Characterization of hospital-associated lineages of ampicillin-resistant *Enterococcus faecium* from clinical cases in dogs and humans

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**INTRODUCTION**

Multiresistant *Enterococcus faecium* has become one of the most important nosocomial pathogens, causing increasing numbers of nosocomial infections worldwide (Willems et al., 2012). They now represent up to one-third of enterococcal infections (Willems and Van Schaik, 2009). A multilocus sequence typing (MLST) study of *E. faecium* isolates revealed the existence of a distinct genetic subpopulation associated with nosocomial infections which was named clonal complex 17 (CC17) (Willems et al., 2011). However, previous studies around the world (Damborg et al., 2008, 2009; De Regt et al., 2008, 2012; Ossiprandi et al., 2008; Jackson et al., 2009; Kwon et al., 2012) have revealed that healthy dogs are known community reservoirs where some of these typical hospital clones seem to reside. Furthermore, HA ARE have been recovered from clinical cases of urinary tract infections in canines from the U.S. (Simjee et al., 2002), Korea (Kwon et al., 2012) and Denmark (Damborg et al., 2008) and from feces of canines leaving the intensive care unit of an American veterinary medicine teaching hospital (Ghosh et al., 2011). In general, only limited data is available on lineages and genotypic content of HA ARE from canine clinical isolates.

A recent report from Canadian human cases of HA ARE (McCracken et al., 2013) has indicated that a shift in ST has occurred from clinical cases, antibiotic resistance, virulence, plasmid, hospital-associated, community-associated, MLST.
occurred after 2006 and that around that time bacteraemia rates began to rise in central and western Canada, suggesting a possible correlation. Prior to 2006, predominant types included ST154, ST16, ST17 and ST80 and after 2006, ST18, ST203, ST412 and ST584 became predominant in Canada (McCracken et al., 2013). In Canada, no data is available on ARE isolates from dogs with ST lineages causing nosocomial infections in humans. Thus, the goal of this study was to characterize canine ARE clinical isolates from Quebec, Canada and to determine their STs. Also, HA ARE strains of human origin were used for comparison to assess possible genetic relationships between the two sets of strains. This study underscores the importance of canines as potential reservoirs of multi-drug resistant HA ARE.

**MATERIALS AND METHODS**

**BACTERIAL ISOLATES AND IDENTIFICATION**

Ten *E. faecium* strains were used in this study (Table 1). Dog strain isolation criteria were the presence of a relatively recent (2007–2012) clinical infection, isolation in pure culture, and PCR identification as *E. faecium* species. Based on these criteria only five canine *E. faecium* isolates were recovered over this period from the diagnostic laboratory of the Faculty of Veterinary Medicine at the University of Montreal and these were from UTI, wounds and cholangiohepatitis infections. The human isolates (*n = 5*) were provided by the “Centre de Recherche Hospitalier de l’Université Laval (CRCHUL)” and randomly chosen from a culture collection of *E. faecium* from hospitalized patients over the same period. Some of the human isolates (CCRI no. 18581, 16717 and 16354) were from colonization/surveillance studies of hospitalized patients. All isolates were identified by multiplex PCR assay using species-specific primer sets for the *dld* gene (*E. faecium* (dld F-5’TTGAGGCGACCCAGATTGACG3’ and dld R-5’TATGCACGGCAGCTCCGATTCC3’) identification gene as previously described (Tremblay et al., 2011). *E. faecium* HA-56038 was used as a positive control.

**ANTI-BACTERIAL SUSCEPTIBILITY TESTING**

Isolates were tested for MIC using the Sensititre plate (Trek™ Diagnostic System Ltd, Cleveland, OH) according to the Clinical and Laboratory Standards Institute (CLSI, M31-A3 and M100-S20) guidelines (Clinical and Laboratory Standards Institute, 2008, 2010). Ciprofloxacin and ampicillin susceptibilities were further analyzed by standard broth macrodilution method (CLSI, M31-A3 and M100-S20). Breakpoints from CLSI and the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) were used (Anonymous, 2008). *Staphylococcus aureus* ATCC29213 and *Enterococcus faecalis* ATCC29212 were used as control strains.

**PFGE AND MLST**

Isolates were analyzed for clonal diversity by pulsed-field gel electrophoresis (PFGE) after *Smnl* (New England Biolabs, Inc., Beverly, Ma, USA) digestion as described by Garcia-Migura et al. (2005). Digested DNA was electrophoresed as previously described (Vankerczkhoven et al., 2008). Computer analysis of PFGE banding patterns was performed with BioNumerics Version 6.5 software (Applied Maths, Austin, TX, USA). Banding pattern similarities were analyzed by the Dice coefficient, and cluster analysis was performed by the unweighted-pair group method using average linkages (UPGMA). PFGE types were determined as ≥ 80% similarity. MLST was based on seven *E. faecium* housekeeping genes (*atpA, ddl, gdh, purk, gyd, pstS, and adk*). Different sequences were assigned allele numbers, and different allelic profiles were assigned STs based on the MLST database (http://www.mlst.net/databases/). Analysis was performed using the eBURST version 3 algorithm implemented as a Java applet at http://eburst.mlst.net.

