Identification of plasmid-mediated extended-spectrum and AmpC β-lactamases in Enterobacter spp. isolated from dogs

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The genetic determinants involved in reduced susceptibility to third-generation cephalosporins and aztreonam were identified in ten canine Enterobacter isolates associated with opportunistic infections in three veterinary hospitals in Brisbane, Australia. All isolates were evaluated by a combination of phenotypic (broth microdilution and disc susceptibility, modified disc diffusion and IEF) and genotypic (PFGE, plasmid analysis, Southern blot hybridization, bacterial conjugation, PCR and sequencing) methods to investigate genetic relatedness and to identify plasmid-mediated resistance genes, in particular β-lactamase genes responsible for extended-spectrum cephalosporin resistance. The ten canine isolates were genotypically diverse based on PFGE and belonged to either Enterobacter cloacae or Enterobacter hormaechei on the basis of 16S rRNA gene sequence analysis. Plasmid profiles were also diverse. Nine isolates contained a transmissible blaSHV-12 -carrying plasmid (~140 kb) that also conferred resistance to chloramphenicol, gentamicin, spectinomycin, tetracycline, trimethoprim and sulfonamides. In all plasmid-mediated extended-spectrum β-lactamase (ESBL)-producing isolates including transconjugants, blaSHV-12 was shown to reside in a ~6.5 kb plasmid fragment. The remaining isolate that was not an ESBL producer possessed an AmpC β-lactamase gene (blaCMY-2) on a ~93 kb transmissible plasmid. This plasmid did not contain any other antimicrobial resistance genes. Additional plasmid-mediated β-lactamases identified in some isolates included blaTEM and blaOXA-10. This is the first report of canine Enterobacter isolates containing transmissible plasmid-mediated blaSHV-12 and blaCMY-2 resistance genes. Therefore, Enterobacter isolated from opportunistic infections in dogs may be an important reservoir of plasmid-mediated resistance genes, which could potentially be spread to other members of the Enterobacteriaceae.

INTRODUCTION

In humans, Enterobacter spp. and especially Enterobacter cloacae have been reported to be the most frequent cause of neonatal sepsis, with most infections being nosocomial in origin (Fernández-Baca et al., 2001; Liu et al., 2004). Originally, resistance of Enterobacter spp. to expanded-spectrum cephalosporins was shown to be mediated by derepressed mutants that overproduced the chromosomal AmpC β-lactamase (Seeberg et al., 1983; Hanson & Sanders, 1999). In the last decade, there has been an increase in the reported incidence of plasmid-mediated extended-spectrum β-lactamases (ESBLs) in Enterobacter spp. Infections caused by ESBL-producing Enterobacter cloacae have become a serious clinical problem in many countries such as the USA, Taiwan and Korea (Levison et al., 2002; Lee et al., 2003; Liu et al., 2004; Park et al., 2005). Alarmingly, ESBL-producing Enterobacter spp. frequently produce multiple β-lactamases, with many recent isolates carrying blaCTX-M in addition to blaTEM and blaSHV (Ho et al., 2005; Ma et al., 2005; Schlesinger et al., 2005). Moreover, there has

Abbreviations: ESBL, extended-spectrum β-lactamase; MDR, multidrug-resistant; MDREC, MDR Escherichia coli.
been a recent report of blaSHV-12 ESBLs co-existing with quinolone resistance determinants on the same Enterobacter cloacae plasmid (Corkill et al., 2005).

