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Prevalence and mechanisms of extended-spectrum cephalosporin resistance in clinical and fecal Enterobacteriaceae isolates from dogs in Ontario, Canada

Pauline L.C. Zhang[a], Xiao Shen[a], Gabhan Chalmers[a], Richard J. Reid-Smith[a,b,c], Durda Slavic[d], Hani Dick[e], Patrick Boerlin[a,*]

[a] Department of Pathobiology, Ontario Veterinary College, University of Guelph, 50 Stone Rd. E, Guelph, Ontario, N1G 2W1, Canada
[b] Department of Population Medicine, Ontario Veterinary College, University of Guelph, 50 Stone Rd. E, Guelph, Ontario, N1G 2W1, Canada
[c] Centre for Food-borne, Environmental and Zoonotic Infectious Diseases, Public Health Agency of Canada, 160 Research Lane, Suite 103, Guelph, Ontario, N1G 5B2, Canada
[d] Animal Health Laboratory, Post Office 3612, Guelph, Ontario, N1H 6R8, Canada
[e] IDEXX Laboratories, 1345 Denison St., Markham, Ontario, L3R 5V2, Canada

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ABSTRACT

There is little information on the genetic basis of resistance to the critically important extended-spectrum cephalosporins (ESCs) in Enterobacteriaceae from dogs in Canada. This study assessed the frequency of ESC resistance in Enterobacteriaceae isolated from dogs in Ontario and the distribution of major ESC resistance genes in these bacteria. A total of 542 Enterobacteriaceae were isolated from 506 clinical samples from two diagnostic laboratories in Ontario. Eighty-eight ESC-resistant Enterobacteriaceae and 217 *Escherichia coli* were isolated from 234 fecal samples from dogs collected at leash-free dog parks. These fecal isolates were tested for ESC resistance along with the clinical isolates. Isolates with reduced ESC susceptibility were screened for bla_{CMY}, $bla_{CTX\text{-}M}$, and bla_{SHV}, and all CTX-M-positive isolates underwent whole-genome sequencing. The prevalence of ESC resistance in clinical Enterobacteriaceae was 10.4%. The average frequency of fecal carriage of ESC-resistant Enterobacteriaceae in healthy dogs was 26.5%. The majority of ESC-resistant isolates were *E. coli* and the other major Enterobacteriaceae carrying ESC resistance genes were *Klebsiella pneumoniae* and *Proteus mirabilis*. The results show that the same ESC resistance genes can be found in clinical and fecal Enterobacteriaceae in dogs. The identified *E. coli* sequence types (including ST131 and ST648) and CTX-M variants (including CTX-M-14, −15, and −27) support the hypothesis of transfer of resistant bacteria between humans and dogs. CTX-M-1 was frequently found in canine fecal Enterobacteriaceae, while it is still rare in human Enterobacteriaceae in Canada, thus suggesting transfer of resistant bacteria to dogs from food animals or other sources.

1. Introduction

Enterobacteriaceae cause a variety of infections in dogs, with urinary tract infections (UTIs) being one of the most frequent (Barsanti, 2012). Enterobacteriaceae such as *Klebsiella pneumoniae* and *Proteus mirabilis* are regularly isolated from canine UTIs, but the most common pathogen is *Escherichia coli* (Barsanti, 2012). Successful treatment of such infections often requires antimicrobials, in particular β-lactams, but antimicrobial resistance (AMR) is increasing (Ball et al., 2008). β-lactam resistance is mostly caused by the production of β-lactamases, of which a variety can hydrolyze and inactivate extended-spectrum cephalosporins (ESCs). This is especially concerning because ESCs are considered critically important antimicrobials for human and veterinary medicine (OIE, 2015; WHO, 2017).

ESC resistance is mainly caused by the expression of AmpC β-lactamases, extended-spectrum β-lactamases (ESBLs), and more recently carbapenemases (Courtice et al., 2016). ESC-resistant Enterobacteriaceae that produce β-lactamases such as CMY, TEM-52, CTX-M, and OXA-48 have been observed in dogs throughout North America (Khashayar, 2009; O'Keefe et al., 2010; Liu et al., 2016). Additionally, multi-drug resistance (MDR) has been increasingly reported in *E. coli* isolated from canine UTIs (Liu et al., 2016).

