Extended-spectrum beta-lactamase producing *Enterobacteriaceae* in companion animals

Valérie Baede

Extended-spectrum beta-lactamase producing *Enterobacteriaceae* in companion animals

Honours programme 2013-2014

Thesis

Valérie Baede
3501493

Supervisor: Dr. Joost Hordijk

Faculty of Veterinary Medicine, Utrecht University
Department Infectious diseases and Immunology – Clinical Infectiology

Contents

Chapter 1

General Introduction

Penicillin was the first antibiotic drug, discovered in 1929 by Alexander Flemming. Many other antibiotics have been developed since, acting against bacteria through various mechanisms which lead to cell death or inhibition of replication. Nowadays, use of antibiotics is incorporated in our daily lives. However, higher levels of bacterial resistance against antibiotics are reported every day. The rapid emergence of antibiotic resistance threatens future use, leading to treatment failures already. For years, antibiotic resistance has been the main focus of many research projects contributing to our growing knowledge of this field.

Antibiotic resistance

In an evolutionary perspective, resistance mechanisms made survival of bacterial species possible, long before antibiotics were developed.[1] Since the use of antibiotics increased, natural selection pressed the spread of antibiotic resistance genes through bacterial populations. In the last decades, emergence of antibiotic resistance has been the focus of many research studies. Methicillin-resistance *Staphylococcus aureus*, or MRSA, is probably most well-known of all types of antibiotic resistant bacteria.

Resistance to antibiotic drugs can occur through various mechanisms.[2] An antibiotic drug can be degraded or modified by bacterial enzymes, hereby inactivating the antibiotic agent. Secondly, the antibiotic target, e.g. ribosomes, can be protected against interaction with the antibiotic agent by another molecule. This applies to both surface-exposed targets, as well as intracellular targets. Furthermore, the outer bacterial membrane can express lack of permeability, or absorbed antibiotic agents can be transported out of the bacterial cell actively, e.g. by efflux pumps. Resistance mechanisms can be present in bacteria naturally, i.e. intrinsic resistance, or acquired through genetic mutation or uptake of resistance genes. Generally, uptake of exogenous resistance genes takes place through transformation, transduction or conjugation. Transformation is the process of uptake of genetic elements out of the environment. In transduction, a bacteriophage is responsible for the transfer of DNA between bacteria. In conjugation, mobile genetic elements, e.g. plasmids, are exchanged between bacteria through direct contact. In general, plasmids contain genes that may be beneficial for bacterial survival, like metabolism enhancing genes, toxin-encoding genes as well as resistance genes. The benefit of acquiring the plasmid with accompanying genes spreads this plasmid and its genes through the bacterial population. Especially this way of genetic modification in bacteria is responsible for the spread of resistance genes.[3]

In this thesis, extended-spectrum beta-lactamase producing *Enterobacteriaceae* are the main subject of investigation. Extended-spectrum beta-lactamases (ESBL) are bacterial enzymes, capable of degrading beta-lactam antibiotics through hydrolysis. Beta-lactam antibiotics act on bacterial cell walls, where the beta-lactam ring is incorporated in the peptidoglycan layer causing an imbalance of this layer which leads to disruption of the bacterial cell wall. Beta-lactamases hydrolyse the beta-lactam ring (Figure 1), making beta-lactam antibiotics ineffective.[4] In contrast to beta-lactamases which target 1st and 2nd generations of cephalosporins and penicillins, targets of extended-spectrum beta-lactamases include 3rd and 4th generations of cephalosporins. ESBL function can be inhibited by clavulanic acid. ESBLs are mainly seen in *Enterobacteriaceae* isolates.

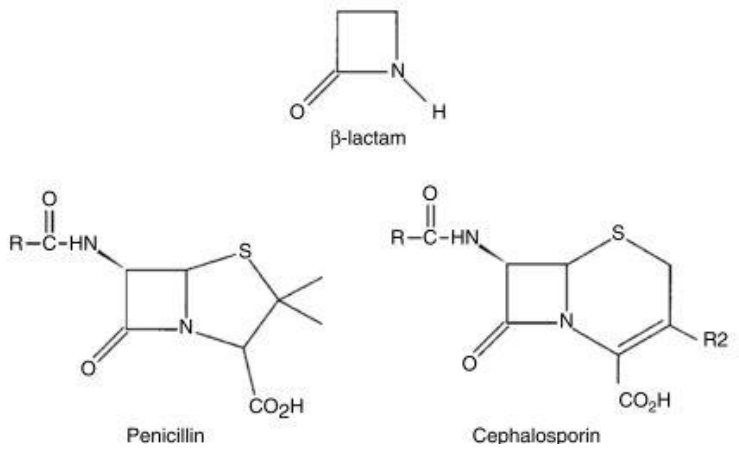


Figure 1. Prescott F in *Antimicrob. Ther. Vet. Med. (2013)* [5]

Extended-spectrum beta-lactamases

Bacterial isolates with an ESBL phenotype present themselves as resistant against penicillins and all generations of cephalosporins (e.g. cefotaxime and ceftazidime) combined with susceptibility to clavulanic acid in disk diffusion tests and are classified as beta-lactamase group 2be, as defined by Bush and Jacoby.[6] Ideally, the ESBL phenotype will be confirmed genotypically through PCR and/or sequence analysis of ESBL genes. In ESBL epidemiology, also AmpC enzymes are investigated, because of their resistance against cefotaxime. AmpC enzymes show resistance against penicillins and 1st, 2nd and 3rd generations of cephalosporins, but are not inhibited by clavulanic acid.[7] In this thesis, the term ESBL is used for both ESBLs and AmpC enzymes.

As mentioned above, plasmids are important in the spread of resistance genes. Therefore, plasmid-mediated ESBL genes are the focus of this research project.

A large amount of ESBL genes are mutated beta-lactamase-encoding genes, of the TEM and SHV gene families.[8] Especially, *bla*$_{\text{TEM-52,}}$ *bla*$_{\text{SHV-2}}$ and *bla*$_{\text{SHV-12}}$ are reported frequently.[9] Another important plasmid-mediated ESBL gene family, is the CTX-M gene family.[8] CTX-M genes are grouped into five major groups.[10] Genes classified as group 1 resemble *bla*$_{\text{CTX-M-1.}}$ In group 2, genes resembling *bla*$_{\text{CTX-M-2}}$ are gathered. Further, group 8 gathers genes resembling *bla*$_{\text{CTX-M-8}}$ and group 9 those resembling *bla*$_{\text{CTX-M-9.}}$ The last group contains CTX-M genes resembling *bla*$_{\text{CTX-M-25.}}$ Frequently reported are *bla*$_{\text{CTX-M-1,}}$ *bla*$_{\text{CTX-M-2,}}$ *bla*$_{\text{CTX-M-9,}}$ *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-15.}}$[9] Other beta-lactamase derivatives accepted in ESBL nomenclature are variants of OXA genes.[11] These include variants of *bla*$_{\text{OXA-1}}$, *bla*$_{\text{OXA-2}}$ and *bla*$_{\text{OXA-10}}$. As mentioned above, also several AmpC beta-lactamases are included. An important gene family belonging to the plasmid-mediated AmpC-type enzymes is the *bla*$_{\text{CMY}}$ genes. These were also included in this study due to their global spread and abundance in beta-lactam resistant strains.[9]

Global spread of ESBLs

The first ESBL-producing strain was isolated from a human clinical isolate in the 1980s, characterised as a SHV-2 carrying *Klebsiella pneumonia.*[12] Since then, ESBL-producing strains are disseminated around the world.

Carriage of ESBL-producing *Enterobacteriaceae* in the human community increased hugely since 2002, especially in South-East Asia, the Western Pacific and the Eastern Mediterranean.[13] Suggestions have been made about associations between ESBL carriage and farming activities or high population density. Furthermore, wastewater is recognized as a major reservoir for ESBL transmission.[13,14] In general, the environment plays an important role as reservoir of ESBL-producing

Enterobacteriaceae. Isolates were found in effluents from wastewater treatment plants, hospital wastewater and in direct surroundings of broiler chicken farms.[15–17]

In humans, ESBL-producing strains were isolated from clinical samples since 1980s, but only since 2001 from the community.[13] During this period, main isolated ESBL/*AmpC* types were CTX-M-15, CTX-M-14, SHV-12, CMY-2 and CTX-M-1.[9]

Only a few prevalence studies have been done in horses. Prevalence ranged from 6.3% in healthy horses to 34.2% in hospitalised horses.[18,19] In asymptomatic cattle, found prevalence ranged from 0.7% to 37% in Europe.[20–23] Low prevalence was shown in Japan; 1.5%.[24] In beef meat samples, a prevalence of 21.7% was found in Tunisia.[25] Prevalence found in asymptomatic pigs were similar to those in cattle, ranging from 2.5% to 32%.[23,26–31] A lot more prevalence studies were done in poultry, especially in Europe, reporting prevalence ranging from 1.7% to 44.7%.[32–39] Besides beef, ESBL-producing *Enterobacteriaceae* were also discovered in pork and chicken meat samples.[40–42]

In the Netherlands, herd prevalence of ESBL-producing *Enterobacteriaceae* was reported for both veal calves and broiler chickens. Herd prevalence in veal calves was 66%, with a within-farm prevalence up to 90%.[43] In broiler chickens, herd prevalence was 100%, with a within-farm prevalence up to 80%.[44] Differences in culture method explain the difference in prevalence between the studies mentioned above and the two Dutch studies, where selective enrichment was used for ESBL detection, resulting in a higher sensitivity.

Both inside and outside of Europe, average prevalence of ESBL-producing *Enterobacteriaceae* were lower in dogs and cats compared with average prevalence found in livestock. In Portugal, faecal samples originating from 39 healthy dogs and 36 healthy cats were investigated, ESBL-positive isolates were found in 10% of dogs and 3% of cats.[45] Later, Costa et al. (2008) found a lower prevalence of 2.6% in healthy dogs.[46] Carattoli et al. (2005) found 4 of 49 healthy dogs ESBL-positive in Italy.[47] Also low prevalence in ESBL-positive animals was found in Switzerland, 2.9% in dogs and 2% in cats. [48] In France, 16 of 90 companion animals in France, 14 dogs and 2 cats, produced ESBL-producing isolates. [49] Outside Europe, the situation seems similar. Harada et al. 2011 found 5.8% ESBL-positive isolates in healthy dogs in Japan.[50] In Canada, isolates derived from 4 of 188 dogs harbored a bla_{CMY-2} gene.[51] High prevalence of ESBL-shedding animals were found in Tunesia by Sallem et al. 2013, in 14.6% of healthy dogs and 20.5% in healthy cats.[52] Also in Africa, Albrechtova et al. 2014, investigated stray dogs in rural Angola and found a surprisingly 75% ESBL-positive animals.[53] Sun et al. 2010 found 24.5% ESBL-positive healthy companion animals in a study in China.[54]

In the Netherlands, 45% of healthy dogs was found positive for carriage of ESBL-producing *Enterobacteriaceae*.[55] All healthy cats in the same study were negative, but 12.5% of diarrheic cats showed ESBL-shedding. Although prevalence in companion animals overall is lower than in livestock, these reports show the relevance of companion animals in ESBL epidemiology.

Relevance for veterinary practice and public health

Before the prevalence of ESBL-producing *Enterobacteriaceae* in healthy companion animals was examined, several reports discussed the presence of these bacteria in veterinary clinical samples.[54,56–61] ESBL-producing *Enterobacteriaceae* are mainly seen in infections of the urinary tract and wounds. Infections with ESBL-producing *Enterobacteriaceae* request a special treatment and spread of the resistance strains should be prevented.

As companion animals live in close contact to their owners, the carriage of ESBL-producing *Enterobacteriaceae* in pets forms a risk of exposure to humans. This shows the importance of surveillance of ESBL-producing *Enterobacteriaceae* in companion animals and urges for further research to unravel the contribution of companion animals to public health threats with regard to ESBL-producing *Enterobacteriaceae*.

Objectives of this project

The aim of this project is to elucidate the role of companion animals in ESBL epidemiology. Four studies were carried out in the scope of this project to gain knowledge about the significance of ESBL-producing *Enterobacteriaceae* in dog and cat populations, and to examine the role of companion animals in relation to humans, other animals and the environment. Chapter 2 describes a longitudinal study in dogs, addressing colonisation dynamics, colonisation rates and dominant ESBL types to estimate the significance of ESBL-producing *Enterobacteriaceae* in dogs and the risk for exposure of ESBL-producing *Enterobacteriaceae* to dog owners. Chapter 3 describes a similar longitudinal study in cats, also addressing colonisation dynamics, colonisation rates and dominant ESBL types, to estimate the significance of ESBL-producing *Enterobacteriaceae* in cats, the difference in ESBL colonisation between dogs and cats and the risk for exposure of ESBL-producing *Enterobacteriaceae* to cat owners.

In order to investigate the role of companion animals in ESBL epidemiology, the interaction of companion animals with their surroundings in relation to spread of ESBL-producing *Enterobacteriaceae* should be addressed. Chapter 4 describes environmental contamination with ESBL-producing *Enterobacteriaceae* in operating theatres of veterinary clinics after admittance of ESBL-positive companion animals, to assess the risk for exposure of ESBL-producing *Enterobacteriaceae* to susceptible animals.

To further address the risk of exposure of ESBL-producing *Enterobacteriaceae* to companion animals, risk factors associated in relation to ESBL-carriage or ESBL-shedding should be determined. Chapter 5 describes a case-control study investigating the prevalence of ESBL-producing *Enterobacteriaceae* in cats with a raw-food diet, compared to a control group.

This project is embedded in the Strategic Infection Biology (SIB) research programme. It is part of research line 3: infection dynamics. The practical work was carried out at the department Infectious Diseases and Immunology – Clinical Infectiology.

References

1. D'Costa, V. M. *et al.* Antibiotic resistance is ancient. *Nature* **477,** 457–61 (2011).

2. Boerlin, P. & White, D. G. in *Antimicrob. Ther. Vet. Med.* 21–40 (2013).

3. Bennett, P. M. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *Br. J. Pharmacol.* **153 Suppl ,** S347–57 (2008).

4. Prescott, J. F. in *Antimicrob. Ther. Vet. Med.* 175–187 (2013).

5. Prescott, J. F. in *Antimicrob. Ther. Vet. Med.* 135–152 (2013).

6. Bush, K. & Jacoby, G. A. Updated functional classification of beta-lactamases. *Antimicrob. Agents Chemother.* **54,** 969–76 (2010).

7. Jacoby, G. a. AmpC beta-lactamases. *Clin. Microbiol. Rev.* **22,** 161–82, Table of Contents (2009).

8. Bradford, P. A. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev.* **14,** 933–951 (2001).

9. Ewers, C., Bethe, A., Semmler, T., Guenther, S. & Wieler, L. H. Extended-spectrum β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. *Clin. Microbiol. Infect.* **18,** 646–55 (2012).

10. Naseer, U. & Sundsfjord, A. The CTX-M conundrum: dissemination of plasmids and *Escherichia coli* clones. *Microb. Drug Resist.* **17,** 83–97 (2011).

11. Livermore, D. M. Defining an extended-spectrum beta-lactamase. *Clin. Microbiol. Infect.* **14 Suppl 1,** 3–10 (2008).

12. Kliebe, C., Nies, B. A., Meyer, J. F., Tolxdorff-Neutzling, R. M. & Wiedemann, B. Evolution of plasmid-coded resistance to broad-spectrum cephalosporins. *Antimicrob. Agents Chemother.* **28,** 302–307 (1985).

13. Woerther, P.-L., Burdet, C., Chachaty, E. & Andremont, A. Trends in human fecal carriage of extended-spectrum β-lactamases in the community: toward the globalization of CTX-M. *Clin. Microbiol. Rev.* **26,** 744–58 (2013).

14. Blaak, H. *et al.* Prevalence and characteristics of ESBL-producing *E. coli* in Dutch recreational waters influenced by wastewater treatment plants. *Vet. Microbiol.* **171,** 448–59 (2014).

15. Laube, H., Friese, A., von Salviati, C., Guerra, B. & Rösler, U. Transmission of ESBL/AmpC-producing *Escherichia coli* from broiler chicken farms to surrounding areas. *Vet. Microbiol.* **172,** 519–27 (2014).

16. Korzeniewska, E. & Harnisz, M. Extended-spectrum beta-lactamase (ESBL)-positive *Enterobacteriaceae* in municipal sewage and their emission to the environment. *J. Environ. Manage.* **128,** 904–11 (2013).

17. Korzeniewska, E. & Harnisz, M. Beta-lactamase-producing *Enterobacteriaceae* in hospital effluents. *J. Environ. Manage.* **123,** 1–7 (2013).

18. Dolejska, M. *et al.* Plasmids carrying blaCTX-M-1 and qnr genes in *Escherichia coli* isolates from an equine clinic and a horseback riding centre. *J. Antimicrob. Chemother.* **66,** 757–64 (2011).

19. Maddox, T. W. *et al.* Cross-sectional study of antimicrobial-resistant bacteria in horses. Part 1: Prevalence of antimicrobial-resistant *Escherichia coli* and methicillin-resistant Staphylococcus aureus. *Equine Vet. J.* **44,** 289–96 (2012).

20. Liebana, E. *et al.* Longitudinal Farm Study of Extended-Spectrum Beta-Lactamase-Mediated Resistance. *J. Clin. Microbiol.* **44,** 1630–1634 (2006).

21. Madec, J.-Y. *et al.* Prevalence of fecal carriage of acquired expanded-spectrum cephalosporin resistance in *Enterobacteriaceae* strains from cattle in France. *J. Clin. Microbiol.* **46,** 1566–7 (2008).

22. Wieler, L. H. *et al.* No evidence of the Shiga toxin-producing E. coli O104:H4 outbreak strain or enteroaggregative *E. coli* (EAEC) found in cattle faeces in northern Germany, the hotspot of the 2011 HUS outbreak area. *Gut Pathog.* **3,** 17 (2011).

23. Geser, N. *et al.* Fecal carriage of extended-spectrum β-lactamase-producing *Enterobacteriaceae* in swine and cattle at slaughter in Switzerland. *J. Food Prot.* **74,** 446–9 (2011).

24. Shiraki, Y., Shibata, N., Doi, Y. & Arakawa, Y. *Escherichia coli* producing CTX-M-2 beta-lactamase in cattle, Japan. *Emerg. Infect. Dis.* **10,** 69–75 (2004).

