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Identification of Qnr and AAC(6′)-1b-cr plasmid-mediated fluoroquinolone resistance determinants in multidrug-resistant *Enterobacter* spp. isolated from extraintestinal infections in companion animals

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ABSTRACT

Fluoroquinolone resistance is becoming more common in veterinary medicine. Resistance is due to a combination of chromosomal and plasmid-mediated fluoroquinolone resistance (PMQR) mechanisms. The aim of the present study was to screen 17 multidrug-resistant *Enterobacter* isolates obtained from opportunistic infections in companion animals for chromosomal and plasmid-mediated fluoroquinolone resistance determinants and to determine if they are co-located with other antimicrobial resistance genes including β-lactamases.

Phenotypic tests (biochemical identification, organic solvent tolerance testing) were combined with genotypic analysis (PCR, pulsed field gel electrophoresis, sequencing, plasmid isolation and southern blot hybridization) to characterize the molecular basis for fluoroquinolone resistance. Antimicrobial susceptibility was determined by broth microdilution for fluoroquinolone antimicrobials (enrofloxacin, ciprofloxacin, moxifloxacin, marbofloxacin and pradofloxacin) and by disk diffusion for other antimicrobials.

Sixteen isolates were resistant to at least one of the five fluoroquinolones tested. Fourteen isolates possessed PMQR determinants which were identified as *qnrA1* ($n = 3$) or *qnrB2* ($n = 11$), often in combination with *aac(6′)-1b-cr* ($n = 6$). The PMQR genes were localized to large, transferable MDR plasmids often associated with an extended-spectrum β-lactamase and quinolone resistance was co-transferred with bla_{SHV-12} for 10 of the 14 *qnr*-positive strains. Three isolates had wild-type topoisomerases, 11 had a single point mutation in *gyrA* (Ser83Phe or Tyr), and three had two mutations; one in *gyrA* (Ser83Ile) and one in *parC* (Ser80Ile).

PMQR genes in clinical veterinary *Enterobacter* isolates are co-located with β-lactamases and other resistance genes on large transferable plasmids. PMQR genes contribute to fluoroquinolone resistance when combined with topoisomerase mutations and efflux.

1. Introduction

Fluoroquinolones are broad-spectrum antimicrobial agents used for treating a variety of bacterial infections (Hopkins et al., 2005), but, resistance is emerging in both

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production (Webber and Piddock, 2001) and companion animals (Cohn et al., 2003; Pomba et al., 2009). In Gram-negative bacteria, resistance to fluoroquinolones primarily occurs due to chromosomal mutations in the drug target enzymes and/or by decreasing accumulation of the drug through active drug efflux with or without decreased expression of outer membrane porins (Hopkins et al., 2005). In addition, a number of plasmid-mediated quinolone resistance (PMQR) mechanisms have been described including fluoroquinolone-specific efflux through QepA (Cattoir et al., 2008; Perichon et al., 2007); drug target protection though QnrA, QnrB, QnrS, QnrC and QnrD (Cavaco et al., 2009; Martinez-Martinez et al., 1998; Robicsek et al., 2006a; Wang et al., 2009) and enzymatic modification through AAC(6′)-1b-cr (Robicsek et al., 2006b). The expression of the *qnr* gene only confers low level resistance, however, it can facilitate the selection of resistant mutants (Robicsek et al., 2006b; Rodriguez-Martinez et al., 2007). β-Lactamases are often associated with *qnr* genes and plasmids containing QnrA and QnrB determinants often harbor additional antimicrobial resistance genes (Cattoir and Nordmann, 2009).

Our laboratory recently examined 10 multidrug-resistant canine *Enterobacter* clinical isolates for the production of extended-spectrum (ESBL) and AmpC β-lactamases. Nine contained a plasmid-mediated bla_{SHV-12} ESBL and one a plasmid-mediated AmpC bla_{CMY-2}. All isolates except one showed reduced susceptibility to fluoroquinolones (Sidjabat et al., 2007). In this study we examined these and an additional six isolates obtained from dogs and a single isolate from a cat with the aim of determining their molecular mechanisms of fluoroquinolone resistance and identifying if the PMQR genes are co-located on β-lactamase bearing plasmids.

