Identification of bla<sub>CMY-7</sub> and associated plasmid-mediated resistance genes in multidrug-resistant <i>Escherichia coli</i> isolated from dogs at a veterinary teaching hospital in Australia

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**Objectives:** To determine clonality and identify plasmid-mediated resistance genes in 11 multidrug-resistant <i>Escherichia coli</i> (MDREC) isolates associated with opportunistic infections in hospitalized dogs in Australia.

**Methods:** Phenotypic (MIC determinations, modified double-disc diffusion and isoelectric focusing) and genotypic methods (PFGE, plasmid analysis, PCR, sequencing, Southern hybridization, bacterial conjugation and transformation) were used to characterize, investigate the genetic relatedness of, and identify selected plasmid-mediated antimicrobial resistance genes, in the canine MDREC.

**Results:** Canine MDRECs were divided into two clonal groups (CG 1 and 2) with distinct restriction endonuclease digestion and plasmid profiles. All isolates possessed bla<sub>CMY-7</sub> on an ~93 kb plasmid. In CG 1 isolates, bla<sub>TEM</sub>, catA1 and class 1 integron-associated dfrA17-aadA5 genes were located on an ~170 kb plasmid. In CG 2 isolates, a second ~93 kb plasmid contained bla<sub>TEM</sub> and unidentified class 1 integron genes, although a single CG 2 strain carried dfrA5. Antimicrobial susceptibility profiling of <i>E. coli</i> K12 transformed with CG 2 large plasmids confirmed that the bla<sub>CMY-7</sub>-carrying plasmid did not carry any other antimicrobial resistance genes, whereas the bla<sub>TEM</sub>/class 1 integron-carrying plasmid carried genes conferring resistance to tetracycline and streptomycin also.

**Conclusions:** This is the first report on the detection of plasmid-mediated bla<sub>CMY-7</sub> in animal isolates in Australia. MDREC isolated from extraintestinal infections in dogs may be an important reservoir of plasmid-mediated resistance genes.

Keywords: multidrug-resistant <i>E. coli</i>, bla<sub>CMY-7</sub>, bla<sub>TEM</sub>, class 1 integrons

**Introduction**

The increasing prevalence of antimicrobial resistance is a serious concern for both human and veterinary medicine. Horizontal gene transfer among bacteria by mobile genetic elements such as plasmids, transposons and integrons has facilitated the widespread distribution of multiple antibiotic resistance genes among Enterobacteriaceae. In human clinical isolates, resistance to extended-spectrum β-lactam antimicrobial agents is generally due to the production of extended-spectrum β-lactamases.

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Plasmid-mediated resistance genes in canine MDREC

(ESBLs) which can be variants of the classical TEM-1, TEM-2 and SHV-1 enzymes. ESBL prevalence has increased dramatically over the past 15 years in human medicine, with many isolates producing multiple combinations of enzymes. Instead of opportunistic infections due to ESBL-producing *Escherichia coli* have been reported in companion animals.3,4 Instead, the most commonly identified β-lactamaes that confer extended-spectrum resistance in veterinary isolates are plasmid-mediated AmpC β-lactamas, in particular the cephalosporinase *bla*<sub>CMY-2</sub>. In North America, *bla*<sub>CMY-2</sub> has been detected with increased frequency in multidrug-resistant *E. coli* (MDREC) and *Salmonella enterica* serotype Newport (NewportMDR-AmpC) isolated from food-producing and companion animals, pet treats and associated human infections.5–7

Sanchez *et al*.6 described the emergence of MDREC associated with nosocomial infections in dogs in the United States. The isolates were shown to contain *flo* and *bla*<sub>CMY</sub> genes conferring chloramphenicol/Hofenicm and extended-spectrum cephalosporin resistance, respectively, as well as to contain class 1 integrons. Our laboratory also identified MDREC as a cause of opportunistic infections in dogs in Australia at approximately the same time.8 The isolates were resistant to all major classes of antimicrobials used in veterinary medicine, including extended-spectrum β-lactams and β-lactamase inhibitors. The aim of the present study was to determine β-lactam, chloramphenicol and class 1 integron-assocated resistance mechanisms of 11 MDREC isolates obtained from 10 cases of clinical infection in hospitalized dogs, the majority of which occurred at the University of Queensland Veterinary Teaching Hospital (UQVTH) between 1999 and 2001. Isolates were also typed using PFGE and plasmid analysis to identify clonal relationships between strains, and selected resistance genes were mapped to specific plasmids using Southern hybridization and transformation experiments.