25. Jouini, A. *et al.* Characterization of CTX-M and SHV extended-spectrum beta-lactamases and associated resistance genes in *Escherichia coli* strains of food samples in Tunisia. *J. Antimicrob. Chemother.* **60,** 1137–41 (2007).

26. Blanc, V. *et al.* ESBL- and plasmidic class C beta-lactamase-producing *E. coli* strains isolated from poultry, pig and rabbit farms. *Vet. Microbiol.* **118,** 299–304 (2006).

27. Machado, E., Coque, T. M., Cantón, R., Sousa, J. C. & Peixe, L. Antibiotic resistance integrons and extended-spectrum beta-lactamases among *Enterobacteriaceae* isolates recovered from chickens and swine in Portugal. *J. Antimicrob. Chemother.* **62,** 296–302 (2008).

28. Gonçalves, A. *et al.* Genetic characterization of extended-spectrum beta-lactamases in *Escherichia coli* isolates of pigs from a Portuguese intensive swine farm. *Foodborne Pathog. Dis.* **7,** 1569–73 (2010).

29. Tian, G.-B. *et al.* Detection of CTX-M-15, CTX-M-22, and SHV-2 extended-spectrum beta-lactamases (ESBLs) in *Escherichia coli* fecal-sample isolates from pig farms in China. *Foodborne Pathog. Dis.* **6,** 297–304 (2009).

30. Kozak, G. K., Boerlin, P., Janecko, N., Reid-Smith, R. J. & Jardine, C. Antimicrobial resistance in *Escherichia coli* isolates from swine and wild small mammals in the proximity of swine farms and in natural environments in Ontario, Canada. *Appl. Environ. Microbiol.* **75,** 559–66 (2009).

31. Akujobi, C. O., Ogbulie, J. N. & Alisi, C. S. Occurrence of extended-spectrum β-lactamases in *Escherichia coli* isolated from piggery farms in Imo State, Nigeria. *World J. Microbiol. Biotechnol.* **24,** 2167–2170 (2008).

32. Brinas, L. *et al.* Detection of CMY-2, CTX-M-14, and SHV-12 -Lactamases in *Escherichia coli* Fecal-Sample Isolates from Healthy Chickens. *Antimicrob. Agents Chemother.* **47,** 2056–2058 (2003).

33. Girlich, D. *et al.* Extended-spectrum beta-lactamase CTX-M-1 in *Escherichia coli* isolates from healthy poultry in France. *Appl. Environ. Microbiol.* **73,** 4681–5 (2007).

34. Smet, A. *et al.* Diversity of extended-spectrum beta-lactamases and class C beta-lactamases among cloacal *Escherichia coli* Isolates in Belgian broiler farms. *Antimicrob. Agents Chemother.* **52,** 1238–43 (2008).

35. Costa, D. *et al.* Prevalence of extended-spectrum beta-lactamase-producing *Escherichia coli* isolates in faecal samples of broilers. *Vet. Microbiol.* **138,** 339–44 (2009).

36. Kolar, M. *et al.* ESBL and AmpC beta-lactamase-producing *Enterobacteriaceae* in poultry in the Czech Republic. *Vet. Med. (Praha).* **55,** 119–124 (2010).

37. Dierikx, C., van Essen-Zandbergen, A., Veldman, K., Smith, H. & Mevius, D. Increased detection of extended spectrum beta-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. *Vet. Microbiol.* **145,** 273–278 (2010).

38. Giufrè, M. *et al. Escherichia coli* of human and avian origin: detection of clonal groups associated with fluoroquinolone and multidrug resistance in Italy. *J. Antimicrob. Chemother.* **67,** 860–7 (2012).

39. Kojima, A. *et al.* Extended-spectrum-beta-lactamase-producing *Escherichia coli* strains isolated from farm animals from 1999 to 2002: report from the Japanese Veterinary Antimicrobial Resistance Monitoring Program. *Antimicrob. Agents Chemother.* **49,** 3533–7 (2005).

40. Leverstein-van Hall, M. a *et al.* Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clin. Microbiol. Infect.* **17,** 873–880 (2011).

41. Egervärn, M. *et al. Escherichia coli* with extended-spectrum beta-lactamases or transferable AmpC beta-lactamases and *Salmonella* on meat imported into Sweden. *Int. J. Food Microbiol.* **171,** 8–14 (2014).

42. Ojer-Usoz, E. *et al.* Prevalence of extended-spectrum β-lactamase-producing *Enterobacteriaceae* in meat products sold in Navarra, Spain. *Meat Sci.* **93,** 316–21 (2013).

43. Hordijk, J. *et al.* Cross-sectional study on prevalence and molecular characteristics of plasmid mediated ESBL/AmpC-producing *Escherichia coli* isolated from veal calves at slaughter. *PLoS One* **8,** e65681 (2013).

44. Dierikx, C. *et al.* Extended-spectrum-β-lactamase- and AmpC-β-lactamase-producing *Escherichia coli* in Dutch broilers and broiler farmers. *J. Antimicrob. Chemother.* **68,** 60–7 (2013).

45. Costa, D. *et al.* Detection of CTX-M-1 and TEM-52 beta-lactamases in *Escherichia coli* strains from healthy pets in Portugal. *J. Antimicrob. Chemother.* **54,** 960–1 (2004).

46. Costa, D. *et al.* Prevalence of antimicrobial resistance and resistance genes in faecal *Escherichia coli* isolates recovered from healthy pets. *Vet. Microbiol.* **127,** 97–105 (2008).

47. Carattoli, A. *et al.* Extended-spectrum beta-lactamases in *Escherichia coli* isolated from dogs and cats in Rome, Italy, from 2001 to 2003. *Antimicrob. Agents Chemother.* **49,** 833–5 (2005).

48. Gandolfi-Decristophoris, P., Petrini, O., Ruggeri-Bernardi, N. & Schelling, E. Extended-spectrum β-lactamase-producing *Enterobacteriaceae* in healthy companion animals living in nursing homes and in the community. *Am. J. Infect. Control* **41,** 831–5 (2013).

49. Poirel, L. *et al.* Extended-spectrum β-lactamase CTX-M-15-producing *Klebsiella pneumoniae* of sequence type ST274 in companion animals. *Antimicrob. Agents Chemother.* **57,** 2372–5 (2013).

50. Harada, K., Morimoto, E., Kataoka, Y. & Takahashi, T. Clonal spread of antimicrobial-resistant *Escherichia coli* isolates among pups in two kennels. *Acta Vet. Scand.* **53,** 11 (2011).

51. Murphy, C. *et al.* Occurrence of antimicrobial resistant bacteria in healthy dogs and cats presented to private veterinary hospitals in southern Ontario: A preliminary study. *Can. Vet. journal.* **50,** 1047–53 (2009).

52. Sallem, R. Ben *et al.* First detection of CTX-M-1, CMY-2, and QnrB19 resistance mechanisms in fecal *Escherichia coli* isolates from healthy pets in Tunisia. *Vector Borne Zoonotic Dis.* **13,** 98–102 (2013).

53. Albrechtova, K. *et al.* High Prevalence and Variability of CTX-M-15-Producing and Fluoroquinolone-Resistant *Escherichia coli* Observed in Stray Dogs in Rural Angola. *Microb. drug Resist.* doi:10.1089/mdr.2013.0177 (2014). doi:10.1089/mdr.2013.0177

54. Sun, Y. *et al.* High prevalence of blaCTX-M extended-spectrum β-lactamase genes in *Escherichia coli* isolates from pets and emergence of CTX-M-64 in China. *Clin. Microbiol. Infect.* **16,** 1475–81 (2010).

55. Hordijk, J. *et al.* High prevalence of fecal carriage of extended-spectrum β-lactamase/AmpC-producing *Enterobacteriaceae* in cats and dogs. *Front. Microbiol.* **4,** 242 (2013).

56. Teshager, T. *et al.* Isolation of an SHV-12 β -Lactamase-Producing *Escherichia coli* Strain from a Dog with Recurrent Urinary Tract Infections. *Antimicrob. Agents Chemother.* **44,** 3483–3484 (2000).

57. Sidjabat, H. E. *et al.* Identification of plasmid-mediated extended-spectrum and AmpC beta-lactamases in *Enterobacter* spp. isolated from dogs. *J. Med. Microbiol.* **56,** 426–34 (2007).

58. Ewers, C. *et al.* Emergence of human pandemic O25:H4-ST131 CTX-M-15 extended-spectrum-beta-lactamase-producing *Escherichia coli* among companion animals. *J. Antimicrob. Chemother.* **65,** 651–60 (2010).

59. O'Keefe, A., Hutton, T. A., Schifferli, D. M. & Rankin, S. C. First detection of CTX-M and SHV extended-spectrum beta-lactamases in *Escherichia coli* urinary tract isolates from dogs and cats in the United States. *Antimicrob. Agents Chemother.* **54,** 3489–92 (2010).

60. Shaheen, B. W. *et al.* Molecular characterization of resistance to extended-spectrum cephalosporins in clinical *Escherichia coli* isolates from companion animals in the United States. *Antimicrob. Agents Chemother.* **55,** 5666–75 (2011).

61. Pomba, C. *et al.* Within-lineage variability of ST131 *Escherichia coli* isolates from humans and companion animals in the south of Europe. *J. Antimicrob. Chemother.* **69,** 271–3 (2014).

Chapter 2

Extended-Spectrum Beta-Lactamase and AmpC-producing *Enterobacteriaceae* in household dogs; a longitudinal study

Valérie O. Baede[1], Jaap A. Wagenaar[1, 2], Els M. Broens[1], Birgitta Duim[1], Wietske Dohmen[3], Rolf Nijsse[1], Arjen J. Timmerman[1], Joost Hordijk[1]

1. Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, PO Box 80.165, 3508 TD Utrecht, the Netherlands
2. Central Veterinary Institute of Wageningen UR, PO Box 65, 8200 AB Lelystad, the Netherlands
3. Division of Environmental Epidemiology, Institute for Risk Assessment Sciences, Utrecht University, PO Box 80.178, 3508 TD Utrecht, the Netherlands

This chapter is modified from a manuscript submitted for publication

Abstract

Objectives

A longitudinal study was performed (i) to investigate persistence of extended-spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* in asymptomatic dogs, (ii) to identify dominant plasmid-mediated ESBL genes and (iii) to quantify ESBL-producing *Enterobacteriaceae* in faeces.

Methods

Faecal samples of 38 dogs were collected monthly for 6 months. From 7 included dogs, additional samples were collected on a weekly basis for a six-week period. CFU/g faeces were determined for non-wild-type *Enterobacteriaceae* on MacConkey agar supplemented with 1 mg/L cefotaxime and total number of *Enterobacteriaceae* on MacConkey agar. Cefotaxime-resistant isolates were screened by PCR and sequence analysis for presence of $bla_{\text{CTX-M}}$, bla_{CMY}, bla_{SHV}, bla_{OXA} and bla_{TEM} gene families. Species identification was carried out with MALDI-TOF MS analysis. PCR-negative isolates were tested by double disk synergy test for enhanced AmpC expression.

Results

259 samples were screened, of which 126 were culture-positive, resulting in 352 isolates. Nine dogs were continuously positive during this study and 6 dogs were continuously negative. Monthly or weekly shifts in faecal shedding were observed in 23 dogs. Genotyping showed a high variety of ESBL genes and gene combinations at single sampling moments and consecutive sampling moments. 327 isolates were *E. coli*. 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. CFU of non-wild-type *Enterobacteriaceae* was $6.11*10^8$ cfu/g faeces.

Conclusions

This study showed an abundance of ESBL-producing *Enterobacteriaceae* in asymptomatic dogs in the Netherlands, mostly in high concentrations. Faecal shedding showed to be highly dynamic over time which is important to consider when studying ESBL epidemiology.

Introduction

Extended-spectrum beta-lactamases are globally disseminated, throughout human and livestock population. So far, little is known about ESBL-producing *Enterobacteriaceae* in companion animals. Hordijk et al. showed high prevalences of ESBL-producing *Enterobacteriaceae* in Dutch companion animals, i.e. 45% in asymptomatic dogs and 55% in diarrheic dogs.[1] As companion animals live in close contact with humans, they might contribute substantially to the exposure of ESBL-producing *Enterobacteriaceae* to humans. Similar ESBL gene types, i.e. CTX-M-14, CTX-M-15, SHV-12, CMY-2, were found in strains originating from humans and companion animals.[2] Additionally, transmission between dogs and humans of CTX-M-15-carrying ST131 and ST648 *Escherichia coli* strains has been suggested.[3,4] Therefore the importance of investigating the role of dogs in the epidemiology of ESBL-producing *Enterobacteriaceae* is clear.

So far, data on ESBL-producing *Enterobacteriaceae* in companion animals has only been collected through cross-sectional studies. However, no data on persistence of colonisation with ESBL-producing *Enterobacteriaceae* in companion animals is available yet. Longitudinal information is essential to estimate the exposure of ESBL-producing *Enterobacteriaceae* to humans by colonised 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 (i) to investigate persistence of extended-spectrum beta-lactamase producing *Enterobacteriaceae* in asymptomatic dogs, (ii) to identify dominant plasmid-mediated ESBL genes and (iii) to quantify ESBL-producing *Enterobacteriaceae* in faecal samples.

Methods

Dogs

To assess persistence of colonisation in this longitudinal study, a minimum sample size of 25 dogs was calculated with a precision of 20%, a 95% confidence interval and an estimated prevalence of 50% among dogs, deduced from Hordijk *et al.*[1] Dogs were selected from a study of healthy dogs, which contributed monthly on a voluntary basis to a longitudinal survey of intestinal pathogens. All dogs were older than 6 months. Animal sampling was in accordance with the Dutch Law on Animal Health and Welfare, based on EU Directive 2010/63/EU. In total, 38 healthy dogs of 24 owners from different parts of the Netherlands were included in this study, to ensure enough power and engaging clustered dogs i.e. dogs housed in the same household. All owners returned an initial questionnaire and monthly questionnaires about their pet's health, drug usage, diet and other notable affairs (in open question to the owner). Faecal samples were collected monthly in the period July 2013 until January 2014. To distinguish between short and long term persistence, additional weekly faecal samples of a convenient number of 7 included dogs were collected for at least six consecutive weeks.

Faecal samples were either deposited at the institute or sent by regular mail service. Within this study an experiment was carried out to assess deterioration by the used transport method. Four freshly collected faecal samples from different dogs were packaged and mailed at different moments during the study according to the protocol. Sending took up to three days, but no differences in cfu/g faeces were measured after shipment compared to the number of cfu/g faeces determined before shipment (data not shown). It was therefore presumed that the used transport method had no influence on quantitative analysis of these samples.

Bacterial isolates

Of each sample 0.5 grams faeces was suspended in 4.5 ml 0.9% NaCl. To quantify viable ESBL-producing *Enterobacteriaceae*, the track dilution method as described by Jett *et al*. was used to inoculate square MacConkey agar plates (MC) (Oxoid, the Netherlands) and square MacConkey agar plates supplemented with 1 mg/L cefotaxime (MCC) (Oxoid, the Netherlands).[5] Six 10-folds serial dilutions in 0.9% NaCl were made and 20 µl of each dilution inoculated on MC and MCC, which

were then cultured overnight. Additionally, 100 µl of 10^{-1} dilution was inoculated in 1 ml LB-broth supplemented with 1 mg/L cefotaxime (LBC) (Oxoid, the Netherlands) for enrichment. After overnight incubation, 1 µl of LBC was streaked onto MCC and cultured overnight. All incubation steps were performed at 37 °C.

When, after direct inoculation of the sample, growth occurred on MCC, 3 typical *E. coli*-suspected, pink colonies were selected for further analysis. If applicable, morphological different pink colonies were selected, otherwise colonies were chosen randomly. In case of presence of colourless colonies, 1 colony was picked for every morphological different phenotype. When growth on MCC occurred after enrichment only, just one typical *E. coli*-suspected colony was selected. All isolates cultured on MCC were designated as isolates with a non-wild-type susceptibility to cefotaxime, according to Schwarz *et al*.[6]

The detection limit in this study was 10^2 cfu/g faeces. MC and MCC cfu/g values were calculated for each phenotypic positive faecal sample, based on number of colonies grown in the lowest dilution.

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 case of unidentifiable isolates, API identification system (BioMérieux SA, Marcy-l'Etoile, France) was used to identify bacterial species.

ESBL identification

Plasmid-mediated ESBL genes were the focus of this study. Boiled isolates with Chelex 100 Molecular Biology Grade Resin (BioRad, Veenendaal, the Netherlands) were screened by conventional PCR for presence of $bla_{\text{CTX-M}}$, bla_{CMY}, bla_{SHV}, bla_{OXA}, bla_{TEM} gene families using primers as listed in Table S1. Additional primers TEM-seq, CMY-F-838 and CMY-R-857 were used in sequence analysis. Also groups of bla_{OXA} with carbapenamase expression and chromosomal *ampC* gene were included. PCR mix consisted of 20 µL, containing 5 µL DNA lysate, 2x GoTaq Hotstart Green Master Mix (Promega Benelux BV, Leiden, the Netherlands), 0.5 µM of each forward and reverse primers and molecular grade water. Presence of ESBL genes was visualised by gel electrophoresis. PCR products were purified with ExoSAP-IT (Affymetrix, Santa Clara, USA) according to the manufacturer's protocol and subsequently sent for sequence analysis (Baseclear, Leiden, the Netherlands) to identify the ESBL genes. Sequences were compared to reference sequences provided on www.lahey.org (last accessed 09-07-2014) and analysed using Bionumerics v7.1 (Applied Maths NV, Sint-Martens-Latem, Belgium).

All ESBL-negative isolates were investigated with sequence analysis for presence of expression-enhancing mutations in promoter regions of chromosomal *ampC* genes to clarify growth on MCC. AmpC types were assigned according to Mulvey *et al*.[7] For confirmation of enhanced AmpC expression, double disk synergy tests were carried out when mutations in promoter regions of chromosomal *ampC* gene were found. A 0.5 McFarland suspension was made of each strain and these were subsequently inoculated on Mueller-Hinton agar (Oxoid, the Netherlands). BD BBL Sensi-Discs (Becton, Dickinson and Company, Breda, the Netherlands) were used, containing cefotaxime (30 µg), cefotaxime/clavunalate (30/10 µg), ceftazidime (30 µg), ceftazidime/clavunalate (30/10 µg) and cefoxitin (30 µg). Isolates showing less than 5 mm growth difference between cefotaxime and cefotaxime/clavunalate and ceftazidime and ceftazidime/clavunalate were considered AmpC phenotype, according to CLSI guidelines.[8] Cefoxitin was used for confirmation of the AmpC phenotype.