AMR in bacteria from dogs is also a potential public health risk because pets can serve as reservoirs of resistant bacteria to which

* Corresponding author.
E-mail address: pboerlin@uoguelph.ca (P. Boerlin).

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humans are exposed (Guardabassi et al., 2004). The potential for transmission of *E. coli* between humans and companion animals is illustrated for instance by the isolation of an identical *E. coli* clone from a dog's UTI and from its household members' feces (Johnson et al., 2008). The transmission potential is also shown by the sequence type (ST) 131, which has successfully spread worldwide in humans and animals, and causes millions of resistant infections each year (Pitout and DeVinney, 2017). CTX-M-producing *E. coli* ST131 have been recovered from companion animals in North America, Europe and Asia (Khashayar, 2009; Pomba et al., 2009; Ewers et al., 2014; Kawamura et al., 2017). Most of the previous studies that investigated AMR in Enterobacteriaceae from dogs in Canada did not examine the associated resistance genes, and the information available is essentially limited to *E. coli* and *Salmonella* (Prescott et al., 2002; Authier et al., 2006; Ogeer-Gyles et al., 2006b; Ball et al., 2008; Murphy et al., 2009; Courtice et al., 2016). More complete knowledge in this area would help guide antimicrobial treatment, particularly since therapy is usually initiated before receiving susceptibility results and some treatment guidelines suggest that if resistance to a particular antimicrobial exceeds 10%, it should not be used for first-line treatment (Weese et al., 2011). In addition, UTIs are often caused by Enterobacteriaceae from the host's own fecal flora, and there is evidence that AMR genes can be transferred from commensal to pathogenic Enterobacteriaceae (Blake et al., 2003). Therefore, knowing the frequency of AMR in canine fecal bacteria, without the bias present in passive diagnostic data, would be of interest as well.

The goal of this work was to study AMR among Enterobacteriaceae in dogs within the province of Ontario, with an emphasis on ESC resistance. It was focused on *E. coli*, *Salmonella enterica, Klebsiella* spp., *Proteus* spp., *Citrobacter* spp., *Enterobacter* spp., *Serratia* spp., and *Pantoea* spp. The study had four main objectives. The first was to assess the prevalence of ESC resistance in Enterobacteriaceae isolated from canine clinical samples submitted to diagnostic laboratories. The second was to determine the prevalence of resistance to ESCs and other clinically relevant antimicrobials in fecal *E. coli* from dogs. The third objective was to assess the frequency of fecal carriage of ESC-resistant Enterobacteriaceae in the local dog population using enrichment cultures and selective media. The last objective was to assess and compare the distribution of three major ESC resistance genes and of CTX-M variants between Enterobacteriaceae species as well as between clinical and fecal isolates.

2. Materials and methods

2.1. Clinical isolate collection

A total of 542 clinical isolates were collected from the Animal Health Laboratory (AHL), University of Guelph, Guelph, Ontario (n = 210) and IDEXX Laboratories Inc., Markham, Ontario (n = 332) from November 2015 to October 2016. For both laboratories, Enterobacteriaceae from the species of interest were collected from cases of UTIs (n = 227; 42%), otitis (n = 37; 7%), dermatitis (n = 26; 5%), wound infections (n = 51; 9%), surgical site infections (n = 21; 4%), septicemia or multiple organ systems infection (n = 4; 1%) and other miscellaneous infections (n = 176; 32%). All the isolates available during the period of investigation were collected from AHL, while for IDEXX, a stratified systematic sampling strategy was used to collect the first ten *E. coli* isolates of each month, and the first ten non-*E. coli* Enterobacteriaceae isolates of each month, in order to determine the general prevalence of ESC resistance in *E. coli* and other Enterobacteriaceae (Fig. 1). Furthermore, in order to more fully assess resistance determinants, purposive sampling was used to collect the first ten ESC-resistant Enterobacteriaceae of the month as identified using AST susceptibility testing cards and the VITEK® 2 system (bioMérieux, Marcy-l'Étoile, France) (Fig. 1). These ten isolates were further categorized into three ESC-resistant *E. coli* isolates and seven ESC-resistant non-*E. coli* Enterobacteriaceae isolates (Fig. 1). All clinical isolates were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Bremen, Germany).