**DNA MICROARRAYS**

Microarray hybridization experiments were performed as previously described (Champagne et al., 2011). This enterococcal virulence microarray was developed (Diaatra et al., 2010) at the National Research Council in Montreal and carries 70 taxonomic probes for species identification as well as 15 virulence factors, and 173 antibiotic resistance probes for a total of 262 probes. The microarray was used for the detection of putative target genes. Briefly, bacterial DNA isolated from single colonies was labeled with Cy5-dCTP (GE Healthcare, Little Chalfont, UK). Hybridization, washing, scanning, image processing, scoring, and data analysis were done as previously described (Champagne et al., 2011). Arrays were scanned by a ScanArray Express microarray Scanner (Packard Biosciences, Billerica, MA, USA). Oligonucleotide spots with a signal-to-noise fluorescence ratio above 3 were considered positive. Positive microarray results were confirmed by PCR with specific primers for the following genes *tet(M), tet(O), tet(L), erm(B), aag* and *esp* (Table 2). Since *erm(AM)* and *erm(B)* shared over 80% similarity and are perfectly correlated, they were considered as the same in this study as previously proposed (Roberts et al., 1999).

**PCR DETECTION OF VIRULENCE AND ANTIBIOTIC RESISTANCE GENES**

PCR amplifications of virulence genes *acm* and *hyl* and of the quinolone resistance-determining regions (QRDR) genes were performed as previously described (Nallapareddy et al., 2003; Vankerczkoven et al., 2004; Billstrom et al., 2008; Werner et al., 2010). The N-terminal and C-terminal regions of the *pbp5* gene were amplified by PCR (Aarestrup et al., 2002; Poeta et al., 2007). Both strands of the purified QRDRs and *pbp5* ampiclon products were sequenced. Sequences of *gyrA/B* and *parC/E* were compared with the *E. faecium* DO genome (GenBank accession no. CP003583) whereas sequences of the C- and N-terminal regions of *pbp5* gene were compared with *pbp5* gene reference sequence (GenBank accession no. X84860).

**PCR FOR REP GENES (PLASMID FAMILIES)**

All isolates were screened for *rep*-like sequences by PCR as previously described (Jensen et al., 2010) with few modifications (Table 2). Briefly, fourteen different *rep*-family plasmids (*rep*1–2, *rep*4, *rep*6, *rep*8–9, *rep*11, *rep*13–15, *rep*16–18 and *rep*Chi) were tested for their presence in *E. faecium* isolates. The PCR amplified and sequenced ampiclons from selected strains (*E. faecium* strain no. 07-5598, M2146-08, 18581 and
Table 1 | Characterization of *E. faecium* isolates from human and canine enterococcal infections.

| Isolate no. | Hosta | Sources | Phenotypic resistanceb | Antibiotic and related resistance genec | Virulence gene | Biofilm formation | Rep-family | PFGE pattern | ST group | MLST allelic profile |
|-------------|-------|---------|------------------------|------------------------------------------|----------------|------------------|------------|--------------|----------|-----------------------|
|             |       |         |                        |                                          |                |                  |            |              |          | aptA ddi gdh purK gyd pstS adk       |
| 07-5598     | D     | Wound   | CHL, CIP, ERY, GEN, KAN, LIN, AMP, PEN, STR, TET, TYL | aac(6’)-I, tet(M), tet(L), trans, trans1 | acm            | –                | 2, 11, 18 | A1 202       | 1 1 1 1 1 7 1 |
| M2971-08    | D     | Wound   | CIP, ERY, GEN, KAN, LIN, AMP, PEN, STR, TET, TYL | aac(6’)-I, aadE, aac(6’)-le-aph(2”)-Ia, mscC, tet(M), tet(L), tet(O), trans, trans1, IS150 | efaA_{I_{cp}}, acm | –                | 2, 6, 11 | A2 202       | 1 1 1 1 1 7 1 |
| M2146-08    | D     | Bile    | CIP, LIN, AMP, PEN, TET | aac(6’)-I, tet(M), tet(L), msrC, trans, trans1, IS150 | efaA_{I_{cp}}, acm | –                | 2, 6, 11, 14 | E1 803       | 1 1 1 1 1 57 1 |
| M5853-09    | D     | Urine   | CIP, LIN, AMP, PEN, TET | aac(6’)-I, msrC, tet(M), tet(L), trans, trans1 | agg, efaA_{I_{cp}}, acm | –                | 2, 6, 11, 14 | E2 803       | 1 1 1 1 1 57 1 |
| M20638-11   | D     | Urine   | CIP, LIN, AMP, PEN | aac(6’)-I, msrC, trans1 | efaA_{I_{cp}}, acm | –                | 6, 11, 14 | F 803        | 1 1 1 1 1 57 1 |
| CCRI-18581  | H     | Anal    | CIP, ERY, KAN, LIN, AMP, PEN, STR, TET, TYL, VAN | aac(6’)-I, aadE, aph(3’)-lIIa, emr(AM), sat(4), emr(B), mscC, tet(M), vanA, vanH, vanR, vanS, vanX, vanY, trans, trans1, IS1182, IS150 | esp, acm | –                | 11, 14, 17 | Unique       | 7 1 1 1 1 5 1 |
| CCRI-16354  | H     | Rectal  | CIP, LIN, AMP, PEN, VAN | aac(6’)-I, msrC, tet(M), vanB1, trans, trans1, IS150 | efaA_{I_{cp}}, acm | –                | 1, 2, 14 | B 18         | 7 1 1 1 1 5 1 |
| CCRI-16717  | H     | Stools  | CIP, ERY, GEN, KAN, LIN, AMP, NIT, STR, TET, TYL, VAN | aac(6’)-I, aadE, aph(3’)-lIIa, emr(AM), emr(B), sat(4), mscC, tet(M), tet(L), vanA, vanH, vanR, vanS, vanX, vanY, res, trans, IS1182 | efaA_{I_{cp}}, acm | –                | 1, 2, 4, 6, 11 | D 1          | 8 4 5 7 1 1 |
| CCRI-18707  | H     | Pus     | CIP, ERY, GEN, KAN, LIN, AMP, PEN, STR, TYL | aac(6’)-I, aac(6’)-le-aph(2”)-Ia, aaeE, aph(3’)-lIIa, emr(AM), sat(4), emr(B), mscC, trans, trans1, IS1182, IS150 | efaA_{I_{cp}}, esp, acm, hyl | –                | 17, Unique | C 65         | 1 2 1 20 1 1 |

