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

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**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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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 bacteria 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 multi-drug 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-clavulinate [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 ºC 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,

| Position | CM18-216 | CM18-242-2 | Nucleotide difference | Protein/region |
|----------|----------|------------|----------------------|----------------|
| 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  |
| 425269   | 425268   | 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     |
| 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  |
| 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        | Non-coding region     |
| 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  |
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 blaSHV-12, qnrB2, mcr-9.1, bla-TEM, catII, tetD, sul1, dfrA19, ereA, arr, aac(3)-II, aac(6′)-Iic, aph(6)-Id, aph(3″)-Ib and ant(3″)-Ia.

Table 1 (continued)

| Position | Nucleotide difference | Protein/region |
|----------|-----------------------|----------------|
| 3658054  | A/-                   | Hypothetical protein |
| 4438341  | A/-                   | Hypothetical protein |
| 4516448  | A/-                   | Hypothetical protein |
| 4641736  | A/-                   | Hypothetical protein |

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

| Position | Nucleotide difference | Protein/region |
|----------|-----------------------|----------------|
| 22872    | T/G                   | ISS family transposase |
| 22873    | T/C                   | ISS family transposase |
| 23080    | A/T                   | ISS family transposase |
| 23081    | C/G                   | ISS family transposase |
| 23111    | A/G                   | ISS family transposase |
| 23117    | A/C                   | ISS family transposase |
| 23166    | G/A                   | ISS family transposase |
| 23181    | G/A                   | ISS family transposase |
| 23183    | G/T                   | ISS family transposase |
| 23184    | C/A                   | ISS family transposase |
| 23185    | A/G                   | ISS family transposase |
| 23254    | T/C                   | ISS family transposase |
| 23255    | G/A                   | ISS family transposase |
| 218820   | G/A                   | ISS family transposase |
| 22869    | CT/-                  | ISS family transposase |
| 22889    | TTCCGA/------         | ISS family transposase |
| 22924    | -/A                   | ISS family transposase |
| 22950    | T/-                   | ISS family transposase |
| 22968    | CGGATTAACCGTTCC/-----| ISS family transposase |
| 23054    | TG/--                 | ISS family transposase |
| 23066    | GCGCTT/------         | ISS family transposase |
| 23085    | A/-                   | ISS family transposase |
| 23093    | T/-                   | ISS family transposase |
| 23118    | -/T                   | ISS family transposase |
| 23157    | CAG/---               | ISS family transposase |
| 23183    | --/ACC                | ISS family transposase |
| 23242    | CC/--                 | ISS family transposase |
| 23253    | -/A                   | ISS family transposase |
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. 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, merB, merT and merR, were located between nucleotide positions 101821 and 105561. The arsenic resistance operon contained arsH, arsR, arsB and arsC and was located...
between nucleotide positions 199610 and 201790. The operon was co-located with an ISNCY family transposase, to the left of \textit{arsH}. In addition to these plasmid operons, two complete copper and silver resistance loci, \textit{pcoABCDRSE} and \textit{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).

| Gene    | Start   | End     | Strand | % Cov. | % Id. | Resistance                        |
|---------|---------|---------|--------|--------|------|-----------------------------------|
| Chromosome |     |         |        |        |      |                                   |
| \textit{bacA} | 652522 | 653339  | +      | 99.51  | 83.37 | Peptide                          |
| \textit{emrB} | 1048287 | 1049816 | −      | 99.42  | 84.25 | Fluoroquinolone                   |
| \textit{emrR} | 1051153 | 1051683 | −      | 100    | 84.18 | Fluoroquinolone                   |
| \textit{axpA} | 1315626 | 1316800 | +      | 99.91  | 87.08 | Phenicol Quinolone                |
| \textit{axpB} | 1316824 | 1319943 | +      | 98.95  | 89.14 | Phenicol Quinolone                |
| \textit{acrD} | 1356278 | 1359376 | −      | 99.52  | 81.41 | Aminoglycoside                    |
| \textit{yojL} | 1537237 | 1538876 | +      | 99.64  | 78.93 | Peptide                          |
| \textit{baeR} | 1652616 | 1653326 | −      | 98.34  | 82.98 | Aminocoumarin Aminoglycoside      |
| \textit{mdtC} | 1656139 | 1659216 | −      | 99.94  | 82.44 | Aminocoumarin                     |
| \textit{mdtB} | 1659217 | 1662339 | −      | 99.9   | 80.23 | Aminocoumarin                     |
| \textit{H-NS} | 2063229 | 2063642 | −      | 100    | 85.51 | Cephalosporin Fluoroquinolone     |
|           |         |         |        |        |      | Macrolide Penam Tetacycline       |
| Plasmid  |         |         |        |        |      |                                   |
| \textit{blaTEM-1} | 112126  | 112986  | −      | 100.00 | 100.00 | Beta-lactam                       |
| \textit{carO2} | 122343  | 122984  | +      | 100.00 | 100.00 | Chloramphenicol                   |
| \textit{tet(M)} | 124596  | 125780  | +      | 100.00 | 99.92  | Tetracycline                      |
| \textit{sulI} | 128882  | 129721  | −      | 100.00 | 100.00 | Sulfonamide                       |
| \textit{ere(A)} | 130245  | 131304  | −      | 86.31  | 99.44  | Macrolide                         |
| \textit{arr} | 132166  | 132579  | −      | 100.00 | 100.00 | Rifamycin                         |
| \textit{aac(3)-II} | 132707  | 133516  | −      | 100.00 | 100.00 | Gentamicin                        |
| \textit{aac(6″)-IIc} | 135601  | 136182  | −      | 100.00 | 100.00 | Gentamicin Kanamycin Tobramycin   |
| \textit{mcr-9.1} | 219313  | 220932  | +      | 100.00 | 100.00 | Colistin                          |
| \textit{aph(6)-Id} | 224139  | 224975  | +      | 100.00 | 100.00 | Streptomycin                      |
| \textit{aph(3″)-Ib} | 224975  | 225777  | −      | 99.88  | 100.00 | Streptomycin                      |
| \textit{dfrA19} | 227552  | 228121  | −      | 100.00 | 100.00 | Trimethoprim                      |
| \textit{sulI} | 230833  | 231672  | −      | 100.00 | 100.00 | Sulfonamide                       |
| \textit{qnmB} | 232163  | 232807  | +      | 100.00 | 100.00 | Quinolone                         |
| \textit{sulII} | 236554  | 237393  | −      | 100.00 | 100.00 | Sulfonamide                       |
| \textit{aadA2} | 237898  | 238689  | −      | 100.00 | 100.00 | Streptomycin                      |
| \textit{blaSHV-12} | 242434  | 243294  | +      | 100.00 | 100.00 | Cephalosporin                     |