2.2. Fecal isolate collection

A total of 234 canine fecal samples were collected from leash-free dog parks in ten different cities in Southern Ontario between May and July of 2016. Locations were selected based on major urban areas within a driving distance from the laboratory of less than two hours (Supplementary files, Fig. S1). A minimum of 20 samples were collected from two different dog parks at each location, with only one exception. The dog owners were provided with new unopened plastic bags and requested to provide their dog's feces to the research team. Information on demographics, health status, and previous antimicrobial treatment were not collected. Samples were kept at 4 °C and processed within 24 h. To determine the frequency of resistance toward a range of antimicrobials in fecal *E. coli*, the samples were directly plated onto Rapid Enterobacteriaceae *Escherichia coli* Coliform Agar (REBECCA) (bioMérieux, Marcy-l'Étoile, France) without antimicrobials and one presumptive *E. coli* colony was isolated per sample, when present (Fig. 1). The fecal samples were also enriched for ESC-resistant Enterobacteriaceae (Fig. 1) using a modification of a previously described procedure (Cormier et al., 2016). Briefly, 1.0 g of feces was used to inoculate 9.0 mL of EE Broth Mossel Enrichment (Becton Dickinson [BD], Cockeysville, MD, USA) supplemented with 2 µg/mL cefotaxime. After overnight 37 °C incubation with agitation, 10 µL of the broth culture was streaked onto REBECCA supplemented with 1 µg/mL ceftriaxone (Sigma-Aldrich, St. Louis, MO, USA). Each different colony morphotype seen on this primary plate was subcultured and purified onto REBECCA supplemented with ceftriaxone. Oxidase tests (Sigma-Aldrich) and catalase tests were conducted on isolates to confirm presumptive Enterobacteriaceae identification. Further identification at the genus and species level was done using MALDI-TOF MS (Bruker Daltonics LTD., East Milton, Canada).

2.3. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) for ampicillin, ticarcillin, cefazolin, amoxicillin/clavulanic acid, ticarcillin/clavulanic acid, cefoxitin, cefpodoxime, imipenem, trimethoprim/sulfamethoxazole, chloramphenicol, doxycycline, amikacin, and gentamicin were determined for the fecal *E. coli* isolates using the Sensititre COMPAN1F plate and quality controls as specified by the manufacturer (TREK Diagnostics, Cleveland, OH, USA). Results were interpreted using the manufacturer's instructions.

All clinical isolates, as well as fecal isolates from enrichment, were tested for cephamycin and ESC susceptibility by the disk diffusion method according to the CLSI guidelines (CLSI, 2015) using cefoxitin (FOX-30), cefotaxime (CTX-30), and ceftazidime (CAZ-30) (Becton Dickinson [BD]) disks (Becton Dickinson [BD]). Ertapenem (ETP-10) was also used to test for susceptibility to carbapenems. The reference strains *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as quality controls. Isolates classified as intermediate or resistant to the antimicrobials tested were defined as having reduced susceptibility for the purpose of this study. An overview of the different sample collections and work flow are shown in Fig. 1.

2.4. PCR screening of ESC resistance genes

All isolates with reduced susceptibility to cefoxitin and/or a third-generation cephalosporin (cefotaxime, ceftazidime or cefpodoxime) were screened for the presence of three main ESC resistance genes, bla_{CMY}, $bla_{CTX\text{-}M}$, bla_{SHV}, using single and multiplex PCR amplification, as described previously (Kozak et al., 2009; Cottell et al., 2013). Lysates