In vitro efficacy of 16 antimicrobial drugs against a large collection of β-lactamase-producing isolates of extraintestinal pathogenic Escherichia coli from dogs and cats

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Abstract

Purpose. The aim of this study was to assess the in vitro efficacy of candidate antimicrobials against extended-spectrum β-lactamase (ESBL)-producing isolates of extraintestinal pathogenic Escherichia coli (ExPEC) from companion animals.

Methodology. A total of 90 ESBL-producing ExPEC isolates from dogs and cats were tested for susceptibility to 16 antimicrobials with the agar dilution method. We also identified the ESBLs and AmpC β-lactamases of these isolates with PCR and DNA sequencing.

Results/Key findings. All isolates were susceptible to meropenem, tebipenem and amikacin (AMK), and various proportions were susceptible to latamoxef (LMX, 97.8 %), fosfomycin (FOM, 97.8 %), faropenem (FPM, 96.7 %), nitrofurantoin (NFT, 96.7 %), flomoxef (FMX, 93.3 %), piperacillin/tazobactam (PTZ, 92.2 %), ceftibuten (CMZ, 91.1 %), chloramphenicol (80.0 %), trimethoprim/sulfamethoxazole (64.4 %), amoxicillin/clavulanic acid (63.3 %), ceftibuten (60.0 %), tetracycline (52.2 %) and enrofloxacin (10.0 %). A genetic analysis showed that 83 of the 90 (92.2 %) isolates were positive for CTX-M-type genes: CTX-M-14 (n=26), CTX-M-27 (n=20), CTX-M-55 (n=17), CTX-M-15 (n=12), CTX-M-2 (n=5), CTX-M-24 (n=2), CTX-M-104 (n=2) and CTX-M-3 (n=1). Eight isolates also expressed AmpC β-lactamase phenotypes.

Conclusion. This study demonstrates that the susceptibility rates to PTZ, CMZ, LMX, AMK, FOM, FPM, NFT and FMX were similar to those to carbapenems (>90 %), implying that these drugs are available alternatives to carbapenems for the treatment of companion animals infected with ExPEC-producing CTX-M-type ESBLs. Further in vivo studies of the effective use of these antimicrobials are required.

INTRODUCTION

Escherichia coli is a pathogenic organism that causes extraintestinal infections at various anatomical sites, including the urinary tract, genitals, skin and respiratory tract, in dogs and cats [1, 2]. Antimicrobial treatment is required for companion animals infected with extraintestinal pathogenic E. coli (ExPEC) [3, 4], and the development of antimicrobial resistance in ExPEC has increased the risk of antimicrobial treatment failure in infected animals.

Extended-spectrum β-lactamase (ESBL)-producing isolates have emerged globally among ExPEC from companion animals and humans [5, 6]. Although ESBLs are usually involved in resistance to oxyimino-cephalosporins, in addition to penicillins and narrow-spectrum cephalosporins, ESBL-producing bacteria are often resistant to the other classes of antimicrobials [7]. These multidrug-resistant phenotypes of ESBL-producing bacteria have major implications for the selection of adequate empirical therapy regimens [7]. Many candidate antimicrobials for the...
treatment of ESBL-producing bacterial infections have been discussed in human medicine [8–10], but are yet to be considered in companion animal medicine.

Although most ESBLs can be divided into four families (TEM, SHV, CTX-M types and OXA), CTX-M enzymes have become the most prevalent types of ESBLs in both companion animals and humans [6, 7]. CTX-M-type ESBLs have been reported in bacteria, mostly ExPEC, from companion animals in many countries [6], including Japan [11]. However, most of these studies have been based on a limited number of ESBL-producing isolates, and therefore the antimicrobial susceptibility and distribution of ESBLs in ExPEC from companion animals have not been fully clarified.

Like ESBLs, AmpC β-lactamases hydrolyse third-generation or extended-spectrum cephalosporins, whereas unlike ESBLs, they are also active against cephemycins and are resistant to inhibition by clavulanate or other β-lactamase inhibitors [12]. The coexistence of ESBLs and AmpC β-lactamases has been found among Enterobacteriaceae from companion animals [11, 13, 14], and poses therapeutic challenges in infected animals.

The purpose of this study was to assess the in vitro efficacy of candidate antimicrobials against a large collection of ESBL-producing ExPEC isolates from dogs and cats. We also investigated the distribution of ESBLs and AmpC among these ExPEC isolates.