The Clinical and Laboratory Standards Institute currently recommends ESBL screening and confirmatory testing only in Escherichia coli, Klebsiella pneumoniae and Klebsiella oxytoca and in some cases Proteus mirabilis. Therefore, comparatively less is known about the incidence of ESBLs in Enterobacter. Detection of ESBLs in Enterobacter spp. can be complicated due to the high-level production of chromosomal AmpC by de-repressed mutants (Thomson et al., 1999). A study in Greece during 1998 and 1999 showed that ESBL rates of 25 and 58% were identified in consecutive isolates of Enterobacter cloacae and Enterobacter aerogenes, respectively (Tzolefi et al., 2000). Similarly, Pai et al. (2004) tested blood isolates of Enterobacter spp. in a Korean tertiary hospital from 1994 to 2001 and reported an overall ESBL prevalence of 43%. Several reports have identified SHV variants in members of the Enterobacteriaceae such as blaSHV-2a, blaSHV-5, blaSHV-7 and blaSHV-12 in many countries (Levison et al., 2002; Liu et al., 2004; Chia et al., 2005; Corkill et al., 2005; Schlesinger et al., 2005). These findings highlight the need to test for ESBL production in Enterobacter spp. in order to determine the spread of these resistance mechanisms. The prevalence of ESBL detection in Enterobacter cloacae isolated in Australia between 1998 and 2001 was 4% (Bell et al., 2003). This is a much lower percentage than that found in some Asian countries, in which the percentage of ESBL-producing Enterobacter cloacae was reported to be 19–44% (Bell et al., 2003). Resistant Enterobacter spp. have been reported to cause bacteraemias in Australia (Gottlieb & Wolfson, 2000), and the cefepime MIC50 and MIC90 values of these Enterobacter spp. were alarmingly high (16 and >256 µg cefepime ml–1, respectively). Recently, K. pneumoniae isolates producing SHV-2a and SHV-12 have been identified in a hospital in Brisbane, Australia (Howard et al., 2002). To date, the only report of an ESBL detected in isolates from dogs has been the identification of blaSHV-12 in Escherichia coli (Teshager et al., 2000; Carattoli et al., 2005). In the present study, we examined a collection of multidrug-resistant (MDR) canine Enterobacter spp. isolates from Brisbane, Australia, for the production of ESBLs, AmpC and other plasmid-mediated resistance genes. This is the first report of Enterobacter isolates from dogs that produce plasmid-mediated ESBLs and AmpC.

**METHODS**

**Bacterial strains.** A collection of ten isolates was evaluated. These were identified provisionally as Enterobacter cloaceae and were isolated from clinical opportunistic infections in dogs between 2001 and 2005. All isolates were obtained from clinical swabs or urine specimens submitted to The University of Queensland Veterinary Diagnostic Laboratory (UQVDL). Each isolate was obtained from a single dog. One isolate obtained from a tarsal joint swab of a dog with osteomyelitis following surgical repair of a fracture (Ec1) has been described previously as C10a (Warren et al., 2001; Sidjabat et al., 2006a). The other nine isolates were obtained from various clinical specimens submitted by three veterinary referral hospitals in Brisbane, Australia (Table 1). The species assignment of the isolates was confirmed biochemically using the Microba 24E system (Medvet Diagnostics). On the basis of disc diffusion susceptibility testing, all ten isolates displayed resistance to extended-spectrum cephalosporins and co-amoxiclavulanate.

**PCR and sequencing of 16S rRNA genes.** DNA was extracted by a heat lysis protocol and the 16S rRNA gene of each isolate was amplified using primers 27F and 1492R (Meadows et al., 1992). The conserved primers 27F, 803F, 907R and 1492R were used to determine the sequences. Amplicons were purified using the QiAquick PCR purification kit (Qiagen) according to the manufacturer’s instructions. The ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit version 3.1 (Applied Biosystems) was used to sequence PCR amplification products and sequences were obtained using an Applied Biosystems/Hitachi 3130XL Genetic Analyser. Sequencing analyses were performed online using BLAST (www.ncbi.nlm.nih.gov/BLAST/).

**Susceptibility testing and IEF.** The MICs of 15 antimicrobials (Table 2) were determined using the CLSI microbroth dilution method (NCCLS, 2002). The combination of clavulanic acid with either cefotaxime or ceftriaxone was determined by Etest (AB Biodisk). Escherichia coli ATCC 25922 and ATCC 35218 were used as reference strains. Phenotypic detection of ESBL production was performed using the modified double-disc test (Pitout et al., 2003). Isolates that were resistant to third-generation cephalosporins and also resistant to the combination of third-generation cephalosporins with clavulanic acid were suspected to overexpress chromosomal AmpC or possibly produce a plasmid-mediated AmpC. β-Lactamases were also identified by IEF of sonic extracts of each isolate, as described previously (Sidjabat et al., 2006a).

**PFGE and plasmid analysis.** Genomic DNA for PFGE was prepared as described previously (Sidjabat et al., 2006a) and separated using a GeneNavigator system (Pharmacia). Fingerprinting were generated by XbaI digestion and electrophoresed using a 5–50 s pulse time and 200 V for 25 h in 0.5 × TBE buffer at 12 °C. The genetic relatedness among isolates was interpreted by the number of band differences among the PFGE patterns (Tenover et al., 1995). Isolates with identical or related PFGE patterns were considered to be derived from a common clone. Plasmids were extracted using the alkaline-lysis method as described previously (Sidjabat et al., 2006a). Plasmid sizes were estimated by comparison with plasmids of known size from the reference strains Escherichia coli R1 (93 kb) and Escherichia coli R27 (169 kb) and using a BAC Tracker Supercoiled DNA ladder (Epicentre Biotechnologies).

