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Longitudinal Study of Extended-Spectrum-β-Lactamase- and AmpC-Producing *Enterobacteriaceae* in Household Dogs

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A longitudinal study was performed to (i) investigate the continuity of shedding of extended-spectrum-beta-lactamase (ESBL)-producing *Enterobacteriaceae* in dogs without clinical signs, (ii) identify dominant plasmid-mediated ESBL genes, and (iii) quantify ESBL-producing *Enterobacteriaceae* in feces. Fecal samples from 38 dogs were collected monthly for 6 months. Additional samples were collected from 7 included dogs on a weekly basis for 6 weeks. Numbers of CFU per gram of feces for non-wild-type *Enterobacteriaceae* were determined by using MacConkey agar supplemented with 1 mg/liter cefotaxime (MCC), and those for total *Enterobacteriaceae* were determined by using MacConkey agar. Cefotaxime-resistant isolates were screened by PCR and sequence analysis for the presence of $bla_{\text{CTX-M}}$, bla_{CMY}, bla_{SHV}, bla_{OXA}, and bla_{TEM} gene families. Bacterial species were identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) analysis. PCR-negative isolates were tested by a double-disk synergy test for enhanced AmpC expression. A total of 259 samples were screened, and 126 samples were culture positive on MCC, resulting in 352 isolates, 327 of which were *Escherichia coli*. Nine dogs were continuously positive during this study, and 6 dogs were continuously negative. Monthly or weekly shifts in fecal shedding were observed for 23 dogs. Genotyping showed a large variety of ESBL genes and gene combinations at single and multiple consecutive sampling moments. The ESBL genes $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-12}}$, and $bla_{\text{CMY-2}}$ were most frequently found. The mean number of CFU of non-wild-type *Enterobacteriaceae* was 6.11×10^8 CFU/g feces. This study showed an abundance of ESBL-producing *Enterobacteriaceae* in dogs in the Netherlands, mostly in high concentrations. Fecal shedding was shown to be highly dynamic over time, which is important to consider when studying ESBL epidemiology.

A substantial share of the present global emergence of antimicrobial resistance is represented by extended-spectrum-beta-lactamase (ESBL)-producing *Enterobacteriaceae*. So far, these bacteria have been isolated from a large variety of sources, including humans, animals, and the environment. Together, these sources seem to form a complex network of reservoirs and transmission routes where ESBL-producing *Enterobacteriaceae* are circulating (1).

High prevalences of ESBL-producing *Enterobacteriaceae* were found in Dutch poultry, pigs, and cattle (2). High prevalences of ESBL-producing *Enterobacteriaceae* were also found in Dutch companion animals, i.e., 45% in dogs without clinical signs and 55% in diarrheic dogs (3). As companion animals live in close contact with humans, they might contribute substantially to the exposure of humans to ESBL-producing *Enterobacteriaceae*. Similar ESBL gene types, i.e., CTX-M-14, CTX-M-15, SHV-12, and CMY-2, were found in strains originating from humans and companion animals (1). Additionally, transmission of CTX-M-15-carrying ST131 and ST648 *Escherichia coli* strains between dogs and humans has been suggested (4, 5). Therefore, the importance of investigating the role of companion animals in the epidemiology of ESBL-producing *Enterobacteriaceae* is clear.

So far, data on ESBL-producing *Enterobacteriaceae* in companion animals have been collected only through cross-sectional studies. However, no data on the continuity of shedding of ESBL-producing *Enterobacteriaceae* in companion animals are available yet. Longitudinal information is essential to estimate the exposure of humans to ESBL-producing *Enterobacteriaceae* by colonized animals and to assess the value of data for the identification of risk factors for dogs to be positive for ESBL-producing *Enterobacteriaceae*.

The aims of this study were to (i) investigate the continuity of shedding of extended-spectrum-beta-lactamase-producing *Enterobacteriaceae* in dogs without clinical signs, (ii) identify dominant plasmid-mediated ESBL genes, and (iii) quantify ESBL-producing *Enterobacteriaceae* in fecal samples.

MATERIALS AND METHODS

Dogs and background information. To assess the continuity of ESBL shedding in this longitudinal study, a minimum sample size of 25 dogs was calculated with a precision of 20%, a 95% confidence interval (CI), and an estimated prevalence of 50% among dogs, deduced from data described previously by Hordijk et al. (3). Dogs were selected from a pool of dogs (~800) who contributed monthly samples (with little to no interruption) on a voluntary basis to a longitudinal 2-year survey of intestinal

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pathogens. Animal sampling was performed in accordance with the Dutch Animals Act (stb-2011-345; https://zoek.officielebekendmakingen.nl/stb-2011-345.html [in Dutch]), based on European Union directive 2010/63/EU.