**Materials and methods**

**Bacterial strains, culture conditions and phenotypic tests**

The species assignment of 11 MDREC isolates was confirmed biochemically using the Microbact 24E system (Medvet Diagnostics, Thebarton, SA, Australia) and using species-specific PCR amplification of *E. coli* uspA.9 The collection represented clinical isolates from 10 cases of opportunistic infection in hospitalized dogs (cystitis, post-surgical and miscellaneous wound infections) obtained from clinical specimens submitted to The University of Queensland Veterinary Diagnostic Laboratory for culture and sensitivity test (Table 1). All cases occurred at UQVTH, except for Case 7, which occurred at a private veterinary referral hospital in Brisbane. Isolates obtained from Cases 1–9 have been described previously.8 The additional isolates were obtained from a surgical wound site (C11) and urine (C12a). Isolates from Case 8 lost their viability and isolates from Case 10 were subsequently shown to belong to *Enterobacter cloacae* and were excluded from the study. The only multiple isolates that were included in the study came from Case 2 and were obtained 22 days apart from the same surgical site (osteomyelitis following a fracture repair). The MICs of 20 antimicrobials (Table 2) were determined for the 11 isolates using broth microdilution as described by the CLSI, using *E. coli* ATCC 25922 as a control.10 Phenotypic examination for ESBLs was performed using the modified double-disc test (MDDT) developed by Pitout *et al*.11 This test has the ability to demonstrate ESBLs regardless of the conflicting presence of AmpC β-lactams. Sonic extracts from four of the 11 isolates (C1, C3, C5a, C7a) were subjected to isoelectric focusing (IEF) as described previously.7 Following IEF, β-lactamase bands were visualized by staining with nitrocefin.

**Table 1. Restriction endonuclease digestion, and plasmid and antimicrobial resistance gene profiles of 11 clinical MDREC isolates**

<table>
<thead>
<tr>
<th>Isolate&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Date isolated</th>
<th>REDP&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Clonal group</th>
<th>PP&lt;sup&gt;c&lt;/sup&gt;</th>
<th>β-Lactamase&lt;sup&gt;d,e&lt;/sup&gt; (PCR)</th>
<th>Chloramphenicol resistance gene&lt;sup&gt;e&lt;/sup&gt;</th>
<th>intI&lt;sup&gt;f&lt;/sup&gt;</th>
<th>Integron cassette array&lt;sup&gt;f&lt;/sup&gt;</th>
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<tr>
<td>C1</td>
<td>28/10/1999</td>
<td>1a</td>
<td>1</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td>catA1</td>
<td>+</td>
</tr>
<tr>
<td>C2a&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17/04/2000</td>
<td>1b</td>
<td>1</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td>catA1</td>
<td>+</td>
</tr>
<tr>
<td>C2b&lt;sup&gt;b&lt;/sup&gt;</td>
<td>08/05/2000</td>
<td>2a</td>
<td>2</td>
<td>IIa</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>C3</td>
<td>02/05/2000</td>
<td>2a</td>
<td>2</td>
<td>IIb</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>C4</td>
<td>12/05/2000</td>
<td>2a</td>
<td>2</td>
<td>IIa</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>C5a</td>
<td>06/07/2000</td>
<td>2a</td>
<td>2</td>
<td>IIa</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>27/07/2000</td>
<td>1b</td>
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<td>I</td>
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<td>+</td>
<td>catA1</td>
<td>+</td>
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<tr>
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</tr>
<tr>
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<td>08/12/2000</td>
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<td>catA1</td>
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<tr>
<td>C11</td>
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<td>2c</td>
<td>2</td>
<td>IIc</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>C12a</td>
<td>22/08/2001</td>
<td>2d</td>
<td>2</td>
<td>IIa</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup>C, clinical isolate.

<sup>b</sup>Restriction endonuclease digestion profile.

<sup>c</sup>Plasmid profile.

<sup>d</sup>β-Lactamase identification by PCR amplification using known β-lactamase-specific primers.

<sup>e</sup>+/- indicates the presence or absence of the genes.

