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 *bla*_{SHV-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, *bla*_{SHV-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 (*bla*_{CMY-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 *bla*_{TEM} and *bla*_{OXA-10}. This is the first report of canine *Enterobacter* isolates containing transmissible plasmid-mediated *bla*_{SHV-12} and *bla*_{CMY-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*.

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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 de-repressed 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). Alarming, ESBL-producing *Enterobacter* spp. frequently produce multiple β -lactamases, with many recent isolates carrying *bla*_{CTX-M} in addition to *bla*_{TEM} and *bla*_{SHV} (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 *bla*_{SHV-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 (Tzelepi *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 *bla*_{SHV-2a}, *bla*_{SHV-5}, *bla*_{SHV-7} and *bla*_{SHV-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. also 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 *bla*_{SHV-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 cloacae* 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.*, 2006b). 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 Microbact 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 (Milinovich *et al.*, 2006). 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 ceftazidime 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). Fingerprints were generated by *Xba*I 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 *bla*_{TEM}, *bla*_{SHV} and *bla*_{OXA} 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 *bla*_{CMY-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.

Isolate	Isolation date	History	Referral	β-Lactamase genotype*				Chloramphenicol resistance gene	Integron cassette array‡	PFGE pattern
				ESBL			Plasmid-mediated AmpC†			
				<i>bla</i> _{SHV}	<i>bla</i> _{TEM}	<i>bla</i> _{OXA}				
Ec1§	01/02/2001	Osteomyelitis	UQVTH	+ (<i>bla</i> _{SHV-12})	+ (<i>bla</i> _{TEM-1})	—	—	<i>catA2</i>	<i>aadA2</i>	A
Ec2	08/04/2002	UTI	BH1	+ (<i>bla</i> _{SHV-12})	+ (<i>bla</i> _{TEM-1})	—	—	<i>catA2</i>	<i>aadA2</i>	A
Ec3	16/10/2002	UTI	UQVTH	+ (<i>bla</i> _{SHV-12})	+ (<i>bla</i> _{TEM-1})	—	—	<i>catA2</i>	<i>aadA2</i>	B
Ec4	18/06/2003	Post-surgery infection	BH1	+ (<i>bla</i> _{SHV-12})	+	—	—	—	<i>aadA2</i>	C
Ec5	19/06/2003	UTI	BH1	+ (<i>bla</i> _{SHV-12})	+	—	—	<i>catA2</i>	<i>aadA2</i>	D
Ec6	19/05/2004	UTI	BH1	+ (<i>bla</i> _{SHV-12})	+	+ (<i>bla</i> _{OXA-10})	—	<i>catA2</i>	<i>aadA2</i>	A
Ec7	05/10/2004	UTI	UQVTH	+ (<i>bla</i> _{SHV-12})	+	—	—¶	<i>catA2</i>	<i>aadA2</i>	E
Ec8	24/11/2004	UTI	BH1	—	+	—	+ (<i>bla</i> _{CMY-2})	<i>catA2</i>	<i>aadA2</i> , <i>dfrV</i>	F1
Ec9	20/07/2005	Multiple abscesses	BH1	+ (<i>bla</i> _{SHV-12})	+	—	—	<i>catA2</i>	<i>aadA2</i>	F2
Ec10	25/08/2005	UTI	BH2	+ (<i>bla</i> _{SHV-12})	+	—	—	—	<i>aadA2</i>	G

