Characterization of multidrug-resistant *Escherichia coli* isolated from extraintestinal clinical infections in animals

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Multidrug-resistant (MDR) *Escherichia coli* causes extraintestinal infections in both humans and animals. This study aimed to determine whether MDR *E. coli* isolates cultured from extraintestinal infections in several animal species were clonal and crossed host-species boundaries, as suggested by initial characterization of a subset of canine and human isolates, or whether they represented a diverse group of host-specific strains. Isolates were obtained either from The University of Queensland Veterinary Diagnostic Laboratory or from an independent diagnostic laboratory between October 1999 and December 2007. Ninety-six MDR *E. coli* isolates cultured from extraintestinal clinical infections in 55 animals comprising dogs (*n* = 45), cats (*n* = 5), horses (*n* = 4) and a koala (*n* = 1) were analysed by phylogenetic grouping, antimicrobial susceptibility testing and PFGE. The isolates were cultured from the urinary tract (*n* = 61), reproductive tract (*n* = 11), wounds (*n* = 11), surgical site infections (*n* = 4) and other sites (*n* = 9). Isolates from the same *E. coli* phylogenetic group with 100% PFGE similarity and the same antimicrobial susceptibility pattern were considered to be repeat clones and excluded from further analysis. Three of the four *E. coli* phylogenetic groups (A, *n* = 19; B1, *n* = 8; and D, *n* = 49) were represented. Analysis of PFGE similarity identified clusters of related phylogenetic group A isolates [clonal group (CG) 1] and group D isolates (CG2 and CG3), with the remainder of the isolates demonstrating diversity. The majority of CG2 isolates contained a plasmid-borne AmpC β-lactamase, imparting resistance to cefoxitin and third-generation cephalosporins, and were obtained between 2000 and 2003. CG3 isolates were sensitive to these antimicrobial agents and appeared to replace CG2 isolates as the dominant clones from 2003 to 2007. Apart from several canine and feline isolates that demonstrated clonality, PFGE profiles tended to be divergent across species. Whilst MDR *E. coli* isolates from extraintestinal infections in different animal species are diverse, some dominant CGs may persist over several years.

INTRODUCTION

Extraintestinal pathogenic *Escherichia coli* (ExPEC) causes infections of the central nervous and circulatory systems, as well as the urinary, respiratory and reproductive tracts (Kaper *et al.*, 2004; Oluoch *et al.*, 2001; Russo & Johnson, 2003). ExPEC isolates possess virulence factors for invasion and colonization of extraintestinal sites (Russo & Johnson, 2003) and typically belong to *E. coli* phylogenetic groups B2 and D (Johnson & Russo, 2002). Commensal *E. coli* isolates are derived from phylogenetic groups A and B1 and lack the specialized virulence genes associated with B2 and D strains (Johnson & Russo, 2002). However, commensal *E. coli* with limited virulence gene repertoires can cause extraintestinal infections in susceptible hosts (Russo & Johnson, 2003).

Similarities in virulence factors, phylogenetic background and genetic profile have been noted among ExPEC isolates from animals and humans (Cherifi *et al.*, 1994; DebRoy *et al.*, 2008; Johnson *et al.*, 2003, 2008b). The sharing of ExPEC clones amongst humans and pets within the same household has also been demonstrated recently (Damorg *et al.*, 2009; Johnson *et al.*, 2008a; Murray *et al.*, 2004). ExPEC strains are becoming increasingly resistant to antimicrobial agents (Cooke *et al.*, 2002; Smith *et al.*, 2007). Antimicrobial resistance was typically associated with less virulent strains from phylogenetic groups A, B1 or D (Johnson *et al.*, 2009a) until the recent emergence of the phylogenetic group B2 ST131 clonal lineage (Nicolas-Chanoine *et al.*, 2008).

**Abbreviations:** CG, clonal group; ESBL, extended spectrum β-lactamase; ExPEC, extraintestinal pathogenic *E. coli*; MDR, multidrug-resistant; UQVTL, University of Queensland Veterinary Teaching Hospital; UTVTH, University of Queensland Veterinary Teaching Hospital; UTI, urinary tract infection.
In this study, we preliminarily characterized 11 multidrug-resistant (MDR) canine ExPEC isolates divided into two clonal groups (CGs), with CG1 corresponding to phylogenetic group A and CG2 to group D (Sidjabat et al., 2009). All isolates contained a plasmid-borne AmpC bla\text{CMY-2} gene and showed resistance to fluoroquinolones (Sidjabat et al., 2006). Both CGs were also isolated from rectal swabs of hospitalized dogs over an 18-month period, and CG1 isolates were cultured from the hospital environment and CG2 strains from rectal swabs of veterinary hospital personnel (Sidjabat et al., 2006).