<sup>f</sup>Class 1 integron cassettes were identified using primers HS317 and HS320. NP, no product was amplified with these primers.

<sup>g</sup>The integron gene cassette array from this isolate was sequenced fully, then specific primers were designed to identify these genes in other isolates.

<sup>h</sup>Samples were from the same animal.
Tracker™ Supercoiled DNA Ladder (Epicentre, Madison, Wisconsin).

Sizes were estimated by comparison with plasmids of known size from the reference strains R27 (112 MDa = 93 kb) and RP4 (54 kb). Gels were also overlaid with clavulanate (for detection of non AmpC-type enzymes) and cloxacillin (AmpC-type enzymes).

PFGE and plasmid analysis

Genomic DNA of the 11 isolates was prepared and digested with the restriction endonuclease XhoI (New England Biolabs, Australia) as described previously.12 A low range lambda ladder (New England Biolabs) was used as a DNA size marker. The resulting fragments were separated in a GeneNavigator system (Pharmacia) at 200 volts with pulse times of 5–50 s and linear ramping at a temperature of 12°C for 25 h. Relatedness between the restriction endonuclease digestion profiles (REDP) was determined by pair-wise comparison as described previously.13 Plasmid DNA for the 11 isolates was isolated using the alkaline lysis method.14 Plasmids were visualized by electrophoresis in 0.5% agarose prepared in 0.5 · TBE. Plasmid digestion profiles (REDP) was determined by pair-wise comparison as described previously.13 Plasmid DNA for the 11 isolates was isolated using the alkaline lysis method.14 Plasmids were visualized by electrophoresis in 0.5% agarose prepared in 0.5 · TBE.

PCR detection of resistance genes

The isolates were tested for the presence of genes encoding blaTEM and blsSTH, β-lactamases using PCR as described previously.15 DNA sequencing of blaTEM products was also undertaken using the same primers. Plasmid-mediated AmpC β-lactamase gene amplification was carried out using multiplex PCR as described previously.16 Oligonucleotide primers CMY25F1 (nt 1736–1754) and CMYDR1 (nucleotides 3167–3147) (Table 3) were designed by Hanson et al.17 to flank the entire structural gene of Citrobacter freundii (nucleotides 3167–3147) (Table 3) was designed by Hanson et al.17 to flank the entire structural gene of Citrobacter freundii (nucleotides 3167–3147) (Table 3). The isolates were also tested for the presence of class 1 integron and chloramphenicol resistance genes using primer-specific PCR screening (Table 3). Oligonucleotide primer pairs used for the amplification of integrases and integron-associated gene cassette arrays were as follows: HS464 and HS463A, HS317 and HS320, respectively (Table 3).18,19 Primers were also designed specifically to detect the presence of dfrA5 and dfrA17 gene cassettes in the isolates. MDREC strains that demonstrated chloramphenicol resistance were tested for the presence of known chloramphenicol resistance genes (i.e. catA1, catA2, catA3, flo and cmlA).20–23 Template DNA was prepared as described previously.15 The total volume of the PCR mixture was 25 μL. Amplifications of products were performed using the GeneAmp™ PCR system 2400 (Perkin-Elmer), in a reaction mixture containing 3.2 pmol of each primer, 200 μM of each dNTP, 1× PCR buffer, 2 mM MgCl₂, and 0.25 U of Red Hot™ PCR system 2400 (Perkin-Elmer). PCR purification kit (QIAGEN). Nucleotide sequences were determined using the ABI BigDye Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit, Version 2.0 (Applied Biosystems), and the reactions analysed using an ABI 377 automatic DNA sequencer at the Australian Genome Research Facility, Brisbane, Australia.