**PCR detection and sequence analysis of resistance genes.** Details of the preparation of bacterial lysates, specific primer pairs and PCR conditions for identification of blaTEM, blaSHV and blaCTX genes have been described previously (Vahaboglu et al., 1998; Sidjabat et al., 2006a). DNA sequencing was performed using the same primers that generated the amplified products. Chloramphenicol resistance genes, integrase and integron cassette resistance genes were detected by PCR and the DNA was sequenced using primers and PCR conditions as described previously (Sidjabat et al., 2006a). All isolates were subjected to identification of plasmid-mediated AmpC β-lactamase genes by multiplex PCR (Pérez-Pérez & Hanson, 2002). The primer pair CMY25F1 and CMY2DR1 (Hanson et al., 2002) was used to amplify and sequence the entire structural gene of blaCTX-M-2. Long-template amplification of all products for sequence analysis was performed using Platinum Taq DNA Polymerase (Invitrogen), as directed by the manufacturer, and purified using the High Pure PCR Product Purification kit (Roche Diagnostics). Sequencing and sequence analyses were performed as described above.
Table 1. Antimicrobial resistance gene profiles and PFGE patterns of ten clinical Enterobacter isolates from dogs

UTI, Urinary tract infection; UQVTH, The University of Queensland Veterinary Teaching Hospital; BH1 and BH2, Brisbane Referral Veterinary Hospital 1 and 2, respectively.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Isolation date</th>
<th>History</th>
<th>Referral</th>
<th>β-Lactamase genotype*</th>
<th>Chloramphenicol resistance gene</th>
<th>Integron cassette array†</th>
<th>PFGE pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ec1§</td>
<td>01/02/2001</td>
<td>Osteomyelitis</td>
<td>UQVTH</td>
<td>+ (blaSHV-12)</td>
<td>–</td>
<td>catA2</td>
<td>A</td>
</tr>
<tr>
<td>Ec2</td>
<td>08/04/2002</td>
<td>UTI</td>
<td>BH1</td>
<td>+ (blaSHV-12)</td>
<td>+ (blaTEM-1)</td>
<td>catA2</td>
<td>A</td>
</tr>
<tr>
<td>Ec3</td>
<td>16/10/2002</td>
<td>UTI</td>
<td>UQVTH</td>
<td>+ (blaSHV-12)</td>
<td>+ (blaTEM-1)</td>
<td>catA2</td>
<td>B</td>
</tr>
<tr>
<td>Ec4</td>
<td>18/06/2003</td>
<td>Post-surgery infection</td>
<td>BH1</td>
<td>+ (blaSHV-12)</td>
<td>+</td>
<td>–</td>
<td>C</td>
</tr>
<tr>
<td>Ec5</td>
<td>19/06/2003</td>
<td>UTI</td>
<td>BH1</td>
<td>+ (blaSHV-12)</td>
<td>–</td>
<td>catA2</td>
<td>D</td>
</tr>
<tr>
<td>Ec6</td>
<td>19/05/2004</td>
<td>UTI</td>
<td>BH1</td>
<td>+ (blaSHV-12)</td>
<td>+ (blaTEM-1)</td>
<td>catA2</td>
<td>A</td>
</tr>
<tr>
<td>Ec7</td>
<td>05/10/2004</td>
<td>UTI</td>
<td>UQVTH</td>
<td>+ (blaSHV-12)</td>
<td>+</td>
<td>catA2</td>
<td>E</td>
</tr>
<tr>
<td>Ec8</td>
<td>24/11/2004</td>
<td>UTI</td>
<td>BH1</td>
<td>–</td>
<td>+</td>
<td>catA2</td>
<td>F1</td>
</tr>
<tr>
<td>Ec9</td>
<td>20/07/2005</td>
<td>Multiple abscesses</td>
<td>BH1</td>
<td>+ (blaSHV-12)</td>
<td>–</td>
<td>catA2</td>
<td>F2</td>
</tr>
<tr>
<td>Ec10</td>
<td>25/08/2005</td>
<td>UTI</td>
<td>BH2</td>
<td>+ (blaSHV-12)</td>
<td>–</td>
<td>aadA2</td>
<td>G</td>
</tr>
</tbody>
</table>