*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

Strain*	β -Lactamase pI(s)	MIC (mg l ⁻¹)†													
		FOX	CTX	CTX/ CLA‡	CAZ	CAZ/ CLA‡	ATM	CHL	GEN	ENR	SPC	STR	TET	TRI	SXT
Ec1	5.4+8.2	128	32	1	256	2	256	>512	64	32	>512	256	512	>512	32/608
T-Ec1	5.4+8.2	4	16	0.032	32	0.094	128	512	64	<0.5	256	256	512	>512	>32/608
Ec2	5.4+8.2	256	128	1	512	4	512	>512	128	16	>512	128	>512	>512	>32/608
T-Ec2	5.4+8.2	8	4	0.023	64	0.064	128	512	32	<0.5	256	256	256	>512	>32/608
Ec3	5.4+7.9+8.2	256	32	ND	128	ND	128	512	128	8	>512	256	>512	>512	>32/608
T-Ec3	5.4+8.2	4	4	0.032	64	0.125	64	256	128	<0.5	256	256	>256	>512	>32/608
Ec4	5.4+8.2	256	32	1	256	0.5	256	16	64	2	>512	128	>512	>512	>32/608
T-Ec4	5.4+8.2	4	4	0.032	32	0.064	64	4	32	<0.5	128	128	0.5	>512	>32/608
Ec5	5.4+8.2	128	32	0.75	256	0.25	256	512	128	0.125	>512	256	>512	>512	>32/608
T-Ec5	5.4+8.2	8	4	0.023	64	0.064	128	512	32	<0.5	256	256	256	>512	>32/608
Ec6	5.4+6.3+8.2	>256	64	ND	256	ND	512	>512	64	32	>512	256	>512	>512	>32/608
T-Ec6	5.4+6.3+8.2	4	8	0.032	64	0.094	64	512	64	0.5	>512	256	256	>512	>32/608
Ec7	5.4+8.2+8.4	256	128	ND	512	ND	512	512	64	1	>512	16	>512	<1	2/38
T-Ec7	5.4+8.2	16	16	0.047	64	0.19	512	512	128	<0.5	512	16	>256	<1	0.25/4.75
Ec8	5.4+8.9	>256	128	ND	512	ND	512	>512	64	128	>512	256	>512	>512	32/608
T-Ec8	5.4+8.9	256	32	ND	512	ND	64	4	1-2	<0.5	8	4	1	<1	<0.06/1.18
Ec9	5.4+8.2	128	128	1	256	0.25	256	512	64	64	>512	256	>512	>512	>32/608
Ec10	5.4+7.9+8.2	256	32	ND	256	ND	512	32	256	16	16	256	512	>512	>32/608
T-Ec10	5.4+8.2	8	4	0.032	64	0.064	128	4	128	<0.5	512	256	512	>512	>32/608
<i>Escherichia coli</i> J53Az ^R §	—	8	0.125	<1	0.5	<1	0.25	4	1	0.03	8	8	0.5	0.125	0.125/2.37

*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 μ g ml⁻¹ and CAZ/CLA > 4 μ g ml⁻¹.§*Escherichia coli* J53Az^R MICs to ampicillin, piperacillin and ticarcillin were 8, 4 and 8 μ g ml⁻¹, respectively.

Southern blot hybridization analysis. DNA (uncut plasmids and BamHI-digested plasmid) was hybridized to DNA probes generated from PCR products using *bla*_{SHV} (885 bp) and *bla*_{CMY} (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*

J53Az^R as the recipient (Sidjabat *et al.*, 2006a). Transconjugants were selected on Mueller–Hinton agar supplemented with 120 μ g sodium azide ml⁻¹ and 50 μ g ampicillin ml⁻¹. Plasmid transfer from *Enterobacter* to the *Escherichia coli* J53Az^R transconjugants was confirmed by *bla*_{SHV}, *bla*_{CMY} and *bla*_{OXA} 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).

Fig. 1. PFGE patterns of ten clinical *Enterobacter* isolates from dogs. Lanes M, low-range lambda ladder (kb) (New England Biolabs).

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

Fig. 2. Uncut plasmid profiles (a) and *Bam*HI-digested plasmids (b) of ten canine *Enterobacter* isolates and their corresponding transconjugants, analysed by Southern blot hybridization. Lanes M, plasmid marker (BAC-Tracker Supercoiled DNA ladder; Epicentre Biotechnologies). R1 and R27 are *Escherichia coli* reference strains that contain plasmids of known size. Chr, Chromosomal DNA.