Southern hybridization analysis

DNA probes were prepared from PCR products generated for blsCMY (462 bp), blsTEM (971 bp), catA1 (581 bp) and class 1 integron-associated dfrA17-aadA5 (652 bp) using a digoxigenin nucleic acid labelling and detection system (Roche Diagnostic GmbH). Plasmid DNA was transferred onto positively charged nylon membranes (Amersham) on a vacuum blotter (Bio-Rad) at 5 mm Hg. Membrane-bound DNA was hybridized to each probe according to conditions described previously.14 The hybridization was carried out overnight at 42°C. Hybridization to the four different probes was also carried

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Table 2. MICs for 11 clinical MDREC isolates

<table>
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<tr>
<th>Clonal group</th>
<th>Isolate no.</th>
<th>CIP</th>
<th>STR</th>
<th>SPT</th>
<th>CHL</th>
<th>TZP</th>
<th>FOX</th>
<th>CRO</th>
<th>CAZ</th>
<th>CAZ/CLA</th>
<th>CTX</th>
<th>CTX/CLA</th>
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<tr>
<td>C1</td>
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<td>128</td>
<td>32</td>
<td>128</td>
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<td>128</td>
<td>32</td>
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<td>512</td>
<td>32</td>
<td>32</td>
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</table>

aAll 11 isolates showed resistance to tetracycline (MIC >32 mg/L), enrofloxacin (>32 mg/L), ampicillin (>512 mg/L), sulfamethoxazole/trimethoprim (32/608 mg/L), ticarcillin (>512 mg/L), piperacillin (>256 mg/L) and amoxicillin/clavulanic acid (>32/16 mg/L). All 11 isolates were susceptible to amikacin and imipenem.
bCIP, ciprofloxacin; STR, streptomycin; SPT, spectinomycin; CHL, chloramphenicol; TZP, piperacillin/tazobactam; FOX, cefotaxime; CRO, ceftriaxone; CAZ, ceftazidime; CAZ/CLA, ceftazidime/clavulanic acid; CTX, cefotaxime; CTX/CLA, cefotaxime/clavulanic acid; ATM, aztreonam; ATM/CLA, aztreonam/clavulanic acid.
cTested in the presence of a constant concentration of clavulanic acid (2 mg/L).
dTested in the presence of a constant concentration of tazobactam (4 mg/L).

References

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out using plasmid DNA that had been digested with the *Bam*HI restriction endonuclease enzyme (Roche Diagnostic GmbH).

### Conjugation and transformation experiments

Conjugation experiments were carried out using a liquid mating procedure and on filters, as described previously. Donor strains from both CG 1 (C1, C2a, C6a) and CG 2 (C2b, C12a) were chosen, and *E. coli* J53AzR and *S. enterica* serotype Typhimurium (*Salmonella* Typhimurium) strain LT2 were the recipients. Transconjugants were selected on MacConkey and Mueller–Hinton agar plates containing sodium azide (150 mg/L) and ampicillin (50 mg/L) for counter-selection of *E. coli* J53AzR. Possible transconjugants of *S. Typhimurium* strain LT2 were selected on Mueller–Hinton agar plates containing ampicillin (50 mg/L). Antimicrobial susceptibility testing of putative transconjugants was conducted for 16 antimicrobials using the CLSI disc diffusion methodology.

Transformation of plasmid DNA from the same CG 1 and CG 2 strains that were used for conjugation was performed using electroporation with *E. coli* K12 as the recipient. Transformants were selected on Mueller–Hinton agar containing 50 mg/L ampicillin or 16 mg/L cefotaxime. Transfer of canine MDREC plasmids into *E. coli* K12 was verified by plasmid profiling, Southern hybridization, PCR detection of *bla*CMY-7 and *bla*TEM, and disc diffusion antimicrobial susceptibility tests described by the CLSI.

### Results

#### Antimicrobial resistance profile, MDDT and IEF

All canine MDREC isolates were resistant to tetracycline, enrofloxacin, ciprofloxacin, streptomycin, trimethoprim/sulfamethoxazole, ampicillin, ticarcillin, piperacillin, amoxicillin/clavulanic acid and ticarcillin/clavulanic acid. Inclusion of the β-lactamase inhibitor clavulanic acid (2 mg/L) had no effect on the susceptibility of isolates to ceftazidime, cefotaxime and ceftazidime.