**METHODS**

**Bacterial strains**

A total of 90 ESBL-producing E. coli strains were collected from dogs (n=62) and cats (n=28) with different owners who visited veterinary hospitals located at 14 prefectures in Japan between 2004 and 2016. The isolates were obtained from different anatomical sites, assessed as sites of bacterial infection by clinical veterinarians, including the urinary tract (n=56), pus from unspecified locations (n=8), respiratory organs (n=7), skin (n=5), genitals (n=4) and other sites (n=10). Bacteria were identified by assessing their growth on CHROMagar Orientation Medium [15], using the API 20E kit (Sysmex; bioMérieux). Isolates with both similar antibiograms and enterobacterial repetitive intergenic consensus-PCR profiles [16] were excluded.

**Detection of ESBL and AmpC production**

ESBL production was screened with the cefpodoxime (CPD) disc test (zone diameter ≤17 mm) [17], and was confirmed, together with the AmpC production, using AmpC and ESBL Detection Set (MAST Group). Briefly, differences in zone diameter of ≥5 mm between the CPD plus ESBL inhibitor disc and the CPD disc, and those of ≤4 mm between the CPD plus AmpC inhibitor disk and the CPD disc were only considered positive for ESBL production. On the other hand, differences in zone diameter of ≥5 mm between the CPD plus ESBL and AmpC inhibitor disc and the CPD plus AmpC inhibitor disc, and that of ≤4 mm between the CPD plus ESBL inhibitor disc and the CPD disc were considered positive for production of both ESBL and AmpC.

**Antimicrobial susceptibility testing**

Bacterial susceptibility to amoxicillin/clavulanic acid (ACV), piperacillin/tazobactam (PTZ), ceftriaxone (CTB), cefmetazole (CMZ), flomoxef (FMX), latamoxef (LMX), faropenem (FPM), meropenem (MPM), amikacin (AMK), tetracycline (TET), enrofloxacin (ENR), nitrofurantoin (NFT), fosfomycin (FOM), chloramphenicol (CHL) and trimethoprim/sulfamethoxazole (TMS) was determined with the agar dilution method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [18]. These tested drugs were selected from antimicrobials whose microbiological efficacy against ESBL-producing bacteria from humans has previously been validated [19–22], and those generally available in veterinary medicine. MICs were interpreted based on the CLSI breakpoint for all antimicrobials [17, 23], except FMX and TBP, for which breakpoints of LMX and MPM, respectively, were used instead. E. coli ATCC 25922 was used as the control strain.

**Identification of β-lactamase types**

Genomic DNA from each of the isolates was prepared by suspending several colonies in 0.5 ml of water and boiling for 10 min. These samples were used as the templates for further genetic analysis. CTX-M-type β-lactamase genes were detected in all isolates with multiplex PCR [24]. In the positive isolates, the genes were amplified and sequenced to identify the CTX-M subtype using group-specific PCR primers [25, 26]. Class A β-lactamase genes (i.e. blaTEM and blaSHV) were identified using PCR and DNA sequencing, as previously described [25]. In AmpC-positive strains, plasmidic AmpC β-lactamase genes (i.e. ACC, FOX, MOX, DHA, CIT and EBC groups) were screened with multiplex PCR [27], and then amplified and bidirectionally sequenced with specific primers [25].

**Statistical analysis**

The antimicrobial susceptibility rates of two groups were compared with Fisher’s exact test. A P-value lower than 0.05 was considered significant.

**RESULTS**

**Antimicrobial susceptibility of ESBL-producing ExPEC isolates**

Table 1 shows the rates of susceptibility to the 16 tested antimicrobials of ESBL-producing ExPEC isolates from dogs and cats. All isolates were susceptible to MPM, TBP and AMK, and various proportions of the isolates were susceptible to LMX (97.8 %), FOM (97.8 %), FPM (96.7 %), NFT (96.7 %), FMX (93.3 %), PTZ (92.2 %), CMZ (91.1 %), CHL (80.0 %), TMS (64.4 %), ACV (63.3 %), CTB (60.0 %), TET (52.2 %) and ENR (10.0 %). The rates of susceptibility to CTB and TET were significantly higher in the isolates from dogs [42/62 (67.7 %) and 40/62 (64.5 %), respectively]
than those from cats [12/28 (42.9 %) and 7/28 (25.0 %), respectively] (P<0.05).