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 *Bam*HI-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 *bla*_{TEM}, and in isolates Ec1, Ec2 and Ec3, the sequence alignments were identical with *bla*_{TEM-1}, which was in accordance with the identified β -lactamase pI of 5.4. Meanwhile, alignments of the *bla*_{SHV} nucleotide sequences from all ESBL-producing isolates (Ec1–Ec7, Ec9 and Ec10) were identical with the *bla*_{SHV-12} sequence, which correlated with the β -lactamase pI of 8.2. In isolate Ec6, the OXA-specific amplified product was identified as *bla*_{OXA-10} and production of this gene product correlated with the β -lactamase with a pI of ~6.3. 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 *bla*_{CMY-2} or *bla*_{CMY-7} (CMY25F1 and CMY2DR1). Sequence analysis of the amplified product from Ec8 was identical to *bla*_{CMY-2} (GenBank accession no. X91840); this was in accordance with an identified β -lactamase with a pI of 8.9. In addition, IEF detected bands with a pI of 7.9 in two

isolates (Ec3 and Ec10) and a band with a pI 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 streptomycin/spectinomycin resistance gene (*aadA2*) (Table 1). Interestingly, Ec8, which possessed *bla*_{CMY-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 *bla*_{SHV-12}-carrying plasmids of eight isolates (Ec1–Ec7 and Ec10), the *bla*_{CMY-2}-carrying plasmid of Ec8 and the *bla*_{OXA-10}-carrying plasmid of Ec6 were readily transferred into *Escherichia coli* J53Az^R 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 *bla*_{SHV-12} gene was carried on a large ~140 kb plasmid, whereas the plasmid carrying *bla*_{CMY-2} was ~93 kb in size (data not shown). Furthermore, Southern blot hybridization of the *Bam*HI-digested plasmids of the transconjugants with a *bla*_{SHV} probe confirmed that *bla*_{SHV-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 *bla*_{CMY} probe confirmed the location of *bla*_{CMY-2} on an ~23 kb plasmid fragment within the ~93 kb plasmid (data not shown). In addition, *bla*_{SHV-12} and *bla*_{CMY-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 *bla*_{SHV-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 *bla*_{CMY-2}. Transfer of tetracycline resistance was also demonstrated for all *bla*_{SHV-12}-producing isolates except Ec4. Resistance to enrofloxacin was not transferable by conjugation for any of the isolates. In addition, the transmissible *bla*_{CMY-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 $\sim$ 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 $\sim$ 93 kb plasmid containing a single resistance gene (*bla*_{CMY-2}), whereas Ec9 contained the $\sim$ 140 kb *bla*_{SHV-12} plasmid. Interestingly, Ec9 was the only isolate for which a transconjugant was not obtained.

Previously, *bla*_{SHV-2} was documented as the most prevalent ESBL throughout the world (Paterson *et al.*, 2003). However, the prevalence of *bla*_{SHV-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, *bla*_{SHV-2a} and *bla*_{SHV-12} were found commonly to be present in hospital isolates of *K. pneumoniae* (Howard *et al.*, 2002). The presence of a combination of *bla*_{SHV-12} and *bla*_{TEM} 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 *bla*_{SHV-12} and *aadA2* in *Enterobacter cloacae* has been reported in isolates identified in Liverpool, UK (Corkill *et al.*, 2005).

Recently, *bla*_{CMY-7} was identified in canine MDREC isolated from dogs in Brisbane (Sidjabat *et al.*, 2006a). Interestingly, the $\sim$ 93 kb MDREC *bla*_{CMY-7} plasmid and the $\sim$ 93 kb *bla*_{CMY-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 *Bam*HI-digested profiles of the *Enterobacter cloacae* and MDREC plasmid profiles were distinct (Sidjabat *et al.*, 2006a). Furthermore, the *bla*_{CMY-2}-carrying plasmid in *Enterobacter* spp. was transferable by conjugation, whilst *bla*_{CMY-7} in *Escherichia coli* was not. The presence of *bla*_{CMY-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 *bla*_{SHV-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 cefepime 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 cefepime. 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 methicillin-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 *bla*_{SHV-12} and *bla*_{CMY-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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REFERENCES