### Table 3. List of oligonucleotide primers used in the study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Target gene</th>
<th>Primer sequence (5′→3′)</th>
<th>Amplicon size (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Reference or accession numbera</th>
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*aGenBank accession number for the sequence used for primer design.*

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843
aztreonam. Piperacillin in combination with tazobactam (4 mg/L) increased the susceptibility of all isolates at least 8-fold compared with their susceptibility to piperacillin alone, although one isolate (C12a) still remained resistant (piperacillin MIC data not shown). Four isolates showed resistance to both chloramphenicol and spectinomycin (Table 2). All the isolates were sensitive to cefepime, imipenem and amikacin (data not shown). All isolates were shown to possess an AmpC β-lactamase using the MDDT method, with no indication of ESBL activity. This was confirmed by demonstration of resistance to third-generation cephalosporins and the lack of a ‘keyhole effect’ between amoxicillin/clavulanate and/or tazobactam/piperacillin with any third- or fourth-generation cephalosporin. Representative isolates had identical IEF profiles showing a pI >8.2 enzyme inhibited by cloxacillin and a pI ~5.4 enzyme inhibited by clavulanate, suggesting that they harboured AmpC and TEM-like β-lactamases, respectively.

**PFGE and plasmid analysis**

Based on PFGE, the 11 isolates were subdivided into six distinct REDPs located within 2 unrelated *E. coli* clonal groups (CG 1 and 2). Four isolates were located in CG 1 and were subdivided into two closely related REDPs (1a–1b) (Table 1 and Figure 1). These isolates were resistant to chloramphenicol, had reduced susceptibility to spectinomycin and shared an identical plasmid profile (Figure 2a). The remaining seven isolates in CG 2 were divided into 4 REDPs (2a–2d) (Table 1 and Figure 1). These isolates shared similar plasmid profiles, Ila–c (Table 1 and Figure 2a).

A large plasmid of similar size to a 93 kb plasmid from *E. coli* strain R1 was present in both CG 1 and CG 2 isolates. Isolates belonging to CG 1 possessed an additional large plasmid which was slightly larger than a ~169 kb plasmid possessed by *E. coli* strain R27 (Figure 2a and c), whereas CG 2 isolates possessed a second ~93 kb plasmid that was slightly larger than the plasmid common to both clonal groups. When compared with CG 1 isolates, CG 2 isolates differed in their susceptibility not only to chloramphenicol and spectinomycin but also to ciprofloxacin (respective MICs of 16 and 128 mg/L).

The first recorded clinical case was a 14-year-old Welsh Pembroke corgi with pyelonephritis that was hospitalized in 1999 for 49 days and treated with multiple classes of antimicrobials. A CG 1 strain was isolated from the urine of this animal. The second clinical case, a 2-year-old St Bernard admitted with chronic osteomyelitis following surgical repair of a fracture, did not develop a MDREC infection until 6 months after Case 1. This animal was infected with a CG 1 MDREC strain initially, whereas a second culture from an orthopaedic screw removed 22 days after the first admission yielded the first CG 2 strain. CG 1 MDRECs were subsequently isolated from Cases 6 and 9. The same or related strains of CG 2 MDREC were responsible for the remaining cases of clinical infection (Cases 3, 4, 5, 7, 11, and 12).

**PCR detection of β-lactam, chloramphenicol and class 1 integron-associated resistance genes**

Direct PCR amplification generated products for all 11 isolates with primers specific for *bla*<sub>TEM</sub>. No product was obtained using primers specific for *bla*<sub>SHV</sub>. Nucleotide sequence analysis identified the gene in two of the isolates as *bla*<sub>TEM.1B</sub>. The multiplex AmpC PCR suggested the presence of a *Citrobacter*-based plasmid-mediated AmpC gene in all isolates. Following detection of a CIT-like AmpC, the full-length PCR-amplified product from each isolate was amplified using primers designed to flank the entire structural gene for *bla*<sub>CMY-2</sub> or *bla*<sub>CMY-7</sub>. Nucleotide sequencing analysis of both strands identified the genes as *bla*<sub>CMY-7</sub>. The sequence was identical with the *bla*<sub>CMY-7</sub> from *S. Typhimurium* strain 100 (GenBank accession number: AY324388).

PCR were carried out using primers designed to detect the presence of class 1 integrons and to recover class 1 integron-associated cassette arrays. All isolates produced a product with the primers HS463A and HS464 designed to amplify a region internal to *intI1*, implying that all isolates possess a class 1 integron. However, long-template PCR amplification using the primers HS317 and HS320 (designed to amplify integron-associated gene cassettes) produced products only for the four CG 1 isolates and one CG 2 isolate (Table 1).