These initial results suggested that MDR E. coli isolates in veterinary settings are clonal, persistent and may cross host-species boundaries. To test this hypothesis further, we characterized additional MDR E. coli isolates obtained from extraintestinal infections in dogs, cats, horses and a koala and compared them with the 11 original MDR CG1 and CG2 canine isolates.

METHODS

Bacterial isolates. Ninety-six MDR E. coli isolates obtained from clinical extraintestinal infections from 55 animals between October 1999 and December 2007 were eligible for selection in this study. These comprised 82 MDR E. coli isolates from 42 animals (34 dogs, five cats and three horses) that were obtained from extraintestinal specimens submitted for bacteriological culture to The University of Queensland Veterinary Diagnostic Laboratory (UQVDL) and 14 MDR E. coli isolates from 13 animals (11 dogs, one horse and one koala) obtained from an independent diagnostic laboratory and forwarded to the UQVDL. The selection criteria for multidrug resistance were resistance to fluoroquinolones and/or cefoxitin and additional resistance to at least three of five commonly used antimicrobials in veterinary species (tetracycline, gentamicin, amoxicillin/clavulanic acid, sulfonamide/trimethoprim and cephalothin). Multiple isolates from the same host were included in this study, as in the preliminary studies one dog yielded two isolates (CG2a and CG2b) that differed in phylogenetic group, CG and resistance profile (Sidjabat et al., 2006, 2009). However, isolates from the same animal and of the same E. coli phylogenetic group and with 100% PFGE similarity and the same resistance profile were considered to be a repeat clone and removed from the data analysis. This resulted in 76 MDR E. coli isolates being used in the final analyses.

Different isolates from the same animal collected over time or at the same time point were given the same animal number and then additional notation, e.g. ‘C20b’ indicates the second isolate from canine sample 20 from the UQVDL. Isolates obtained from the independent diagnostic laboratory were designated with the prefix ‘VP’ prior to species identification and then the sample number, e.g. ‘VPC1’ indicates independent laboratory canine sample 1.

The UQVDL receives most of its samples from veterinary referral centres and the majority of isolates originated from five small-animal and two equine referral hospitals in south-east Queensland. The isolates obtained from the independent diagnostic laboratory were from Queensland (n=10) and New South Wales (n=4). All specimens were obtained from animals with clinical signs of extraintestinal infection. The isolates were cultured from urinary tract infections (UTIs) (n=61), surgical site infections (n=4), wounds (n=11), the reproductive tract (n=11) and other sites (n=9). Stock cultures were stored in Luria–Bertani broth with 15% (v/v) glycerol at ~80 °C.

Isolate identification and phylogenetic grouping. E. coli isolates were isolated and identified using standard veterinary diagnostic techniques including the Microbact 24E system (Medvet Diagnostics) and by species-specific PCR amplification of the E. coli uspA gene (Chen et al., 2003). Isolates were assigned to one of the four major E. coli phylogenetic groups (A, B1, B2 and D) using an established triplex PCR-based assay for the chuA, yjaA and tsPE4.C2 genes (Clermont et al., 2000).

Antimicrobial susceptibility testing. Disc diffusion susceptibility testing for 16 antimicrobial agents was performed on all isolates using Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2008a, b). The susceptibility was interpreted according to the guidelines (CLSI, 2008a) for amikacin, amoxicillin/clavulanic acid, ampicillin, cefpodoxime, cephalothin, chloramphenicol, enrofloxacin, gentamicin, imipenem, sulfamethoxazole/trimethoprim, timentin and tetracycline, and the CLSI guidelines (CLSI, 2008b) for cefoxitin, cefazidime, cefepime and ciprofloxacin. All discs were obtained from Oxoid. E. coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 were used as control strains.