*The presence (+) or absence (−) of the ESBL and AmpC genotypes are indicated, as determined by PCR and sequencing using β-lactamase specific primers.
†The presence of plasmid-mediated AmpC β-lactamase genes was determined by multiplex PCR (Pérez-Pérez & Hanson, 2002).
‡The integron gene cassette arrays were fully sequenced.
§The previous number of this isolate was C10a (Warren et al., 2001).
¶Ec7 was suggested to produce AmpC by the CLSI method and an ESBL by the modified disc diffusion method, but was negative in the multiplex AmpC PCR assay.
||Sequencing of the integron consisted of a partial sequence of the *aadA2* gene and the sequence of an IS5 transposase.
Table 2. MICs for ten clinical Enterobacter isolates from dogs and their corresponding transconjugants

<table>
<thead>
<tr>
<th>Strain*</th>
<th>β-Lactamase pl(s)</th>
<th>FOX</th>
<th>CTX</th>
<th>CTX/CLA</th>
<th>CAZ</th>
<th>CAZ/CLA</th>
<th>MIC (mg l⁻¹)†‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ec1</td>
<td>5.4 + 8.2</td>
<td>128</td>
<td>32</td>
<td>1</td>
<td>256</td>
<td>256</td>
<td>&gt;512, 64, 32</td>
</tr>
<tr>
<td>T-Ec1</td>
<td>5.4 + 8.2</td>
<td>4</td>
<td>16</td>
<td>0.032</td>
<td>32</td>
<td>0.094</td>
<td>128, 512, &lt;0.5</td>
</tr>
<tr>
<td>Ec2</td>
<td>5.4 + 8.2</td>
<td>256</td>
<td>128</td>
<td>1</td>
<td>512</td>
<td>4</td>
<td>&gt;512, 128, 16</td>
</tr>
<tr>
<td>T-Ec2</td>
<td>5.4 + 8.2</td>
<td>8</td>
<td>4</td>
<td>0.023</td>
<td>64</td>
<td>0.064</td>
<td>128, 512, &lt;0.5</td>
</tr>
<tr>
<td>Ec3</td>
<td>5.4 + 7.9 + 8.2</td>
<td>256</td>
<td>32</td>
<td>ND</td>
<td>128</td>
<td>ND</td>
<td>128, 8</td>
</tr>
<tr>
<td>T-Ec3</td>
<td>5.4 + 8.2</td>
<td>4</td>
<td>4</td>
<td>0.032</td>
<td>64</td>
<td>0.125</td>
<td>64, 256, &lt;0.5</td>
</tr>
<tr>
<td>Ec4</td>
<td>5.4 + 8.2</td>
<td>256</td>
<td>32</td>
<td>1</td>
<td>256</td>
<td>0.5</td>
<td>256, 16, 2</td>
</tr>
<tr>
<td>T-Ec4</td>
<td>5.4 + 8.2</td>
<td>4</td>
<td>4</td>
<td>0.032</td>
<td>32</td>
<td>0.064</td>
<td>64, 4, 0.3</td>
</tr>
<tr>
<td>Ec5</td>
<td>5.4 + 8.2</td>
<td>128</td>
<td>32</td>
<td>0.75</td>
<td>256</td>
<td>0.25</td>
<td>256, 512, 0.125</td>
</tr>
<tr>
<td>T-Ec5</td>
<td>5.4 + 8.2</td>
<td>8</td>
<td>4</td>
<td>0.023</td>
<td>64</td>
<td>0.064</td>
<td>128, 512, &lt;0.5</td>
</tr>
<tr>
<td>Ec6</td>
<td>5.4 + 6.3 + 8.2</td>
<td>&gt;256</td>
<td>64</td>
<td>ND</td>
<td>256</td>
<td>ND</td>
<td>512, 64, 32</td>
</tr>
<tr>
<td>T-Ec6</td>
<td>5.4 + 6.3 + 8.2</td>
<td>4</td>
<td>8</td>
<td>0.032</td>
<td>64</td>
<td>0.094</td>
<td>64, 512, 0.5</td>
</tr>
<tr>
<td>Ec7</td>
<td>5.4 + 8.2 + 8.4</td>
<td>256</td>
<td>128</td>
<td>ND</td>
<td>512</td>
<td>ND</td>
<td>512, 64, 1</td>
</tr>
<tr>
<td>T-Ec7</td>
<td>5.4 + 8.2</td>
<td>16</td>
<td>16</td>
<td>0.047</td>
<td>64</td>
<td>0.19</td>
<td>512, 128, &lt;0.5</td>
</tr>
<tr>
<td>Ec8</td>
<td>5.4 + 8.9</td>
<td>&gt;256</td>
<td>128</td>
<td>ND</td>
<td>512</td>
<td>ND</td>
<td>512, 64, 128</td>
</tr>
<tr>
<td>T-Ec8</td>
<td>5.4 + 8.9</td>
<td>256</td>
<td>32</td>
<td>ND</td>
<td>512</td>
<td>ND</td>
<td>64, 4, 1-2, &lt;0.5</td>
</tr>
<tr>
<td>Ec9</td>
<td>5.4 + 8.2</td>
<td>128</td>
<td>128</td>
<td>1</td>
<td>256</td>
<td>0.25</td>
<td>256, 512, 64</td>
</tr>
<tr>
<td>Ec10</td>
<td>5.4 + 7.9 + 8.2</td>
<td>256</td>
<td>128</td>
<td>ND</td>
<td>512</td>
<td>ND</td>
<td>512, 32, 16</td>
</tr>
<tr>
<td>T-Ec10</td>
<td>5.4 + 8.2</td>
<td>8</td>
<td>4</td>
<td>0.032</td>
<td>64</td>
<td>0.064</td>
<td>128, 4, 0.25</td>
</tr>
<tr>
<td>Escherichia coli J53AzR§</td>
<td>–</td>
<td>8</td>
<td>0.125</td>
<td>&lt;1</td>
<td>0.5</td>
<td>&lt;1</td>
<td>0.25</td>
</tr>
</tbody>
</table>