Results

Phenotypic results

A total of 259 faecal samples were analysed: 204 monthly faecal samples and 55 weekly faecal samples (Table 1). Ninety-five faecal samples showed growth directly on MacConkey agar supplemented with 1 mg/L cefotaxime (MCC) and 31 faecal samples showed growth after enrichment.

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

CFU/g faeces of MCC-positive *Enterobacteriaceae* and total count of *Enterobacteriaceae* were calculated for every faecal sample with non-wild-type isolates. The number of cfu/g and corresponding fractions in individual samples are given in supplementary Table S2. Mean cfu/g faeces of *Enterobacteriaceae* with non-wild-type phenotype 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 non-wild-type phenotype compared to total count of *Enterobacteriaceae* was 0.02 within a range of $2.00*10^{-9}$ to 1.00. Of 61 faecal samples, this fraction was equal to or higher than 0.001.

Species identification

The 126 MCC-positive faecal samples resulted in 352 isolates with non-wild-type susceptibility to cefotaxime. Species determination using MALDI-TOF MS identified 327 isolates as *E. coli*, 13 isolates as *Acinetobacter* spp, 8 isolates as *Pseudomonas* spp, 1 isolates as *Enterobacter* spp and 1 isolates as *Ochrobactrum* spp. Two isolates could not be identified by MALDI-TOF MS and were analysed with API 20 NE. This test identified one isolate as *Pseudomonas* spp and the species of one isolate could not be identified.

ESBL identification

ESBL genes were detected in 269 *E. coli* isolates. Fifty-seven 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 was PCR-negative for all screened genes. No ESBL genes were detected in 24 non-*E. coli* isolates. One isolate identified as *Acinetobacter radioresistens*, carried a $bla_{\text{OXA-23-like}}$ gene, with 2 functional mutations of substitutions of G to A on position +452 and A to G on position +578. Genotyping results are summarised in Table 2 for monthly samples and Table 3 for weekly samples.

Of 22 faecal samples, only one strain could be retrieved. Of these, 19 isolates were obtained through direct plating and 3 isolates after enrichment. Of the remaining 106 faecal 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 faecal sample. Also, consecutive faecal samples often had isolates with different ESBL gene combinations (Table 2).

Twenty-four cases with matching ESBL gene combinations were found at the same sampling within households, eight of these matches were seen in household 9 (Table 2). Dog 9A and 9C both had isolates harbouring $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1varA}}$ in the first month. At sampling moment 2, all three dogs had isolates harbouring a $bla_{\text{CTX-M-2-like}}$ gene. Dog 9A and 9B both showed isolates harbouring $bla_{\text{CTX-M-1}}$ at sampling moment 3. At sampling moment 4, this $bla_{\text{CTX-M-1}}$ was found in dog 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 harbouring *bla*$_{CTX-M-1.}$

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% a *bla*$_{CTX-M}$ gene of group 8.

One isolate harboured a mutant of *bla*$_{CMY-2}$ with a substitution of C to T on position +505. Another isolate, harboured both a *bla*$_{CMY-2}$ gene and a mutant of *bla*$_{TEM-33}$ (Genbank no. GU371926) with a substitution of A to G on position +820. Effect of this functional mutation has not been phenotypically confirmed.

Eight isolates, originating from 5 different dogs in 4 different households, carried a variant of *bla*$_{TEM-1}$ (*bla*$_{TEM-1varA}$) combined with *bla*$_{CTX-M-15.}$ The *bla*$_{TEM-1varA}$ variant showed a synonymous mutation of C to T on position +537 compared to reference *bla*$_{TEM-1b}$ (GenBank AB263754). Another 2 isolates, originating from a dog from a different household, harboured the same gene variant in combination with *bla*$_{TEM-1b}$. Furthermore, one isolate from yet another household, harboured only the *bla*$_{TEM-1varA}$ variant.

Of 57 ESBL-negative *E. coli* isolates, 39 isolates carried chromosomal *amp*C types with mutations in the promoter region. One *E. coli* isolate, was PCR-negative when screening for the chromosomal *ampC* gene, but showed an AmpC phenotype. Another isolate was identified as *ampC*-WT. Thirty-seven isolates carried an *ampC*-3-type variant, two isolates an *ampC*-18-variant and one isolate an *ampC*-11 variant, as designated by Mulvey *et al*.[7] Three new *ampC* types were found. All new variants showed most resemblance to *ampC* type-3.[7] One new variant showed a substitution of G to A on position +23 compared to *ampC* type-3. Another new variant showed a substitution of C to A on position +31 compared to *ampC* type-3. The third new variant showed a deletion of G on position +32 compared to *ampC* type-3. Enhanced AmpC expression could be confirmed with disk diffusion tests for all *ampC*-mutants, except three isolates that were genotypically confirmed as *ampC*-11 and *ampC*-18. One *ampC*-11 and one *ampC*-18 isolate were both confirmed by disk diffusion tests as ESBL. The second *ampC*-18 isolate could not be confirmed phenotypically by disk diffusion and was therefore designated as false positive.

Discussion

In several countries, cross-sectional studies were carried out to investigate ESBL-producing *Enterobacteriaceae* in companion animals. Prevalence of ESBL-producing *Enterobacteriaceae* found in most of these studies was below 20%.[9–15] Based on a single point in time, 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 as reported by Hordijk *et al.*[1] However, considering this entire longitudinal study, 82% of participating dogs were at least once ESBL-positive. 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 faecal shedding of ESBL-producing *Enterobacteriaceae* in dogs. Within the study presented here, faecal samples were both collected with weekly and monthly time intervals, to distinguish between short and long term persistence and shifts. This led to the observation of shifts in faecal shedding of ESBL-producing *Enterobacteriaceae* in the majority of screened dogs. Weekly screened dogs showed short term shifts, as various dogs showed 3 or more shifts in a six-week period. This could not have been observed in monthly screening of samples only. The observed shifts in ESBL shedding may be due to significant differences in colonisation or even uptake and loss of strains, caused by factors that still need to be identified. High fractions of ESBL-producing *Enterobacteriaceae* compared to total count of

Enterobacteriaceae were found, 0.02 on average, 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, also the ESBL genes found in consecutive MCC- positive faecal samples differed often. An explanation for this variation could be a difference in relative proportion for each type present in the gut. While screening consecutive faecal samples, new ESBL types were found at almost every consecutive sampling moment. This supports the uptake of new strains and loss of strains with different ESBL types more than differences in colonisation with recurrence of previous types.

The shifts in faecal shedding and the high variety of ESBL genes as shown in this longitudinal study demonstrate the complexity of ESBL epidemiology, which means that e.g. estimation of risk factors for being ESBL-positive cannot be determined easily.

The observation of matching ESBL genes combinations in several dogs in the same household at different time point, led to a presumption of a common source or transmission between these dogs. A common source could serve as a risk factor for uptake of ESBL-producing *Enterobacteriaceae*. Examples could be feed, a shared walking environment or a shared living environment. As the number of raw food eaters in this study was relatively high (21/38 dogs), feed should be considered as a common source for uptake of ESBL-producing *Enterobacteriaceae*. As mentioned above, phenotypic shifts and gene characterisation showed the complexity of ESBL epidemiology and high diversity of ESBL types. To determine potential clonality of ESBL-carrying isolates between clustered animals, plasmid characterisation and determination of *E. coli* sequence types and phylogenetic groups is needed. Nevertheless, this may very well lead to an even higher diversity.

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.[2] Surprising were the findings of three $bla_{\text{CTX-M-55/57}}$ isolates and two $bla_{\text{CTX-M-65}}$ gene isolates, ESBL types that are only reported in dogs in Asia.[16–21] Furthermore, to our knowledge, this is the first report of $bla_{\text{CTX-M-8}}$ and $bla_{\text{CTX-M-32}}$ isolated from dogs. The two b$la_{\text{CTX-M-8}}$ carrying isolates originated from the same faecal sample, one also carried $bla_{\text{TEM-1b}}$. The seven $bla_{\text{CTX-M-32}}$ carrying isolates originated from 5 different faecal samples, which were not epidemiologically linked.

Another interesting finding was the detection of a $bla_{\text{TEM-1}}$ variant ($bla_{\text{TEM-1varA}}$) in combination with $bla_{\text{CTX-M-15}}$ in 10 isolates. The presence of this gene combination in six epidemiologically unrelated dogs may suggest the existence of a specific clone in the dog population.

No carbapenamase-producing *Enterobacteriaceae* originating from animals have been found in the Netherlands yet. All isolates collected in this study were screened for carriage of carbapenemase-encoding bla_{OXA} genes, including groups OXA-23, OXA-24, OXA-51 and OXA-58. Only one isolate, identified as *Acinetobacter radioresistens*, carried a $bla_{\text{OXA-23-like}}$ gene. Expression of carbapenamase could not be confirmed with disk diffusion test containing imipenem, meropenem, ertapenem, cloxacillin and aztreonam, which may be explained by two functional mutations in the gene.

An explanation for growth on MCC of ESBL-negative isolates could be the carriage of a chromosomal *ampC* gene with expression-enhancing mutations in the promoter region. In *amp*C type-18 mutations led to an alternate displaced promoter and in *amp*C type-11 mutations were located outside of the promoter region, which means overexpression of AmpC is not seen in these *amp*C types.[22] Therefore isolates harbouring these *amp*C types could not be confirmed as AmpC phenotype, but the isolates were confirmed as ESBL phenotype by double disk synergy tests. Growth on MCC by these isolates can be explained by the carriage of an unscreened ESBL gene, e.g. PER, or contribution of other mechanisms, as enhanced expression of efflux pumps or pore deficiencies, in combination with a beta-lactamase encoding gene. Two ESBL PCR-negative isolates, the isolates harbouring *amp*C-WT and *amp*C type-18, could not be confirmed in having an ESBL or AmpC phenotype using double disk synergy test and were therefore designated as false positive.

Conclusion

The high concentrations of ESBL-producing *Enterobacteriaceae* in faecal samples and high diversity of ESBL genes in combination with frequent shifts in faecal 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 is required. Also, no longitudinal data comprising persistence of ESBL-producing *Enterobacteriaceae* in asymptomatic humans has been reported. These steps have to be made to assess the contribution of companion animals in exposure and possible risk of infection for human health.

References

1. Hordijk, J. *et al.* High prevalence of fecal carriage of extended-spectrum β-lactamase/AmpC-producing *Enterobacteriaceae* in cats and dogs. *Front. Microbiol.* **4,** 242 (2013).

2. Ewers, C., Bethe, A., Semmler, T., Guenther, S. & Wieler, L. H. Extended-spectrum β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. *Clin. Microbiol. Infect.* **18,** 646–55 (2012).

3. Ewers, C. *et al.* Emergence of human pandemic O25:H4-ST131 CTX-M-15 extended-spectrum-beta-lactamase-producing *Escherichia coli* among companion animals. *J. Antimicrob. Chemother.* **65,** 651–60 (2010).

4. Ewers, C. *et al.* CTX-M-15-D-ST648 *Escherichia coli* from companion animals and horses: another pandemic clone combining multiresistance and extraintestinal virulence? *J. Antimicrob. Chemother.* **69,** 1224–30 (2014).

5. Jett, B. D., Hatter, K. L., Huycke, M. M. & Gilmore, M. S. Simplified agar plate method for quantifying viable bacteria. *Biotechniques* **23,** 648–50 (1997).

6. Schwarz, S. *et al.* Editorial: assessing the antimicrobial susceptibility of bacteria obtained from animals. *J. Antimicrob. Chemother.* **65,** 601–4 (2010).

7. Mulvey, M. R. *et al.* Molecular Characterization of Cefoxitin-Resistant *Escherichia coli* from Canadian Hospitals. *Antimicrob. Agents Chemother.* **49,** 358–365 (2005).

8. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twentieth Informational Supplement M100-S20. CLSI, Wayne, PA, USA, 2010. **30,**

9. Costa, D. *et al.* Detection of CTX-M-1 and TEM-52 beta-lactamases in *Escherichia coli* strains from healthy pets in Portugal. *J. Antimicrob. Chemother.* **54,** 960–1 (2004).

10. Costa, D. *et al.* Prevalence of antimicrobial resistance and resistance genes in faecal *Escherichia coli* isolates recovered from healthy pets. *Vet. Microbiol.* **127,** 97–105 (2008).

11. Carattoli, A. *et al.* Extended-spectrum beta-lactamases in *Escherichia coli* isolated from dogs and cats in Rome, Italy, from 2001 to 2003. *Antimicrob. Agents Chemother.* **49,** 833–5 (2005).

12. Gandolfi-Decristophoris, P., Petrini, O., Ruggeri-Bernardi, N. & Schelling, E. Extended-spectrum β-lactamase-producing *Enterobacteriaceae* in healthy companion animals living in nursing homes and in the community. *Am. J. Infect. Control* **41,** 831–5 (2013).

13. Poirel, L. *et al.* Extended-spectrum β-lactamase CTX-M-15-producing *Klebsiella pneumoniae* of sequence type ST274 in companion animals. *Antimicrob. Agents Chemother.* **57,** 2372–5 (2013).

14. Harada, K., Morimoto, E., Kataoka, Y. & Takahashi, T. Clonal spread of antimicrobial-resistant *Escherichia coli* isolates among pups in two kennels. *Acta Vet. Scand.* **53,** 11 (2011).

15. Murphy, C. *et al.* Occurrence of antimicrobial resistant bacteria in healthy dogs and cats presented to private veterinary hospitals in southern Ontario: A preliminary study. *Can. Vet. journal.* **50,** 1047–53 (2009).

16. Sun, Y. *et al.* High prevalence of blaCTX-M extended-spectrum β-lactamase genes in *Escherichia coli* isolates from pets and emergence of CTX-M-64 in China. *Clin. Microbiol. Infect.* **16,** 1475–81 (2010).

17. Tamang, M. D. *et al.* Molecular characterization of extended-spectrum-β-lactamase-producing and plasmid-mediated AmpC β-lactamase-producing *Escherichia coli* isolated from stray dogs in South Korea. *Antimicrob. Agents Chemother.* **56,** 2705–12 (2012).

18. Harada, K., Niina, A., Nakai, Y., Kataoka, Y. & Takahashi, T. Prevalence of antimicrobial resistance in relation to virulence genes and phylogenetic origins among urogenital *Escherichia coli* isolates from dogs and cats in Japan. *Am. J. Vet. Res.* **73,** 409–17 (2012).

19. Lv, L. *et al.* Genetic characterization of IncI2 plasmids carrying blaCTX-M-55 spreading in both pets and food animals in China. *Antimicrob. Agents Chemother.* **57,** 2824–7 (2013).

20. Choi, M. J., Lim, S. K., Jung, S. C. & Ko, K. S. Comparisons of CTX-M-Producing *Escherichia coli* Isolates from Humans and Animals in South Korea. *J. Bacteriol. Virol.* **44,** 44–51 (2014).

21. Hou, J. *et al.* Dissemination of the fosfomycin resistance gene fosA3 with CTX-M β-lactamase genes and rmtB carried on IncFII plasmids among *Escherichia coli* isolates from pets in China. *Antimicrob. Agents Chemother.* **56,** 2135–2138 (2012).

22. Tracz, D. M. *et al.* ampC gene expression in promoter mutants of cefoxitin-resistant *Escherichia coli* clinical isolates. *FEMS Microbiol. Lett.* **270,** 265–71 (2007).

23. Dierikx, C., van Essen-Zandbergen, A., Veldman, K., Smith, H. & Mevius, D. Increased detection of extended spectrum beta-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. *Vet. Microbiol.* **145,** 273–278 (2010).

24. Carattoli, A. *et al.* Molecular epidemiology of *Escherichia coli* producing extended-spectrum beta-lactamases isolated in Rome, Italy. *J. Clin. Microbiol.* **46,** 103–108 (2008).

25. Jiang, X. *et al.* Detection of extended-spectrum beta-lactamases in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **50,** 2990–2995 (2006).

26. Paauw, A., Fluit, A. C., Verhoef, J. & Leverstein-van Hall, M. A. *Enterobacter cloacae* outbreak and emergence of quinolone resistance gene in Dutch hospital. *Emerg. Infect. Dis.* **12,** 807–812 (2006).

27. Dierikx, C. M. *et al.* Occurrence and characteristics of extended-spectrum-β-lactamase- and AmpC-producing clinical isolates derived from companion animals and horses. *J. Antimicrob. Chemother.* **67,** 1368–1374 (2012).

28. Hordijk, J. *et al.* Within-farm dynamics of ESBL/AmpC-producing *Escherichia coli* in veal calves: a longitudinal approach. *J. Antimicrob. Chemother.* **68,** 2468–76 (2013).

29. Voets, G. M., Fluit, A. C., Scharringa, J., Cohen Stuart, J. & Leverstein-van Hall, M. A. A set of multiplex PCRs for genotypic detection of extended-spectrum β-lactamases,

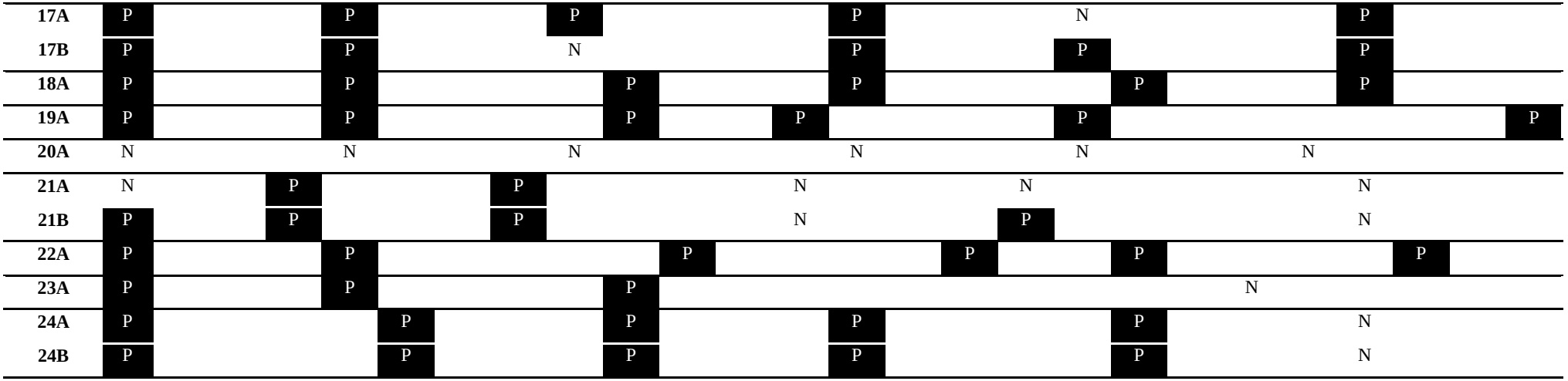
carbapenemases, plasmid-mediated AmpC β-lactamases and OXA β-lactamases. *Int. J. Antimicrob. Agents* **37,** 356–359 (2011).