- Bell, J. M., Turnidge, J. D., Jones, R. N. & the SENTRY Asia-Pacific Participants (2003). Prevalence of extended-spectrum β -lactamase-producing *Enterobacter cloacae* in the Asia-Pacific region: results from the SENTRY Antimicrobial Surveillance Program, 1998 to 2001. *Antimicrob Agents Chemother* **47**, 3989–3993.
- Bradford, P. A. (2001). Extended-spectrum β -lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev* **14**, 933–951.
- Carattoli, A., Lovari, S., Franco, A., Cordaro, G., Di Matteo, P. & Battisti, A. (2005). Extended-spectrum β -lactamases in *Escherichia coli* isolated from dogs and cats in Rome, Italy, from 2001 to 2003. *Antimicrob Agents Chemother* **49**, 833–835.
- Chia, J.-H., Chu, C., Su, L.-H., Chiu, C.-H., Kuo, A.-J., Sun, C.-F. & Wu, T.-L. (2005). Development of a multiplex PCR and SHV melting-curve mutation detection system for detection of some SHV and CTX-M β -lactamases of *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* in Taiwan. *J Clin Microbiol* **43**, 4486–4491.
- Corkill, J. E., Anson, J. J. & Hart, C. A. (2005). High prevalence of the plasmid-mediated quinolone resistance determinant *qnrA* in multi-drug-resistant *Enterobacteriaceae* from blood cultures in Liverpool, UK. *J Antimicrob Chemother* **56**, 1115–1117.
- Fernández-Baca, V., Ballesteros, F., Hervás, J. A., Villalón, P., Domínguez, M. A., Benedi, V. J. & Alberti, S. (2001). Molecular epidemiological typing of *Enterobacter cloacae* isolates from a neonatal intensive care unit: three-year prospective study. *J Hosp Infect* **49**, 173–182.
- Gottlieb, T. & Wolfson, C. (2000). Comparison of the MICs of cefepime for extended-spectrum β -lactamase-producing and non-extended-spectrum β -lactamase-producing strains of *Enterobacter cloacae*. *J Antimicrob Chemother* **46**, 330–331.
- Hanson, N. D. & Sanders, C. C. (1999). Regulation of inducible AmpC β -lactamase expression among *Enterobacteriaceae*. *Curr Pharm Des* **5**, 881–894.
- Hanson, N. D., Moland, E. S., Hossain, A., Neville, S. A., Gosbell, I. B. & Thomson, K. S. (2002). Unusual *Salmonella enterica* serotype Typhimurium isolate producing CMY-7, SHV-9 and OXA-30 β -lactamases. *J Antimicrob Chemother* **49**, 1011–1014.
- Ho, P. L., Shek, R. H. L., Chow, K. H., Duan, R. S., Mak, G. C., Lai, E. L., Yam, W. C., Tsang, K. W. & Lai, W. M. (2005). Detection and characterization of extended-spectrum β -lactamases among blood-stream isolates of *Enterobacter* spp. in Hong Kong, 2000–2002. *J Antimicrob Chemother* **55**, 326–332.
- Hoffmann, H., Stindl, S., Ludwig, W., Stumpf, A., Mehlen, A., Monget, D., Pierard, D., Ziesing, S., Heesemann, J. & other authors (2005). *Enterobacter hormaechei* subsp. *oharae* subsp. nov., *E. hormaechei* subsp. *hormaechei* comb. nov., and *E. hormaechei* subsp. *steigerwaltii* subsp. nov., three new subspecies of clinical importance. *J Clin Microbiol* **43**, 3297–3303.
- Howard, C., van Daal, A., Kelly, G., Schooneveldt, J., Nimmo, G. & Giffard, P. M. (2002). Identification and minisequencing-based discrimination of SHV β -lactamases in nosocomial infection-associated *Klebsiella pneumoniae* in Brisbane, Australia. *Antimicrob Agents Chemother* **46**, 659–664.
- Kim, J., Lim, Y.-M., Rheem, I., Lee, Y., Lee, J.-C., Seol, S.-Y., Lee, Y.-C. & Cho, D.-T. (2005). CTX-M and SHV-12 β -lactamases are the most common extended-spectrum enzymes in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* collected from 3 university hospitals within Korea. *FEMS Microbiol Lett* **245**, 93–98.
- Lee, S. H., Kim, J. Y., Shin, S. H., An, Y. J., Choi, Y. W., Jung, Y. C., Jung, H. I., Sohn, E. S., Jeong, S. H. & Lee, K. J. (2003). Dissemination of SHV-12 and characterization of new AmpC-type β -lactamase genes among clinical isolates of *Enterobacter* species in Korea. *J Clin Microbiol* **41**, 2477–2482.
- Levison, M. E., Mailapur, Y. V., Pradhan, S. K., Jacoby, G. A., Adams, P., Emery, C. L., May, P. L. & Pitsakis, P. G. (2002). Regional occurrence of plasmid-mediated SHV-7, an extended-spectrum β -lactamase, in *Enterobacter cloacae* in Philadelphia Teaching Hospitals. *Clin Infect Dis* **35**, 1551–1554.
- Liu, C.-P., Wang, N.-Y., Lee, C.-M., Weng, L.-C., Tseng, H.-K., Liu, C.-W., Chiang, C.-S. & Huang, F.-Y. (2004). Nosocomial and community-acquired *Enterobacter cloacae* bloodstream infection: risk factors for and prevalence of SHV-12 in multiresistant isolates in a medical centre. *J Hosp Infect* **58**, 63–77.
- Ma, L., Chang, F.-Y., Fung, C.-P., Chen, T.-L., Lin, J.-C., Lu, P.-L., Huang, L.-Y., Chang, J.-C. & Siu, L. K. (2005). Variety of TEM-, SHV-, and CTX-M-type β -lactamases present in recent clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* from Taiwan. *Microb Drug Resist* **11**, 31–39.
- Milinovich, G. J., Trott, D. J., Burrell, P. C., van Eps, A. W., Thoenner, M. B., Blackall, L. L., Al Jassim, R. A. M., Morton, J. M. & Pollitt, C. C. (2006). Changes in equine hindgut bacterial populations during oligofructose-induced laminitis. *Environ Microbiol* **8**, 885–898.
- NCCLS (2002). *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals: Approved Standard*, 2nd edn. Approved Standard M31-A2. Wayne, PA: National Committee for Clinical Laboratory Standards.
- Pai, H., Hong, J. Y., Byeon, J.-H., Kim, Y.-K. & Lee, H.-J. (2004). High prevalence of extended-spectrum β -lactamase-producing strains among blood isolates of *Enterobacter* spp. collected in a tertiary hospital during an 8-year period and their antimicrobial susceptibility patterns. *Antimicrob Agents Chemother* **48**, 3159–3161.
- Park, Y.-J., Park, S. Y., Oh, E.-J., Park, J.-J., Lee, K.-Y., Woo, G.-J. & Lee, K. (2005). Occurrence of extended-spectrum β -lactamases