Nucleotide sequence analysis of the products amplified using HS317 and HS320 primers indicated that the four CG 1 isolates possessed a *dfrA17-aadA5* gene cassette array. These genes respectively confer resistance to trimethoprim and spectinomycin/streptomycin. The amplicon of the variable region of class 1 integrons from isolate C5a contained *dfrA5*, which encodes a dihydrofolate reductase and confers resistance to...
trimethoprim. Integron-associated gene cassettes could not be identified in any of the remaining CG 2 isolates using primer pairs HS317 and HS320 or HS458 and HS459. Use of a longer annealing temperature also did not result in production of amplicons for these isolates (data not shown).

All four chloramphenicol-resistant CG 1 isolates were positive for catA1 and were negative for PCR amplification using primers designed to amplify other phenicol resistance genes.

Southern blot hybridization

The blaCMY-7 gene was shown to reside on the ~93 kb plasmid that was present in both CG 1 and CG 2 (Figure 2b). Following restriction endonuclease digestion of plasmids by BamHI, Southern hybridization revealed that blaCMY-7 was present on the same size plasmid fragment (~12 kb) possessed by all MDREC isolates (Figure 2c and d), confirming that there was
no detectable difference in the location of this gene on the common ~93 kb plasmid shared by CG 1 and CG 2. In CG 1 isolates, blaCMY-7 and catA1 and the class 1 integron dfrA17-aadA5 gene cassette were confirmed to be located on the large ~170 kb plasmid (data not shown). Of the two ~93 kb plasmids present in CG 2 isolates, the second, slightly larger plasmid was hybridized using the class 1 integron dfrA17-aadA5 and blaTEM probes. It is possible that the remaining isolates within CG 2 may contain a single dfb gene encoding trimethoprim resistance. However, none of the remaining CG 2 isolates was positive in PCR amplifications designed to amplify either dfrA5 or dfrA17 genes.

Conjugation and transformation experiments
Despite multiple attempts, blaCMY-7 and blaTEM-containing plasmids could not be transferred by conjugation to the sodium azide-resistant E. coli strain and S. Typhimurium strain LT2. The only colonies appearing on selective agar containing ampicillin and sodium azide were confirmed to be CG 1 and CG 2 MDREC mutants that had developed spontaneous sodium azide resistance (data not shown). However, transformation of each of the ~93 kb plasmids from E. coli CG 2 strain C12a into E. coli K12 using electroporation was achieved successfully. The rate of transformation was very low, since only four isolates were obtained from a total population of 10^8 E. coli K12, and each of the transformants was shown to possess a single ~93 kb plasmid. Two transformants designated E. coli K12:pCMY-7HSA and K12:pCMY-7HSB were confirmed to contain blaCMY-7 by PCR and had a typical AmpC β-lactamase phenotype. They were resistant to all β-lactams and third-generation cephalosporins tested, including their combination with clavulanic acid, although they showed intermediate susceptibility to aztreonam. They were susceptible to all other antimicrobials tested. The two remaining transformants (E. coli K12:pTEMHSA and K12:pTEMHSB) were confirmed by PCR to contain blaTEM, and they showed resistance to ampicillin, tetracycline, streptomycin and sulfamethoxazole/trimethoprim. The presence of blaCMY-7 in the E. coli K12:pCMY-7HSA transformant and its absence in the other E. coli K12:pTEMHS transformants also was confirmed by Southern hybridization (Figure 2c and d).

Discussion
In this study, we report the identification of plasmid-mediated β-lactamases, chloramphenicol and class 1 integron-associated resistance genes in two clonal groups of MDREC isolated from clinical infections in dogs at a veterinary teaching hospital in Australia. Apart from the ~93 kb plasmid demonstrated to contain blaCMY-7 that appeared to be common to both clonal groups, there was a marked difference in the distribution of plasmid-mediated resistance genes between CG 1 and CG 2. CG 1 strains shared the same plasmid profile, were resistant to chloramphenicol and spectinomycin, and possessed catA1, dfrA17-aadA5 and blaTEM genes on a large ~170 kb plasmid. In contrast, CG 2 isolates were sensitive to chloramphenicol and spectinomycin and possessed class 1 integron-associated and blaTEM genes on a second ~93 kb plasmid that was not present in CG 1 strains. Southern blotting of plasmids digested with BamHI and hybridized using a blaCMY-7 probe confirmed that the blaCMY-7 gene was present on a ~12 kb fragment common to both MDREC clonal groups, suggesting that the ~93 kb plasmid that carries blaCMY-7 is highly conserved between CG 1 and CG 2. These results confirm that opportunistic infections at UQVTH were caused by clonal expansion of two distinct genetic groups of MDREC, rather than by the transfer and spread of a single multidrug-resistant plasmid between genetically unrelated isolates. Epidemiological relationships between the 11 canine MDRECs characterized in the present study and a large collection of MDR coliforms obtained from rectal swabs of hospitalized dogs and the hospital environment over the same period are the subject of a separate study (H. E. Sidjabat, N. D. Hanson and D. J. Trott, unpublished results).