30. Aubert, D., Naas, T., Héritier, C., Poirel, L. & Nordmann, P. Functional characterization of IS1999, an IS4 family element involved in mobilization and expression of beta-lactam resistance genes. *J. Bacteriol.* **188,** 6506–6514 (2006).

31. Caroff, N., Espaze, E., Bérard, I., Richet, H. & Reynaud, A. Mutations in the ampC promoter of *Escherichia coli* isolates resistant to oxyiminocephalosporins without extended spectrum ß-lactamase production. *FEMS Microbiol. Lett.* **173,** 459–465 (1999).

Table 1. Time points of analysis of faecal samples and results of culturing on MC and MCC

	t (weeks)																								
DogID	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				



P, faecal sample with non-wild-type susceptible colonies; N, faecal sample with wild-type colonies. Dogs with the same numbers in dogID shared the same household.

Table 2. Genotypic ESBL characteristics of non-wild-type isolates produced by monthly collected faecal samples

	t(months)					
DogID	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&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&52c		CTX-M-15 CMY-2 (2)	CTX-M-15 (3)	SHV-2 SHV-12 TEM-1b&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-2mutant NE(1)	CTX-M-15 (3)

15B	TEM-1b&52c (3)	x	x	CTX-M-1		
15C	CTX-M-1 CTX-M-14 CMY-2 TEM-1b&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&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&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&135 (2) OXA-23-like			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

x, faecal sample without growth on MCC; NE, ESBL-negative isolates with non-wild-type susceptibility to cefotaxime. Dogs with the same numbers in dogID shared the same household. If a faecal sample produced >1 isolate containing similar genes, the number of isolates is shown in brackets. 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, CTX-M-14/18 is shown as CTX-M-14, CMY 2/61 is shown as CMY-2, SHV-12/129 is shown as SHV-12

Table 3. Genotypic ESBL characteristics of non-wild-type isolates produced by weekly collected faecal samples

	t(weeks)																
DogID	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	x		
12A	x	x	CTX-M-1 SHV-12 (3)			CMY-2 NE(3)	x	x	CTX-M-1 (3)	CMY-2 TEM-33mut	x	x	x			x	
12B	x	x	x			NE(3)	NE(3)	CMY-2	x	x	x	x	x			x	

X, faecal sample without growth on MCC; NE, ESBL-negative isolates with non-wild-type susceptibility to cefotaxime. Dogs with the same numbers in dogID shared the same household. If a faecal sample produced >1 isolate containing similar genes, the number of isolates is shown in brackets. 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, CTX-M-14/18 is shown as CTX-M-14, CMY 2/61 is shown as CMY-2, SHV-12/129 is shown as SHV-12

Table 4. Frequency of characterised gene combinations originating from non-wild-type isolates

Gene combination	#	
$bla_{\text{CTX-M}}$	90	
$bla_{\text{CTX-M}}$ + $bla_{\text{TEM(ESBL)}}$	1	
$bla_{\text{CTX-M}}$ + $bla_{\text{TEM(beta-lactam)}}$	70	Including 1 isolate also containing bla_{CMY}
$bla_{\text{CTX-M}}$ + bla_{OXA}	11	
$bla_{\text{CTX-M}}$ + bla_{OXA} + $bla_{\text{TEM(beta-lactam)}}$	7	
bla_{SHV}	22	
bla_{SHV} + $bla_{\text{TEM(beta-lactam)}}$	7	
bla_{CMY}	25	
bla_{CMY} + $bla_{\text{TEM(beta-lactam)}}$	11	
$bla_{\text{TEM(ESBL)}}$	25	
Other*	81	

* $bla_{\text{TEM(beta-lactam)}}$, $bla_{\text{OXA(Carbapenamase)}}$, *ampC* promoter mutants with enhanced expression or unidentified (25)

Chapter 3

Extended-Spectrum Beta-Lactamase producing *Enterobacteriaceae* in household cats; a longitudinal study

Valérie O. Baede[1], Joost Hordijk[1]

1. Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, the Netherlands

Abstract

Objectives

A longitudinal study was performed (i) to investigate variation in time of faecal shedding of extended-spectrum beta-lactamase producing *Enterobacteriaceae* in cats, (ii) to identify dominant plasmid-mediated ESBL genes and (iii) to quantify ESBL-producing *Enterobacteriaceae* in cat faeces.

Methods

In a period of six months, monthly faecal samples of 23 cats were screened for ESBL-producing *Enterobacteriaceae* by culturing on MacConkey agar supplemented with 1 mg/L cefotaxime. Additionally, 6 weekly collected faecal samples from 13 cats were screened. CFU/g faeces were determined for non-wild-type *Enterobacteriaceae* on MacConkey agar supplemented with 1 mg/L cefotaxime and total number of *Enterobacteriaceae* on MacConkey agar. Cefotaxime-resistant isolates were screened by PCR and sequence analysis for presence of $bla_{\text{CTX-M}}$, bla_{CMY}, bla_{SHV}, bla_{OXA} and bla_{TEM} gene families. Species identification was carried out with MALDI-TOF MS analysis.

Results

10 of 189 collected feline faecal samples showed growth on MacConkey agar supplemented with 1 mg/L cefotaxime. Mean CFU/g faeces of non-wild-type *Enterobacteriaceae* was $7.52*10^5$ cfu/g. 24 of 25 isolates originating from cats were *E. coli*. In 15 isolates, $bla_{\text{CMY-2}}$ was found. Also one isolate harbouring $bla_{\text{CTX-M-55}}$ was found. Additionally, two canine samples and two feed samples were screened; one canine and three feed isolates were obtained. One feed isolate harboured $bla_{\text{CTX-M-55}}$ simultaneously with the corresponding feline isolate.

Conclusions

Variation over time in ESBL-shedding and concentrations of ESBL-producing *Enterobacteriaceae* in cats are low. Feed may play a role in uptake of ESBL-producing *Enterobacteriaceae*.

Introduction

In ESBL epidemiology, the role of companion animals is poorly understood. Prevalence of ESBL-producing *Enterobacteriaceae* in the Dutch dog population is high.[1] A longitudinal study in asymptomatic dogs showed a large variety over time in ESBL-shedding, high concentrations of ESBL-producing *Enterobacteriaceae* and high diversity of ESBL types in dogs (Chapter 2). As pets live in close contact with their owner, the contribution of cats and dogs to exposure of ESBL-producing *Enterobacteriaceae* to humans should be investigated thoroughly.

Hordijk *et al*. showed a prevalence of 0% in healthy cats in the Netherlands.[1] However, ESBL-producing *Enterobacteriaceae* have been found in healthy cats in several other studies.[2–6] As ESBL-shedding was shown to be highly dynamic over time in a longitudinal study in dogs, it was suggested that cross-sectional studies may cause a distorted view addressing ESBL epidemiology (Chapter 2). As all the studies mentioned above investigated ESBL-producing *Enterobacteriaceae* in cats based on a cross-sectional study design, occurrence of shifts in ESBL-shedding in cats could not be shown and therefore the role of cats in ESBL epidemiology is still unclear.

A longitudinal study was performed (i) to investigate variation in time of faecal shedding of extended-spectrum beta-lactamase producing *Enterobacteriaceae* in asymptomatic cats, (ii) to identify dominant plasmid-mediated ESBL genes and (iii) to quantify ESBL-producing *Enterobacteriaceae* in cat faeces.

Methods

23 asymptomatic cats from different parts of the Netherlands were included in this study. Eleven cats were male, 12 were female. Age ranged from 4.5 months up to 15 years, at the start of the study. Two households participated with a pair of cats, therefore these cats were epidemiologically clustered. An initial questionnaire and subsequent monthly questionnaires about the cat's health, drug usage, diet and other notable affairs were returned by all cat owners. Faecal samples were collected monthly in the period November 2013 until April 2014. Additionally, 6 consecutive weekly faecal samples of 13 cats were collected within this study, to observe weekly shifts in faecal shedding of ESBL-producing *Enterobacteriaceae*. Animal sampling was in accordance with the Dutch Law on Animal Health and Welfare, based on EU Directive 2010/63/EU.

Faecal samples were deposited at the institute or sent by regular mail service as described in Chapter 2. Culturing of faecal samples, calculation of CFU/g faeces on MC and MCC, species identification, genotyping and double disk synergy testing was also carried out as described in Chapter 2. All isolates cultured on MCC were designated as isolates with a non-wild-type susceptibility to cefotaxime, according to Schwarz *et al*.[7]

Within this study, feed and clustered animals of one consecutive ESBL-positive cat were investigated for the presence or shedding of ESBL-producing *Enterobacteriaceae.* Culturing of the faecal samples was carried out as mentioned above. Broth enrichment of 0.5 g feed and subsequent inoculation on MCC was used for culturing of feed samples. The same criteria as mentioned above were used for selection of bacterial isolates obtained from faecal and feed samples. Obtained isolates were included in species identification and genotyping.

Results

Phenotypic results

Twenty-three cats were included in this study. A total of 189 faecal samples originating from household cats were cultured on MC and MCC, 129 faecal samples were collected at monthly sampling moment and another 60 samples were collected in weekly sampling moments (Table 1).

Ten faecal samples showed growth on MacConkey agar supplemented with 1 mg/L cefotaxime. Eight of these MCC-positive samples originated from cat 9A, of which 6 samples were collected with weekly time intervals. Other MCC-positive samples originated from cat 4A and cat 6A.

Of every faecal sample with non-wild-type isolates, CFU/g faeces was calculated for both MC and MCC. Mean CFU/g faeces of *Enterobacteriaceae* with non-wild-type phenotype was $7.52*10^5$ cfu/g within a range of $1.00*10^2$ to $5.45*10^6$ cfu/g. The average fraction of *Enterobacteriaceae* with non-wild-type phenotype compared to total count of *Enterobacteriaceae* was 0.001 within a range of 0.00002 to 0.004.

Of 179 MCC-negative feline faecal samples, 24 faecal samples did not show growth of *Enterobacteriaceae* on MC either.

Besides cat 9A, household 9 also owned two dogs. Within this study, both dogs (9B and 9C) were investigated on shedding of ESBL-producing *Enterobacteriaceae* by culturing of one faecal sample in week 7. Also the cat's feed (9D) was cultured in week 17 and week 20. One dog sample, of dog 9B, was MCC-positive. Also both feed samples were MCC-positive.

Species identification

In total, 29 isolates with non-wild-type susceptibility to cefotaxime were obtained (Table 2). Twenty-five isolates originated from feline faecal samples, one isolate from a canine faecal sample and three isolates from feed samples. Bacterial species were determined with MALDI-TOF. Twenty-five isolates were identified as *E. coli*. Two isolates were identified as *Pseudomonas* spp. One isolate was determined as *Myroides* spp and the species of one isolate could not be identified.

ESBL identification

ESBL genes were detected in 24 *E. coli* isolates. One remaining *E. coli* isolate harboured an *ampC* gene with expression-enhancing promoter region mutations. No ESBL genes were found in non-*E. coli* isolates. The most frequently found ESBL gene in 15 feline isolates was $bla_{\text{CMY-2}}$. Three isolates expressed $bla_{\text{TEM-1b}}$ combined with ESBL gene $bla_{\text{CMY-2}}$ or $bla_{\text{SHV-2}}$ (Table 2). From three faecal samples, isolates with different ESBL genes could be obtained.

Discussion

Hordijk *et al*. reported low prevalence of ESBL-producing *Enterobacteriaceae* in household cats.[1] This longitudinal study also showed low prevalence, only 9 of 189 feline faecal samples were ESBL-positive. From a tenth feline faecal sample, an isolate with non-wild-type susceptibility to cefotaxime could be obtained, but this isolate was PCR-negative for the screened ESBL genes.

Mean cfu/g faeces of *Enterobacteriaceae* with non-wild-type phenotype was $7.52*10^5$ in cats, whereas the fraction compared to total count of *Enterobacteriaceae* was 0.001. These figures are much lower when compared to mean cfu/g of *Enterobacteriaceae* with non-wild-type phenotype and fraction compared to total count of *Enterobacteriaceae* in dogs (Chapter 2). Also 24 of 179 MCC-negative faecal samples did not show any growth of *Enterobacteriaceae* on MC. This suggests that average numbers of *Enterobacteriaceae* in faeces are lower in cats than in dogs.

Surprising was the founding of a $bla_{\text{CTX-M-55}}$ gene in one cat isolate. Previously, this ESBL gene was mainly found in Asia.[2,8] Recently, this gene has also been reported in the Netherlands, in 2 human isolates and 1 calf isolate and in a longitudinal ESBL study in dogs (Chapter 2).[9]

Table 1. Time points of faecal sample analysis and results of culturing on MCC

		t (weeks)																								
SampleID		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
Cat	1A	N	N	N	N	N	N					N				N					N				N		
Cat	2A			N	N	N	N	N	N			N				N				N				N			
Cat	3A			N	N	N	N	N	N	N			N				N					N					
Cat	4A	N	N	N	N	N	P	N					N				N				N	N				N	
Cat	5A			N	N	N	N	N	N				N				N					N					N
Cat	6A				N				N						N			N					P				N
Cat	6B				N				N						N			N						N			N
Cat	7A		N	N	N	N	N	N				N				N						N			N		
Cat	8A			N	N	N	N	N	N				N				N					N				N	
Cat	9A		P	P	P	P	P	P				P						P			N						N
Dog	9B							P																			
Dog	9C							N																			
Feed	9D																	P			P						
Cat	10A			N	N	N	N	N	N				N						N		N						
Cat	11A		N	N	N	N	N	N					N				N					N				N	
Cat	12A		N	N	N	N	N	N						N				N			N						N
Cat	13A				N				N				N					N			N					N	
Cat	14A			N					N			N						N			N					N	
Cat	15A		N				N					N				N				N					N		
Cat	16A			N	N	N	N	N	N						N			N					N				
Cat	17A			N				N				N					N					N			N		
Cat	18A			N					N						N				N				N		N		
Cat	19A		N				N						N			N					N					N	
Cat	20A			N		N	N	N			N	N	N						N		N	N	N		N	N	N
Cat	21A						N																		N		
Cat	21B						N						N		N						N				N		

P, faecal samples with non-wild-type susceptible colonies; N, faecal sample with wild-type colonies. Samples with the same numbers in sampleID originated from the same household.

Table 2. ESBL gene characteristics of non-wild-type isolates

SampleID	Origin	t-value	ESBL/*amp*C type [+]	Species
9A	Cat	2	CMY 2	*Escherichia coli*
9A	Cat	2	CMY 2	*Escherichia coli*
9A	Cat	2	CMY 2	*Escherichia coli*
9A	Cat	3	CMY 2	*Escherichia coli*
9A	Cat	3	SHV 12	*Escherichia coli*
9A	Cat	3	SHV 12 TEM 1b	*Escherichia coli*
9A	Cat	3	CMY 2 TEM 1b	*Escherichia coli*
9A	Cat	4	CMY 2	*Escherichia coli*
9A	Cat	4	*amp*C 11	*Escherichia coli*
9A	Cat	4	CMY 2	*Escherichia coli*
9A	Cat	5	CMY 2	*Escherichia coli*
9A	Cat	5	CMY 2	*Escherichia coli*
9A	Cat	5	CMY 2	*Escherichia coli*
4A	Cat	6	NE	*Pseudomonas* spp
9A	Cat	6	CMY 2 TEM 1b	*Escherichia coli*
9A	Cat	6	CMY 2 TEM 1b	*Escherichia coli*
9A	Cat	6	CTX-M 1	*Escherichia coli*
9A	Cat	6	CTX-M 1	*Escherichia coli*
9A	Cat	7	CMY 2	*Escherichia coli*
9A	Cat	7	CMY 2	*Escherichia coli*
9A	Cat	7	CMY 2	*Escherichia coli*
9B	Dog	7	NE	*Pseudomonas* spp
9A	Cat	11	SHV 12	*Escherichia coli*
9A	Cat	11	SHV 12	*Escherichia coli*
9A	Cat	17	CTX-M 55	*Escherichia coli*
9D	Feed	17	CTX-M 55	*Escherichia coli*
9D	Feed	20	NE	Unidentified
9D	Feed	20	NE	*Myroides* spp
6A	Cat	22	TEM 52StPaul	*Escherichia coli*

[+] NE, ESBL/ampC-negative isolates with non-wild-type susceptibility to cefotaxime. Gene type CTX-M-1/61 is shown as CTX-M-1, CTX-M-55/57/79 is shown as CTX-M-55, CMY-2/61 is shown as CMY-2, SHV-12/129 is shown as SHV-12.

To investigate ESBL-shedding through suggested persistence or uptake from a common source in cat 9A further, additional information about household 9 was obtained. Besides cat 9A, the household owned two dogs. As simultaneous ESBL-shedding in clustered animals was frequently seen in a longitudinal study in dogs (Chapter 2), faecal samples of the two dogs clustered with cat 9A were investigated for shedding of ESBL-producing *Enterobacteriaceae* in week 7. Both cat 9A and dog 9B were ESBL-positive at this sampling moment. Dog 9C was not able to come in contact with cat 9A. This dog was ESBL-negative at the sampling moment. Isolates retrieved from cat 9A and dog 9B at this sampling moment did not contain similar ESBL genes. Therefore, it was assumed that these isolates were not related and not obtained via interspecies transmission or a common source.

Cat 9A was the only cat in this study to be fed with raw food. The feed of cat 9A was sampled and cultured to investigate for the presence of ESBL-producing *Enterobacteriaceae* in week 17 and 20. In week 17, ESBL-producing *Enterobacteriaceae* were present in both the cat's faeces and in feed. Both isolates retrieved at these time points harboured $bla_{\text{CTX-M-55}}$, an ESBL gene which is rarely seen in the Netherlands. This finding may suggest feed as a source for uptake of ESBL-producing *Enterobacteriaceae*.
Cat 4A did not live with any clustered animals in the same household. Cat 6A shared a household with cat 6B, but this cat did not shed ESBL-producing *Enterobacteriaceae* during the study. As these cats were ESBL-positive at one single sampling moment, it seems likely that uptake and subsequent loss of ESBL-producing *Enterobacteriaceae* occurred without colonisation of ESBL-producing strains in the gut.