among chromosomal AmpC-producing *Enterobacter cloacae*, *Citrobacter freundii*, and *Serratia marcescens* in Korea and investigation of screening criteria. *Diagn Microbiol Infect Dis* 51, 265–269.

Paterson, D. L., Hujer, K. M., Hujer, A. M., Yeiser, B., Bonomo, M. D., Rice, L. B., Bonomo, R. A. & the International *Klebsiella* Study Group (2003). Extended-spectrum β -lactamases in *Klebsiella pneumoniae* bloodstream isolates from seven countries: dominance and widespread prevalence of SHV- and CTX-M-type β -lactamases. *Antimicrob Agents Chemother* 47, 3554–3560.

Pérez-Pérez, F. J. & Hanson, N. D. (2002). Detection of plasmid-mediated AmpC β -lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol* 40, 2153–2162.

Pitout, J. D. D., Reisbig, M. D., Venter, E. C., Church, D. L. & Hanson, N. D. (2003). Modification of the double-disk test for detection of *Enterobacteriaceae* producing extended-spectrum and AmpC β -lactamases. *J Clin Microbiol* 41, 3933–3935.

Ramphal, R. & Ambrose, P. G. (2006). Extended-spectrum β -lactamases and clinical outcomes: current data. *Clin Infect Dis* 42 (Suppl. 4), S164–S172.