The isolates characterized in the present study shared highly similar resistance mechanisms with canine MDREC from a previous study in the United States, although the US isolates showed greater genetic diversity.6 Canine MDREC from the United States and Australia possessed a plasmid-mediated Citrobacter-type AmpC and class 1 integron-associated gene cassettes conferring resistance to trimethoprim (dfrA17 and dfrA5) and streptomycin/spectinomycin (aadA5). Interestingly, analysis of the GenBank submission (accession no. AF475279) of blacEM identified in the study of Sanchez et al.6 showed that this AmpC β-lactamase was also blacEM for and not blacEM as reported. In the Australian isolates, chloramphenicol resistance in the CG 1 isolates was mediated by catA1. However, chloramphenicol resistance in the US isolates was in part due to the presence of the florfenicol resistance gene (flo), which mediates resistance to both chloramphenicol and florfenicol, an antimicrobial registered for use in food-producing animals only.

There has been only one other report demonstrating the presence of plasmid-mediated blacEM in a clinical isolate from Australia. This concerns an unusual isolate of S. enterica serotype Typhimurium that also produced an ESBL (SHV-9) and OXA-30.17 The Salmonella strain 100 plasmid encoding blacEM could be transferred into a sodium azide-resistant recipient E. coli strain.24 However, in the present study, attempts to transfer blacEM-carrying plasmids from CG 1 and 2 into the same sodium azide-resistant recipient E. coli strain or into S. Typhimurium strain LT2 (data not shown) were unsuccessful. Several previous studies have documented difficulties in transferring very large AmpC-containing plasmids by conjugation, though greater success has been obtained by direct transformation of plasmid DNA.5,25 In our study, the transformation of the two ~93 kb plasmids possessed by CG 2 strain C12a into E. coli K12 confirmed the array of resistance genes carried by each plasmid. The plasmid conferring an AmpC β-lactamase phenotype was shown to contain only blacEM, conferring the AmpC resistance phenotype, whereas the plasmid carrying blaTEM was shown to carry in addition resistance to tetracycline, streptomycin and sulfamethoxazole/trimethoprim and conferred reduced susceptibility to gentamicin (data not shown).

In Italy, Carattoli et al.1 suggested that increased expanded spectrum cephalosporin resistance detected in E. coli isolates from dogs and cats was due to diffuse off-label veterinary use of extended-spectrum cephalosporins. Extended-spectrum cephalosporins are rarely used by veterinarians in companion animal practice in Australia, although first-generation cephalosporins are rarely used by veterinarians in companion animal practice in Australia. Apart from the ~93 kb plasmid shared by CG 1 and CG 2. These results confirm that opportunistic infections at UQVTH were caused by clonal expansion of two distinct genetic groups of MDREC, rather than by the transfer and spread of a single multidrug-resistant plasmid between genetically unrelated isolates. Epidemiological relationships between the 11 canine MDRECs characterized in the present study and a large collection of MDR coliforms obtained from rectal swabs of hospitalized dogs and the hospital environment over the same period are the subject of a separate study (H. E. Sidjabat, N. D. Hanson and D. J. Trott, unpublished results).

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Plasmid-mediated resistance genes in canine MDREC

Dr Myat Kyaw-Tanner (School of Veterinary Science, The University of Queensland) for her excellent technical assistance. This work was supported by the Australian Companion Animal Health Foundation, the New South Wales Canine and Veterinary Foundation and a University of Queensland Research Development Grant. H. E. Sidjabat was the recipient of an AusAID postgraduate scholarship.

Transparency declarations
None to declare.

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