(Continued)
Table 1 | Continued

| Isolate no. | Host | Sources | Phenotypic resistance | ST group | MLST allelic profile | PAF family | PFGE pattern | Biofilm formation | Rep-family | CRISPR-Cas |
|-------------|------|---------|-----------------------|----------|----------------------|------------|--------------|------------------|------------|------------|
| CCRI-18231  | H    | Wound   |                       |          |                      | 205        | G            | ++               |           |           |
|             | H    | Wound   |                       |          |                      | 17         | Unique       | + + +            |           |           |
|             | H    | Wound   |                       |          |                      | 117        | IS1182       | + + +            |           |           |
|             | H    | Wound   |                       |          |                      | 112        | IS150        | + + +            |           |           |

aD, dogs; H, humans.

bAMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; KAN, kanamycin; LIN, lincomycin; NIT, nitrofurantoin; PEN, penicillin; STR, streptomycin; TET, tetracycline; TYL, tylosin; VAN, vancomycin.

cIdentified by microarray; aac(6′)-Ii is intrinsic resistance in E. faecium.

Weak biofilm producer (+) and strong biofilm producer (++++), based upon the previously calculated OD values (see Materials and Methods).

DETECTION OF CRISPR-Cas
The CRISPR1-Cas and CRISPR3-Cas loci were screened by PCR as previously described (Palmer and Gilmore, 2010) with slight modifications. Briefly, the PCR reactions were performed in a total of 50 µl, using 80 pmol of each primer, 1.5 mM MgCl₂, 10 mM each of dNTPs, 2 U of Taq DNA, 1X Buffer mix (New England Biolabs) and sample DNA. Amplification reactions were carried out using a Whatman Biometra thermocycler (Montreal Biotech Inc, Québec, Canada) programmed as follows: an initial denaturation step of 94°C for 2 min, 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min and extension at 72°C for 1 min, followed by a final elongation at 72°C for 5 min. For visualization, 5 µl of the PCR reaction were subjected to electrophoresis in 1.2% agarose gel stained with ethidium bromide. A 100 bp ladder (TrackIt, Invitrogen, Ontario, Canada) was used as a marker.

BIOFILM FORMATION
Isolates were inoculated in tryptic soy broth (TSB) supplemented with 1% glucose in 96 well microtiter plates (Fisher scientific) for bacterial growth and biofilm formation as described elsewhere (Zoletti et al., 2011). Biofilm was quantified using crystal violet staining method as described by Zoletti et al. (2011). Staphylococcus epidermidis ATCC 35984 (biofilm producer) and wells containing uninoculated medium were used as controls. ODs were obtained using a microplate reader Biotek Synergy HT (Bio-tek Instruments Inc., Winooski, VT, USA) at a wavelength of 570 nm. Analyses were based on two different experiments where isolates were tested in triplicate. The quantification of biofilm formation in microtiter plates was performed as previously described (Stepanovic et al., 2000, 2007). Briefly, strains were divided into the following categories: no biofilm producer (−), weak biofilm producer (+), moderate biofilm producer (+++) and strong biofilm producer (+++), based upon the previously calculated OD values: OD ≤ ODc = no biofilm producer; ODc < OD ≤ 2X ODc = weak biofilm producer; 2X ODc < OD ≤ 4X ODc = moderate biofilm producer; 4X ODc < OD = strong biofilm producer. ODc is defined as three standard deviations (SD) above the mean OD of the negative control.

