High prevalence of fecal carriage of extended-spectrum β-lactamase/AmpC-producing Enterobacteriaceae in cats and dogs

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Extended-spectrum-β-lactamase (ESBL)/AmpC producing Enterobacteriaceae have been reported worldwide amongst isolates obtained from humans, food-producing animals, companion animals, and environmental sources. However, data on prevalence of fecal carriage of ESBL/AmpC producing Enterobacteriaceae in healthy companion animals is limited. This pilot study describes the prevalence of ESBL/AmpC encoding genes in healthy cats and dogs, and cats and dogs with diarrhea. Twenty fecal samples of each group were cultured on MacConkey agar supplemented with 1 mg/L cefotaxime and in LB-enrichment broth supplemented with 1 mg/L cefotaxime, which was subsequently inoculated on MacConkey agar supplemented with 1 mg/L cefotaxime. ESBL/AmpC genes were identified using the Check-Points CT103 micro array kit and subsequently by sequencing analysis. Chromosomal ampC promoter mutations were detected by PCR and sequencing analysis. From the healthy and diarrheic dogs, respectively 45 and 55% were positive for Escherichia coli with reduced susceptibility to cefotaxime. From the healthy and diarrheic cats, the estimated prevalence was respectively 0 and 25%. One diarrheic cat was positive for both reduced susceptible E. coli and Proteus mirabilis. The ESBL/AmpC genes found in this study were mainly BlaCTX-M-1, but also BlaCTX-M-14, BlaCTX-M-15, BlaTEM-12, BlaOXA-12, and BlaTEM-1 were detected. This pilot study showed that the prevalence of ESBL/AmpC producing Enterobacteriaceae in healthy and diarrheic dogs, and diarrheic cats was relatively high. Furthermore, the genes found were similar to those found in isolates of both human and food-producing animal origin. However, since the size of this study was relatively small, extrapolation of the data to the general population of cats and dogs should be done with great care.

Keywords: ESBL, AmpC, companion animals, fecal carriage, enterobacteriaceae, cat, dog

INTRODUCTION

Extended-spectrum-β-lactamase (ESBL) and plasmid mediated (p)AmpC-producing Enterobacteriaceae have been isolated from humans, different animal species, and the environment worldwide. ESBL/AmpC-producing Enterobacteriaceae in both humans and animals have also been reported increasingly (Coque et al., 2008; Wieler et al., 2011). One of the main concerns is that resistance caused by these enzymes may result in reduced efficacy of antimicrobial therapy or therapy failure. Despite many studies on ESBL/AmpC-producing bacteria from different sources, clear data especially on routes of transmission is still lacking. Therefore the epidemiology of ESBL/AmpC is poorly understood. One of the driving forces behind the increased resistance is the use of 3rd and 4th generation cephalosporins in both humans and animals (Dutil et al., 2010; Damborg et al., 2011, 2012). Resistance to these compounds may appear very quickly in case food-producing animals receive antimicrobial treatment resulting in a subsequent increase in resistance in the human population, as was shown in poultry and humans (Dutil et al., 2010). A similar increase was also shown within individual dogs and horses treated with antimicrobials (Damborg et al., 2011, 2012). In companion animals, among others, the 1st generation cephalosporin cephalexin and 3rd generation, long acting cephalosporin cefovecin are commonly used and licensed in Europe. Companion animals have also been suggested as potential reservoir for antimicrobial resistant bacteria (Guardabassi et al., 2004). Several studies have reported the presence of ESBL/AmpC-producing Enterobacteriaceae in clinical samples from companion animals (Schink et al., 2011; Dierikx et al., 2012b; Evers et al., 2012), however, knowledge about intestinal carriage of ESBL/AmpC in healthy companion animals is limited (Costa et al., 2008; Murphy et al., 2009; Gandolfi-Decristophoris et al., 2013). In these studies, all using different isolation methods, the prevalence of Escherichia coli with reduced susceptibility to 3rd generation cephalosporins in dogs varied from 0 to 17% and in cats from 0 to 12%. The present study combined the analysis of intestinal carriage of Enterobacteriaceae with reduced susceptibility to cefotaxime in both healthy dogs and cats, and dogs and cats with diarrhea.

