]. As highlighted in the Australia's first National Antimicrobial Resistance Strategy, 'Responding to the Threat of Antimicrobial Resistance' [4], the considerable diversity of animal health care settings necessitates the identification and closure of any gap in the infection prevention and control protocols implemented in these settings. Such gaps can be effectively identified using practice-specific environmental surveillance programmes.

The U-Vet animal hospital described in this thesis is one of the largest tertiary care veterinary facilities in Australia. The Infection Control Committee (ICC) of the U-Vet Animal Hospital has implemented a routine environmental surveillance programme, under which various environmental samples are collected at regular intervals and sent to the Veterinary Microbiology Laboratory for isolation and identification of potential pathogens in the environment. The main focus of current routine environmental surveillance programme is to track methicillin-resistant *Staphylococcus* species, ESBL-producing *Enterobacteriaceae* and vancomycin-resistant *Enterococcus* species, but environmental isolates are not routinely assessed for their antimicrobial susceptibility using a full panel of the drugs used in veterinary clinical practice. As non-pathogenic environmental bacteria can harbour ARGs conferring resistance to clinically important antimicrobials and transfer those genes to pathogens in the same environment [5, 6], it is important to monitor such risks. However, it is impractical to track all the bacteria that reside in a veterinary hospital environment and their AMR given the current resource limitations and the conventional microbiological techniques employed by the

Veterinary Microbiology Laboratory.

The work described in this thesis emphasises the utility of novel sequencing strategies to track drug-resistant organisms in veterinary clinical environments. Metagenomic sequencing and whole genome sequencing approaches were developed to support important aspects of a routine environmental surveillance programme. Both approaches can detect important ARGs, their arrangement, including their association with MGEs, and the organisms that carry them. Additionally, the metagenomic sequencing based approach targets a large number of organisms simultaneously and can identify environmental sites that may be high risks sources of AMR. Therefore, it is a strategy that has considerable utility for routine application, as outlined later in this discussion. In contrast, a whole genome sequencing based approach is suitable for determining the local and global molecular epidemiology of important drug-resistant pathogens found in the clinical environment.

This is the first study to successfully employ ONT nanopore metagenomic sequencing to profile the ARGs present in veterinary clinical environments and to predict potential clinical risks associated with them. The protocol developed in this study could be used to explore potential risks caused by antimicrobial-resistant organisms present in the environment of other sections of the U-Vet animal hospital and could be adapted to other veterinary hospitals. Hospital-wide investigation of environmental resistomes would result in identification of locations with high prevalences of ARGs and the predominant types of ARGs in these locations, allowing risk assessments to be conducted and targeted interventions to be introduced to reduce the risks associated with specific locations in the hospital. This evidence-based refinement of the surveillance programme could lead to more efficient allocation of resources [7].

The ONT nanopore metagenomic sequencing based environmental resistome surveillance could be performed biannually or annually to regularly update the existing environmental

surveillance programme of the U-Vet animal hospital. Recently, a user-friendly web-based interface called NanoARG has been introduced to enable rapid exploration of Nanopore sequence reads for the presence and context of ARGs [8]. This tool has the potential to accelerate data analysis in future studies. This online tool is available to wider scientific community and can be implemented by uploading ONT metagenomic sequence reads from environmental samples in a simple text format, negating the need for extensive bioinformatic resources and skills.

Another advantage of generating metagenomic surveillance data using a rapid and convenient methodology is that such information can be used to increase awareness among veterinary personnel about the potential risks associated with drug-resistant organisms within the facility. Awareness among veterinary staff of the AMR risks on the environment in which they work is likely to increase adherence to infection control and prevention strategies [9].

The approach described here is preferable to other contemporary approaches as it combines the advantages of several different methods into a single workflow and provides some additional benefits, a favourable feature for monitoring AMR risks in hospital environments. In previous studies, ARG detection and quantification methods, such as qPCR arrays, had to be combined with microbial identification methods, such as 16S rRNA sequencing, to determine potential bacterial hosts of the ARGs [10]. However, this approach only yields a correlation between bacterial taxa and ARGs, using complex statistics, and the correlations are not conclusive evidence of carriage of specific ARGs by particular bacterial species, nor are they conclusive evidence of carriage of multiple resistance genes by a single organism [10]. Bioinformatic tools such as SEAR [11] and SRST2 [12] can rapidly detect ARGs (SEAR can also estimate ARG abundance) directly from raw short-read sequences, but SEAR cannot identify the bacterial host of the ARG [13], and SRST2 is designed to work on single genome datasets [12], so its performance with metagenomic data is not clear. Neither qPCR array nor raw short-read