Conclusion

Prevalence and concentrations of ESBL-producing *Enterobacteriaceae* in cats shown in this study are low, which indicates a small role for cats in ESBL epidemiology. However, since the number of screened cats is relatively low, extrapolation of these findings to the total Dutch domestic cat population should be done with great care. Continuous ESBL-shedding in one cat fed with raw food and the finding of a rare ESBL type in both a faecal sample and feed at the same sampling moment suggests feed as a potential source for ESBL-producing *Enterobacteriaceae*.

References

1. Hordijk, J. *et al.* High prevalence of fecal carriage of extended-spectrum β-lactamase/AmpC-producing *Enterobacteriaceae* in cats and dogs. *Front. Microbiol.* **4,** 242 (2013).

2. Sun, Y. *et al.* High prevalence of blaCTX-M extended-spectrum β-lactamase genes in *Escherichia coli* isolates from pets and emergence of CTX-M-64 in China. *Clin. Microbiol. Infect.* **16,** 1475–81 (2010).

3. Gandolfi-Decristophoris, P., Petrini, O., Ruggeri-Bernardi, N. & Schelling, E. Extended-spectrum β-lactamase-producing *Enterobacteriaceae* in healthy companion animals living in nursing homes and in the community. *Am. J. Infect. Control* **41,** 831–5 (2013).

4. Liao, X.-P. *et al.* Comparison of plasmids coharboring 16s rrna methylase and extended-spectrum β-lactamase genes among *Escherichia coli* isolates from pets and poultry. *J. Food Prot.* **76,** 2018–23 (2013).

5. Sallem, R. Ben *et al.* First detection of CTX-M-1, CMY-2, and QnrB19 resistance mechanisms in fecal *Escherichia coli* isolates from healthy pets in Tunisia. *Vector Borne Zoonotic Dis.* **13,** 98–102 (2013).

6. Poirel, L. *et al.* Extended-spectrum β-lactamase CTX-M-15-producing *Klebsiella pneumoniae* of sequence type ST274 in companion animals. *Antimicrob. Agents Chemother.* **57,** 2372–5 (2013).

7. Schwarz, S. *et al.* Editorial: assessing the antimicrobial susceptibility of bacteria obtained from animals. *J. Antimicrob. Chemother.* **65,** 601–4 (2010).

8. Harada, K., Niina, A., Nakai, Y., Kataoka, Y. & Takahashi, T. Prevalence of antimicrobial resistance in relation to virulence genes and phylogenetic origins among urogenital *Escherichia coli* isolates from dogs and cats in Japan. *Am. J. Vet. Res.* **73,** 409–17 (2012).

9. MARAN 2013. Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2012. at <http://www.wageningenur.nl/nl/Expertises-Dienstverlening/Onderzoeksinstituten/Central-Veterinary-Institute/Publicaties-CVI/MARAN-Rapporten.htm>

Chapter 4

No environmental contamination of veterinary operating theatres with ESBL-producing *Enterobacteriaceae* when admitting companion animals shedding these bacteria

Valérie O. Baede[1], Joost Hordijk[1]

1. Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, the Netherlands

Abstract

Objectives

To investigate contamination of veterinary operating theatres with ESBL-producing *Enterobacteriaceae* after admittance of ESBL-positive companion animals.

Methods

Four operating theatres and a preparation room of two veterinary hospitals were investigated for the presence of ESBL-producing *Enterobacteriaceae* before and after scheduled surgeries. Environmental wipes were taken from operating tables, anaesthesia machines, operating room lights, horizontal surfaces and floors. Rectal swabs and fur wipes were taken from every admitted patient. Environmental wipes and fur wipes were cultured in LB-broth supplemented with 1 mg/L cefotaxime (LBC). Rectal swabs were directly inoculated on MacConkey supplemented with 1 mg/L cefotaxime (MCC) and cultured again after enrichment in LBC. Species identification of bacterial isolates was determined with MALDI-TOF MS. Presence of ESBL genes in *E. coli* isolates was investigated with PCR and sequence analysis.

Results

In clinic A, ESBL-producing *E. coli* were obtained from 1 of 9 admitted patients. No ESBL-producing *E.coli* were isolated from the environmental wipes. In clinic B, ESBL-producing *E. coli* were obtained from 1 of 2 admitted patients. No ESBL-producing *E. coli* were isolated from the environmental wipes in clinic B. Culturing of 11 fur wipes and 50 environmental wipes lead to the isolation of cefotaxime-resistant isolates, the majority of these isolates were identified as *Acinetobacter* (25) and *Pseudomonas* (21).

Conclusions

Contamination of the clinical environment with ESBL-producing *E. coli* during surgical procedures was not found. *Pseudomonas* spp and *Acinetobacter* spp isolates were obtained from fur and environmental wipes before and after surgery.

Introduction

Various antibiotic resistant bacterial species have been shown to survive in household or clinical settings, including methicillin-resistant *Staphylococcus aureus*, methicillin-resistant *Staphylococcus pseudintermedius* and ESBL-producing *Enterobacteriaceae*.[1–4] Persistence on surfaces of *E. coli* can take up to several weeks.[5–7] Environmental contamination of veterinary clinics after admittance of ESBL-positive animals could form an indirect transmission route of ESBL-producing strains between ESBL-positive animals and susceptible patients. The risk of obtaining a nosocomial infection is especially high in invasive procedures. Therefore, contamination of operating theatres with ESBL-producing *Enterobacteriaceae* should be monitored and prevented.

However, the extent of contamination with ESBL-producing *Enterobacteriaceae* after admittance of an ESBL-positive animal in veterinary operating theatres is still unknown. Therefore, it is difficult to perform an accurate risk assessment for the association of post-surgical infections due to these bacteria with contamination of the operating theatre environment.

In this study contamination with ESBL-producing *Enterobacteriaceae* in veterinary operating theatres after admitting and performing surgery on ESBL-positive patients was investigated.

Methods

Study design

Two small animal hospitals were selected based on low incidence of postoperative wound infections. Both clinics provide specialised animal health care and maintain separate operating theatres for internal surgery and orthopaedic surgery. These operating theatres were monitored to investigate differences in environmental contamination with ESBL-producing *Enterobacteriaceae* in clean and clean-contaminated surgical procedures. The operating theatre for internal surgery in clinic A (AOT3) was used for ophthalmology simultaneously. Two sets of operating tables, anaesthesia machines and operating room lights were available in AOT3. In clinic A, preparation rooms for both the internal surgery department and the orthopaedic surgery department were separated. In clinic B, the preparation room was used for patients from all surgical departments including radiology.

From all patients receiving surgery, rectum swabs and fur wipes were taken in the corresponding preparation room after anaesthetic induction. Dry sterile cotton swabs (Copan, Italy) were used to take rectum samples. A 5x5cm piece of simple household cleaning cloth (85% viscose, 15% polypropylene) was used to take fur wipes of back and hindquarters. At the start and end of the day, all operating theatres were sampled on the following spots: top and sides of operating table, anaesthesia machine, operating light, a horizontal surface and part of the floor. Approximately 50x50cm of these spots was wiped thoroughly. When taking samples, latex gloves were worn. To prevent cross-contamination in between sampling, latex gloves were repeatedly changed between samples. All samples were further processed on the sampling day.

Bacterial isolates

Rectal swabs were streaked directly onto MacConkey agar supplemented with 1 mg/L cefotaxime (MCC) and again after subsequent enrichment in 1 ml LB-broth supplemented with 1 mg/L cefotaxime (LBC). Both MCC plates were cultured overnight. Fur and environmental wipes were cultured overnight in 25 ml LBC and subsequently inoculated on MCC. MCC plates were screened for growth of cefotaxime-resistant colonies. If applicable, one colony for every morphological different phenotype was picked for further analysis. All overnight incubations were carried out at 37°C.

Species identification

Bacterial species of all isolates was identified by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker, Delft, the Netherlands).

Results

In clinic A, four dogs were admitted to the orthopaedic operating theatres. Two dogs were scheduled for surgery in operating theatre 1 (AOT1) and the other two dogs were scheduled for surgery in operating theatre 2 (AOT2). On the same day, 2 dogs and 1 cat were admitted to operating theatre 3 for internal surgery (AOT3a), and two cats for ophthalmologic surgery (AOT3b). One rectal swab, obtained from a dog in AOT1, showed growth on MCC. Fur wipes showed growth on MCC for all canine patients. No cefotaxime-resistant isolates could be obtained from feline samples.

Cefotaxime-resistant isolates could be obtained from environmental wipes of various spots in these operating theatres, for both wipes taken before and after surgical procedures (Table 1). In AOT3, both sets of operating table, anaesthesia machine and operating room lights were wiped.

Table 1. Environmental wipes in clinic A and results of culturing on MCC

	AOT1		AOT2		AOT3a		AOT3b	
Timepoint	1	2	1	2	1	2	1	2
Operating table				■		■		
Anaesthesia machine	■		■		■		■	■
Operating room lights				■	■	■	■	■
Horizontal surface	■			■	■	■	⊠	⊠
Floor				■			⊠	⊠

Black squares represent growth on MCC. Timepoint 1: before start first surgery; timepoint 2: after end last surgery. AOT1: operating theatre 1 of clinic A; AOT2: operating theatre 2 of clinic A; AOT3a: operating theatre 3 of clinic A, internal surgery; AOT3b: operating theatre 3 of clinic A, ophthalmologic surgery.

In clinic B, two canine patients were admitted to the orthopaedic operating theatre (BOT1). One rectal swab showed growth on MCC. Cefotaxime-resistant isolates could not be obtained from fur wipes. Two pre-operative environmental wipes of BOT1 showed growth on MCC (Table 2). No patients were scheduled for internal surgery on the sampling day. Instead, additional environmental wipes were taken in the preparation room (BPR). MCC-positive wipes were found in 3 of 5 pre-operative wipes and 4 of 5 post-operative wipes.

Table 2. Environmental wipes in clinic B and results of culturing on MCC

	BOT1		BPR	
Timepoint	1	2	1	2
Operating table				■
Anaesthesia machine	■		■	■
Operating room lights			■	
Horizontal surface		■		■
Floor	■		■	■

Black squares represent growth on MCC. Timepoint 1: before start first surgery; timepoint 2: after end last surgery. BOT1: operating theatre 1 of clinic B; BPR: preparation room of clinic B.

In total 63 cefotaxime-resistant isolates were obtained. Identified species are shown in Table 3. Cefotaxime-resistant *E. coli* were only identified in two canine isolates, one originating from a patient in clinic A and one from a patient in clinic B. All other cefotaxime-resistant isolates were identified as non-*Enterobacteriaceae*. Twenty-one isolates were identified as *Pseudomonas* spp, 19 isolates were identified as *Acinetobacter* spp, 6 isolates were identified as *Acinetobacter baumannii* and 5 isolates

were identified as *Enterococcus* spp. One isolate was obtained for each of the following species: *Achromobacter* spp, *Bacillus* spp, *Clostridium* spp and *Stenotrophomonas* spp.

Discussion

During this study, two cefotaxime-resistant *E. coli* isolates were found, obtained from two canine faecal swabs. One cefotaxime-resistant *E. coli* isolate was obtained from the first patient admitted to AOT1. Contamination of the operating theatre with this strain was not found, as none of the post-operative environmental wipes picked up this strain. The second cefotaxime-resistant *E. coli* isolate was obtained from the first patient admitted to BOT1. Also in this operating theatre, no contamination with this strain could be found. This suggests a low risk for contamination of a large area with ESBL-producing *E. coli*, when there is no direct contact with contaminated faeces.

However, cefotaxime-resistant non-*Enterobacteriaceae* isolates were obtained from 11 fur wipes and 50 environmental wipes in total. MCC-positive environmental wipes were found both before and after admitting patients to the operating theatres. No clear association could be found between presence of strains on certain spots before and after surgeries or presence of certain strains before or after surgeries. Most frequently found bacterial species in fur and environmental wipes were of genera *Pseudomonas* and *Acinetobacter*. Species of these bacterial genera are frequently seen in nosocomial infections and have specialised mechanisms of survival. *Acinetobacter* species have the ability to acquire resistance mechanisms rapidly.[8] *Pseudomonas* species are known for their resistance against many disinfectants and are capable of forming biofilms.[9] Moreover, Kramer *et al.* showed the ability of these bacteria to survive for several months on dry surfaces.[10]

The finding of *Pseudomonas* and *Acinetobacter* in a large number of fur wipes and environmental wipes should be interpreted with great care. The detection method was focused on cefotaxime-resistant isolates to detect ESBL-producing *Enterobacteriaceae* and has a very low detection limit. As data on total number of bacteria and composition of the bacterial flora was not available, the exact role of *Pseudomonas* and *Acinetobacter* cannot be determined.

Conclusion

In this study, the risk of contamination with ESBL-producing *Enterobacteriaceae* of operating theatres by faecal shedding and thereby spread of these bacteria to susceptible patients, was found to be low. However, interpretations of these data should be done with care, as only two ESBL-producing isolates were obtained. Other bacterial species as *Acinetobacter* spp and *Pseudomonas* spp were able to survive in a surgical environment and presented resistance against cefotaxime. These bacterial species can play an important role in nosocomial infections.

Table 3. Species determination of cefotaxime-resistant isolates obtained from operating theatres in 2 veterinary clinics

Clinic	Department	Theatre	Subject	Timepoint	Species (# isolates)
A	Orthopaedics	AOT1	Anaesthesia machine	1	*Pseudomonas spp (2)*
			Horizontal surface	1	*Pseudomonas spp (1)*
			Patient 1 rectal (dog)	**1**	***Escherichia coli (1)***
			Patient 1 fur (dog)	1	*Acinetobacter spp (1), unidentified (1)*
			Patient 2 fur (dog)	1	*Enterococcus spp (1), Clostridium spp (1), Pseudomonas spp (1)*
A	Orthopaedics	AOT2	Anaesthesia machine	1	*Pseudomonas spp (2)*
			Patient 1 fur (dog)	1	*Unidentified (1)*
			Patient 2 fur (dog)	1	*Acinetobacter spp (1)*
			Operating table	2	*Acinetobacter spp (2)*
			Operating room lights	2	*Pseudomonas spp (1)*
			Horizontal surface	2	*Achromobacter spp (1)*
			Floor	2	*Acinetobacter baumannii (1)*
			Dust computer	2	*Pseudomonas spp (1)*
A	General surgery/ Soft tissue	AOT3a	Anaesthesia machine	1	*Pseudomonas spp (1)*
			Operating room lights	1	*Pseudomonas spp (1), Enterococcus spp (1)*
			Horizontal surface	1	*Pseudomonas spp (1), Enterococcus spp (1)*
			Patient 1 fur (dog)	1	*Pseudomonas spp (1)*
			Patient 2 fur (dog)	1	*Acinetobacter baumannii (3)*
			Operating table	2	*Pseudomonas spp (1)*
			Operating room lights	2	*Acinetobacter spp (1)*
			Horizontal surface	2	*Pseudomonas spp (1), Acinetobacter spp? (1)*
A	Ophthalmology	AOT3b	Anaesthesia machine	1	*Pseudomonas spp (2)*
			Operating room lights	1	*Acinetobacter spp (1)*
			Anaesthesia machine	2	*Acinetobacter spp (1)*
			Operating room lights	2	*Acinetobacter spp (2), Bacillus spp (1)*

B	Orthopaedics	BOT1	Anaesthesia machine	1	*Pseudomonas (2)*
			Floor	1	*Acinetobacter spp (1)*
			Patient 1 rectal (dog)	**1**	***Escherichia coli (1)***
			Horizontal surface	2	*Pseudomonas spp (1)*
B	Surgery prep room	BPR	Anaesthesia machine	1	*Acinetobacter spp (2), unidentified (1)*
			Operating room lights	1	*Acinetobacter spp (2)*
			Floor	1	*Stenotrophomonas spp (1)*
			Operating table	2	*Acinetobacter baumannii (1), Pseudomonas spp (1)*
			Anaesthesia machine	2	*Acinetobacter spp (3), unidentified (1)*
			Horizontal surface	2	*Acinetobacter baumannii (1), Enterococcus spp (1), unidentified (1)*
			Floor	2	*Acinetobacter spp (1), Pseudomonas spp (1), Enterococcus spp (1), unidentified (1)*

Timepoint 1: before start first surgery sampling day; timepoint 2: after end last surgery sampling day.

References

1. Guet-Revillet, H. *et al.* Environmental contamination with extended-spectrum β-lactamases: is there any difference between *Escherichia coli* and *Klebsiella* spp? *Am. J. Infect. Control* **40,** 845–8 (2012).

2. Laarhoven, L. M. *et al.* Longitudinal study on methicillin-resistant *Staphylococcus pseudintermedius* in households. *PLoS One* **6,** e27788 (2011).

3. Van Duijkeren, E. *et al.* Transmission of methicillin-resistant *Staphylococcus pseudintermedius* between infected dogs and cats and contact pets, humans and the environment in households and veterinary clinics. *Vet. Microbiol.* **150,** 338–43 (2011).

4. Boyce, J. M. Environmental contamination makes an important contribution to hospital infection. *J. Hosp. Infect.* **65 Suppl 2,** 50–4 (2007).

5. Williams, A. P., Avery, L. M., Killham, K. & Jones, D. L. Persistence of *Escherichia coli* O157 on farm surfaces under different environmental conditions. *J. Appl. Microbiol.* **98,** 1075–83 (2005).

6. Wilks, S. A., Michels, H. & Keevil, C. W. The survival of *Escherichia coli* O157 on a range of metal surfaces. *Int. J. Food Microbiol.* **105,** 445–54 (2005).

7. Starlander, G., Yin, H., Edquist, P. & Melhus, Å. Survival in the environment is a possible key factor for the expansion of *Escherichia coli* strains producing extended-spectrum β-lactamases. *APMIS* **122,** 59–67 (2014).

8. Bergogne-Bérézin, E., Towner, K. J. & Bergogne-Berezin, E. *Acinetobacter* spp. as nosocomial pathogens: microbiological , clinical , and epidemiological features. *Clin. Microbiol. Rev.* **9,** 148–165 (1996).