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MATERIALS AND METHODS

SAMPLE COLLECTION
Fecal samples were collected from December 2011 to March 2012 from healthy dogs (n = 20) and cats (n = 20) from different parts of the Netherlands. The samples from the healthy dogs were randomly selected from a longitudinal study on the presence of enteropathogens. Fecal samples from dogs (n = 20) and cats (n = 20) with diarrhea which were submitted for bacteriological and parasitological examination between December 2011 and February 2012 to the Veterinary Microbiological Diagnostic Center (VMDC) of the Faculty of Veterinary Medicine of Utrecht University were included. A cotton swab was used to inoculate each sample both onto MacConkey agar supplemented with 1 mg/L ceftoxime (MacC+) and in 1 ml LB-medium supplemented with 1 mg/L cefotaxime (LB+) for enrichment. After overnight incubation 10 μL LB+ was plated on MacC+. From samples showing growth on MacC+ without previous selective enrichment, five colonies were selected. From samples showing growth on MacC+ after selective enrichment, one colony was selected.

SPECIES IDENTIFICATION
Species identification was performed biochemically on all isolates that were selected for further analysis using triple sugar iron (TSI) and ornithine decarboxylase (ODC) and additionally checked for urease production and tryptophan reduction (indole).

ESBL/AmpC CHARACTERIZATION
To identify ESBL/AmpC-producing phenotypes, all selected isolates were screened with a double disk synergy test including ceftoxime (30 μg), ceftriaxone with clavulanic acid (30/10 μg), cefotaxime (30 μg), ceftriaxone with clavulanic acid (30/10 μg) and cefoxitin (30 μg; Beckton, Dickinson and Company, Breda, Netherlands). Results were interpreted according to CLSI guidelines (CLSI, 2006). From each animal positive for ESBL/AmpC-producing Enterobacteriaceae, one isolate was selected for molecular characterization. If an animal harbored isolates with different phenotypes, based on biochemical analysis and disk diffusion, one isolate of each phenotype was selected. These isolates (n = 46) were first screened for the epidemiologically most relevant beta-lactamase genes using the Check-MDR CT-103 tube array according to manufacturers’ protocol (Check-Points, Wageningen, Netherlands). These targets include: blaCTX-M, blaTEM, blaSHV, blaOXA−1, blaOXA−3, blaKPC, blaNDM, blaVIM, blaDHA, blaIMI, blaGES, blaGES−2, and a blaOXA−24 variant. Subsequently, PCR products of the identified genes were sent for sequence analysis (Macrogen, Amsterdam, Netherlands) using primers previously described (Hendriksen et al., 2012b). The online database for ESBL/AmpC Genes was used as a reference (www.lahey.org/studies), last accessed 10 July 2013) with additional references for blaOXA−12 variants (GenBank AF126444 and AY884111). Furthermore, sequence analysis of the chromosomal ampC promoter region was performed, using primers designed by Caroff et al. (1999). The ampC classification was performed as described by Mulvey et al. (2005).

STATISTICAL ANALYSIS
Confidence intervals for the prevalence estimates were determined by calculating Exact Binomial Confidence Intervals using SAS v9.2 (SAS Institute Inc., NC, USA).

RESULTS

SAMPLE COLLECTION AND SPECIES IDENTIFICATION
After direct plating, nine healthy dogs (45%) were positive for cefotaxime reduced susceptible (CTX-RS) E. coli (Table 1). After selective enrichment, no additional dogs were found positive. From the diarrheic dogs, 11 animals were positive for CTX-RS E. coli (55%), of which eight were positive after direct plating and three more were obtained after selective enrichment. No CTX-RS Enterobacteriaceae were found in healthy cats. Four cats with diarrheaha (20%) were positive for CTX-RS E. coli after direct plating and one animal (5%) was positive for both CTX-RS E. coli and Proteus mirabilis (25% in total).