sequence based approaches are able to determine the genetic context of the ARGs including their association with MGEs, critical information required to assess the risks associated with ARGs in a clinical context. Short-read sequencing based strategies that involve metagenomic assembly are able to overcome this drawback, but assembled contigs cannot be used to estimate ARG abundance [14]. This necessitates the analysis of both the raw reads and assembled contigs separately [15]. Furthermore, metagenomic assembly is a complex process which requires considerable expertise, resources and time. In contrast, the workflow applied here allows simultaneous detection and quantification of ARGs, determination of the bacterial hosts carrying those ARGs, identification of co-located ARGs (MDR genotypes) and association of ARGs with MGEs in a short period of time. This can be achieved with relatively limited resources at an affordable cost, making it an environmental surveillance tool that is suitable for application in clinical settings.

This study has found genotypic evidence for the presence of MGE-associated ARGs and for the organisms they were associated with in the U-Vet ICU environment and some of them carried multiple ARGs. This suggests a high risk of dissemination of these resistance determinants and of nosocomial infection with MDR organisms carrying these determinants. Screening for infections caused by bacterial isolates with corresponding resistance phenotypes and characterising them would confirm these potential risks. Analysis of the Veterinary Microbiology Laboratory records of bacteria isolated from U-Vet clinical samples during 2016 to 2017 (the period in which the environmental swabs analysed in this study were collected) revealed the presence of clinical *E. coli* isolates with MDR phenotypes corresponding to the MDR genotypes identified in environmental samples in this study. Moreover, infections caused by *E. coli* with similar resistance phenotypes have been reported in other Australian veterinary hospitals [16, 17]. Future studies to characterise U-Vet patient isolates genotypically and to employ metagenomic assembly methods to completely assemble MDR environmental isolates

would allow direct comparisons between environmental and patient isolates (the Veterinary Microbiology Laboratory maintains a collection of clinical bacterial isolates). Alternatively, MDR environmental bacteria could be isolated on selective agar plates containing antimicrobials of interest from the frozen environmental samples collected for this study. The environmental bacteria isolated using these selective culture techniques could then be characterised genotypically to compare them with the genomes of the clinical isolates.

At the beginning of this study, the ICU cages were cleaned daily, followed by thorough cleaning and washing with detergents, before treatment with commercial disinfectants after the discharge of each patient. The laundry trolley kept in the corridor outside the ICU to collect dirty laundry was not cleaned or disinfected routinely. The mop bucket was cleaned in the morning and filled with a disinfectant solution. The mop head was immersed in this solution after each use. The results of the studies described here have indicated that the cleaning of the ICU cages, the immediate environment of the patients, was adequate. However, the high abundance of high-risk ARGs and elevated proportions of MDR genotypes in the waste collection points suggested that interventions should be introduced to improve the biosecurity practices employed at these sites.

The ICC of the U-Vet animal hospital implemented regular cleaning and disinfection of the laundry trolley and it was removed from the busy corridor. In addition, cleaning and drying of the mop bucket and mop head after each use and preparing the disinfection solution freshly for each use have been enforced as a result of the findings of the studies described here. The U-Vet animal hospital underwent extensive renovation a few months after the implementation of these strategies and the new ICU is in a new building. The design of this new ICU allows for better waste management practices, with separate areas designated for patient cages, examination tables, surgical procedures, washing equipment and waste collection.

Although the ICU sink has not been included in the environmental resistome studies described

in Chapter 2, swabs from this site are sent to the veterinary microbiology laboratory as part of the routine environmental surveillance programme of the U-Vet animal hospital. As a result, an ESBL-producing MDR *Enterobacter* species isolate was identified. This and follow-up environmental screening guided thorough and repeated disinfection to remove the organism from the sink environment. Further characterisation of this organism by genome sequencing revealed the presence of an *mcr-9.1* gene, which can confer resistance to colistin, a last resort antimicrobial used in human medicine. This finding was coincidental and resulted from its genetic co-location with the ESBL gene *blaSHV-12*, as the current routine environmental surveillance programme targets ESBL producers. However, the *mcr-9.1* gene was located in between *IS903* and *IS26* transposable elements, making its independent transfer possible. Therefore, routine monitoring for colistin resistance should be included in the current surveillance programme. This exemplifies the value of sequencing based methods to supplement conventional environmental surveillance. However, full genome sequencing and characterisation is not always feasible, so the use of ONT nanopore metagenomic sequencing to explore environmental resistomes provides a rapid and convenient alternative.