STATISTICAL ANALYSIS
A chi-square exact test was used to examine the association between isolate, origin, antibiotic resistance, virulence genes, biofilm formation, plasmid families and CRISPR genes. Statistical analyses were carried out using SAS software v. 9.2 (SAS Institute Inc., Cary, N.C., USA). The level of statistical significance was set at 0.05.
### Table 2 | Primers and conditions used in PCR for confirmation of microarray results and for rep-like genes identification.

| Gene detected or Rep-family no. | Primers (5’ to 3’)
|---------------------------------|---------------------------------|
| **tet**<sup>1</sup>(M) | F-GTGGCAAAAGGTACACACGAGG<br>R-CGTTAAGGGTCATGATGAC<br>406<br>55<br>De Leener et al., 2004 |
| **tet**<sup>1</sup>(O) | F-GATGCGCATACCGACAGAC<br>R-CAAATGACGAGCAAGAGCT<br>614<br>62<br>Aarestrup, 2000 |
| **tet**<sup>1</sup>(L) | F-ATAAATGTTCTGACGGTGAAT<br>R-AACCCAGGCTCAATTGACAGG<br>1077<br>52<br>De Leener et al., 2004 |
| **erm**<sup>2</sup> | F-GAAAAGTACCTCAACCAAAATA<br>R-AGTAAACGCGACTTAAAAATTGT<br>639<br>52<br>Poeta et al., 2005 |
| **agg** | F-GTGGCCACATACTAATAGG<br>R-GATGCTGAGTGTGTTGAAACG<br>380<br>46<br>Seno et al., 2005 |
| **esp** | F-TTTGCTAAGTCTGCTCAGGACC<br>R-GCGTCAACACTTTGCGAA<br>955<br>63<br>Seno et al., 2005 |
| 1 (pIP501) | F-TGGCCTAATCAGCAGCAACGC<br>R-CTTGAAGCTAGAAACACC<br>624<br>52<br>X17655 |
| 9 (pCF10) | F-GTGGCATCATTTCTCAGAG<br>R-CGCAAAACATTTGCWATT<br>201<br>U26268 |
| 4 (pMBB1) | F-ACATGTCGGTGGTTCAATGCT<br>R-AGCAACAGTGAATAATCT<br>430<br>52<br>U26268 |
| 14 (pR11) | F-RATTTGRCCTTCTSTSYYTCA<br>R-TGAAAGTTRGATAGT<br>164<br>EU327398 |
| 17 (pRUM) | F-TACTAACTGTGTGTTAATCGTGAAAT<br>R-ATGCGACTCCAACCGTAAT<br>502<br>EU376117 |
| Unique (pMG1/pHTβ) | F-GTATTTACACACTGGAGCTC<br>R-TCAGTGTAGGCAAAAC<br>199<br>52<br>AB206333 |
| 2 (pRE25) | F-GAGAACCTAAGGCGGAAT<br>R-ACCAGATAAAGTGAATCT<br>630<br>56<br>X92945 |
| 6 (pS66) | F-AAGAACAAAAAGAGGAGTAG<br>R-CCAGACGCTGGATATCT<br>551<br>AJ223161 |
| 8 (pAM373) | F-CGTAATCGTAGTGAAGAGACT<br>R-TGAAAGTTRGATAGG<br>394<br>AE002565 |
| 18 (pEF418) | F-ACACAGATGCAAGATT<br>R-AAGAAATACCTGAAATCT<br>462<br>AF408195 |
| 13 (pC194) | F-TACCGAATATYYYYGCAGCT<br>R-GGCAATCAGATCT<br>402<br>54<br>VD4777 |
| 15 (pUSA03) | F-CAGTAGAGAAAGGAAAAAT<br>R-CTTATAGGT<br>327<br>CP000258 |
| 16 (pSAS) | F-CTTTATATCTACATATGC<br>R-CAGGAAACACTGTTT<br>592<br>BX571858 |
| 11 (pEF1071) | F-CTTGAATGCGTGAAGGAGG<br>R-CCTTTGAAGATWGCR<br>500<br>54<br>AF164559 |

<sup>a</sup>Rep-family numbers are associated with plasmid in parenthesis.

<sup>b</sup>F, forward; R, reverse.

### RESULTS

#### MLST AND PFGE

MLST allelic profiles are presented in **Table 1**. A total of six STs were found among the *E. faecium* isolates, ST1 (n = 1), ST17 (n = 2), ST65 (n = 1), ST202 (n = 2), ST205 (n = 1) and one novel, ST803 (isolates M2146-08, M5853-09 and M20638-11) (**Table 1**).