Characterization and identification of \(\beta\)-lactamas. Characterization and identification of \(\beta\)-lactamas for new isolates was conducted as described previously for isolates C1–C12a (Sidjabat et al., 2006). Briefly, a modified double-disc diffusion test was performed on the isolates to screen phenotypically for the presence of extended-spectrum \(\beta\)-lactamas (ESBLs) (Sidjabat et al., 2006). PCR amplification of \text{bla\text{ESBL}} and \text{bla\text{OXA}} was performed on isolates, and positive results were confirmed by direct sequencing (Sidjabat et al., 2007). Plasmid-borne AmpC \(\beta\)-lactamase gene amplification was conducted using a previously described multiplex PCR (Sidjabat et al., 2007). Sequencing of the entire structural gene of \text{Citrobacter freundii} origin plasmid-borne AmpC was performed as described previously (Sidjabat et al., 2006).

PFGE. Genomic DNA from each isolate was analysed by PFGE after \(XbaI\) (New England Biolabs) digestion (Sidjabat et al., 2007). PFGE patterns were analysed using GelComparII (Applied Maths). Dice similarity coefficients were calculated and the unweighted pair group method with arithmetic averages was used for cluster analysis. Optimization of 2% and position tolerance of 2% were used. Isolates with a similarity of \(\geq 85\%\) were considered a cluster.

RESULTS AND DISCUSSION

Study population

The MDR isolates from the UQVDL were obtained from extraintestinal infections. This represents a prevalence of 2.0% of all submissions to the diagnostic laboratory that yielded a positive culture and 10.9% of all E. coli isolates. This high prevalence may in part be due to the UQVDL obtaining most of its isolates from diagnostic submissions from veterinary referral centres, which treat a higher proportion of animals with complicated underlying medical or surgical conditions (Gibson et al., 2008). In addition, most have intensive care facilities, where treatment with multiple classes of antimicrobial agents for prolonged periods is not uncommon. The proportion of the population represented by the MDR isolates (n=14) obtained from the independent diagnostic laboratory was unknown, but this laboratory supports both primary accession and referral veterinary practices and may not
have forwarded all MDR *E. coli* isolates obtained over the 7-year period.

**Phylogenetic grouping**

Isolates were divided into phylogenetic groups A (*n* = 19), B1 (*n* = 8) and D (*n* = 49). None of the isolates corresponded to *E. coli* phylogenetic group B2, which is most frequently associated with ExPEC isolates of greater virulence (Johnson & Russo, 2002). The majority of isolates belonged to phylogenetic group D, members of which can contain a broad range of virulence factors (Johnson & Russo, 2002). In a previous study, a subset of these isolates (Figs 1 and 2) was examined for virulence potential, and phylogenetic group D isolates were shown to contain four extraintestinal virulence genes (*iutA, ibeA, fimH* and *kpsMT K5*), whereas phylogenetic group A isolates contained only two virulence genes (*iutA* and *traT*) (Sidjabat et al., 2009).

**Antimicrobial susceptibility testing and \( \beta \)-lactamase identification**

The number and percentage of isolates in each phylogenetic group that were non-susceptible to 16 antimicrobial agents is detailed in Table 1. All isolates were resistant to fluoroquinolones, except for one isolate from phylogenetic group D and one from phylogenetic group A. Isolates from non-ExPEC phylogenetic groups A and B1 had a higher proportion of isolates showing reduced susceptibility to cefoxitin and third-generation cephalosporins (84 % and 50 %, respectively) compared with ExPEC phylogenetic group D (39 %). In phylogenetic group A, 53 % of isolates were also resistant to chloramphenicol. This phenomenon...
Fig. 2. PFGE profiles of MDR *E. coli* isolated from extraintestinal infections in animals belonging to phylogenetic group D (*n*=49). *Two main clonal clusters (CG2 and CG3) are identified.* 1UQVTH, The University of Queensland Veterinary Teaching Hospital; BH, Brisbane Veterinary Hospital; EqH1, Equine Veterinary Hospital 1; for samples submitted to the independent diagnostic laboratory, origins are at a state level (Qld, Queensland; NSW, New South Wales). 2Extended-spectrum and AmpC β-lactamase characterization, phylogenetic grouping and virulence genes of these isolates have been described previously (Sidjabat et al., 2006, 2009). 3Considered the same clone. 4Two clones present, with differing antimicrobial susceptibility profiles. *Isolate C14d possessed an ESBL *bla* _SHV-12_ gene. **Isolate C27h possessed an AmpC β-lactamase *bla* _CMY-2_ gene. ND, Not detected; +, positive.
has been noted previously in canine fluoroquinolone-resistant *E. coli* strains (Johnson *et al.*, 2009a). Fifteen isolates from phylogenetic group A contained an AmpC β-lactamase, with more recent isolates possessing *bla*CMY-2 and older isolates *bla*CMY-7. One isolate from phylogenetic group A possessed an ESBL *bla*OXA-10 gene (Fig. 1). Half of the phylogenetic group B1 isolates (*n* = 4) possessed the AmpC β-lactamase *bla*CMY-2 (Fig. 1). Isolates from phylogenetic group D were divided into two groups: those that showed reduced susceptibility to cefoxitin and third-generation cephalosporins (*n* = 19) due to the presence of AmpC β-lactamases (*bla*CMY-7, *bla*CMY-2 or other) and those that were sensitive to these agents (*n* = 30) and did not contain an AmpC β-lactamase (Fig. 2).