Fecal samples were collected monthly during the period of July 2013 to January 2014. To distinguish between short- and long-term shedding, additional weekly fecal samples from a convenient number of 7 included dogs were collected for at least six consecutive weeks. Basic background information on the dogs regarding housing, diet, health, and drug treatment was obtained through an initial questionnaire and subsequent monthly questionnaires to check if anything had changed in the preceding month. All questionnaires were filled out online by the owners.

Fecal samples were either deposited at the institute or sent by regular mail service (non-temperature controlled). Within this study, an experiment was carried out to assess deterioration by the transport method used. Four freshly collected fecal samples from different dogs were packaged and mailed at different times during the study according to the protocol. Sending of samples by mail took up to 3 days, but no significant differences in the number of CFU per gram of feces were measured after shipment compared to the number of CFU per gram of feces determined before shipment (data not shown). It was therefore presumed that the transport method used had no significant influence on the quantitative analysis of these samples.

Bacterial isolates. Dog owners were instructed to collect fecal samples immediately after defecation. Fecal material used for analysis was taken from the center of the sample. Of each sample, 0.5 g feces was suspended in 4.5 ml 0.9% NaCl. To quantify viable ESBL-producing *Enterobacteriaceae*, the track dilution method, as described previously by Jett et al. (6), was used to inoculate square plates containing MacConkey agar (MC) (Oxoid, the Netherlands) and square plates containing MacConkey agar supplemented with 1 mg/liter cefotaxime (MCC) (Oxoid, the Netherlands). Six 10-fold serial dilutions in 0.9% NaCl were made, and 20 μl of each dilution was inoculated onto MC and MCC, which were then cultured overnight. Additionally, 100 μl of a 10^{-1} dilution was inoculated in 1 ml LB broth supplemented with 1 mg/liter cefotaxime (LBC) (Oxoid, the Netherlands) for enrichment. After incubation overnight, 1 μl of LBC was streaked onto MCC and cultured overnight. All incubation steps were performed at 37°C.

When growth occurred on MCC after direct inoculation of the sample, 3 typical pink colonies suspected to be *E. coli* were selected for further analysis. If applicable, morphologically different pink colonies were selected; otherwise, colonies were chosen randomly. In the case of the presence of colorless colonies, 1 colony was picked for every morphologically different phenotype. When growth on MCC occurred after enrichment only, just one typical suspected *E. coli* colony was selected. All isolates cultured on MCC were designated isolates with non-wild-type susceptibility to cefotaxime (nWT), according to data reported previously by Schwarz et al. (7).

The detection limit in this study was 10^2 CFU/g feces. Numbers of CFU per gram in MC and MCC were calculated for each phenotypically positive fecal sample. Also, the average number of CFU per gram was calculated for *Enterobacteriaceae* with the nWT phenotype. To determine the fraction of *Enterobacteriaceae* with the nWT phenotype compared to the total number of *Enterobacteriaceae*, the numbers of CFU per gram on MC and MCC were compared.

Species identification. The species of each isolate was determined by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker, Delft, the Netherlands). In the case of unidentifiable isolates, the API identification system (bioMérieux SA, Marcy l'Etoile, France) was used to identify bacterial species.

ESBL identification. This study focused on plasmid-mediated ESBL genes. Isolates were screened by conventional PCR for the presence of $bla_{CTX\text{-}M}$, bla_{CMY}, bla_{SHV}, bla_{OXA}, and bla_{TEM} gene families by using the primers listed in Table S1 in the supplemental material. Additional primers, TEM-seq, CMY-F-838, and CMY-R-857, were used for sequence analysis. DNA lysates were prepared by boiling in Chelex 100 molecular-biology-grade resin (Bio-Rad, Veenendaal, the Netherlands). PCR mix consisted of 20 μl containing 5 μl DNA lysate, 2× GoTaq Hotstart Green master mix (Promega Benelux BV, Leiden, the Netherlands), 0.5 μM each forward and reverse primers, and molecular-grade water. The presence of ESBL genes was visualized by gel electrophoresis. PCR products were purified with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol and subsequently sent for sequence analysis (Baseclear, Leiden, the Netherlands) to identify ESBL genes. Sequences were compared to reference sequences provided by the Lahey Clinic (http://www.lahey.org/studies [last accessed 26 February 2015]) and analyzed by using Bionumerics v7.1 (Applied Maths NV, Sint-Martens-Latem, Belgium).

Potential novel allele variants were additionally sequenced by using an Illumina MiSeq platform, and contigs were assembled by using SPAdes v3.1.1 (Algorithmic Biology Lab, Russia). The sequences were verified by the Lahey Clinic database curators (http://www.lahey.org/studies) and submitted to GenBank.