* T-Ec denotes transconjugant.
† FOX, cefoxitin; CTX, cefotaxime; CTX/CLA, cefotaxime/clavulanic acid; CAZ, ceftazidime; CAZ/CLA, ceftazidime/clavulanic acid; ATM, aztreonam; CHL, chloramphenicol; GEN, gentamicin; ENR, enrofloxacin; SPC, spectinomycin; STR, streptomycin; TET, tetracycline, TRI, trimethoprim; SXT, sulfamethoxazole/trimethoprim. MICs were obtained by the broth microdilution method (CLSI), except for CTX/CLA and CAZ/CLA. All clinical isolates and transconjugants showed resistance to ampicillin, piperacillin and ticarcillin (≥512 mg l⁻¹).
‡ MICs were obtained using Etest (AB Biodisk); ND, non-determinable CTX/CLA >1 µg ml⁻¹ and CAZ/CLA >4 µg ml⁻¹.
§ Escherichia coli J53AzR MICs to ampicillin, piperacillin and ticarcillin were 8, 4 and 8 µg ml⁻¹, respectively.

Southern blot hybridization analysis. DNA (uncut plasmids and BamHI-digested plasmid) was hybridized to DNA probes generated from PCR products using blaSHV (885 bp) and blaCMY (462 bp) template and a digoxigenin nucleic acid labelling and detection system (Roche Diagnostics).

Conjugation experiments. Conjugation experiments were performed for all isolates using sodium azide-resistant Escherichia coli J53AzR as the recipient (Sidjabat et al., 2006a). Transconjugants were selected on Mueller-Hinton agar supplemented with 120 µg sodium azide ml⁻¹ and 50 µg ampicillin ml⁻¹. Plasmid transfer from Enterobacter to the Escherichia coli J53AzR transconjugants was confirmed by blaSHV, blaCMY and blablaOXA primer-specific PCR, plasmid analysis, Southern blot hybridization and IEF. Transconjugants were confirmed to be Escherichia coli using biochemical tests and species-specific PCR amplification of Escherichia coli uspA (Sidjabat et al., 2006a).
RESULTS

Species identification, antimicrobial susceptibility profile and phenotypic detection of β-lactamases

All isolates were identified as *Enterobacter cloacae* on the basis of their biochemical reactions using the Microbact 24E system. PCR and sequencing of the 16S rRNA gene confirmed that each isolate belonged to the genus *Enterobacter*; however, it was difficult to determine the exact species identity using this method. Alignments of the 16S rRNA gene sequences of Ec1, Ec2, Ec6, Ec8 and Ec9 were identical and produced significant identities (99%) with both *Enterobacter hormaechei* and *Enterobacter cloacae*. However, the 16S rRNA gene sequences of the remaining isolates (Ec3, Ec4, Ec5, Ec7 and Ec10) differed from the above isolates by 5–7 nt. Although these isolates also had approximately 99% identity with both *Enterobacter hormaechei* and *Enterobacter cloacae*, the identity with *Enterobacter hormaechei* was slightly higher. Because of recent changes in *Enterobacter* taxonomy and classification, *Enterobacter hormaechei* is now divided into several subspecies, which together with *Enterobacter cloacae* and several other *Enterobacter* species and genovars are combined within the so-called *Enterobacter cloacae* complex (Hoffmann et al., 2005).