ESBL/AmpC CHARACTERIZATION
Molecular analysis showed that within each group of animals there was a high variety of ESBL/pAmpC genes present, especially within diarrheic cats (Table 2). In this group four out of five animals harbored more than one ESBL/pAmpC type. The diarrheic cats were positive for blaCTX-M, –1, –12, blaTEM, –35, and blaOXA−22 genes, of which three animals harbored more than one type of ESBL/AmpC (Table 2). The strain containing blaTEM−35, which is an inhibitor resistant TEM (Zhao et al., 1994), also harbored blaOXA−22. An AmpC phenotype was shown for this strain by a double disk synergy test. Healthy dogs were positive for blaCTX-M, –11, and blaOXA−22 genes (Table 2). One healthy dog harbored three ESBL/pAmpC determinants, respectively blaCTX-M, –1, blaOXA−22, and an isolate with reduced susceptibility to cefotaxime in which no resistance gene was found. Further molecular typing performed after sequence analysis of the chromosomal ampC promoter region. Healthy dogs, diarrheic dogs, and diarrheic cats all harbored the ampC-type-3 variant (Mulvey et al., 2005). Chromosomal ampC-type-3 variant (Mulvey et al., 2005). Chromosomal ampC-type-3 variant (Mulvey et al., 2005). Chromosomal ampC-type-3 variant (Mulvey et al., 2005). Chromosomal ampC-type-3 variant (Mulvey et al., 2005). Chromosomal ampC-type-3 variant (Mulvey et al., 2005).

Table 1 | Prevalence of cats and dogs carrying ESBL/AmpC producing Enterobacteriaceae.

| Group | n screened | Prevalence % (n) | Confidence interval (95 %) |
|-------|------------|------------------|---------------------------|
| Dogs healthy | 20 | 45.0 (9) | 23.1-68.5 |
| Dogs diarrheic | 20 | 55.0 (11) | 31.5-76.9 |
| Dogs total | 40 | 50.0 (20) | 33.8-66.2 |
| Cats healthy | 20 | 0.0 (0) | 0.0-16.8 |
| Cats diarrheic | 20 | 25.0 (5) | 8.7-49.1 |
| Cats total | 40 | 12.5 (5) | 4.2-26.8 |
Table 2 | Number of animals carrying ESBL/AmpC genes in Enterobacteriaceae isolated from healthy cats and dogs, and diarrheic cats and dogs in Netherlands.

| ESBL/AmpC coding gene | Healthy Dog | Diarrheic Dog | Healthy Cat | Diarrheic Cat |
|-----------------------|------------|--------------|-------------|--------------|
| blaCTX-M-15           | 4          | 2            | –           | –            |
| blaCTX-M-15           | –          | –            | –           | 1            |
| blaKPC-3              | –          | 1            | –           | –            |
| blaKPC-3              | 1          | –            | –           | –            |
| blaKPC-2              | –          | –            | –           | 1            |
| CMY-2                 | –          | –            | –           | –            |
| CMY-2                 | 2          | 2            | –           | –            |
| ampC type-3           | 1          | 1            | 1           | –            |
| ampC type-3           | 0          | 1            | –           | –            |
| Unknown                | 1          | 1            | –           | –            |
| >1 R gene             | 1          | 3            | 0           | 4            |

| E. coli               | 9          | 11           | 0           | 4            |
| E. coli + P. mirabilis| 0          | 0            | 0           | 1            |

a This animal carried one E. coli harboring both a blaCTX-M-15 gene and a blaKPC-2 gene. The blaCTX-M-15 gene and another blaKPC-2 gene were found in separate E. coli.
b Isolates negative on Check-MDR CT-103 array, and no ampC type-2.
c Classification as previously described (Mulvey et al., 2005).
d New ampC variant compared to previously described variants (Mulvey et al., 2005).