Canine ARE belonged to ST202 (n = 2) and ST803 (n = 3), both SLV of ST17. Human clinical isolates belonged to ST65 (n = 1), a singleton known to be found only among clinical strains (Werner et al., 2011), and ST205 (n = 1), a SLV of ST17 (**Table 1**).
Human surveillance isolates belonged to ST18 (n = 2), and ST1 (n = 1, linked to CC1). Therefore, all these STs were linked to HA-ARE except for ST1 which was shown to belong to a cluster containing primarily isolates from calves in the Netherlands (http://efaecium.mlst.net/). Following genomic Smal digestion and PFGE, the E. faecium isolates (n = 10) of human and canine origins produced ten macro-restriction clusters clustered into eight PFGE types, termed A through H (Table 1). Canine E. faecium isolates of subtypes A and E were ≥ 80% similar whereas the remaining E. faecium isolates were considered unrelated (≤ 80%). The E. faecium PFGE types B and H were set as a same group by MLST (ST18) whereas the new ST803 was grouped as PFGE types E and F (Table 1).

**ANTIBIOTIC RESISTANCE**

Chloramphenicol, ciprofloxacin, erythromycin, lincomycin, tylosin, gentamicin, kanamycin, streptomycin, penicillin, ampicillin, and tetracycline resistances were observed in E. faecium clinical isolates of canine origin with no resistance observed to daptomycin, linezolid, nitrofurantoin, quinupristin/dalfopristin, vancomycin and tigecycline (Tables 1, 3). Resistances to ciprofloxacin, erythromycin, lincomycin, tylosin, gentamicin, kanamycin, streptomycin, nitrofurantoin, penicillin, ampicillin, tetracycline and vancomycin but not to chloramphenicol, daptomycin, linezolid, quinupristin/dalfopristin and tigecycline were observed in human E. faecium clinical isolates (Tables 1, 3). All E. faecium isolates were resistant to ampicillin, ciprofloxacin and lincomycin. Seven isolates were considered as high-level ciprofloxacin-resistant (MICs of > 16 µg/ml) whereas nine isolates showed high-level ampicillin resistance (MICs of ≥ 256 µg/ml) (Table 3). Also, erythromycin resistance (n = 6) was positively associated (p-value < 0.05) with aminoglycoside resistance (n = 6), whereas ciprofloxacin resistance (n = 10) correlated (p-value < 0.05) with ampicillin (n = 9) resistances. All vancomycin resistant E. faecium (n = 3) isolates were of human origin.

**MICROARRAY AND PCR ANALYSIS OF VIRULENCE AND ANTIBIOTIC RESISTANCE GENES**

Virulence and antibiotic resistant genotypes are presented in Table 1. Results demonstrated all canine ARE isolates were acm-positive but were negative for esp and hyl genes. The majority of these isolates were shown to harbor efaAfm gene, encoding for a cell wall adhesin. One canine ARE isolate also had the agg gene encoding for an aggregation substance of pheromone-responsive plasmid. The efaAfm gene was detected in all human isolates but one. The two human clinical isolates (CCRI-18707 and -18231) were also positive for the glycosyl hydrolase gene (hyl). Acquisition of multiple antibiotic resistance genes was observed ranging from 2 to 14 per isolate. Correlation between MICs and the presence of resistance genes has indicated that tetracycline resistance was attributable to tet(M) (n = 2); tet(M) and tet(L) (n = 3); and tet(M), tet(L) and tet(O) (n = 2). Resistance to erythromycin, lincomycin and tylosin could be explained by the presence of msrC, erm(AM)/erm(B) in all strains with the exception of one (07-5598). Streptomycin resistance was associated with the aadE gene in all strains except one (07-5598). Kanamycin resistance was explained by aph(3′)-IIIa in all strains except two (07-5598 and M2971-08) whereas gentamicin resistance was attributable to aac(6′)-Ie-aph(2′)-Ia in all strains but two (07-5598 and CCRI-16717). Chloramphenicol resistance (n = 1) could not be explained because both cat and floR genes were absent in the two canine ARE resistant isolates. The gene aac(6′)-Ii is intrinsic in E. faecium and confers resistance to chloramphenicol, ciprofloxacin, erythromycin, lincomycin, tylosin, gentamicin, kanamycin, streptomycin, nitrofurantoin, penicillin, ampicillin, tetracycline and vancomycin but not to chloramphenicol, daptomycin, linezolid, quinupristin/dalfopristin and tigecycline were observed in human E. faecium clinical isolates (Tables 1, 3).