All ESBL-negative *Escherichia coli* isolates were investigated by sequence analysis for the presence of expression-enhancing mutations in promoter regions of chromosomal *ampC* genes to clarify growth on MCC. AmpC types were assigned according to methods described previously by Mulvey et al. (8). For confirmation of enhanced AmpC expression, double-disk synergy tests were carried out when mutations in promoter regions of the chromosomal *ampC* gene were found. A 0.5 McFarland standard suspension was made for each strain, and these suspensions were subsequently inoculated onto Mueller-Hinton agar (Oxoid, the Netherlands). BD BBL Sensi-Discs (Becton, Dickinson and Company, Breda, the Netherlands) containing cefotaxime (30 μg), cefotaxime-clavulanate (30 and 10 μg, respectively), ceftazidime (30 μg), ceftazidime-clavulanate (30 and 10 μg, respectively), and cefoxitin (30 μg) were used. Isolates showing a <5-mm growth difference in the inhibition zone diameters between cefotaxime and cefotaxime-clavulanate and between ceftazidime and ceftazidime-clavulanate were considered to have the AmpC phenotype, according to CLSI guidelines (9). Cefoxitin was used for confirmation of the AmpC phenotype.

Statistical analysis. Statistical analyses were conducted by using SAS software 9.4 (SAS Institute, Inc., Cary, NC, USA). Descriptive analysis was undertaken, and risk factors for ESBL carriage were identified by logistic regression analysis using the generalized linear model (GENMOD) procedure. The hierarchical structure of the data was taken into account to adjust for the fact that repeated observations within dogs and for dogs with the same owner may not be independent.

Nucleotide sequence accession number. The sequence for the novel CMY variant $bla_{CMY\text{-}134}$ was submitted to the GenBank database under accession no. KP860987.

RESULTS

Dogs and background information. In total, 38 dogs belonging to 24 owners from different parts of the Netherlands were included in this study. All dogs were >6 months of age. Eleven households owned one dog, eight households owned two dogs, and three households owned three dogs. For two other multiple-dog households, only one dog was included in the study; one of these households owned two dogs, and the other one owned three dogs. Basic background information that was obtained through the questionnaires is given in Table S2 in the supplemental material (columns A to W for the initial questionnaire and column Y for the subsequent monthly questionnaires). Potentially relevant factors that may have influence on the presence of ESBLs, such as antimicrobial treatment, diet, and coprophagy, were included. Four dogs were treated with antimicrobials during the study. Dog 16B was treated orally with cephalexin from weeks 4 to 7 for a skin condition (Table 1). Dog 18A was treated with sulfasalazine, met-

TABLE 1 Time points of analysis of fecal samples and results of culturing on MC and MCC

Dog[b]	Result at wk[a]:																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1A			P					N						N							N					
1B			P					P					N								N					
2A	N	N	N	N	N	N	N	N	P				P				P				N					
3A	N	N	N	N	N					N	P	N	P	P	N	N	N					N				
4A			N				N						N					N					N			
5A	N	N	N	N	N	N	P	N	N	N	N	N	N					N				N				
5B	N	N	N	P	P	P	N	P	P	P	N	P	N					N				P				
6A	N				N					P				N				N					P			
6B	N				N					N				N				N					P			
7A			N					P				N					P									
7B			N					P				N					P									
7C			N					N				N					N									
8A					N	N	N	N		N	N	N	N	N	N								N			
9A				P				P					P				P				P					P
9B				P				P					P				N				N					P
9C				P				P					P				P				P					P
10A		P			P								P					P				P				
11A					P				P			P					P				P					P
12A	N	N		P		P	N	N	P	P	N	N	N			N					N					N
12B	N	N		N		P	P	P	N	N	N	N	N			N					N					N
13A		N				N							N					N				N				
14A	N						N					N					N				N					
15A	P				P					P				P				P				P				
15B	P				N					N				P												
15C	P				P					P				P				P				P				
16A	P						N						P									P				
16B	P						P						N									P				
17A	P				P				P					P				N					P			
17B	P				P				N					P				P					P			
18A	P				P					P				P					P				P			
19A	P				P					P			P					P								P
20A	N				N				N					N				N				N				
21A	N			P				P					N				N						X			
21B	P			P				P					N				P						N			
22A	P				P						P					P			P					P		
23A	P				P					P											N					
24A	P					P				P				P					P				N			
24B	P					P				P				P					P				N			

[a] P, fecal sample with non-wild-type susceptible colonies; N, fecal sample with wild-type colonies; X, dog died.
[b] Dogs with the same numbers shared the same household.

ronidazole, and spiramycin after week 8 for severe diarrhea. Dog 24A and dog 24B were treated for a skin condition with amoxicillin-clavulanic acid on weeks 3 and 17, respectively (see Table S2, column Y, in the supplemental material). All treated dogs were positive for ESBL-producing *E. coli* upon subsequent sampling. Three of these dogs also tested positive in the period preceding antimicrobial treatment. Furthermore, 18 dogs (47%) ate feces (see Table S2, column P, in the supplemental material). However, a negative association between ESBL carriage and coprophagy was observed (odds ratio [OR] = 0.42 [95% CI, 0.20 to 0.86]). In total, 23 dogs (60%) were fed raw meat products (frozen when purchased) as part of their diet (see Table S2, columns R and S, in the supplemental material). Feeding of raw meat was positively associated with the presence of ESBL-producing *E. coli* (OR = 2.08 [95% CI, 1.22 to 3.57]).