### PFGE

PFGE confirmed that the isolates were diverse, with a number of distinct CGs of genetically related strains identified and some clustering apparent both within and across species, origin and date of isolation (Figs 1 and 2). Similar to a previous study examining the genetic relationships of fluoroquinolone-resistant *E. coli* causing UTIs in dogs by PFGE (Cooke *et al.*, 2002), this study also suggested that the MDR *E. coli* extraintestinal infections in veterinary species were not due to the dissemination of a single, dominant clone. However, PFGE alone may not be sufficient to identify broader CGs. In MDR ExPEC infections in humans, the isolates often belong to globally disseminated CGs, such as trimethoprim/sulfamethoxazole-resistant ‘clonal group A’ (Manges *et al.*, 2001) and the recently described fluoroquinolone-resistant O25:H4-ST131 clonal lineage, which belongs to phylogenetic group B2 (Lau *et al.*, 2008). The PFGE profiles of these clonal groups are often diverse (Lau *et al.*, 2008; Manges *et al.*, 2001; Nicolas-Chanoine *et al.*, 2008), and other molecular techniques such as multilocus sequence typing, which measures conserved regions in the genome, may be a better means of identifying relatedness of *E. coli* clones and broader clonal groups (Feil, 2004).

### Characterization of phylogenetic group A and B1 isolates

A group of 10 phylogenetic group A isolates formed a cluster of potentially related strains (Fig. 1), which contained the original CG1 isolates (C1, C2a, C6a, C6b and C9) from The University of Queensland Veterinary Teaching Hospital (UQVTH) study (Sidjabat *et al.*, 2006). Within this cluster, two isolates from different host species, a cat (F1, peritoneal abscess) and a dog (VPC1, transtracheal wash), were highly related (Tenover *et al.*, 1995). Although these isolates were obtained at similar time points, they were from two different hospitals in south-east Queensland. It has been shown that dogs and cats in the same household can be colonized with the same *E. coli* strain (Johnson *et al.*, 2009b), but it is less likely that a dog and cat, presenting to different referral hospitals with extraintestinal infection, would have direct contact. An equine isolate (VPEq1) was also located within phylogenetic group A and was possibly related to two canine isolates (C40 and C41). The remaining phylogenetic group A and B1 isolates from dogs, horses, a cat and a koala were genetically diverse apart from a small cluster of B1 isolates from the same dog.

### Table 1. Number (%) of non-susceptible MDR *E. coli* isolates within phylogenetic groups A, B1 and D

<table>
<thead>
<tr>
<th>Antimicrobial agent*</th>
<th>Phylogenetic group</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>A (n=19)</td>
<td>B1 (n=8)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>19 (100)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Sulfamethoxazole/trimethoprim</td>
<td>13 (68)</td>
<td>7 (88)</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>18 (95)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>18 (95)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>16 (84)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>10 (52)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>19 (100)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid</td>
<td>19 (100)</td>
<td>7 (88)</td>
</tr>
<tr>
<td>Timentin</td>
<td>16 (84)</td>
<td>6 (75)</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>18 (95)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>16 (84)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>16 (84)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>Cefpodoxime</td>
<td>17 (89)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>Cefepime</td>
<td>1 (5)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*All isolates were susceptible to imipenem (CLSI, 2008a).
Characterization of phylogenetic group D isolates