9. Ogeer-Gyles, J. S., Mathews, K. a. & Boerlin, P. Nosocomial infections and antimicrobial resistance in critical care medicine. *J. Vet. Emerg. Crit. Care* **16,** 1–18 (2006).

10. Kramer, A., Schwebke, I. & Kampf, G. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infect. Dis.* **6,** 130 (2006).

Chapter 5

Raw-feed as a risk factor for ESBL-carriage in domestic cats; a case-control study

Valérie O. Baede[1], Joost Hordijk[1]

1. Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, the Netherlands

This chapter describes temporary results of a study in progress and is not complete yet

Introduction

Prevalence of ESBL-producing *Enterobacteriaceae* in domestic cats is low (Chapter 3).[1–4] In a longitudinal study, described in Chapter 3, only 10 of 189 feline faecal samples showed ESBL-shedding. In this study, one cat showed long-term persistence of ESBL-producing *Enterobacteriaceae* in the gut. Long-term persistence has not been seen in other cats, or in a longitudinal study in dogs (Chapter 2). Instead of all other cats participating in the longitudinal study, the colonised cat had a raw-food diet. As ESBL-producing *Enterobacteriaceae* were discovered in beef, pork and chicken meat samples, consumption of raw meat products could be a risk for colonisation with ESBL-producing *Enterobacteriaceae*.[5–8] At one of two sampling moments where feed and faeces were tested simultaneously, cefotaxime-resistance isolates of raw-feed and faeces harboured the same rare ESBL gene. This suggests feed as a source for uptake of ESBL-producing *Enterobacteriaceae*.

In this study, a case-control study was carried out, to investigate raw-feed as a potential risk factor for the carriage of ESBL-producing *Enterobacteriaceae*.

Methods

Two groups of 12 cats were investigated for faecal shedding of ESBL-producing *Enterobacteriaceae* for three successive weeks. Cat owners were asked to return a questionnaire containing questions on diet, contact animals and the cat's health. Cats were assigned to a group, based on diet. Group A consisted of cats fed with raw-feed. Group B consisted of cats fed with any other kind of feed, and served as control group. Faecal samples were sent to the institute through regular mail service (as described in Chapter 2).

Of each faecal sample, 0.5 grams of faeces was inoculated in LB-broth for enrichment and cultured overnight at 37°C. Inoculated broth was streaked onto MacConkey agar supplemented with 1 mg/L cefotaxime (MCC) and cultured overnight. If growth occurred on MCC, quantitative culturing was performed as described in Chapter 2. All isolates cultured on MCC were designated as isolates with a non-wild-type susceptibility to cefotaxime, according to Schwarz *et al*.[9]

ESBL genes were identified in all obtained isolates with conventional PCR, as described in Chapter 2. Species identification was carried out by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

Results

As this study is still in progress, results are not complete yet. This results section contains all obtained results up to this point. (August 2014)

Phenotypic results

In group A, 24 faecal samples of 8 cats were investigated for the presence of ESBL-producing *Enterobacteriaceae*. Sixteen of 24 samples showed reduced susceptibility to cefotaxime. In group B, 27 faecal samples of 9 cats were investigated for the presence of ESBL-producing *Enterobacteriaceae*. In this group, 5 of 27 samples showed reduced susceptibility to cefotaxime. Results of consecutive faecal samples are shown in Table 1.

Discussion

The phenotypic results of this study have to be confirmed genotypically before any interpretation of results can be done. Also, more cats have to be investigated to confirm the significance of obtained results.

Table 1. Results of culturing on MCC for consecutive faecal samples

		Sampling moment		
Group	CatID	1	2	3
A	K025	N	P	P
A	K027	P	P	P
A	K029	P	N	N
A	K031	P	P	P
A	K033	P	N	P
A	K036	N	P	P
A	K037	P	P	P
A	K042	N	N	N
B	K004	P	N	N
B	K028	N	P	N
B	K032	P	N	N
B	K034	N	N	N
B	K035	N	N	N
B	K038	N	N	N
B	K040	P	N	N
B	K041	N	P	N
B	K043	N	N	N

P, faecal samples with non-wild-type susceptible colonies; N, faecal sample with wild-type colonies

References

1. Costa, D. *et al.* Detection of CTX-M-1 and TEM-52 beta-lactamases in Escherichia coli strains from healthy pets in Portugal. *J. Antimicrob. Chemother.* **54,** 960–1 (2004).

2. Gandolfi-Decristophoris, P., Petrini, O., Ruggeri-Bernardi, N. & Schelling, E. Extended-spectrum β-lactamase-producing Enterobacteriaceae in healthy companion animals living in nursing homes and in the community. *Am. J. Infect. Control* **41,** 831–5 (2013).

3. Poirel, L. *et al.* Extended-spectrum β-lactamase CTX-M-15-producing Klebsiella pneumoniae of sequence type ST274 in companion animals. *Antimicrob. Agents Chemother.* **57,** 2372–5 (2013).

4. Hordijk, J. *et al.* High prevalence of fecal carriage of extended-spectrum β-lactamase/AmpC-producing Enterobacteriaceae in cats and dogs. *Front. Microbiol.* **4,** 242 (2013).

5. Leverstein-van Hall, M. a *et al.* Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clin. Microbiol. Infect.* **17,** 873–880 (2011).

6. Egervärn, M. *et al.* Escherichia coli with extended-spectrum beta-lactamases or transferable AmpC beta-lactamases and Salmonella on meat imported into Sweden. *Int. J. Food Microbiol.* **171,** 8–14 (2014).

7. Jouini, A. *et al.* Characterization of CTX-M and SHV extended-spectrum beta-lactamases and associated resistance genes in Escherichia coli strains of food samples in Tunisia. *J. Antimicrob. Chemother.* **60,** 1137–41 (2007).

8. Ojer-Usoz, E. *et al.* Prevalence of extended-spectrum β-lactamase-producing Enterobacteriaceae in meat products sold in Navarra, Spain. *Meat Sci.* **93,** 316–21 (2013).

9. Schwarz, S. *et al.* Editorial: assessing the antimicrobial susceptibility of bacteria obtained from animals. *J. Antimicrob. Chemother.* **65,** 601–4 (2010).

Chapter 6

General Discussion

Antibiotic resistance is spreading globally, which is causing major threats to treatment possibilities of bacterial infections. ESBL-producing *Enterobacteriaceae* contribute for a significant amount to the emergence of antibiotic resistance and are therefore monitored carefully in human health. A large number of studies has also been done in livestock, thereby leaving the contribution of companion animals in the epidemiology of ESBL-producing *Enterobacteriaceae* behind.[1,2] As companion animals are acknowledged as a potential reservoir for ESBL-producing *Enterobacteriaceae*, their role should be investigated thoroughly. Hereby, several matters should be addressed. First, the significance of ESBL-producing *Enterobacteriaceae* in dog and cat populations should be investigated; this includes the consequences for both healthy and clinically affected animals. Secondly, ESBL-producing *Enterobacteriaceae* in companion animals should be addressed in relation to susceptible humans, animals and the environment.

In order to investigate these matters, several studies were carried out in the outline of this project. Prevalence of ESBL-producing *Enterobacteriaceae* was investigated in longitudinal studies in both dogs and cats (Chapter 2 and 3). Information about colonisation dynamics was also collected in these studies, next to quantities of ESBL-producing *Enterobacteriaceae* in faecal samples. To address the role of companion animals in relation to other populations and the environment, ESBL gene characterisation was carried out. Additional information was gathered through a study in veterinary clinics, investigating contamination of the clinic environment. Raw feed was investigated as a potential source for ESBL-producing *Enterobacteriaceae* in cats (Chapter 5).

Significance in companion animal populations

Previous investigations of prevalence of ESBL-producing *Enterobacteriaceae* in companion animals were mostly based on retrospective analysis, frequently restricted to clinical isolates.[2] High prevalence in healthy dogs was found earlier.[3] However, the longitudinal study in dogs, described in Chapter 2 of this thesis, showed a surprisingly high prevalence in this animal category. The prevalence in healthy cats was much lower, but also in this group ESBL-producing *Enterobacteriaceae* were present. Therefore, healthy companion animals should be recognized as an important reservoir and potential source of transmission of these multi-resistance bacteria. The difference in prevalence between dogs and cats is not clarified yet, but may be explained by differences in colonisation rate or exposure to different sources of ESBL-producing *Enterobacteriaceae*.

Colonisation rates over time were investigated by quantifying shedding of ESBL-producing *Enterobacteriaceae* compared to total count of *Enterobacteriaceae* in faeces. In dogs, ESBL-producing *Enterobacteriaceae* could comprise a large part of the gut microbiota (Chapter 2). A high degree of faecal shedding is expected to increase the spread of these bacteria in the environment and thereby the risk of transmission to other individuals. High levels of ESBL-producing *Enterobacteriaceae* in faeces also suggest a high density of these bacteria in the gut. When this high density leads to easy exchange of resistance genes, a dog could act as a source for genetic shifts, enhancing the diversity of resistance genes in the environment through continuous introduction. The average fraction of ESBL-producing *Enterobacteriaceae* compared to the total count of *Enterobacteriaceae* in cats was much lower than in dogs (Chapter 3). Also the total count of *Enterobacteriaceae* in cats was surprisingly low in some cases; however no previous data is available to compare these counts. From these results, cats seem less engaged than dogs in the spread of ESBL-producing *Enterobacteriaceae* to the environment, however, this difference cannot be explained based on the current information yet.

The monitoring of colonisation dynamics in these studies showed frequent shifts in faecal shedding of ESBL-producing *Enterobacteriaceae* in healthy dogs (Chapter 2), making both long-term and short-term persistence with these bacteria unlikely. This finding stresses the importance of ESBL-producing *Enterobacteriaceae* in the dog population. It suggests easy uptake of these bacteria, which, combined

with unrestrained shedding, would lead to a rapid spread through the population and the environment. In cats, shifts in faecal shedding were less prominent and should be supported by other studies (Chapter 3). However, one cat in this study showed long-term persistence of an ESBL-producing strain, which leads to the presumption of feed being a potential risk factor for colonisation with ESBL-producing *Enterobacteriaceae* in cats. A study addressing this hypothesis is currently in progress (Chapter 5). If feed can be confirmed as a risk factor, this may present the first reason for the difference in ESBL characteristics between dogs and cats. The finding of a high dynamic colonisation indicates that longitudinal data is an important tool in ESBL epidemiological research. The longitudinal data presented in this thesis does not only exhibit new information on a phenotypic level, but also on a genotypic level. High variety in ESBL genes confirmed low persistence and showed similar ESBL genes on certain time points in different dogs in the same household, suggesting clonality in clustered animals. To elucidate the behaviour of ESBL-producing strains in infection, persistence of ESBL-producing strains should also be monitored in animals in which these strains are involved in infection.

Impact on interspecies transmission

A high diversity of ESBL genes was present in ESBL-positive dogs, even in subsequent faecal samples, which confirmed limited persistence of ESBL-producing strains (Chapter 2). In cats, less variety was seen, which may be explained by the little amount of ESBL-positive samples. ESBL genes in dogs and cats resembled those earlier found in humans and livestock, which shows possibilities of interspecies transmission of these strains. [1] Direct transmission between clustered animals was not addressed in this project. In order to show direct transmission, further genotyping should be carried out to determine transmission characteristics. Detailed genotyping should include gene characterisation, plasmid characterisation and sequence type determination of the bacterial species, including all levels of ESBL epidemiology. Confirmation of transmission would depend on the similarity of all levels in compared isolates. Detailed genotyping would also simplify the detection of risk factors, when comparing isolates from related sources as surrounding environment, feed, household members etc.

Implications for veterinary practice

Another way to address the risk of potential transmission of ESBL-producing *Enterobacteriaceae*, is contamination of veterinary clinics (Chapter 4). After admittance of ESBL-positive animals, no contamination of veterinary operating theatres with ESBL-producing E. coli was seen. This suggests a low chance of spreading when anaesthetised animals are handled with care and appropriate hygiene measures. However, as *Enterobacteriaceae* are shown to be able to survive for several weeks on dry surfaces, this stresses the need for information about contamination when animals are not anaesthetised and can express their normal behaviour.[4] Unfortunately, no data is available on contamination of waiting, consultation or recovery rooms after admittance of an ESBL-positive animal. This should be addressed in further research and may require an experimental setting to assure a clear outcome.

Earlier studies showed presence of ESBL-producing strains in clinical samples in dogs and cats.[5–11] This project showed the major spread of these bacteria throughout the Dutch dog population and their presence in the Dutch cat population. Frequent shifts in faecal shedding and high quantities of ESBL-producing *Enterobacteriaceae* predict easy shedding and spread into the environment and indirectly to other species. Veterinarians should be aware of this phenomenon and maintain appropriate hygiene measurements in veterinary clinics, to prevent spread and risk for nosocomial infections. Healthy animals were the focus of this project and the findings show that ESBL-shedding can take place without affecting the animal's health. Circumstances for carrying ESBL-producing strains and acquiring an infection with these bacteria should be compared to address important risk factors for developing an infection with ESBL-producing strains.

Implications for public health

So far, confirmed interspecies transmission between humans and companion animals is undescribed. Potential transmission has been suggested by Ewers. *et al*.[8] However, Bortolaia *et al.* suggested limited exchange between human and dog populations because of heterogeneity of plasmids.[12] This subject should be addressed in further research. To prevent transmission to other individuals or even species, hygiene is important. Pet owners, immunocompromised individuals and health care workers, should be aware of the possibility of pets carrying resistant strains and maintain proper hygiene to protect themselves against bacterial infections with resistant strains. In case of an infection, susceptibility tests should be carried out to determine suitable antibiotic treatment.

Conclusion

This project has shown the importance of the contribution of companion animals to the global spread of antibiotic resistance through ESBL-producing *Enterobacteriaceae*. High prevalence, low persistence, high levels of shedding and high variety of ESBL genes indicate dogs as an important reservoir of ESBL-producing *Enterobacteriaceae*. These matters are less prominent in cats. Furthermore, longitudinal data are crucial in elucidating ESBL epidemiology and should be used in following studies. Feed is proposed as a potential risk factor for carriage of ESBL-producing *Enterobacteriaceae.*

Further research

Several questions need to be answered to provide necessary information about the role of companion animals in transmission of ESBL-producing *Enterobacteriaceae*. To determine the risk of acquiring nosocomial infections, contamination of consultation and recovery rooms or wards in veterinary clinics should be investigated, in relation to normal animal behaviour. An experimental setting would be ideal to carry out a risk assessment in environmental contamination.

Detailed genotyping of ESBL-producing isolates, including gene, plasmid and sequence type characterisation, would assist in determination of ESBL-producing clones in households and may confirm transmission between individuals. Ideally, several households would be monitored for several months and faecal samples of animals and their owners and environmental samples would be investigated at least once a week.

Risk factor investigation would contribute to our current knowledge on circumstances needed for transmission between individuals, populations and the environment. It may also contribute to an explanation for the difference between dogs and cats in ESBL epidemiology. However, because of the frequent shifts and relatively high prevalence in faecal shedding of ESBL-producing *Enterobacteriaceae,* it will be hard to determine risk factors for dogs. Differences between risk factors for ESBL-shedding animals with and without infections with resistant strains may explain necessary circumstances for acquiring an infection with ESBL-producing strains. One potential risk factor that should also be considered is antibiotic treatment. Increased prevalence of ESBL-producing *Enterobacteriaceae* after administration of beta-lactam antibiotics or cephalosporins was reported in horses and presents antibiotic treatment with these classes as a risk factor for ESBL-carriage.[13] In dogs, antibiotic treatment has not been confirmed as a risk factor yet, but decreased susceptibility of *E. coli* isolates to cephalexine and amoxicilline has been shown after treatment with these drugs.[14,15]

To explore the role of cats in ESBL epidemiology further, the longitudinal study described in Chapter 3 should be repeated with a larger sample size. This study already confirmed the presence of ESBL-producing *Enterobacteriaceae* and appearance of similar ESBL genes as found in dogs, livestock and humans. However, a clearer image of ESBL-producing *Enterobacteriaceae* in cats may explain the difference in epidemiology compared to dogs, leading to a clearer view on ESBL epidemiology in companion animals.

References

1. Ewers, C., Bethe, A., Semmler, T., Guenther, S. & Wieler, L. H. Extended-spectrum β-lactamase-producing and AmpC-producing Escherichia coli from livestock and companion animals, and their putative impact on public health: a global perspective. *Clin. Microbiol. Infect.* **18,** 646–55 (2012).

2. Rubin, J. E. & Pitout, J. D. D. Extended-spectrum β-lactamase, carbapenemase and AmpC producing Enterobacteriaceae in companion animals. *Vet. Microbiol.* **170,** 10–18 (2014).

3. Hordijk, J. *et al.* High prevalence of fecal carriage of extended-spectrum β-lactamase/AmpC-producing Enterobacteriaceae in cats and dogs. *Front. Microbiol.* **4,** 242 (2013).

4. Kramer, A., Schwebke, I. & Kampf, G. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infect. Dis.* **6,** 130 (2006).

5. Sun, Y. *et al.* High prevalence of blaCTX-M extended-spectrum β-lactamase genes in Escherichia coli isolates from pets and emergence of CTX-M-64 in China. *Clin. Microbiol. Infect.* **16,** 1475–81 (2010).

6. Teshager, T. *et al.* Isolation of an SHV-12 β -Lactamase-Producing Escherichia coli Strain from a Dog with Recurrent Urinary Tract Infections. *Antimicrob. Agents Chemother.* **44,** 3483–3484 (2000).

7. Sidjabat, H. E. *et al.* Identification of plasmid-mediated extended-spectrum and AmpC beta-lactamases in Enterobacter spp. isolated from dogs. *J. Med. Microbiol.* **56,** 426–34 (2007).

8. Ewers, C. *et al.* Emergence of human pandemic O25:H4-ST131 CTX-M-15 extended-spectrum-beta-lactamase-producing Escherichia coli among companion animals. *J. Antimicrob. Chemother.* **65,** 651–60 (2010).

9. O'Keefe, A., Hutton, T. A., Schifferli, D. M. & Rankin, S. C. First detection of CTX-M and SHV extended-spectrum beta-lactamases in Escherichia coli urinary tract isolates from dogs and cats in the United States. *Antimicrob. Agents Chemother.* **54,** 3489–92 (2010).

10. Shaheen, B. W. *et al.* Molecular characterization of resistance to extended-spectrum cephalosporins in clinical Escherichia coli isolates from companion animals in the United States. *Antimicrob. Agents Chemother.* **55,** 5666–75 (2011).