Phenotypic results. A total of 259 fecal samples were analyzed: 204 monthly fecal samples and 55 weekly fecal samples (Table 1). Ninety-five fecal samples showed growth directly on MCC, and 31 fecal samples showed growth on MCC after enrichment.

Thirty-two dogs (84%) had at least one fecal sample with nWT isolates during the study period. Six dogs were ESBL negative throughout the study period. One ESBL-negative dog (dog 7C) was housed in a household with multiple dogs in which the other dogs were positive. Nine dogs were continuously positive, and the vast majority of 23 dogs showed monthly or even weekly shifts in fecal shedding of *Enterobacteriaceae* with a nWT phenotype. Here, shifts in fecal shedding are defined as shifts between MCC-positive and MCC-negative samples, after culturing. For dogs screened weekly, these shifts were frequently seen. In 3 of these 7 dogs, dogs 3A, 5B, and 12A, even 3 or more shifts in fecal shedding were observed within 6 weeks (Table 1). In some households (households 9, 15, and 24), dogs showed clustered shedding of nWT *Enterobacteriaceae* at the same time, whereas in other households, shedding was not clustered (households 5 and 12) (Table 1).

The numbers of CFU of MCC-positive *Enterobacteriaceae* and total *Enterobacteriaceae* per gram of feces were calculated for every fecal sample with nWT isolates. The numbers of CFU per gram and the corresponding fractions in individual samples are given in Table S3 in the supplemental material. The mean number of CFU of *Enterobacteriaceae* with the nWT phenotype per gram of feces was 6.11×10^8 CFU/g, within a range of 1.00×10^2 to 6.22×10^{10} CFU/g. The average fraction of *Enterobacteriaceae* with the nWT phenotype compared to total *Enterobacteriaceae* was 0.02, within a range of 2.00×10^{-9} to 1.00. For 61 fecal samples, this fraction was ≥0.001.

Species identification. The 126 MCC-positive fecal samples resulted in 352 isolates with non-wild-type susceptibility to cefotaxime. Species determination using MALDI-TOF MS identified 327 isolates as *E. coli*. One isolate was identified as an *Enterobacter* species. Twenty-two isolates were identified as non-*Enterobacteriaceae* (13 *Acinetobacter* species, 8 *Pseudomonas* species, and 1 *Ochrobactrum* species isolates). Two isolates could not be identified by MALDI-TOF MS and were analyzed with the API 20 NE system. This test identified one isolate as *Pseudomonas* species, and the species of one isolate could not be identified. Non-*Enterobacteriaceae* were not included in ESBL identification.

ESBL identification. ESBL genes were detected in 269 *E. coli* isolates. Fifty-six ESBL-negative *E. coli* isolates carried beta-lactamase-encoding bla_{TEM} genes or expression-enhancing promoter region mutations of a chromosomal *ampC* gene. One *E. coli* isolate and one *Enterobacter* species isolate were PCR negative for all screened genes. Another isolate was identified as the *ampC* WT. Genotyping results are summarized in Table 2 for monthly samples and in Table 3 for weekly samples.

For 22 fecal samples, only one isolate could be retrieved. From these samples, 19 isolates were obtained through direct plating, and 3 isolates were obtained after enrichment. Of the remaining 106 fecal samples, 72 samples contained multiple isolates with different combinations of ESBL types and beta-lactamase-encoding bla_{TEM} genes or expression-enhanced *ampC* genes, resulting in a wide variety of resistance genes in one fecal sample. Also, consecutive fecal samples often had isolates with different ESBL gene combinations (Table 2).

Frequencies of ESBL genes combinations are given in Table 4. Of all screened ESBL genes, bla_{CTX-M} genes were most frequently present, in a total of 179 isolates. Eighty percent of these isolates carried a bla_{CTX-M} gene of group 1. Eleven percent of these isolates carried a bla_{CTX-M} gene of group 9, 8% carried a bla_{CTX-M} gene of group 2, and 1% carried a bla_{CTX-M} gene of group 8.