According to the result of the combination disc kit, 82 isolates contained only ESBLs, whereas eight isolates contained both ESBLs and AmpC β-lactamases. The rates of susceptibility to ACV, CTB, CMZ, FMX, LMX and FPM were significantly higher in the isolates containing only ESBLs than in those containing both ESBLs and AmpC β-lactamases (P<0.05).

Prevalence of β-lactamases in ESBL-producing isolates
A genetic analysis showed that 83 of the 90 (92.2 %) ESBL-producing ExPEC isolates were positive for CTX-M-type genes (Table 2). The CTX-M-type ESBLs identified were: CTX-M-14 (n=26), CTX-M-27 (n=20), CTX-M-55 (n=17), CTX-M-15 (n=12), CTX-M-2 (n=5), CTX-M-24 (n=2), CTX-M-104 (n=2) and CTX-M-3 (n=1). The other types of ESBLs (i.e. TEM and SHV families) were not detected in our collection. The CTX-M-1 group ESBLs were significantly more prevalent among the isolates from cats (14/28, 50.0 %) than those from dogs (14/62, 22.6 %, P<0.05), whereas the CTX-M-9 group ESBLs were significantly more prevalent among the isolates from dogs (39/62, 62.9 %) than those from cats (11/28, 39.3 %, P<0.05).

Five of the eight AmpC-positive isolates contained CMY-2 genes, and the remaining three isolates were negative for all plasmidic AmpC genes, implying the overexpression of chromosomal AmpC. Of the other β-lactamases, the TEM-1 gene was detected in 42 isolates.

Comparison of antimicrobial susceptibility rates between isolates containing the main CTX-M groups
In this study, we confirmed the predominance of the CTX-M-1 and CTX-M-9 groups in the ESBLs detected in our collection, and compared the rates of antimicrobial susceptibility of the isolates containing these two CTX-M groups. The susceptibility rate to CTB was significantly higher in the isolates containing CTX-M-9 group ESBLs than in those containing CTX-M-1 group ESBLs (88.9 vs 26.9 %, P<0.05) (Table 3). In contrast, there was no significant difference between the two CTX-M groups in their susceptibility to the remaining antimicrobials.

DISCUSSION
Although infections with ESBL-producing bacteria have been increasing in companion animals, the appropriate drugs for these infections remain to be determined. To the best of our knowledge, this is the first attempt to determine the activity of many candidate antimicrobials in vitro
against a large collection of ESBL-producing ExPEC isolates from companion animals.

In human medicine, carbapenems are recommended as the drugs of choice for the empirical treatment of severe infections with ESBL-producing bacteria, including treatment of bacteraemia, nosocomial pneumonia and meningitis [9, 10]. In this study, we first investigated the susceptibility of ESBL-producing ExPEC from companion animals to TBM, the only oral carbapenem, and to MPM. All bacteria were highly susceptible to both carbapenems, as previously reported among human isolates [19]. Therefore, these carbapenems can be used for the treatment of companion animals infected with ESBL-producing ExPEC. However, these drugs must be used prudently because carbapenemase- 

Table 2. Distribution of ESBLs and AmpC β-lactamases in 90 ESBL-producing ExPEC isolates from dogs and cats

<table>
<thead>
<tr>
<th>Category</th>
<th>ESBL type*</th>
<th>Genes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Only ESBL (n=57/25)</td>
<td>CTX-M-1 group (n=13/13)</td>
<td>CTX-M-55 (n=6/8)</td>
</tr>
<tr>
<td></td>
<td>CTX-M-2 group (n=2/1)</td>
<td>CTX-M-15 (n=6/5)</td>
</tr>
<tr>
<td></td>
<td>CTX-M-9 group (n=36/9)</td>
<td>CTX-M-3 (n=1/0)</td>
</tr>
<tr>
<td></td>
<td>CTX-M-2 (n=2/1)</td>
<td>CTX-M-2 (n=2/1)</td>
</tr>
<tr>
<td></td>
<td>CTX-M-14 (n=20/2)</td>
<td>CTX-M-14 (n=20/2)</td>
</tr>
<tr>
<td></td>
<td>CTX-M-27 (n=12/7)</td>
<td>CTX-M-27 (n=12/7)</td>
</tr>
<tr>
<td></td>
<td>CTX-M-24 (n=2/0)</td>
<td>CTX-M-24 (n=2/0)</td>
</tr>
<tr>
<td></td>
<td>CTX-M-104 (n=2/0)</td>
<td>CTX-M-104 (n=2/0)</td>
</tr>
<tr>
<td>More than one CTX-M groups (n=1/1)</td>
<td>CTX-M-2 and CTX-M-55 (n=0/1)</td>
<td>–</td>
</tr>
<tr>
<td>Not identified (n=5/1)</td>
<td>CTX-M-55 and CMY-2 (n=0/1)</td>
<td>–</td>
</tr>
<tr>
<td>Both ESBL and AmpC (n=5/3)</td>
<td>CTX-M-1 group (n=1/1)</td>
<td>CTX-M-15 and nonidentified AmpC (n=1/0)</td>
</tr>
<tr>
<td></td>
<td>CTX-M-2 group (n=1/0)</td>
<td>CTX-M-2 and nonidentified AmpC (n=1/0)</td>
</tr>
<tr>
<td></td>
<td>CTX-M-9 group (n=2/2)</td>
<td>CTX-M-14 and CMY-2 (n=1/2)</td>
</tr>
<tr>
<td></td>
<td>Not identified (n=1/0)</td>
<td>Nonidentified ESBL and CMY-2 (n=1/0)</td>
</tr>
</tbody>
</table>