### Table 3 | Antibiotic resistance of E. faecium clinical isolates from humans and dogs based on MICs.

| Antibiotic agent | Number of isolates at a MIC (µg/ml) ofa | % Resistant isolates (b) | Human | Dog |
|------------------|----------------------------------------|------------------------|-------|-----|
| Chloramphenicol  | 8, 1, 1                                 | 0 (0)                  | 20 (1) |
| Daptomycin       | 7, 3, 6                                 | 80 (4)                 | 40 (2) |
| Erythromycin     | 2, 2, 6                                 | 60 (3)                 | 40 (2) |
| Gentamicin       | 5, 4, 6                                 | 100 (5)                | 100 (5) |
| Kanamycin        | 10, 8, 3                                | 80 (4)                 | 100 (5) |
| Lincomycin       | 5, 4, 6                                 | 80 (4)                 | 100 (5) |
| Linezolid        | 10, 3, 6                                | 100 (5)                | 100 (5) |
| Ciprofloxacin    | 1, 2, 4                                 | 100 (5)                | 100 (5) |
| Nitrofurantoin   | 1, 8, 6                                 | 20 (1)                 | 0 (0)  |
| Ampicillin       | 1, 3, 6                                 | 80 (4)                 | 100 (5) |
| Streptomycin     | 4, 1, 1                                 | 80 (4)                 | 100 (5) |
| Quinupristin/    | 6, 3, 1                                 | 0 (0)                  | 0 (0)  |
| Dalfopristin     | 4, 1, 5                                 | 80 (4)                 | 40 (2) |
| Tetracycline     | 3, 1, 6                                 | 60 (3)                 | 0 (0)  |
| Tylosin          | 2, 5, 3                                 | 0 (0)                  | 0 (0)  |

aCiprofloxacin and ampicillin MICs from the standard broth macrodilution method.

bNumber of isolates; |, MIC breakpoint; bold numbers, numbers of resistant isolates; |, susceptible breakpoint; unshaded areas, range of dilutions tested; numbers in the shaded area, isolates with MICs greater than the highest tested concentrations.
resistance to low-level aminoglycosides. Also, the transposase genes \textit{trans} and \textit{trans1} (both GenBank accession no. AF516335) associated with transposons Tn1546 and Tn5405-like on the plasmid pUW786 carrying multidrug resistance gene cluster were identified in many isolates (n = 9). A correlation (p-value < 0.05) was observed between \textit{aadE} (high-level streptomycin resistance), \textit{aph(3')-IIIa} (aminoglycoside resistance), \textit{sat(4)} (streptothricin acetyltransferase) and IS1182 transposase gene (also associated with plasmid pUW786) in human isolates of \textit{E. faecium} (Diarra et al., 2010). The antibiotic resistance genes \textit{erm(AM)/erm(B)} (macrolide-lincosamide-streptogramin B, MLS\(_{B}\)), \textit{aadE}, \textit{sat(4)}, \textit{aph(3')-IIIa} were significantly (p-value < 0.05) associated with human isolates. Positive associations (p-value < 0.05) were observed between \textit{trans1} and \textit{IS150} transposase genes, \textit{aac(6')-Ie-aph(2')-Ia} (encoding for a bi-functional aminoglycoside-modifying enzyme) and \textit{erm(AM)/erm(B)}. No beta-lactamase genes were detected in this study.

**SCREENING FOR gyrA/B AND parC/E MUTATIONS**

Enterococcus \textit{faecium} isolates spanned a range of ciprofloxacin MICs from 4 \(\mu\)g/ml to > 256 \(\mu\)g/ml with seven isolates considered as high-level ciprofloxacin-resistant (MICs of > 16 \(\mu\)g/ml) (Table 3). High-level ciprofloxacin resistance was attributable to ParC (Ser80 \rightarrow Arg, Ile or Asp; and Asn73 \rightarrow Asp) and GyrA (Ser83 \rightarrow Arg, Tyr, or Ile; Met127 \rightarrow Trp; and Glu87 \rightarrow Lys) mutations (Table 4). All high-level ciprofloxacin-resistant isolates had mutations in both GyrA (Ser83 \rightarrow Arg or Tyr or Ile) and ParC (Ser80 \rightarrow Arg or Ile or Asp). Corresponding fragments of subunits B (parE/gyrB) were also investigated. Two isolates demonstrated mutations in GyrB (Asp436 \rightarrow Asn, Leu371 \rightarrow Trp and Pro455 \rightarrow Ser) whereas no changes in ParE were detected (Table 4). Isolates with MICs of < 16 \(\mu\)g/ml did not harbor mutational changes.

**SEQUENCE ANALYSIS OF \textit{pbp5}**

Nine isolates showed high-level ampicillin resistance (MICs of ≥ 256 \(\mu\)g/ml) with all canine ARE being high-level. Both the N-terminal and C-terminal regions were analyzed and revealed that PBP5 contained 25 amino acid changes, as shown in Table 5. The insertion of aspartic acid at position 466 was mostly observed in isolates of animal origin. Alleles were designated 1-5 based on important amino acid substitutions in the C-terminal region with none showing 100% identity with the reference sequence (GenBank accession no. X84860). The same \textit{pbp5} alleles (alleles 1 and 2) were observed in clonal isolates with PFGE patterns A1 and A2 and E1 and E2.