One novel allele variant was identified and submitted to the Lahey Clinic database (http://www.lahey.org/studies). This isolate harbored a novel CMY variant designated $bla_{CMY-134}$.

Eight isolates, originating from 5 different dogs in 4 different households, carried a variant of bla_{TEM-1} ($bla_{TEM-1varA}$)combined with $bla_{CTX-M-15}$. $bla_{TEM-1varA}$ showed a synonymous mutation of C to T on position +537 compared to the reference bla_{TEM-1b} sequence (GenBank accession no. AB263754). Another 2 isolates, originating from a dog from a different household, harbored the same gene variant in combination with bla_{TEM-1b}. Furthermore, one isolate from yet another household harbored only $bla_{TEM-1varA}$.

Twenty-four cases with matching ESBL gene combinations were found for the same sampling within households; eight of these matches were seen in household 9 (Table 2). Both dogs 9A and 9C had isolates harboring $bla_{CTX-M-15}$ and $bla_{TEM-1varA}$ in the first month. At sampling moment 2, all three dogs had isolates harboring a $bla_{CTX-M-2}$-like gene. Dogs 9A and 9B both showed isolates harboring $bla_{CTX-M-1}$ at sampling moment 3. At sampling moment 4, $bla_{CTX-M-1}$ was found in dogs 9A and 9C. At sampling moment 5, bla_{SHV-12} was found in isolates originating from these two dogs. In the final month, dogs 9B and 9C produced isolates harboring $bla_{CTX-M-1}$.

Of 56 ESBL-negative *E. coli* isolates, 18 isolates carried beta-lactamase-encoding bla_{TEM} genes, and 38 isolates carried chromosomal *ampC* types with mutations in the promoter region.

DISCUSSION

In several countries, cross-sectional studies were carried out to investigate ESBL-producing *Enterobacteriaceae* in companion animals. The prevalence of ESBL-producing strains in clinical *Enterobacteriaceae* isolates derived from dogs and cats ranged between 3.1% and 54.4% (10–16). In samples obtained from healthy companion animals, the percentage of ESBL-producing *Enterobacteriaceae* found in most of these studies was <20% (16–22). Based on a single point in time, the prevalence in the study presented here would range from 45% to 63%, depending on the chosen time point. This corresponds to the estimated prevalence in the Dutch dog population reported previously by Hordijk et al. (3). However, considering this entire longitudinal study, 84% of participating dogs were ESBL positive at least once. This difference between cross-sectional and longitudinal data shows the relevance of longitudinal data when studying ESBL epidemiology.

Moreover, longitudinal data revealed the occurrence of frequent shifts in fecal shedding of ESBL-producing *Enterobacteriaceae* in dogs. Within the study presented here, fecal samples were collected at both weekly and monthly time intervals, to distinguish between short- and long-term shedding and shifts. This led to the observation of shifts in the fecal shedding of ESBL-producing *Enterobacteriaceae* in the majority of screened dogs. Dogs screened weekly showed short-term shifts, as various dogs showed 3 or more shifts in a 6-week period. This could not have been observed in the monthly screening only. The observed shifts in ESBL shedding may be due to significant differences in colonization or even uptake and loss of strains, caused by factors that still need to be identified.

The microbiota of colon and rectum in dogs comprises $\sim 10^8$ *Enterobacteriaceae* (23). Large fractions of ESBL-producing *Enterobacteriaceae* compared to the total number of *Enterobacteriaceae* were found in this study. A fraction of 0.02 equals an average carriage of 2.00×10^6 ESBL-producing *Enterobacteriaceae* in dogs. In two cases, the number of CFU per gram of ESBL-producing *Enterobacteriaceae* was equal to that of total *Enterobacteriaceae*, which shows that ESBL-producing *Enterobacteriaceae* can comprise a large part of the gut microbiota.

Besides the shifts in MCC-positive and MCC-negative samples in time, the ESBL genes found in consecutive MCC-positive fecal samples also often differed. An explanation for this variation could be a difference in the relative proportions of each type present in the gut. During screening of consecutive fecal samples, new ESBL types were found at almost every consecutive sampling moment. This supports the uptake of new strains and the loss of strains with different ESBL types more than differences in colonization with a recurrence of previous types.