*The numbers in parentheses are the values of mean number of isolates from dogs/number of isolates from cats.

Table 3. Antimicrobial susceptibility rates in ExPEC producing only group CTX-M-1 or CTX-M-9 ESBLs from dogs and cats

<table>
<thead>
<tr>
<th>Antimicrobials</th>
<th>CTX-M-1 group (n=26)</th>
<th>CTX-M-9 group (n=45)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC₅₀ (µg ml⁻¹)</td>
<td>MIC₉₀ (µg ml⁻¹)</td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid</td>
<td>8/4</td>
<td>16/8</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>4/4</td>
<td>16/4</td>
</tr>
<tr>
<td>Cefibuten</td>
<td>32</td>
<td>128</td>
</tr>
<tr>
<td>Cefmetazole</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Flomoxef</td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>Latamoxef</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>Faropenem</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Meropenem</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Tebipenem</td>
<td>0.015</td>
<td>0.03</td>
</tr>
<tr>
<td>Amikacin</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>1</td>
<td>128</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>32</td>
<td>128</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>4</td>
<td>128</td>
</tr>
<tr>
<td>Trimethoprim/sulfamethoxazole</td>
<td>0.125/2.375</td>
<td>&gt;64/1216</td>
</tr>
</tbody>
</table>

*Significantly higher susceptibility rates in the isolates containing CTX-M-9 group ESBLs.
producing bacteria in animals constitute a public health risk [28]; therefore, alternative drugs are desirable.

Faropenem sodium, which was developed in Japan in 1985, is the only oral penem [29], and cephemyls are stable to hydrolysis by ESBLs [7, 30]. These drugs have shown excellent in vitro antimicrobial activity against ESBL-producing bacteria from humans [21, 31, 32]. Similarly, our results show high rates of susceptibility to FPM, FMX and LMX (>90%) in our collection, similar to those for carbapenem. Therefore, these drugs may be appropriate for the treatment of companion animals infected with ESBL-producing ExPEC. However, the rates of susceptibility to these drugs, especially FMX, were lower in isolates containing both ESBL and AmpC, which must be considered when using these antimicrobials. To the best of our knowledge, this is the first report of the susceptibility of animal-origin bacteria to FPM, FMX and LMX.

β-Lactam–β-lactamase inhibitor combinations are also recommended as alternative drugs for infections with ESBL-producing bacteria. In human medicine, ACV and PTZ may be clinically efficacious against infections of ESBL-producing bacteria if the organism is susceptible to these drugs [33]. In the present study, the in vitro activity of ACV was lower than that of the carbapenems, and decreased greatly in the presence of AmpC β-lactamases. In contrast, PTZ displayed in vitro activity similar to that of the carbapenems, which was negligibly affected by AmpC β-lactamases. These findings imply that PTZ may be a more successful alternative drug than ACV. However, the clinical efficacy of PTZ against infections of ESBL-producing bacteria is controversial in human medicine, because both positive and negative results have been reported [34–36]. The inoculum effect may also affect the clinical efficacy of PTZ [37]. Clinical trials in companion animals are required to verify the efficacy of PTZ.

CTB is an oral third-generation cephalosporin, with relatively high activity against ESBL-producing bacteria compared to other cephalexins [38]. We first assessed the in vitro activity of CTB against animal-origin bacteria. More than half our collection was susceptible to CTB. Notably, most of the isolates producing CTX-M-9 group ESBLs were susceptible to CTB, similar to findings reported elsewhere [20, 39]. Therefore, CTB may be appropriate for the treatment of infections with ESBL-producing bacteria in countries where the CTX-M-9 group ESBLs are prevalent, such as Japan, although the in vitro activity of the drug is inferior to that of the carbapenems.