MIC testing showed that all of the canine *Enterobacter* spp. isolates were resistant to ampicillin, piperacillin, ticarcillin, gentamicin, spectinomycin, cefoxitin, cefotaxime, ceftazidime and aztreonam (Table 2). The majority of the isolates...
were also resistant to enrofloxacin (8/10 isolates), chloramphenicol (8/10 isolates) and sulfamethoxazole/trimethoprim (9/10 isolates). All ten isolates were sensitive to amikacin and imipenem (data not shown).

Nine isolates (Ec1–Ec7, Ec9 and Ec10) were shown to produce an ESBL by the modified double-disc test method. Ec7 and Ec8 showed resistance to ceftazidime and cefotaxime and showed no inhibition to these respective antimicrobials when they were combined with clavulanic acid.

**PFGE and plasmid analysis**

The PFGE patterns of the ten Enterobacter isolates comprised seven distinct PFGE profiles, designated A–G (Fig. 1 and Table 1). Three isolates had an indistinguishable PFGE pattern (Ec1, Ec2 and Ec6) and therefore were considered to represent the same strain, designated PFGE pattern A. Two isolates (Ec8 and Ec9) were considered to be closely related, as these PFGE patterns differed by only three different bands. The remaining isolates, Ec3–Ec5, Ec7 and Ec10, were shown to be genotypically unrelated to any other strains, as their PFGE patterns differed considerably from the PFGE patterns of other strains (Fig. 1). All Enterobacter isolates possessed large plasmids, which were larger than or equal to the ~93 kb plasmid of Escherichia coli R1 (Fig. 2). All but two isolates (Ec7 and Ec8) possessed a large ~140 kb plasmid, whereas the plasmids from Ec7 and Ec8 were ~93 kb in size. Six isolates (Ec4, Ec5, Ec7 and Ec8–Ec10) also had small plasmids (~<23 kb). The BamHI-digested plasmid profiles of eight isolates (Ec1–Ec7 and Ec10) were relatively similar, although there were differences in band intensity. The digested plasmid profiles of Ec8 and Ec9 were closely related and differed significantly from the other isolates.

**Antimicrobial resistance genes and IEF**

Antimicrobial resistance genes identified from sequence analysis of the amplified products are listed in Table 1. All isolates possessed blaTEM, and in isolates Ec1, Ec2 and Ec3, the sequence alignments were identical with blaTEM-1, which was in accordance with the identified β-lactamase pl1 of 5.4. Meanwhile, alignments of the blaSHV nucleotide sequences from all ESBL-producing isolates (Ec1–Ec7, Ec9 and Ec10) were identical with the blaSHV12 sequence, which correlated with the β-lactamase pl1 of 8.2. In isolate Ec6, the OXA-specific amplified product was identified as blaOXA-10 and production of this gene product correlated with the β-lactamase with a pl of ~6.5. The ampC multiplex PCR indicated the presence of a Citrobacter-type gene in only one isolate, Ec8. The AmpC gene was amplified from this isolate with primers designed to flank the entire structural gene for either blaCMY-2 or blaCMY-7 (CMY25F and CMY2DR1). Sequence analysis of the amplified product from Ec8 was identical to blaCMY-2 (GenBank accession no. X91840); this was in accordance with an identified β-lactamase with a pl of 8.9. In addition, IEF detected bands with a pl of 7.9 in two isolates (Ec3 and Ec10) and a band with a pl of 8.4 in isolate Ec7. These bands may represent Enterobacter chromosomal AmpC, as the bands were not inhibited by clavulanic acid and were not apparent in the transconjugants.

Identification of integrase and integron class 1-specific resistance genes indicated that all ten Enterobacter isolates possessed a class 1 integron-associated spectinomycin resistance gene (aadA2) (Table 1). Interestingly, Ec8, which possessed blaCMY-2, also carried two class 1 integrons, each containing a single resistance gene (aadA2 and dfrV). In addition, eight of the ten isolates possessed the chloramphenicol resistance gene catA2.