Extraintestinal infections caused by multiple drug-resistant *Enterobacter* species have been reported in Australian companion animals [18, 19]. Some of these isolates carried a large plasmid containing both *qnr* and *blaSHV-12* genes [18] but complete sequences of these plasmids are not available for comparison. Nevertheless, plasmid-mediated fluoroquinolone resistance and ESBL genes in *Enterobacter* species from companion animal infections underscore the clinical significance of the environmental *Enterobacter hormaechei* strain characterized in this study. Nosocomial infections caused by drug-resistant *Enterobacter* species were not detected during the period that the ESBL-producing MDR *Enterobacter hormaechei* strain was recovered from the ICU sink. However, ESBL-producing *E. coli* strains resistant to penicillins, cephalosporins, aminoglycosides, tetracycline, chloramphenicol,

trimethoprim and sulphonamides were recovered from an infected wound in one canine patient and a urinary tract infection in another canine patient within one month of the recovery of *E. hormaechei* from the ICU sink. It would be interesting to analyse the genomes of these clinical *E. coli* isolates in future studies to see if they harbour the same plasmid as the *E. hormaechei* isolate from the ICU sink. Nevertheless, given the conjugative nature of the multiple-ARG-carrying IncH12 plasmid harboured by the environmental *E. hormaechei* strain, regular monitoring of sinks and other environmental sites in the hospital for the presence of organisms with similar resistance phenotypes is important.

The detection of ARGs that can confer resistance to amikacin, colistin, fluoroquinolones, higher generations of cephalosporins, rifamycins and streptogramins from the environmental sites of a veterinary hospital deserves attention, as these are high importance antimicrobials usually reserved for human use [20]. Amikacin, colistin and rifamycins are not registered for animal therapy in Australia and their use in companion animal medicine is justifiable only when alternative drugs are not available [20]. There are veterinary-specific fluoroquinolones (enrofloxacin), third generation cephalosporins (cefovecin) and streptogramins (virginiamycin) registered for use in animals [20]. The selection pressure imposed by frequent use of some high-importance antimicrobials in companion animal practice [21], use of other antimicrobials that can select for cross-resistance to high importance antimicrobials [20] and co-selection of ARGs as a result of genomic colocation could be some of the factors contributing to detection of these important ARGs in the veterinary hospital environment. The association between these ARGs and MGEs can potentially contribute to their widespread dissemination.

This is particularly true for the *mcr-9* colistin resistance gene. Although this gene was first reported very recently [22], a retrospective analysis of publicly available whole genome sequence data revealed its global distribution, including in *Enterobacteriaceae* from humans, animals and the environment from Australia [23]. IncHI2 plasmids have been identified as a

major reservoir of the *mcr-9* gene, with variable genetic contexts surrounding the gene [23]. This study did not detect potential transposon elements associated with the gene (by comparative analysis of representative *mcr-9* harbouring regions) and therefore concluded that the dissemination of *mcr-9* was mainly a result of conjugation and recombination events [23]. Interestingly, the studies described here found that the *mcr-9* gene region (together with number of other ARGs) was flanked by inverted and intact *IS903* elements in pCM18-216, indicating the presence of a potential transposon. This is an important finding with relevance to understanding the epidemiology of colistin resistance in Australia, as transposons can further accelerate the dissemination of *mcr-9*.

In conclusion, the work described in this thesis demonstrated the applicability of next generation sequencing, particularly ONT nanopore technology, in monitoring potential antimicrobial resistance risks present in the environment of veterinary clinical settings. The protocols described here can be coupled with conventional environmental surveillance programmes to inform and improve infection control strategies. The detection of ARGs encoding resistance to high-importance antimicrobials in the veterinary hospital environment and their potential for dissemination is a significant concern. Expansion of this study to include other environmental sites of the U-Vet animal hospital and to characterise some patient isolates would further enhance our knowledge on antimicrobial resistance risks present in the U-Vet animal hospital environment.

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