Schlesinger, J., Navon-Venezia, S., Chmelnitsky, I., Hammer-Münz, O., Leavitt, A., Gold, H. S., Schwaber, M. J. & Carmeli, Y. (2005). Extended-spectrum beta-lactamases among *Enterobacter* isolates obtained in Tel Aviv, Israel. *Antimicrob Agents Chemother* 49, 1150–1156.

Seeberg, A. H., Tolxdorff-Neutzling, R. M. & Wiedemann, B. (1983). Chromosomal β -lactamases of *Enterobacter cloacae* are responsible for resistance to third-generation cephalosporins. *Antimicrob Agents Chemother* 23, 918–925.

Sidjabat, H. E., Townsend, K. M., Hanson, N. D., Bell, J. M., Stokes, H. W., Gobius, K. S., Moss, S. M. & Trott, D. J. (2006a). Identification of *bla*_{CMY-7} and associated plasmid-mediated resistance genes in multidrug-resistant *Escherichia coli* isolated from dogs at a veterinary teaching hospital in Australia. *J Antimicrob Chemother* 57, 840–848.

Sidjabat, H. E., Townsend, K. M., Lorentzen, M., Gobius, K. S., Fegan, N., Chin, J. J.-C., Bettelheim, K. A., Hanson, N. D., Bensink, J. C. & Trott, D. J. (2006b). Emergence and spread of two distinct clonal groups of multidrug-resistant *Escherichia coli* in a veterinary teaching hospital in Australia. *J Med Microbiol* 55, 1125–1134.

Su, L.-H., Chen, H.-L., Chia, J.-H., Liu, S.-Y., Chu, C., Wu, T.-L. & Chiu, C.-H. (2006). Distribution of a transposon-like element carrying *bla*_{CMY-2} among *Salmonella* and other *Enterobacteriaceae*. *J Antimicrob Chemother* 57, 424–429.

Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 33, 2233–2239.

Teshager, T., Dominguez, L., Moreno, M. A., Saenz, Y., Torres, C. & Cardenosa, S. (2000). Isolation of an SHV-12 β -lactamase-producing *Escherichia coli* strain from a dog with recurrent urinary tract infections. *Antimicrob Agents Chemother* 44, 3483–3484.

Thomson, K. S., Sanders, C. C. & Moland, E. S. (1999). Use of microdilution panels with and without β -lactamase inhibitors as a phenotypic test for β -lactamase production among *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter freundii*, and *Serratia marcescens*. *Antimicrob Agents Chemother* 43, 1393–1400.

Tzelepi, E., Giakkoupi, P., Sofianou, D., Loukova, V., Kemeroglou, A. & Tsakris, A. (2000). Detection of extended-spectrum β -lactamases in clinical isolates of *Enterobacter cloacae* and *Enterobacter aerogenes*. *J Clin Microbiol* 38, 542–546.

Vahaboglu, H., Ozturk, R., Akbal, H., Saribas, S., Tansel, O. & Coskunkan, F. (1998). Practical approach for detection and identification of OXA-10-derived ceftazidime-hydrolyzing extended-spectrum β -lactamases. *J Clin Microbiol* 36, 827–829.

Warren, A., Townsend, K. M., King, T., Moss, S. M., O'Boyle, D. & Trott, D. J. (2001). Multi-drug resistant *Escherichia coli* with extended-spectrum β -lactamase activity and fluoroquinolone resistance isolated from clinical infections in dogs. *Aust Vet J* 79, 621–623.

Weese, J. S., Dick, H., Willey, B. M., McGeer, A., Kreiswirth, B. N., Innis, B. & Low, D. E. (2006). Suspected transmission of methicillin-resistant *Staphylococcus aureus* between domestic pets and humans in veterinary clinics and in the household. *Vet Microbiol* 115, 148–155.

Yan, J.-J., Wu, S.-M., Tsai, S.-H., Wu, J.-J. & Su, I.-J. (2000). Prevalence of SHV-12 among clinical isolates of *Klebsiella pneumoniae* producing extended-spectrum β -lactamases and identification of a novel AmpC enzyme (CMY-8) in Southern Taiwan. *Antimicrob Agents Chemother* 44, 1438–1442.