**Conjugation and Southern blot hybridization experiments**

Only large plasmids were transferred by conjugation (Fig. 2). The Escherichia coli transconjugants were designated according to their corresponding Enterobacter donor strain, i.e. T-Ec1–T-Ec8 and T-Ec10. The blaSHV-12-carrying plasmids of eight isolates (Ec1–Ec7 and Ec10), the blaCMY-2-carrying plasmid of Ec8 and the blaOXA-10-carrying plasmid of Ec6 were readily transferred into Escherichia coli J53AzR by conjugation. This was confirmed by plasmid analysis, Southern blot hybridization, primer-specific PCR and sequencing of β-lactamase genes possessed by donors and their corresponding transconjugants. The blaSHV-12 gene was carried on a large ~140 kb plasmid, whereas the plasmid carrying blaCMY-2 was ~93 kb in size (data not shown). Furthermore, Southern blot hybridization of the BamHI-digested plasmids of the transconjugants with a blaSHV probe confirmed that blaSHV-12 was located on an ~6.5 kb plasmid fragment within the ~140 kb plasmid common to all Enterobacter strains and their respective transconjugants except Ec8 and Ec9 (data not shown). Hybridization of digested plasmids from Ec8 and T-Ec8 with the blaCMY-2 probe confirmed the location of blaCMY-2 on an ~23 kb plasmid fragment within the ~93 kb plasmid (data not shown). In addition, blaSHV-12 and blaCMY-2 genes from the transconjugants were sequenced to confirm their identity with the corresponding genes in the donor isolates.

The susceptibility patterns of 17 antimicrobials for the transconjugants were determined (Table 2). These data indicated that blaSHV-12 contributed to ceftazidime and aztreonam resistance. Resistance to chloramphenicol, gentamicin, streptomycin, trimethoprim/sulfamethoxazole and spectinomycin was co-transferred by conjugation to all recipients, except in the single isolate producing blaCMY-2.

Transfer of tetracycline resistance was also demonstrated for all blaSHV-12-producing isolates except Ec4. Resistance to enrofloxacin was not transferable by conjugation for any of the isolates. In addition, the transmissible blaCMY-2-carrying plasmid in isolate Ec8 did not carry resistance for any of the other antimicrobial agents tested. Even though the susceptibility pattern of Ec8 confirmed that this isolate was resistant to chloramphenicol, gentamicin, spectinomycin, streptomycin, tetracycline and trimethoprim,
these resistance phenotypes were not co-transferred by conjugation.

**DISCUSSION**

This paper describes the first occurrence of an ESBL or AmpC β-lactamase in *Enterobacter* spp. isolated from dogs. The first *Enterobacter* case occurred at the same time as a cluster of MDR *Escherichia coli* (MDREC) nosocomial infections (2000–2001) in The University of Queensland Veterinary Teaching Hospital (Sidjabat et al., 2006a). During a 1 year infection control study conducted from mid-2000 to mid-2001, MDR *Enterobacter* was rarely isolated (1/129 isolates) from rectal swabs of hospitalized dogs compared with MDREC, but was more frequently isolated from swabs of the hospital environment (Sidjabat et al., 2006b).

PFGE confirmed that the ten canine MDR *Enterobacter* isolates were clonally diverse, although it was observed that three isolates (Ec1, Ec2 and Ec6) had the same PFGE pattern (A). These three isolates also had similar plasmid profiles and did not possess plasmids smaller than 23 kb. This indicated that the spread of resistant *Enterobacter* in dogs was largely mediated by horizontal gene transfer of the ~140 kb ESBL-containing plasmid to unrelated strains. However, clonal dissemination of PFGE pattern A was confirmed, as it first emerged at The University of Queensland Veterinary Teaching Hospital (Ec1), but was then isolated from a different referral hospital approximately 3 years after the first case (Ec6). Interestingly, analysis of the β-lactamases possessed by strain Ec6 confirmed the recent acquisition of plasmid-mediated OXA-10. Resistance gene acquisition on plasmids may explain the fact that Ec8 and Ec9 are clonally related based on their PFGE patterns (F1 and F2), with Ec9 isolated 9 months after Ec8 from a different infection site in a different dog at the same hospital. Ec8 possessed an ~93 kb plasmid containing a single resistance gene (*blaCMY-2*), whereas Ec9 contained the ~140 kb *blaSHV-12* plasmid. Interestingly, Ec9 was the only isolate for which a transconjugant was not obtained.