11. Pomba, C. *et al.* Within-lineage variability of ST131 Escherichia coli isolates from humans and companion animals in the south of Europe. *J. Antimicrob. Chemother.* **69,** 271–3 (2014).

12. Bortolaia, V., Hansen, K. H., Nielsen, C. a, Fritsche, T. R. & Guardabassi, L. High diversity of plasmids harbouring blaCMY-2 among clinical Escherichia coli isolates from humans and companion animals in the upper Midwestern USA. *J. Antimicrob. Chemother.* 1–5 (2014). doi:10.1093/jac/dku011

13. Damborg, P., Marskar, P., Baptiste, K. E. & Guardabassi, L. Faecal shedding of CTX-M-producing Escherichia coli in horses receiving broad-spectrum antimicrobial prophylaxis after hospital admission. *Vet. Microbiol.* **154,** 298–304 (2012).

14. Grønvold, A.-M. R. *et al.* Changes in fecal microbiota of healthy dogs administered amoxicillin. *FEMS Microbiol. Ecol.* **71,** 313–26 (2010).

15. Damborg, P., Gaustad, I. B., Olsen, J. E. & Guardabassi, L. Selection of CMY-2 producing Escherichia coli in the faecal flora of dogs treated with cephalexin. *Vet. Microbiol.* **151,** 404–408 (2011).

Appendix 1

Supplementary data Chapter 2

Table S1. Primer characteristics

Name	Gene	Sequence	Size product (bp)	Reference
CTX-M-F	CTX-M-gr25	ATG TGC AGY ACC AGT AAR GTK ATG GC		[23]
CTX-M-R	CTX-M-gr25	TGG GTR AAR TAR GTS ACC AGA AYS AGC GG	592	
CTX-M-1g Fw	CTX-M-gr1	CCC ATG GTT AAA AAA TCA CTG C		[24]
CTX-M-1g Rv	CTX-M-gr1	CAG CGC TTT TGC CGT CTA AG	~1000	
CTX-M-2-F	CTX-M-gr2	ATG ATG ACT CAG AGC ATT CG		[25]
CTX-M-2-R	CTX-M-gr2	TGG GTT ACG ATT TTC GCC GC	865	
CTX-M-9F	CTX-M-gr9	TGG TGA CAA AGA GAG TGC AAC G		[26]
CTX-M-9R	CTX-M-gr9	TCA CAG CCC TTC GGC GAT	874	
CTX-M-9_{792}F	CTX-M-14-like	CTA TTT TAC CCA GCC GCA AC		[27]
CTX-M-9_{1029}R	CTX-M-14-like	GTT ATG GAG CCA CGG TTG AT	238	
TEM-F	TEM	GCG GAA CCC CTA TTT G		[23]
TEM-R	TEM	ACC AAT GCT TAA TCA GTG AG	964	
TEM-seq	TEM	GCC AAC TTA CTT CTG ACA ACG		[28]
SHV-F	SHV	TTA TCT CCC TGT TAG CCA CC		[23]
SHV-R	SHV	GAT TTG CTG ATT TCG CTC GG	795	
CMY-2-F	CMY	ATG ATG AAA AAA TCG TTA TGC TGC		[23]
CMY-2-R	CMY	GCT TTT CAA GAA TGC GCC AGG	1117	
CMY-F-838	CMY	TGG CGT ATT GGC GAT ATG TA		[28]
CMY-R-857	CMY	TAC ATA TCG CCA ATA CGC CA		
O1-GD2M-F	OXA-1-like	CAA CGG ATT AAC AGA AGC ATG GCT CG		[29]
O1-GD2M-R	OXA-1-like	GCT GTR AAT CCT GCA CCA GTT TTC CC	194	
O2-GD2M-F	OXA-2-like	GAC CAA GAT TTG CGA TCA GCA ATG CG		[29]
O2-GD2M-R	OXA-2-like	CYT TGA CCA AGC GCT GAT GTT CYA CC	254	
O10-GDM-F	OXA-10-like	CGC CAG AGA AGT TGG CGA AGT AAG		[29]
O10-GDM-R	OXA-10-like	GAA ACT CCA CTT GAT TAA CTG CGG	138	
OXA-48A	OXA-48-like	TTG GTG GCA TCG ATT ATC GG		[30]
OXA-48B	OXA-48-like	GAG CAC TTC TTT TGT GAT GGC	743	
O23-GDM-F	OXA-23-like	CCT GAT CGG ATT GGA GAA CCA G		[29]
O23-GDM-R	OXA-23-like	GAT GCC GGC ATT TCT GAC CG	512	
O24-GDM-F	OXA-24-like	GGT CGA TAA TTT TTG GTT AGT TGG CCC		[29]
O24-GDM-R	OXA-24-like	CCA TTA GCT TGC TCC ACC CAA CCA G	236	
O51-GDM-F	OXA-51-like	GAC CGA GTA TGT ACC TGC TTC GAC C		[29]
O51-GDM-R	OXA-51-like	GAG GCT GAA CAA CCC ATC CAG TTA ACC	493	
O58-GDM-F	OXA-58-like	GTG CTG AGC ATA GTA TGA GTC GAG C		[29]
O58-GDM-R	OXA-58-like	GGT CTA CAG CCA TTC CCC AGC C	629	
*amp*C1-$_{71}$	Amp-Chromosomal	AAT GGG TTT TCT ACG GTC TG		[31]
*amp*C2-$_{120}$	Amp-Chromosomal	GGG CAG CAA ATG TGG AGC AA	191	

Table S2. Complete quantitative and genotypic characteristics

SampleID	CFU/g	F	Isolate	DogID	t-value	Species	Gene characterisation*
13S00698	1.96E+04	0.0000	13S00698-4	23A	1	*Escherichia coli*	*amp*C type WT
			13S00698-5	23A	1	*Escherichia coli*	*amp*C type 18
13S00699	6.60E+04	0.0000	13S00699-1	19A	1	*Escherichia coli*	CMY 2/61
			13S00699-2	19A	1	*Escherichia coli*	CMY 2/61
			13S00699-3	19A	1	*Escherichia coli*	TEM 1b&52StPaul
13S00700	9.43E+05	0.0003	13S00700-1	24B	1	*Escherichia coli*	TEM 1b
			13S00700-2	24B	1	*Escherichia coli*	TEM 1b
			13S00700-3	24B	1	*Escherichia coli*	TEM 1b
13S00701	8.49E+05	0.0016	13S00701-1	24A	1	*Escherichia coli*	TEM 1b
			13S00701-2	24A	1	*Escherichia coli*	TEM 1b
			13S00701-3	24A	1	*Escherichia coli*	TEM 1b
13S00702	1.73E+06	0.0020	13S00702-1	17A	1	*Escherichia coli*	*amp*C type 3
			13S00702-2	17A	1	*Escherichia coli*	*amp*C type 3
			13S00702-3	17A	1	*Escherichia coli*	*amp*C type 3
13S00703	9.62E+04	0.0000	13S00703-1	17B	1	*Escherichia coli*	*amp*C type 3
			13S00703-2	17B	1	*Escherichia coli*	*amp*C type 18
			13S00703-3	17B	1	*Escherichia coli*	*amp*C type 3
13S00705	1.33E+05	0.0006	13S00705-1	15B	1	*Escherichia coli*	TEM 1b&52c
			13S00705-2	15B	1	*Escherichia coli*	TEM 1b&52c
			13S00705-3	15B	1	*Escherichia coli*	TEM 1b&52c
13S00706	1.36E+05	0.0001	13S00706-1	15A	1	*Escherichia coli*	CTX-M 1/61
			13S00706-2	15A	1	*Escherichia coli*	TEM 1b
			13S00706-3	15A	1	*Escherichia coli*	CTX-M 1/61
13S00707	2.45E+07	0.0006	13S00707-1	15C	1	*Escherichia coli*	TEM 1b&52c
			13S00707-2	15C	1	*Escherichia coli*	CTX-M 1 TEM-1b
			13S00707-3	15C	1	*Escherichia coli*	CTX-M 14/18
			13S00707-4	15C	1	*Escherichia coli*	CMY 2/61
13S00710	9.43E+03	0.0066	13S00710-5	16A	1	*Escherichia coli*	CTX-M 1 TEM 1b
			13S00710-6	16A	1	*Escherichia coli*	CMY 2
13S00711	1.37E+06	0.0482	13S00711-1	16B	1	*Escherichia coli*	CTX-M 1 TEM 1b
			13S00711-2	16B	1	*Escherichia coli*	CTX-M 1 TEM 1b
			13S00711-3	16B	1	*Escherichia coli*	CTX-M 1 TEM 1b
13S00712	1.63E+06	0.0001	13S00712-1	18A	1	*Escherichia coli*	TEM 84
			13S00712-2	18A	1	*Escherichia coli*	CTX-M 15/28 OXA 1/30
			13S00712-3	18A	1	*Escherichia coli*	CTX-M 15/28 OXA 1/30 TEM 1varA
13S00713	5.66E+05	0.0001	13S00713-1	21B	1	*Escherichia coli*	CTX-M 1
			13S00713-2	21B	1	*Escherichia coli*	SHV 12/129
			13S00713-3	21B	1	*Escherichia coli*	SHV 12/129
			13S00713-4	21B	1	*Escherichia coli*	CTX-M 14/18
13S00715	1.92E+06	0.0007	13S00715-1	22A	1	*Escherichia coli*	SHV 12/129
			13S00715-2	22A	1	*Escherichia coli*	CTX-M 55/57/79
			13S00715-3	22A	1	*Escherichia coli*	CTX-M 55/57/79
13S00723	7.00E+05	0.0007	13S00723-1	10A	2	*Escherichia coli*	*amp*C type 3
			13S00723-2	10A	2	*Escherichia coli*	CTX-M 1/61
			13S00723-3	10A	2	*Escherichia coli*	*amp*C type 3
13S00738	3.85E+05	0.0129	13S00738-5	1A	3	*Escherichia coli*	CTX-M 14/18

			13S00738-6	1A	3	*Escherichia coli*	CTX-M 1/61 TEM 1b/104
			13S00738-7	1A	3	*Escherichia coli*	CTX-M 1/61 TEM 1b/104
13S00739	1.82E+04	0.0017	13S00739-4	1B	3	*Escherichia coli*	*amp*C type 3
			13S00739-5	1B	3	*Escherichia coli*	*amp*C type 3
13S00740	2.13E+04	0.0436	13S00740-5	5B	4	*Acinetobacter spp*	
			13S00740-6	5B	4	*Acinetobacter spp*	
			13S00740-7	5B	4	*Pseudomonas spp*	
13S00741	3.33E+05	0.0004	13S00741-4	12A	4	*Escherichia coli*	CTX-M 1/61
			13S00741-5	12A	4	*Escherichia coli*	SHV 12/129 TEM 1b
			13S00741-6	12A	4	*Escherichia coli*	SHV 12/129 TEM 1b
			13S00741-7	12A	4	*Escherichia coli*	SHV 12/129 TEM 1b/104
13S00745	1.28E+05	0.0002	13S00745-5	21A	4	*Escherichia coli*	CTX-M 1/61
			13S00745-6	21A	4	*Escherichia coli*	SHV 12/129
			13S00745-7	21A	4	*Escherichia coli*	SHV 12/129
13S00746	3.23E+06	0.0004	13S00746-5	21B	4	*Escherichia coli*	CTX-M 1 TEM 1b
			13S00746-6	21B	4	*Escherichia coli*	SHV 12/129
			13S00746-7	21B	4	*Escherichia coli*	SHV 12/129
13S00747	2.08E+06	0.0017	13S00747-4	9B	4	*Escherichia coli*	*amp*C type 3
			13S00747-5	9B	4	*Escherichia coli*	*amp*C type 3
			13S00747-6	9B	4	*Escherichia coli*	*amp*C type 3
13S00748	1.09E+07	0.0003	13S00748-5	9A	4	*Escherichia coli*	CTX-M 14/18 TEM 1b/104
			13S00748-6	9A	4	*Escherichia coli*	CMY 2/61 TEM 1b/104
			13S00748-7	9A	4	*Escherichia coli*	CTX-M 15/28 TEM 1varA
			13S00748-8	9A	4	*Escherichia coli*	*amp*C type 3
13S00749	1.17E+07	0.0003	13S00749-5	9C	4	*Escherichia coli*	CTX-M 15/28 TEM 1varA
			13S00749-6	9C	4	*Escherichia coli*	CTX-M 15/28 TEM 1varA
			13S00749-7	9C	4	*Escherichia coli*	*amp*C type 3
			13S00749-8	9C	4	*Escherichia coli*	*amp*C type 3
13S00751	1.00E+04	0.0455	13S00751-3	5B	5	*Acinetobacter spp*	
13S00753	4.81E+07	0.0016	13S00753-4	11A	5	*Escherichia coli*	CTX-M 1/61 TEM 1b/104
			13S00753-5	11A	5	*Escherichia coli*	TEM 52c
			13S00753-6	11A	5	*Escherichia coli*	*amp*C type 3
13S00754	1.94E+06	0.0027	13S00754-4	15A	5	*Escherichia coli*	CTX-M 14/18 TEM 1b
			13S00754-5	15A	5	*Escherichia coli*	CTX-M 32
			13S00754-6	15A	5	*Escherichia coli*	TEM 52c
			13S00754-7	15A	5	*Escherichia coli*	CMY 2/61
13S00756	3.77E+07	0.0363	13S00756-4	15C	5	*Escherichia coli*	*amp*C type 3
			13S00756-5	15C	5	*Escherichia coli*	*amp*C type 3
			13S00756-6	15C	5	*Escherichia coli*	*amp*C type 3 mutant
13S00758	1.04E+04	0.0000	13S00758-4	22A	5	*Escherichia coli*	TEM 1varA
			13S00758-5	22A	5	*Escherichia coli*	CTX-M 1 TEM 1b
			13S00758-6	22A	5	*Escherichia coli*	CTX-M 2/20/44/56/97 TEM 1b&52c
13S00761	5.21E+04	0.0000	13S00761-4	17A	5	*Escherichia coli*	CTX-M 32
			13S00761-5	17A	5	*Escherichia coli*	CTX-M 32
			13S00761-6	17A	5	*Escherichia coli*	CTX-M 32
13S00762	1.57E+05	0.0001	13S00762-4	17B	5	*Escherichia coli*	CTX-M 1 TEM 1b
			13S00762-5	17B	5	*Escherichia coli*	CTX-M 1
			13S00762-6	17B	5	*Escherichia coli*	CTX-M 14/18 TEM 1b

13S00763	4.00E+04	0.0004	13S00763-4	19A	5	*Escherichia coli*	CMY 2/61 TEM 1b
			13S00763-5	19A	5	*Escherichia coli*	SHV 12/129
			13S00763-6	19A	5	*Escherichia coli*	SHV 12/129
13S00769	1.86E+07	0.0024	13S00769-4	10A	5	*Escherichia coli*	TEM 1b&52c
			13S00769-5	10A	5	*Escherichia coli*	CTX-M 1
			13S00769-6	10A	5	*Escherichia coli*	CTX-M 1 TEM 1b
			13S00769-7	10A	5	*Escherichia coli*	CTX-M 1 TEM 1b
13S00770	3.94E+06	0.0015	13S00770-4	18A	5	*Escherichia coli*	CTX-M 1
			13S00770-5	18A	5	*Escherichia coli*	TEM 1d
			13S00770-6	18A	5	*Escherichia coli*	CTX-M 1
			13S00770-7	18A	5	*Escherichia coli*	*amp*C type 3
13S00771	2.88E+04	0.0000	13S00771-4	23A	5	*Escherichia coli*	*amp*C type 3
			13S00771-5	23A	5	*Escherichia coli*	CTX-M 1
			13S00771-6	23A	5	*Escherichia coli*	*amp*C type 3
13S00774	1.00E+02	0.0000	13S00774-4	5B	6	*Escherichia coli*	CMY 2/61
13S00775	2.94E+05	0.0001	13S00775-4	24A	6	*Escherichia coli*	TEM 52c
			13S00775-5	24A	6	*Escherichia coli*	CMY 2/61
			13S00775-6	24A	6	*Escherichia coli*	TEM 52c
13S00776	1.00E+05	0.0000	13S00776-4	24B	6	*Escherichia coli*	CMY 2/61
			13S00776-5	24B	6	*Escherichia coli*	CTX-M 1
			13S00776-6	24B	6	*Escherichia coli*	*amp*C type 3 mutant
13S00777	1.89E+04	0.0067	13S00777-4	12B	6	*Acinetobacter spp*	
			13S00777-5	12B	6	*Acinetobacter spp*	
			13S00777-6	12B	6	*Escherichia coli*	
13S00778	1.00E+02	0.0000	13S00778-4	12A	6	*Escherichia coli*	CMY 2/61 TEM 33
			13S00778-5	12A	6	*Acinetobacter spp*	
			13S00778-6	12A	6	*Acinetobacter spp*	
			13S00778-7	12A	6	*Pseudomonas spp*	
13S00784	1.00E+02	0.0000	13S00784-4	5A	7	*Unidentified species*	
			13S00784-5	5A	7	*Pseudomonas spp*	
13S00788	7.29E+03	0.0001	13S00788-4	12B	7	*Acinetobacter spp*	
			13S00788-5	12B	7	*Pseudomonas spp*	
			13S00788-6	12B	7	*Pseudomonas spp*	
13S00792	6.22E+10	1.0000	13S00792-4	16B	7	*Escherichia coli*	CMY 2/61
			13S00792-5	16B	7	*Escherichia coli*	CMY 2/61
			13S00792-6	16B	7	*Escherichia coli*	CMY 2/61
13S00883	2.12E+05	0.0000	13S00883-4	7A	8	*Escherichia coli*	CTX-M 1/61
			13S00883-5	7A	8	*Escherichia coli*	CTX-M 1/61
			13S00883-6	7A	8	*Escherichia coli*	CTX-M 1/61
			13S00883-7	7A	8	*Escherichia coli*	CTX-M 1/61
			13S00883-8	7A	8	*Escherichia coli*	CTX-M 1
13S00884	1.00E+02	0.0000	13S00884-2	7B	8	*Acinetobacter spp*	
			13S00884-3	7B	8	*Acinetobacter spp*	
13S00887	5.88E+07	0.0010	13S00887-4	9A	8	*Escherichia coli*	CTX-M 2/20/44/56/97 TEM 1b
			13S00887-5	9A	8	*Escherichia coli*	CTX-M 2/20/44/56/97 TEM 1b
			13S00887-6	9A	8	*Escherichia coli*	CTX-M 2/20/44/56/97 TEM 1b
13S00888	2.02E+07	0.0007	13S00888-4	9B	8	*Escherichia coli*	CTX-M 2/20/44/56/97 TEM 1b
			13S00888-5	9B	8	*Escherichia coli*	CTX-M 2/20/44/56/97 TEM 1b