**repA GENES (PLASMID FAMILIES)**

Nine different \textit{rep}-family plasmid genes (\textit{rep1–2}, \textit{rep4}, \textit{rep6}, \textit{rep11}, \textit{rep14}, \textit{rep17–18} and \textit{repUnique}) were detected in \textit{E. faecium} isolates (Table 1). The predominant \textit{rep}-family among \textit{E. faecium} isolates was \textit{rep11} (pEF1071) with seven isolates. Other \textit{rep}-families were also observed: \textit{rep1} (pIP501) (n = 2), \textit{rep2} (pRE25) (n = 6), \textit{rep4} (pMBB1) (n = 1), \textit{rep6} (pS86) (n = 5), \textit{rep14} (pRI1) (n = 5), \textit{rep17} (pRUM) (n = 3), \textit{rep18} (pEF418) (n = 1) and \textit{repUnique} (pUB101) (n = 3). Overall, \textit{rep11} and \textit{rep6} families were significantly (p-value < 0.05) associated with isolates of canine origin. The families’ \textit{rep8–9}, \textit{rep13}, and \textit{rep15–16} were not detected.

**CRISPR-cas GENES**

To determine whether there is an association between CRISPR elements and plasmid family genes, virulence and/or antibiotic resistance genes, CRISPR-cas genes were investigated by PCR. Because potential sequence divergence among \textit{csn1} genes, an internal region of CRISPR-cas locus-specific genes, may lead to false-negative PCR results, isolates with negative PCR results were further screened with primers flanking the conserved locations of the CRISPR1-cas and CRISPR3-cas loci, between homologues of EF0672-EF0673 and EF1760-EF1759, respectively, as compared with the \textit{E. faecalis} genome V583 (Palmer and Gilmore, 2010). According to Palmer and Gilmore (2010), one CRISPR-cas locus was identified in three \textit{E. faecium} genomes. However, this locus could not be detected in our ten ARE strains. No significant correlations could be made with the absence of antibiotic resistance, virulence, and plasmid family genes due to low number of isolates.

### Table 1 | Mutations in \textit{gyrA/B} and \textit{parC} of clinical \textit{E. faecium} isolates with their corresponding ciprofloxacin MIC.

| Ciprofloxacin MIC (\(\mu\)g/ml) | Isolate no. | Host\(^a\) | Amino acid\(^b\) mutation in gene (codon) |
|-------------------------------|-------------|-----------|------------------------------------------|
| 4 | M2146-08 | D | None |
| 8 | M5683-09 | D | None |
| 8 | CCRI-16717 | H | None |
| 64 | M20638-11 | D | I (83) |
| 128 | 07-5598 | D | Y (83), W (127) |
| 128 | CCRI-18231 | H | K (87) |
| >256 | M2971-08 | H | Y (83) |
| >256 | CCRI-18581 | H | Y (83) |
| >256 | CCRI-16354 | H | Y (83) |
| >256 | CCRI-18707 | H | R (83) |

\(^a\)D, dogs; H, humans.

\(^b\)A, aspartic acid; I, isoleucine; K, lysine; N, asparagine; R, arginine; S, serine; W, tryptophan; Y, tyrosine.
Table 5 | Amino acid changes detected in the C- and N-terminal regions of \( \text{pbp5} \) of clinical \( \text{E. faecium} \) isolates.

| MIC ampicillin (µg/ml) | Host | \( \text{E. faecium} \) | Amino acid change at position$^c$ | \( \text{pbp5} \) allele$^d$ |
|------------------------|------|-----------------|---------------------------------|---------------------|
| 256                    | D    | X84860          | G A E E K T L D A T G Q          | −−                  |
| >256                   | H    | CCRI-18581      | T D Q Q A I G S A NA            | k                  |
| >256                   | D    | M2971-08        | Q D Q S M K L T D V A V D 1    | 2                  |
| >256                   | M    | M5853-09        | Q D Q A A K F T D L A V E 2    | 2                  |
| >256                   | M    | M20638-11       | Q D Q A A K F T D L A V D 2    | 2                  |
| 64                     | H    | CCRI-16717      | Q D Q A A K F T D L A V D 2    | 2                  |
| >256                   | H    | CCRI-16354      | T D Q Q A I G S A NA            | K                  |
| >256                   | H    | CCRI-18707      | T D Q Q A I G S A E K          | −−                  |
| 256                    | H    | CCRI-18231      | T D Q Q A I G S A NA            | −−                  |

$^a$D, dogs; H, humans.
$^b$A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; K, lysine; L, leucine; M, methionine; N, asparagine; Q, glutamine; S, serine; T, threonine; V, valine.
$^c$Amino acid changes with respect to the reference sequence (GenBank accession no. X84860) are indicated in bold.
$^d$Alleles were designated 1–5 based on important amino acid substitutions in the C-terminal region.