Previously, *blaSHV-2* was documented as the most prevalent ESBL throughout the world (Paterson et al., 2003). However, the prevalence of *blaSHV-12* in human isolates of members of the *Enterobacteriaceae* is increasing in many countries (Yan et al., 2000; Lee et al., 2003; Kim et al., 2005). Similarly, in Australia, *blaSHV-2* and *blaSHV-12* were found commonly to be present in hospital isolates of *K. pneumoniae* (Howard et al., 2002). The presence of a combination of *blaSHV-12* and *blaTEM* in human *Enterobacter cloacae* isolates has been found in other countries (Chia et al., 2005; Ho et al., 2005; Ma et al., 2005; Schlesinger et al., 2005), but is yet to be confirmed in human isolates from Australia. In addition, the presence of both *blaSHV-12* and *aadA2* in *Enterobacter cloacae* has been reported in isolates identified in Liverpool, UK (Corkill et al., 2005). Recently, *blaCMY-7* was identified in canine MDREC isolated from dogs in Brisbane (Sidjabat et al., 2006a). Interestingly, the ~93 kb MDREC *blaCMY-7* plasmid and the ~93 kb *blaCMY-2* plasmid in *Enterobacter cloacae* isolate Ec8 did not confer resistance to other non-β-lactam antibiotics, suggesting that these plasmids could possibly be related. However, the BamHI-digested profiles of the *Enterobacter cloacae* and MDREC plasmid profiles were distinct (Sidjabat et al., 2006a). Furthermore, the *blaCMY-2*-carrying plasmid in *Enterobacter* spp. was transferable by conjugation, whilst *blaCMY-7* in *Escherichia coli* was not. The presence of *blaCMY-2* in *Enterobacter* spp. was unusual, as most reports identify the presence of this gene in *Escherichia coli*, *Salmonella* sp. and *Klebsiella* sp., and only one very recent report has confirmed the detection of this β-lactamase gene in an *Enterobacter cloacae* human isolate from Taiwan (Su et al., 2006).

There have been very few reports of the occurrence of ESBLs in animal isolates (Teshager et al., 2000; Carattoli et al., 2005). The increasing prevalence of ESBLs in *K. pneumoniae* and *Escherichia coli* isolated from humans is well documented, and the presence of ESBLs in *Enterobacter* spp. that often cause serious systemic nosocomial infections is now a reality in hospitalized patients worldwide (Liu et al., 2004; Ho et al., 2005). The inappropriate use and/or overuse of expanded-spectrum β-lactams has been associated with the emergence and spread of ESBL-producing organisms in human hospitals (Bradford, 2001). However, use of expanded-spectrum β-lactams is extremely limited in companion animal practice in Australia, whereas the use of β-lactam/clavulanate combinations is widespread (Sidjabat et al., 2006a, b). However, the presence of multiple resistance mechanisms on the same *blaSHV-12*-encoding plasmid would ensure co-selection by a number of commonly used veterinary antimicrobials, such as doxycycline and sulfonamide/trimethoprim.

There have been reports on the treatment failure of infections in humans caused by ESBL-producing bacteria using third-generation cephalosporins, whereas the fourth-generation cephalosporin ceftazidime may be of clinical value for the treatment of some infections (Ramphal & Ambrose, 2006). However, an Australian study confirmed that more than 40 % of *Enterobacter* spp. isolates from humans were resistant to ceftazidime. Therefore, treatment using fourth-generation cephalosporins is also not recommended before the susceptibility testing is known (Gottlieb & Wolfson, 2000).

The emergence of MDR *Enterobacter* isolates containing ESBL and AmpC β-lactamases in dogs has not been reported previously, but could theoretically occur by a number of routes and human-to-dog transmission should not be overlooked, especially since the recently documented cases of interspecies transmission of meticillin-resistant *Staphylococcus aureus* between humans and companion animals (Weese et al., 2006). The persistence of MDR *Enterobacter* in the veterinary hospital environment could be a major
contributor to the emergence of clinical infections in dogs caused by these strains, as the majority of environmental isolates obtained during an infection control study (Sidjabat et al., 2006b) belonged to PFGE pattern A (H. E. Sidjabat, D. J. Trott & S. M. Moss, unpublished results). The high percentage of canine clinical isolates (90%) that readily self-transfer their plasmids is a cause for concern and suggests that there is the potential for interspecies spread of blaSHV-12 and blaCMY-2-carrying plasmids among members of the Enterobacteriaceae. Therefore, infection control procedures that prevent the spread of resistant bacteria are equally relevant in the veterinary context and should receive strong consideration.

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