The shifts in fecal shedding and the large variety of ESBL genes, as shown in this longitudinal study, demonstrate the complexity

TABLE 2 Genotypic ESBL characteristics of nWT isolates in fecal samples collected monthly[a]

Dog	ESBL(s) detected (no. of isolates) at mo:					
	1	2	3	4	5	6
1A	CTX-M-1 (2), CTX-M-14	X		X		X
1B	NE (2)	CTX-M-15 (3)		X		X
2A	X	X	CMY-2	NE (1)	CTX-M-3, NE (1)	X
3A	X	X	X	NE (1)	X	X
4A	X	X		X	X	X
5A	X	X	X	X	X	X
5B	X	NE (1)	CTX-M-15 (3)	X	X	CTX-M-15 (3)
6A	X	X	NE (1)	X	X	CTX-M-1
6B	X	X	X	X	X	CTX-M-1, CTX-M-14 (2), SHV-12, TEM-1b and -52StPaul
7A	X	CTX-M-1 (5)	X		NE (2)	
7B	X	NE (2)	X		CMY-2 (2)	
7C	X	X	X		X	
8A	X	X	X		X	
9A	CTX-M-15, CTX-M-14, CMY-2, NE (1)	CTX-M-2 (3)	CTX-M-1 (3)	CTX-M-1, CTX-M-15, CTX-M-14	SHV-12 (3)	NE (3)
9B	NE (3)	CTX-M-2 (3)	CTX-M-1 (2), SHV-12	X	X	CTX-M-1 (3)
9C	CTX-M-15 (2), NE (2)	CTX-M-2 (3)	CMY-2 (3)	CTX-M-1 (2), CTX-M-3	SHV-12 (3)	CTX-M-1
10A	CTX-M-1, NE (2)	CTX-M-1 (3), TEM-1b and -52c		CTX-M-15, CMY-2 (2)	CTX-M-15 (3)	SHV-2, SHV-12, TEM-1b and -52StPaul
11A	CTX-M-1, TEM-52c, NE (1)	CTX-M-1, CTX-M-14, NE (1)	CTX-M-1 (3)	CTX-M-14 (2), NE (1)	CTX-M-1, CTX-M-2, CTX-M-65	CTX-M-1, CTX-M-15 (2)
12A	X	CMY-2, NE (3)	CTX-M-1 (3)	X	X	X
12B	X	NE (3)	X	X	X	X
13A	X	X		X	X	X
14A	X	X	X		X	X
15A	CTX-M-1 (2), NE (1)	CTX-M-32, CTX-M-14, TEM-52c, CMY-2	CTX-M-1 (4)	CTX-M-1 (2), SHV-12 (2)	CTX-M-1, CMY-2, CMY-134, NE (1)	CTX-M-15 (3)
15B	TEM-1b and -52c (3)	X	X	CTX-M-1		
15C	CTX-M-1, CTX-M-14, CMY-2, TEM-1b and -52c	NE (3)	CTX-M-1 (2), SHV-12, CMY-2	CTX-M-1 (2), NE (2)	CTX-M-1, CTX-M-15 (2)	TEM-52StPaul
16A	CTX-M-1, CMY-2	X		NE (1)		CTX-M-2 (2), CTX-M-14, TEM-52c
16B	CTX-M-1 (3)	CMY-2 (3)		X		CTX-M-32
17A	NE (3)	CTX-M-32 (3)	SHV-12	CTX-M-1 (3)	X	CTX-M-14, NE (2)
17B	NE (3)	CTX-M-1 (2), CTX-M-14	X	CTX-M-1, NE (2)	CTX-M-8 (2), CTX-M-14, TEM-52c	CTX-M-14, NE (2)
18A	CTX-M-15 (2), NE (1)	CTX-M-1 (2), NE (2)	CTX-M-1 (3)	CTX-M-1 (2), CTX-M-15	CTX-M-1 (2), CTX-M-32	CMY-2 (2), TEM-52StPaul
19A	CMY-2 (2), TEM-1b and -52StPaul	SHV-12 (2), CMY-2	CTX-M-1 (2), CTX-M-55, CTX-M-14	CTX-M-1 (2), CTX-M-15	NE (1)	CTX-M-1 (3)
20A	X	X	X	X	X	X
21A	X	CTX-M-1, SHV-12 (2)	CTX-M-1	X	X	X
21B	CTX-M-1, CTX-M-14, SHV-12 (2)	CTX-M-1, SHV-12 (2)	CMY-2, NE (1)	X	NE (1)	X
22A	SHV-12, CTX-M-55 (2)	CTX-M-1, CTX-M-2, TEM-1b and -52c, NE (1)	CTX-M-32, CTX-M-2, TEM-52c	CTX-M-1 (2), CTX-M-2	NE (2), CTX-M-1	CTX-M-1, CTX-M-65, NE (1)
23A	NE (2)	CTX-M-1, NE (2)	CTX-M-1 (2), TEM-52c and -135 (2)			X
24A	NE (3)	CMY-2, TEM-52c (2)	CMY-2 (2), TEM-52c, SHV-12	CTX-M-1 (3)	CTX-M-1, SHV-12, TEM-52c, NE (1)	X
24B	NE (3)	CTX-M-1, CMY-2, NE (1)	CMY-2	CTX-M-1 (3)	SHV-12, TEM-52c (3)	X