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

**The multidrug resistance plasmid pCM18-216 is conjugative**

Mating between \textit{E. hormaechei} CM18-216 and a laboratory strain of \textit{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 \textit{E. coli} recipient by conventional biochemistry. Mating performed at the higher temperature of 37 °C did not result in transconjugants.
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 (cefazolin, cefoxitin, 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 *catI* between the first two, the tetracycline resistance gene *tetD* and its regulator *tetR* between the second and third, and a complete class 1 integron between third and fourth IS26 elements. The integron contained the aminoglycoside resistance gene *aph(3′)-Ia*.
resistance gene \( aac(6')-\text{IIc} \) upstream of the integrase gene, an IS1380 family transposase gene, and the aminoglycoside, rifampicin and erythromycin resistance genes \( aac(3)-\text{II}, arr \) and \( ereA \), 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 \( \text{bla-TEM} \) 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 \textit{Enterobacter} isolated from a hospital environment in Taiwan, and pIMP4-SEM1 (KX810825.1) carried by a \textit{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

### Table 5 MIC of \textit{Enterobacter} sink isolates, DH5 alpha transconjugant (TG) and parental recipient strain used in mating experiments

| Antimicrobial | \textit{E. hormachei} 18-216 | \textit{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      |
| Ceftazidine   | > 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                         | = 1.2        | = 1.2    |
| 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      |
| Orifloxacin   | = 4                         | = 4                         | ≤ 1          | ≤ 1      |
| Piperacillin/tazobactam constant 4 | ≤ 8                  | ≤ 8                         | ≤ 8          | ≤ 8      |
| Polymyxin     | ≤ 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 Sensititre plates COMPGN1F and GNX2F. n/d: no data available for the organism
two “insertion sequence common region 1” elements (ISCR1, or IS91 family transposases). Eight ARGs were found in locus 2, including the ESBL blaSHV-12, fluoroquinolone resistance qnrB2 and colistin resistance mcr9.1, 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 blaSHV-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 blaSHV-12 and a class 1 complex integron containing ISCR1 elements and qnrB2, but these components were located distantly on the replicon. Similarly, the plasmid p34977 from Enterobacter hormaechei subsp. steigerwaltii (CP_012170.1) possessed an IS26-blaSHV transposon located 21 kbp away from the class 1 complex integron above described. By contrast, in pCM18-216 ARG locus-2, the IS26-blaSHV-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 (blaSHV-12 or blaOXA1), only 3 plasmids (namely pEC-IMPQ, pIMP4-SEM1
and pMS7884A) also encoded metallo beta lactamases (\textit{bla}IMP-4 or \textit{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
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 qnrB2 gene. However, the E. coli transconjugants carrying pCM18-216 were susceptible to fluoroquinolones. The reason for this is unclear, but the impact of a qnrB2 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 polymixin 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 blaSHV-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 co-linearity 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 blaSHV-12 and the fluoroquinolone resistance gene qnrB2, along with other antimicrobial resistance genes, through a single transposition event. In Australia, blaIMP-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 blaIMP sequences on their plasmids, the presence of the same mobile genetic elements found on blaIMP plasmids and pCM18-216 opens the question whether the organism is able to acquire such resistance. This underlines the importance of early detection of multi-drug 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 colistine 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, colistine and carabenems, 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.
Hospital, Ms Robin Searson, and the U-vet staff for their help in collecting the mum inhibitory concentration; ARG: Antimicrobial resistance gene.

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 M.M.; formal analysis, K.K., H.B. and M.M.; investigation, K.K. FR, MM and GB; visualization, K.K., H.B. and G.B.; formal analysis, K.K., H.B. and M.M.; investigation, K.K. FR, MM and GB; visualization, K.K., 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 SRA11409014: CP05031 (chromosome CM18-216), CP050312 (plasmid pCM18-216); BioSample SRA11449833: CP050506 (chromosome CM18-242-2), CP050507 (plasmid pCM18-242-2).

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Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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