DISCUSSION

Both isolates producing biofilm were recovered from infections, one weak and one strong both harboring the \( \text{esp} \) gene (Table 1). Biofilm formation may be an important route of transmission of \( \text{E. faecium} \) strains with a greater impact on human health (Regnqvist et al., 2012). The presence of biofilm production in human isolates suggests that these strains may have a greater potential for transmission compared to canine isolates.

In humans, the emergence of high-level ampicillin resistance, specifically in US hospitals in the early 1980s, preceded the epidemic rise of vancomycin resistance, which occurred in the 1990s, explaining why virtually all vancomycin-resistant entero- cocci (VRE) recovered from nosocomial infections in humans probably reflects sequential and independent acquisition of resistance genes resulting in the selective dominance of virulent clones currently involved in the nosocomial epidemiology subsequent to the selective advantage in humans probably reflects sequential and independent acquisition of resistance genes resulting in the selective dominance of virulent clones currently involved in the nosocomial epidemiology (McCracken et al., 2013). Similar to a study on Dutch canine ARE isolates (De Reggi et al., 2012), these findings indicate that the \( \text{esp} \) gene is involved in the pathogenesis of human and canine infections. The presence of biofilm production in human isolates suggests that these strains may have a greater potential for transmission compared to canine isolates.
and in PBP5 (Ligozi et al., 1996; Zorzi et al., 1996; Rice et al., 2001; Jureen et al., 2003; Werner et al., 2010). To our knowledge, previous studies did not observe mutations in the gyrB target (Leavis et al., 2006; Werner et al., 2010; Valdezate et al., 2012). The contribution of these amino acid changes in GyrB to ciprofloxacin resistance is difficult to evaluate because amino acid changes in GyrA and ParC alone are sufficient to confer high-level resistance to ciprofloxacin. Other resistance mechanisms may be responsible for (low-level) ciprofloxacin resistance in HA ARE isolates that do not contain amino acid changes in the QRDRs of ParC and GyrA. Recently, characterization of EfmA, a multidrug efflux pump conferring resistance to quinolones and macrolides, from E. faecium has been described (Nishioka et al., 2009) which could explain low-level ciprofloxacin resistance in some of the isolates of this study.

So far, canine ARE have been shown to carry fewer virulence traits (De Regt et al., 2012; Kwon et al., 2012) compared to human HA ARE (Top et al., 2008; Werner et al., 2010). It was also shown in a recent report that canine E. faecium did not form biofilms and lacked the strong gelatinase phenotype as well as esp (Ghosh et al., 2011). Also, as observed in our study, ARE strains that colonize the gastrointestinal tract of humans have been shown to carry less determinants such as the enterococcal surface protein, Esp (Coque et al., 2002; Heikens et al., 2007), genomic islands (Heikens et al., 2008), and insertion sequence elements (Leavis et al., 2007). Recently, a sequence-based classification system for enteroococcal plasmids has been established which targets replicon-specific plasmid DNA to determine 19 plasmid families which are associated with either a very narrow or a broader host range (Jensen et al., 2010). This procedure was attempted to determine whether specific plasmid families were involved in ARE of canine origin vs. human origin. In this study, repB (small cryptic plasmids) and repP1 (toxin producing plasmids) plasmid families were significantly associated with isolates from dogs. Interestingly, it was also observed that these two families seem to be associated with human isolates from colonization (CCRI no. 18581, 16717, and 16354) but due to low number of isolates this statistical significance could not be addressed. No significant association between rep families and antimicrobial resistance genes could be identified for isolates of canine origin whereas the human clinical strains were significantly associated (p < 0.05) with erm(B)/erm(AM), aade, sat(4), aph(3’)-IIIa, IS1182 and with the rep17 plasmid family which has been previously reported (Grady and Hayes, 2003). Interestingly, these genes were also detected in a surveillance human isolate. Recently, a significant correlation between the absence of CRISPR-cas loci and the presence of antibiotic-resistance genes was previously described for E. faecalis (Palmer and Gilmore, 2010). This tendency was also observed in ARE of this study but due to the low number of isolates its significance could not be determined. Further research is needed to assess the virulence and the antimicrobial resistance of canine strains in comparison with that of human strains and, more generally, to quantify the magnitude of this possible emerging zoonotic problem.

In conclusion, the current study provides the first characterization of canine clinical ARE isolates in Canada with STs identical or closely related to human clinical isolates. This analysis, as well as previous reports, further indicates that canine ARE isolates are evolutionarily linked with hospital ARE isolates with some discordance in their multi-drug resistance and virulence attributes. These findings also support the importance of prudent use of antibiotics in veterinary medicine to avoid zoonotic spread and development of vancomycin resistance of canine ARE isolates. Select and spread of HA-strains that can cause human infections is a major concern, independently of being VRE, because in some countries ARE associated with human infections remain vancomycin susceptible. Further studies are needed to understand the significance of dogs in the spread of this nosocomial pathogen in the community. The occurrence of ARE in dogs and other domestic animals could be addressed by national programs in order to explore the importance of the animal reservoir in the evolution of human hospital ARE isolates.

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