[a] X, fecal sample without growth on MCC; NE, ESBL-negative isolates with non-wild-type susceptibility to cefotaxime, including non-*Enterobacteriaceae*. Dogs with the same numbers shared the same household. If a fecal sample produced >1 isolate containing similar genes, the number of isolates is shown in parentheses. Of isolates with combinations of resistance genes, only ESBL-type genes are shown. The CTX-M-1/61 gene type is shown as CTX-M-1, CTX-M-15/28 is shown as CTX-M-15, CTX-M-2/20/44/56/97 is shown as CTX-M-2, CMY 2/61 is shown as CMY-2, and SHV-12/129 is shown as SHV-12. Sequence references were provided by the Lahey Clinic (http://www.lahey.org/studies), and the following GenBank accession numbers were used for characterization: AB263754 for TEM-1b, AY883411 for TEM-52c, and AF126444 for TEM-52StPaul.

TABLE 3 Genotypic ESBL characteristics of nWT isolates in fecal samples collected weekly[a]

Dog	ESBL(s) detected (no. of isolates) at wk:																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
2A	X	X	X	X	X	X	X	X	CMY-2				NE (1)				CTX-M-3, NE (1)
3A	X	X	X	X	X					X	CMY-2 (3)	X	NE (1)	CTX-M-1, CMY-2	X	X	X
5A	X	X	X	X	X	X	NE (2)	X	X	X	X	X	X				
5B	X	X	X	NE (3)	NE (1)	CMY-2	X	CTX-M-1	CTX-M-15 (3)	NE (1)	X	CTX-M-1 (3), TEM-52c	X				
8A			X			X	X	X		X	X	X	X	X	X		
12A	X	X	CTX-M-1, SHV-12 (3)			CMY-2, NE (3)	X	X	CTX-M-1 (3)	CMY-2, TEM-35	X	X	X			X	
12B	X	X	X			NE (3)	NE (3)	CMY-2	X	X	X	X	X			X	

[a] X, fecal sample without growth on MCC; NE, ESBL-negative isolates with non-wild-type susceptibility to cefotaxime. Dogs with the same numbers shared the same household. If a fecal sample produced >1 isolate containing similar genes, the number of isolates is shown in parentheses. Of isolates with combinations of resistance genes, only ESBL-type genes are shown. Gene type CTX-M-1/61 is shown as CTX-M-1, CTX-M-15/28 is shown as CTX-M-15, CTX-M-2/20/44/56/97 is shown as CTX-M-2, CMY 2/61 is shown as CMY-2, and SHV-12/129 is shown as SHV-12. Sequence references were provided by the Lahey Clinic (http://www.lahey.org/studies), and the following GenBank accession numbers were used for characterization: AB263754 for TEM-1b, AY883411 for TEM-52c, and AF126444 for TEM-52StPaul.

TABLE 4 Frequency of characterized gene combinations originating from nWT isolates

Gene or gene combination[a]	No. of isolates
$bla_{\text{CTX-M}}$	
$bla_{\text{CTX-M}}$ (group 1)	77
$bla_{\text{CTX-M}}$ (group 2)	2
$bla_{\text{CTX-M}}$ (group 8)	1
$bla_{\text{CTX-M}}$ (group 9)	10
$bla_{\text{CTX-M}}$ + $bla_{\text{TEM(ESBL)}}$	
$bla_{\text{CTX-M}}$ (group 2) + $bla_{\text{TEM(ESBL)}}$	1
$bla_{\text{CTX-M}}$ + $bla_{\text{TEM(BL)}}$	
$bla_{\text{CTX-M}}$ (group 1) + $bla_{\text{TEM(BL)}}$	48
$bla_{\text{CTX-M}}$ (group 2) + $bla_{\text{TEM(BL)}}$	12
$bla_{\text{CTX-M}}$ (group 8) + $bla_{\text{TEM(BL)}}$	1
$bla_{\text{CTX-M}}$ (group 9) + $bla_{\text{TEM(BL)}}$	8
$bla_{\text{CTX-M}}$ (group 1) + $bla_{\text{CMY-2}}$ + $bla_{\text{TEM(BL)}}$	1
$bla_{\text{CTX-M}}$ + $bla_{\text{OXA(BL)}}$	
$bla_{\text{CTX-M}}$ (group 1) + $bla_{\text{OXA(BL)}}$	10
$bla_{\text{CTX-M}}$ (group 9) + $bla_{\text{OXA(BL)}}$	1
$bla_{\text{CTX-M}}$ + $bla_{\text{OXA(BL)}}$ + $bla_{\text{TEM(BL)}}$	
$bla_{\text{CTX-M}}$ (group 1) + $bla_{\text{OXA(BL)}}$ + $bla_{\text{TEM(BL)}}$	7
bla_{SHV}	
$bla_{\text{SHV-2}}$	1
$bla_{\text{SHV-12}}$	21
bla_{SHV} + $bla_{\text{TEM(BL)}}$	
$bla_{\text{SHV-12}}$ + $bla_{\text{TEM(BL)}}$	7
bla_{CMY}	
$bla_{\text{CMY-2}}$	25
bla_{CMY} + $bla_{\text{TEM(BL)}}$	
$bla_{\text{CMY-2}}$ + $bla_{\text{TEM(BL)}}$	11
$bla_{\text{TEM(ESBL)}}$	
$bla_{\text{TEM-52c}}$	21
$bla_{\text{TEM-52StPaul}}$	4
Other[b]	56