2. Materials and methods

2.1. Bacterial strains

Seventeen MDR *Enterobacter* isolates were obtained from extraintestinal specimens submitted for bacteriological culture to The University of Queensland Veterinary Diagnostic Laboratory between January 2001 and December 2007. This represents a prevalence of 0.35% of all submissions to the diagnostic laboratory that yielded a positive culture and 17% of all *Enterobacter* spp. isolates. To be included in this study the *Enterobacter* spp. had to be resistant to four or more of six antimicrobials (tetracycline, gentamicin, enrofloxacin, amoxicillin/clavulanic acid, sulphonamide/trimethoprim and cephalothin). All specimens were obtained from animals with clinical signs of disease or suspicion of clinical disease in the organ from which the specimens were obtained. The isolates were submitted from four referral hospitals in southeast Queensland.

Nine of the canine isolates were obtained from cases of urinary tract infection, five from orthopedic infection sites post-surgery and two from abscesses. The feline isolate was obtained from the mediastinum of a pleuritis case. Stock cultures were stored in Luria–Bertani broth with 15% (v/v) glycerol at −80 °C.

2.2. Isolate identification and PFGE analysis

Isolates were identified on the basis of their biochemical reactions using the Microbact 24E system (Medvet Diagnositics, Thebarton, SA, Australia) and 16S rRNA gene sequencing (Sidjabat et al., 2007).

Genomic DNA from each isolate was analyzed by PFGE after *Xba*I (New England Bio labs, Ipswich, MA, USA) digestion (Sidjabat et al., 2007). PFGE patterns were analyzed using GelComparII (Applied Maths Inc., Sint-Martens-Latem, Belgium). Dice similarity coefficients were calculated and the unweighted pair group method with arithmetic averages (UPGMA) used for cluster analysis. Two percent optimization and 2% position tolerance were used. Isolates with patterns differing by ≤3 bands (≥94% similarity) were considered clonal, and to be possibly related if they differed by 4–6 bands (Tenover et al., 1995).

2.3. Antimicrobial susceptibility testing

The MICs of five fluoroquinolone antibiotics (enrofloxacin, ciprofloxacin, moxifloxacin, marbofloxacin and pradofloxacin) were determined for original strains, confirmed transconjugants and the *Escherichia coli* recipient strain J53 using the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute (CLSI), 2008a,b). Fluoroquinolone antimicrobial agents were obtained from Bayer, Germany. The tests were performed in microtitre plates. All plates were inoculated and incubated following CLSI guidelines. All MIC determinations were performed in duplicate with *E. coli* ATTC 25922 included in each experiment as a quality control organism. The MIC was read as the lowest concentration without visible growth. CLSI breakpoints were used for enrofloxacin, marbofloxacin (Clinical and Laboratory Standards Institute (CLSI), 2008a) and ciprofloxacin (Clinical and Laboratory Standards Institute (CLSI), 2008b); and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint was used for moxifloxacin (Van Bambeke et al., 2005). Currently there are no published breakpoints for pradofloxacin.

Disk diffusion susceptibility testing for 13 additional non-fluoroquinolone antimicrobial agents was performed on all original isolates and confirmed transconjugants using CLSI guidelines (Clinical and Laboratory Standards Institute (CLSI), 2008a,b).

The susceptibility was interpreted according to the guidelines (Clinical and Laboratory Standards Institute (CLSI), 2008a) for amikacin, amoxicillin/clavulanic acid, ampicillin, cefpodoxime, cephalothin, chloramphenicol, gentamicin, imipenem, sulphamethoxazole/trimethprim and tetracycline, and to CLSI guidelines (Clinical and Laboratory Standards Institute (CLSI), 2008b) for cefoxitin, ceftazidime and cefepime. All discs were obtained from Oxoid (Australia). *E. coli* ATTC 25922 was used as a control strain in all experiments.

2.4. Organic solvent tolerance testing

Strains were tested for organic solvent tolerance, as an indicator of efflux pump activity, as described (White et al.,