Of the antimicrobials, other than the β-lactams tested in this study, AMK, FOM and NFT displayed high activity, similar to that of the carbapenems. In our collection, the rates of susceptibility to these antimicrobials were similar to those in mainly non-ESBL-producing E. coli isolates from companion animals [40–42]. High susceptibility to these three drugs has been confirmed in ESBL-producing bacteria from humans [22]. Because the clinical efficacy of these drugs has been verified in humans, especially in patients with urinary-tract infections of ESBL-producing bacteria [43–45], it is preferable that the veterinary use of these drugs is also limited to urinary-tract infections. Our study showed relatively low susceptibility rates of the bacteria tested to CHL, TMS and TET, so these antimicrobials cannot be used empirically for animals infected with ESBL-producing bacteria, although they may be used for the treatment of infected animals if bacterial susceptibility is confirmed. In contrast, the rate of susceptibility to ENR, one of the veterinary fluoroquinolones, was extremely low, as previously reported [46]. Therefore, it is unlikely that veterinary fluoroquinolones have utility as alternative drugs for the treatment of animals infected with ESBL-producing bacteria.

Ewers et al. [5] previously described the regional distribution of ESBL types among E. coli isolates from companion animals: CTX-M-1, CTX-M-14 and CTX-M-15 are the most prevalent ESBL types in Europe, Asia and the Americas, respectively. In this study, we found a high prevalence of the CTX-M-9 group (mainly CTX-M-14 and CTX-M-27) and the CTX-M-1 group (mainly CTX-M-55 and CTX-M-15) in Japan. Of these main ESBLs, CTX-M-14 and CTX-M-15 have been identified in E. coli isolates from companion animals in many countries, including the USA [47], the Netherlands [48], Korea [49], the UK [50] and China [51]. However, CTX-M-55 has rarely been detected [49, 52], except in China, where this type of ESBL is prevalent [51]. CTX-M-27 has been identified as a minor ESBL in other countries [49–51], but is frequently detected in E. coli isolates from humans in Japan [32], suggesting that this type of ESBL is locally prevalent in this country. We first identified CTX-M-104 in bacteria from companion animals, although this enzyme has previously been found in isolates from food-producing animals [53].

Comparison between ESBL-producing ExPEC isolates from dogs and cats revealed significant differences in susceptibility to CTB and TET, and prevalence of CTX-M-1 and CTX-M-9 groups. These results suggest that animal species differences, as well as geographical differences, play a role in the epidemiology of ESBL-producing ExPEC. Unfortunately, such animal species differences have not yet been confirmed because many of the published studies for companion animals covered a small number of ESBL-producing isolates [6]. Large-scale surveillance of ESBL-producing ExPEC isolates from companion animals would be desirable worldwide.

In conclusion, we evaluated the in vitro activity of candidate drugs against 90 ESBL-producing ExPEC isolates from companion animals. The rates of susceptibility to PTZ, CMZ, FMX, LMX, AMK and FOM (available as parenteral drugs), and FPM, NTF and FOM (available as oral drugs) were similar to the rates of susceptibility to carbapenems. We confirmed the predominance of CTX-M-type enzymes among the ESBL-producing ExPEC isolated from these animals. Therefore, these drugs may be appropriate
alternatives to carbapenems in the treatment of companion animals infected with ExPEC that produce CTX-M-type ESBLs. Unfortunately, few of the data required before companion animals can be treated with these antimicrobials, including their pharmacokinetics/pharmacodynamics, potential adverse effects and clinical efficacy, are available yet. Therefore, further in vivo studies are required to confirm the effective application of the antimicrobials investigated in this study.

Funding information
This work was partly financially supported by JPSP KAKENHI grant number 16K18804. None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of this paper.

Acknowledgements
The authors would like to thank Dr. Kensaku Komatsuzaki (Shionogi) for providing the CTB, FMX and LMX, and Dr. Kazuhiko Hirose (Meiji Seika Pharma) for providing the TBP. We are also grateful to Ms. Emiko Shimoda for technical assistance.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This study did not need ethical approval because the study contained no experiments conducted using animals. Data on dogs and cats were anonymized; therefore individual owner consent was not required.

References