[a] BL, beta-lactamase.

[b] $bla_{\text{TEM(BL)}}$; *ampC* promoter mutants with enhanced or unidentified expression (2).

of ESBL epidemiology. This complexity, especially the variation in time, will cause difficulties in identifying risk factors for the carriage of ESBL-producing *Enterobacteriaceae* in cross-sectional studies. Therefore, repeated sampling should be used when determining risk factors. In this study, eating of raw meat as part of the diet was found to be a potential risk factor in relation to shedding of ESBL-producing *Enterobacteriaceae*. However, this correlation should be interpreted with great care, since we have not shown actual transmission. This also requires a longitudinal approach, including samples from both feed and feces, preferably with an intervention of starting/stopping of feeding raw meat during the study. In addition, a more in-depth molecular analysis, including plasmid and *E. coli* typing, is required.

The most frequently found ESBL genes in this study were $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-12}}$, and $bla_{\text{CMY-2}}$. All these ESBL types were previously found in humans, companion

animals, and livestock (1). The finding of three $bla_{CTX\text{-}M\text{-}55/57}$ isolates and two $bla_{CTX\text{-}M\text{-}65}$ gene isolates, ESBL types that have been reported only for dogs in Asia (10, 24–28), was surprising. Furthermore, to our knowledge, this is the first report of $bla_{CTX\text{-}M\text{-}8}$ and $bla_{CTX\text{-}M\text{-}32}$ isolated from dogs. The two $bla_{CTX\text{-}M\text{-}8}$-carrying isolates originated from the same fecal sample; one also carried $bla_{TEM\text{-}1b}$. The seven $bla_{CTX\text{-}M\text{-}32}$-carrying isolates originated from 5 different fecal samples, which were not epidemiologically linked.

An explanation for the growth of ESBL-negative isolates on MCC could be the carriage of a chromosomal *ampC* gene with expression-enhancing mutations in the promoter region (29). Another explanation could be the presence of ESBL genes that were not screened for, e.g., PER, or the contribution of other mechanisms, such as enhanced expression of efflux pumps or pore deficiencies, in combination with a beta-lactamase-encoding gene.

The high concentrations of ESBL-producing *Enterobacteriaceae* in fecal samples and the high level of diversity of ESBL genes in combination with frequent shifts in fecal shedding illustrate the abundance of these bacteria in dogs and how easily ESBL-producing *Enterobacteriaceae* are acquired and presumably lost. As most dogs live in close contact with humans, transmission of these bacteria between dogs and humans seems plausible. However, to be able to show transmission, additional data are required. Also, no longitudinal data comprising the continuity of shedding of ESBL-producing *Enterobacteriaceae* in asymptomatic humans has been reported. These steps have to be made to assess the contribution of companion animals to exposure and possible risk of infection for human health.

The observation of matching ESBL gene combinations in several dogs in the same household at different time points led to a presumption of a common source or transmission between these dogs. A common source could serve as a risk factor for the uptake of ESBL-producing *Enterobacteriaceae*. Examples could be the type of food (as mentioned above), a shared walking environment, or a shared living environment.

To determine the potential clonality of ESBL-carrying isolates between clustered animals and animals with recurrent carriage of ESBL-carrying isolates, characterization of both plasmids and *E. coli* is essential. The more in-depth data required could be obtained by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) for both *E. coli* and plasmids, together with PCR-based replicon typing for plasmids.

Even though the sample size in this study was very small compared to the total dog population in the Netherlands, the selection of dogs that was included in the study was considered to be a relatively good representation of the Dutch dog population. The only inclusion criteria were a sampling history with no or very limited interruption and that all dogs were >6 months of age. However, since participation was on a voluntary basis, it should be taken into account that there is potential sampling bias.

In summary, this study showed a high prevalence of ESBL-producing *Enterobacteriaceae* among healthy dogs, with a high level of diversity of ESBL variants. Often, multiple ESBL variants were present in one fecal sample. However, there were frequent shifts in carriage, implying that the ESBLs were present in the gut for only a short period of time.

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