Clustering of isolates within distinct clonal lineages was also apparent from the phylogenetic group D dendrogram (Fig. 2). Thirty-three isolates from the UQVDL (C2b–C12b) that originated from three different veterinary referral hospitals in Brisbane and two isolates from the private diagnostic laboratory, also located in Brisbane, formed a distinct cluster corresponding to the original CG2 MDR E. coli isolates from the UQVDL (Sidjabat et al., 2006). Interestingly, most of these isolates were resistant to cefoxitin and contained blaCMY-7 and were all obtained from extraintestinal infections in dogs occurring between 2000 and 2003. In a previous mouse model study, CG2 strain C2b showed greater virulence and prolonged gastrointestinal and urinary tract colonization compared with CG1 (phylogenetic group A) strain C1 (Sidjabat et al., 2009).

A third cluster of clonally related phylogenetic group D isolates (designated clonal group 3) were obtained from dogs and one cat presenting to three different veterinary hospitals between 2003 and 2007. All CG3 isolates except for C27h were sensitive to cefoxitin and third-generation cephalosporins and we originally hypothesized that they may be CG2 MDR E. coli strains that did not possess or had lost the AmpC β-lactamase-containing plasmid. PFGE analysis determined that these isolates were instead representative of a distinct phylogenetic group D clonal lineage first identified in 2003 at the same time as the last of the CG2 isolations. CG3 isolates then became the predominant MDR E. coli isolated from extraintestinal infections at several hospitals. It remains to be determined whether this is related to enhanced virulence, fitness or other epidemiological factors. The phenomenon of one CG of clinical MDR E. coli replacing another has been noted in previous studies amongst hospitalized humans (Mamlouk et al., 2006), but, to the best of our knowledge, it has not been reported in the veterinary literature. Clustering of isolates is more common in outbreaks of Salmonella infection (Zhang et al., 2005) and has been reported in a large animal teaching hospital (Cummings et al., 2010). The remaining phylogenetic group D isolates from cats, dogs and a koala were genetically diverse.

Multiple isolates from the same animal

Isolates from the same animal were all represented by the same clonal type except for three cases. Isolates C2a (CG1) and C2b (CG2) were collected from a surgical site infection after fracture repair 1 month apart and have been reported previously (Sidjabat et al., 2006). Eq1a (phylogenetic group A) and Eq1b (phylogenetic group D) were collected from the same horse at the same time from a mandibular abscess. Isolates C27b–C27p were collected over a 21-month period from the same dog with recurrent chronic UTIs due to impaired colonization resistance (Gibson et al., 2008). Seven clonally related isolates belonging to phylogenetic group D CG3 were cultured sequentially from this animal, followed by the two clonally related isolates from phylogenetic group B1. Three months post-infection, an MDR E. coli isolate was then cultured that was clonally related to the original CG3 isolates. It is not uncommon for dogs to be chronically infected with one or two strains of E. coli, which can result from persistence of the strain within the urinary tract or repeated endogenous infection from the gastrointestinal tract (Drazenovich et al., 2004; Seguin et al., 2003). The isolates from horses and the koala were distinct from the canine and feline isolates.

Conclusions

Characterization of MDR E. coli from extraintestinal infections in four animal species revealed a diverse range of phylogenetic groups (A, B1 and D) and PFGE profiles. Group B2 strains were not identified. The isolate collection contained a single cluster of group A isolates (CG1) and two clusters of group D isolates (CG2 and CG3) that showed distinct differences in the carriage of AmpC β-lactamase and isolation dates, with CG3 dominating in recent isolates and CG2 in older isolates. Apart from PFGE profile similarities for a small number of canine and feline isolates, most strains were genotypically diverse across host species. These results confirm the emergence and persistence of particular MDR clonal lineages within a diverse background of strains which may have a competitive advantage in virulence or fitness under antimicrobial selection pressure compared with previous clonal groups of isolates. Monitoring of the emergence and spread of dominant MDR clonal groups within hospital surveillance programmes may assist veterinarians in developing improved strategies for treatment and prevention of infections for which the choice of antimicrobials is limited.

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REFERENCES