Based on disk diffusion results, these variants showed ESBL as well as AmpC phenotypes, depending on the other ESBL/AmpC gene present in the same isolate. Therefore the contribution of these chromosomal mutations to resistance could not be confirmed. One diarrheic dog harbored an ampC type-1 and one new ampC variant. The new ampC variant showed close resemblance to ampC type-2 (Mulvey et al., 2005), with a substitution of cytosine to guanine at position +20. Disk diffusion results showed this variant had an AmpC phenotype. No other ESBL/AmpC genes were found in this isolate. Furthermore, two healthy dogs were negative in the array and harbored either a wild-type ampC or ampC type-11 gene. Results from the disk diffusion assay were inconclusive. Because reduced cefotaxime susceptibility caused by the isolate only harboring an ampC wild-type or type-11 could not be confirmed phenotypically, these three isolates from the healthy and diarrheic dog were designated “unknown” (Table 2).

**DISCUSSION**

In the animals included in this study there is a high level of intestinal carriage of ESBL/AmpC-producing Enterobacteriaceae in healthy dogs (45%), and both diarrheic dogs (55%) and cats (29%). In all animal groups in most fecal samples large numbers of reduced susceptible E. coli or P. mirabilis were found after direct plating on selective MacConkey agar plates. Only few fecal samples were found positive additionally after inoculation using the more sensitive selective enrichment and subsequent pure culturing on selective MacConkey agar. Even though the proportion of ESBL/AmpC producing E. coli/P. mirabilis as part of the total population of E. coli/P. mirabilis present in the fecal sample was not determined, this suggests that the bacterial cell count of ESBL/AmpC producing E. coli/P. mirabilis in these fecal samples is relatively high. Due to the small size of the study, the confidence intervals for the estimated prevalence were relatively large (Table 1). Despite this fact, the prevalence in the different animal groups, especially for both healthy and diarrheic dogs, observed in the present study was relatively high compared to other studies. Costa et al. (2008) showed in a study on healthy cats (n = 36) and dogs (n = 39) that two E. coli isolates, both from the same dog, were reduced susceptible to cefotaxime, indicating a prevalence of 2.6%. The fact that no selective culturing media were used in that study may contribute to this large difference. In healthy cats they did not find isolates with reduced susceptibility to cefotaxime. Murphy et al. (2008) reported that in two regions in southern Ontario 9% of both cats (n = 39) and dogs (n = 188) were positive for E. coli with reduced susceptibility to cefotaxime. In that study, fecal samples...
were also screened using selective media. Gandolfi-Decristophoris et al. (2013) showed that in Switzerland from faecal swabs that were taken from healthy cats (n = 202) and dogs (n = 174) at nursing homes and at veterinary clinics, 17% of the examined dogs and 12% of the cats were positive for Enterobacteriaceae with reduced susceptibility for the 3rd generation cephalosporin cefepime. In contrast, 2.9% of the dogs and 2% of the cats examined were positive for ESBL genes. This difference in prevalence between cefepime resistance and presence of ESBL genes may be caused by the fact that the presence of AmpC type genes was not determined. As we have shown, bla_{CPE}-like genes were present in more than 10% of all examined isolates. Additionally, we have also shown that promoter mutations in the chromosomal ampC gene were present.

Gandolfi-Decristophoris et al. (2013) also indicated that antimicrobial treatment (not further specified) that was administered during the last three months prior to sampling was identified as a risk factor for the carriage of ESBL producing Enterobacteriaceae. Similar results were shown by Damborg et al. (2011) in a study in which cefalexin was orally administered to dogs. This treatment resulted in selection for the presence of bla_{CPE}-producing *E. coli* in the feces. The treatment history of the animals included in our study is not known. Therefore, any possible contribution of previous antibiotic usage as described above could not be established.

In China, a more or less similar prevalence was observed compared to our study (Sun et al., 2010). In their data set 93% of the animals included in our study is not known. Therefore, any possible contribution of previous antibiotic usage as described above could not be established.

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