			13S00888-6	9B	8	*Escherichia coli*	CTX-M 2/20/44/56/97 TEM 1b
13S00890	5.21E+06	0.0012	13S00890-4	9C	8	*Escherichia coli*	CTX-M 2/20/44/56/97 TEM 1b
			13S00890-5	9C	8	*Escherichia coli*	CTX-M 2/20/44/56/97 TEM 1b
			13S00890-6	9C	8	*Escherichia coli*	CTX-M 2/20/44/56/97 TEM 1b
13S00892	1.00E+02	0.0000	13S00892-3	12B	8	*Escherichia coli*	CMY 2/61
13S00894	1.00E+02	0.0000	13S00894-3	5B	8	*Escherichia coli*	CTX-M 1
13S00896	2.50E+06	0.0375	13S00896-4	1B	8	*Escherichia coli*	CTX-M 15 TEM 1b
			13S00896-5	1B	8	*Escherichia coli*	CTX-M 15 TEM 1b
			13S00896-6	1B	8	*Escherichia coli*	CTX-M 15 TEM 1b
13S00898	9.62E+03	0.0017	13S00898-5	21A	8	*Escherichia coli*	CTX-M 1 TEM 1b
13S00899	1.00E+05	0.0013	13S00899-2	21B	8	*Escherichia coli*	TEM 1b
			13S00899-5	21B	8	*Escherichia coli*	CMY 2/61
13S00902	2.94E+07	1.0000	13S00902-4	5B	9	*Escherichia coli*	CTX-M 15 OXA 1/30
			13S00902-5	5B	9	*Escherichia coli*	CTX-M 15 OXA 1/30
			13S00902-6	5B	9	*Escherichia coli*	CTX-M 15 OXA 1/30
13S00903	2.00E+06	0.0100	13S00903-4	12A	9	*Escherichia coli*	CTX-M 1 TEM 1b
			13S00903-5	12A	9	*Escherichia coli*	CTX-M 1 TEM 1b
			13S00903-6	12A	9	*Escherichia coli*	CTX-M 1 TEM 1b
13S00905	1.00E+02	0.0000	13S00905-3	2A	9	*Escherichia coli*	CMY 2/61
13S00906	1.00E+08	0.0048	13S00906-4	11A	9	*Escherichia coli*	CTX-M 1 TEM 1
			13S00906-5	11A	9	*Escherichia coli*	CTX-M 14/18 TEM 1b
			13S00906-6	11A	9	*Acinetobacter spp*	
13S00911	1.00E+02	0.0000	13S00911-3	17A	9	*Escherichia coli*	SHV 12/129
13S00914	1.00E+02	0.0000	13S00914-3	5B	10	*Pseudomonas spp*	
13S00915	1.30E+10	0.1300	13S00915-4	18A	10	*Escherichia coli*	CTX-M 1
			13S00915-5	18A	10	*Escherichia coli*	CTX-M 1/61
			13S00915-6	18A	10	*Escherichia coli*	CTX-M 1
13S00916	1.12E+07	0.0032	13S00916-4	19A	10	*Escherichia coli*	CTX-M 1
			13S00916-5	19A	10	*Escherichia coli*	CTX-M 1
			13S00916-6	19A	10	*Escherichia coli*	CTX-M 14
			13S00916-7	19A	10	*Escherichia coli*	CTX-M 55/57 TEM 1b
13S00917	3.13E+06	0.0001	13S00917-4	23A	10	*Escherichia coli*	CTX-M 1 TEM 1b
			13S00917-5	23A	10	*Escherichia coli*	CTX-M 1 TEM 1b
			13S00917-6	23A	10	*Escherichia coli*	TEM 52c&135
			13S00917-7	23A	10	*Escherichia coli*	TEM 52c&135
			13S00917-8	23A	10	*Acinetobacter spp*	OXA-23-like
13S00918	3.85E+05	0.0003	13S00918-4	24A	10	*Escherichia coli*	CMY 2
			13S00918-5	24A	10	*Escherichia coli*	CMY 2
			13S00918-6	24A	10	*Escherichia coli*	TEM 52c
			13S00918-7	24A	10	*Escherichia coli*	SHV 12/129
13S00919	1.00E+02	0.0000	13S00919-3	24B	10	*Escherichia coli*	CMY 2
13S00920	1.00E+02	0.0000	13S00920-3	6A	10	*Enterobacter cloacae*	
13S00926	4.17E+06	0.0060	13S00926-4	15A	10	*Escherichia coli*	CTX-M 1
			13S00926-5	15A	10	*Escherichia coli*	CTX-M 1
			13S00926-6	15A	10	*Escherichia coli*	CTX-M 1
			13S00926-7	15A	10	*Escherichia coli*	CTX-M 1 TEM 1b
13S00928	1.76E+06	0.0001	13S00928-4	15C	10	*Escherichia coli*	CMY 2/61

			13S00928-5	15C	10	*Escherichia coli*	SHV 12/129 TEM 1
			13S00928-6	15C	10	*Escherichia coli*	CTX-M 1 TEM 1b
			13S00928-7	15C	10	*Escherichia coli*	CTX-M 1 TEM 1b
13S00929	1.00E+02	0.0000	13S00929-3	12A	10	*Escherichia coli*	CMY 2/61 TEM 33mut
13S00940	9.80E+05	0.0012	13S00940-4	22A	11	*Escherichia coli*	TEM 52c
			13S00940-5	22A	11	*Escherichia coli*	CTX-M 2/97
			13S00940-6	22A	11	*Escherichia coli*	CTX-M 32
13S00944	2.40E+07	0.0277	13S00944-4	3A	11	*Escherichia coli*	CMY 2/61 TEM 1b/104
			13S00944-5	3A	11	*Escherichia coli*	CMY 2/61 TEM 1b/104
			13S00944-6	3A	11	*Escherichia coli*	CMY 2/61 TEM 1b
13S00951	2.94E+08	0.2146	13S00951-4	11A	12	*Escherichia coli*	CTX-M 1 OXA 1/30 TEM 1b&1d
			13S00951-5	11A	12	*Escherichia coli*	CTX-M 1 OXA 1/30 TEM 1b&1d
			13S00951-6	11A	12	*Escherichia coli*	CTX-M 1 OXA 1/30 TEM 1b&1d
13S00956	4.17E+04	0.0001	13S00956-4	5B	12	*Escherichia coli*	CTX-M 1 TEM 1b
			13S00956-5	5B	12	*Escherichia coli*	CTX-M 1
			13S00956-6	5B	12	*Escherichia coli*	CTX-M 1 TEM 1b
			13S00956-7	5B	12	*Escherichia coli*	TEM 52c
13S00969	1.00E+02	0.0002	13S00969-3	16A	13	*Ochrobactrum spp*	
13S00971	9.80E+05	0.0001	13S00971-4	19A	13	*Escherichia coli*	CTX-M 1
			13S00971-5	19A	13	*Escherichia coli*	CTX-M 1
			13S00971-6	19A	13	*Escherichia coli*	CTX-M 15 OXA 1/30 TEM 33
13S00972	4.31E+07	0.0031	13S00972-4	9A	13	*Escherichia coli*	CTX-M 1
			13S00972-5	9A	13	*Escherichia coli*	CTX-M 1
			13S00972-6	9A	13	*Escherichia coli*	CTX-M 1
13S00973	1.47E+06	0.0000	13S00973-4	9B	13	*Escherichia coli*	CTX-M 1
			13S00973-5	9B	13	*Escherichia coli*	SHV 12/129
			13S00973-6	9B	13	*Escherichia coli*	CTX-M 1
13S00974	9.57E+06	0.0002	13S00974-4	9C	13	*Escherichia coli*	CMY 2
			13S00974-5	9C	13	*Escherichia coli*	CMY 2
			13S00974-6	9C	13	*Escherichia coli*	CMY 2
13S00977	1.00E+02	0.0000	13S00977-3	2A	13	*Pseudomonas aeruginosa*	
13S00978	1.00E+02	0.0094	13S00978-3	3A	13	*Acinetobacter spp*	
13S00980	4.79E+07	0.0012	13S00980-4	10A	13	*Escherichia coli*	CMY 2 TEM 1b
			13S00980-5	10A	13	*Escherichia coli*	CTX-M 15 TEM 1varA&1varB
			13S00980-6	10A	13	*Escherichia coli*	CMY 2/61 TEM 1b
13S00982	5.00E+07	0.0071	13S00982-4	15A	14	*Escherichia coli*	SHV 12/129
			13S00982-5	15A	14	*Escherichia coli*	CTX-M 1
			13S00982-6	15A	14	*Escherichia coli*	CTX-M 1
			13S00982-7	15A	14	*Escherichia coli*	SHV 12/129
13S00983	1.00E+02	0.0000	13S00983-3	15B	14	*Escherichia coli*	CTX-M 1
			13S00984-4	15C	14	*Escherichia coli*	CTX-M 1
			13S00984-5	15C	14	*Escherichia coli*	*amp*C type 11
			13S00984-6	15C	14	*Escherichia coli*	CTX-M 1
13S00984	1.54E+08	0.0025	13S00984-7	15C	14	*Pseudomonas aeruginosa*	
13S00986	1.85E+06	0.0003	13S00986-4	24A	14	*Escherichia coli*	CTX-M 1
			13S00986-5	24A	14	*Escherichia coli*	CTX-M 1

			13S00986-6	24A	14	*Escherichia coli*	CTX-M 1
13S00987	4.00E+07	0.0057	13S00987-4	24B	14	*Escherichia coli*	CTX-M 1
			13S00987-5	24B	14	*Escherichia coli*	CTX-M 1/61
			13S00987-6	24B	14	*Escherichia coli*	CTX-M 1
13S00988	6.86E+06	0.0175	13S00988-4	17A	14	*Escherichia coli*	CTX-M 1
			13S00988-5	17A	14	*Escherichia coli*	CTX-M 1
			13S00988-6	17A	14	*Escherichia coli*	CTX-M 1
13S00989	7.14E+04	0.0008	13S00989-4	17B	14	*Escherichia coli*	CTX-M 1
			13S00989-5	17B	14	*Escherichia coli*	TEM 1b
			13S00989-6	17B	14	*Escherichia coli*	TEM 1b
13S00990	1.90E+06	0.0009	13S00990-4	18A	14	*Escherichia coli*	CTX-M 1
			13S00990-5	18A	14	*Escherichia coli*	CTX-M 15
			13S00990-6	18A	14	*Escherichia coli*	CTX-M 1
13S01005	1.00E+02	0.0000	13S01005-3	3A	14	*Escherichia coli*	CTX-M 1 CMY 2/61 TEM 1b
13S01013	1.00E+05	0.0003	13S01013-4	22A	16	*Escherichia coli*	CTX-M 2/20/44/56/97
			13S01013-5	22A	16	*Escherichia coli*	CTX-M 1 TEM 1
			13S01013-6	22A	16	*Escherichia coli*	CTX-M 1 TEM 1b
13S01021	5.56E+05	0.0000	13S01021-4	11A	17	*Escherichia coli*	CTX-M 14/18 TEM 1b
			13S01021-5	11A	17	*Escherichia coli*	*amp*C type 3
			13S01021-6	11A	17	*Escherichia coli*	CTX-M 14/18
13S01027	1.00E+02	0.0000	13S01027-3	2A	17	*Escherichia coli*	CTX-M 3 TEM 1b
			13S01027-6	2A	17	*Pseudomonas aeruginosa*	
13S01028	1.92E+04	0.0001	13S01028-4	7A	17	*Escherichia coli*	TEM 1
			13S01028-5	7A	17	*Escherichia coli*	TEM 1
13S01029	1.00E+04	0.0000	13S01029-4	7B	17	*Escherichia coli*	CMY 2/61 TEM 33
			13S01029-5	7B	17	*Escherichia coli*	CMY 2/61 TEM 33
13S01031	3.13E+06	0.0006	13S01031-4	9A	17	*Escherichia coli*	CTX-M 15
			13S01031-5	9A	17	*Escherichia coli*	CTX-M 14/18 TEM 1b
			13S01031-6	9A	17	*Escherichia coli*	CTX-M 1
13S01033	2.50E+06	0.0004	13S01033-4	9C	17	*Escherichia coli*	CTX-M 1 TEM 1&1b
			13S01033-5	9C	17	*Escherichia coli*	CTX-M 1
			13S01033-6	9C	17	*Escherichia coli*	CTX-M 3
13S01042	1.00E+02	0.0000	13S01042-3	21B	17	*Escherichia coli*	*amp*C type 3 mutant
13S01053	1.63E+07	0.0080	13S01053-4	10A	18	*Escherichia coli*	CTX-M 15 TEM 1varA
			13S01053-5	10A	18	*Escherichia coli*	CTX-M 15 TEM 1varA
			13S01053-6	10A	18	*Escherichia coli*	CTX-M 15 TEM 1varA
13S01061	5.32E+05	0.0001	13S01061-4	15A	18	*Escherichia coli*	CMY 2 mutant
			13S01061-5	15A	18	*Escherichia coli*	CMY 2/61
			13S01061-6	15A	18	*Escherichia coli*	CTX-M 1
			13S01061-7	15A	18	*Escherichia coli*	*amp*C type 3
13S01062	6.67E+07	0.0026	13S01062-4	15C	18	*Escherichia coli*	CTX-M 15 OXA 1/30 TEM 1varA
			13S01062-5	15C	18	*Escherichia coli*	CTX-M 1 TEM 1b
			13S01062-6	15C	18	*Escherichia coli*	CTX-M 15 OXA 1/30 TEM 1b/104
13S01063	1.00E+02	0.0000	13S01063-3	19A	18	*Escherichia coli*	*amp*C type 3
13S01075	6.27E+05	0.0032	13S01075-4	17B	18	*Escherichia coli*	CTX-M 8 TEM 1b/104
			13S01075-5	17B	18	*Escherichia coli*	CTX-M 14/18
			13S01075-6	17B	18	*Escherichia coli*	CTX-M 8

			13S01075-8	17B	18	*Escherichia coli*	TEM 52c
13S01076	3.00E+04	0.0003	13S01076-4	18A	19	*Escherichia coli*	CTX-M 1
			13S01076-5	18A	19	*Escherichia coli*	CTX-M 32
			13S01076-6	18A	19	*Escherichia coli*	CTX-M 1
13S01077	4.35E+05	0.0001	13S01077-4	22A	19	*Escherichia coli*	*amp*C type 3
			13S01077-5	22A	19	*Escherichia coli*	*amp*C type 3
			13S01077-6	22A	19	*Escherichia coli*	CTX-M 1 TEM 1b
13S01078	3.60E+06	0.0008	13S01078-4	24A	19	*Escherichia coli*	*amp*C type 3 mutant
			13S01078-5	24A	19	*Escherichia coli*	CTX-M 1
			13S01078-6	24A	19	*Escherichia coli*	TEM 52c
			13S01078-7	24A	19	*Escherichia coli*	SHV 12/129
13S01079	8.65E+04	0.0003	13S01079-4	24B	19	*Escherichia coli*	TEM 52c
			13S01079-5	24B	19	*Escherichia coli*	TEM 52c
			13S01079-6	24B	19	*Escherichia coli*	TEM 52c
			13S01079-8	24B	19	*Escherichia coli*	SHV 12/129
13S01116	4.35E+05	0.0001	13S01116-4	9A	21	*Escherichia coli*	SHV 12/129
			13S01116-5	9A	21	*Escherichia coli*	SHV 12/129
			13S01116-6	9A	21	*Escherichia coli*	SHV 12/129 TEM 1b
13S01118	4.81E+05	0.0002	13S01118-4	9C	21	*Escherichia coli*	SHV 12/129 TEM 1&1b
			13S01118-5	9C	21	*Escherichia coli*	SHV 12/129
			13S01118-6	9C	21	*Escherichia coli*	SHV 12/129 TEM 1&1b
13S01135	1.00E+06	0.0014	13S01135-4	11A	21	*Escherichia coli*	CTX-M 2/20/44/56/97 TEM 1b
			13S01135-5	11A	21	*Escherichia coli*	CTX-M 1 TEM 1b
			13S01135-6	11A	21	*Escherichia coli*	CTX-M 65
13S01141	9.80E+05	0.0200	13S01141-4	5B	22	*Escherichia coli*	CTX-M 15 OXA 1/30
			13S01141-5	5B	22	*Escherichia coli*	CTX-M 15 OXA 1/30
			13S01141-6	5B	22	*Escherichia coli*	CTX-M 15 OXA 1/30
13S01143	2.14E+08	0.0583	13S01143-4	10A	22	*Escherichia coli*	SHV 12/129
			13S01143-5	10A	22	*Escherichia coli*	SHV 2
			13S01143-6	10A	22	*Escherichia coli*	TEM 1b&52StPaul
13S01151	8.24E+06	0.0047	13S01151-4	16A	22	*Escherichia coli*	TEM 52c
			13S01151-5	16A	22	*Escherichia coli*	CTX-M 14/18 OXA 1/30
			13S01151-6	16A	22	*Escherichia coli*	CTX-M 2/20/44/56/97 TEM 1b
			13S01151-8	16A	22	*Escherichia coli*	CTX-M 2/20/44/56/97 TEM 1b
13S01152	1.00E+02	0.0000	13S01152-3	16B	22	*Escherichia coli*	CTX-M 32
13S01154	5.88E+07	0.0100	13S01154-4	15A	22	*Escherichia coli*	CTX-M 15 OXA 1/30
			13S01154-5	15A	22	*Escherichia coli*	CTX-M 15 OXA 1/30
			13S01154-6	15A	22	*Escherichia coli*	CTX-M 15 OXA 1/30
13S01155	1.00E+02	0.0000	13S01155-3	15C	22	*Escherichia coli*	TEM 52StPaul
13S01164	1.04E+05	0.0005	13S01164-4	18A	23	*Escherichia coli*	CMY 2/61
			13S01164-5	18A	23	*Escherichia coli*	CMY 2/61
			13S01164-6	18A	23	*Escherichia coli*	TEM 52StPaul
13S01165	1.04E+07	0.0010	13S01165-4	17A	23	*Escherichia coli*	CTX-M 14/18
			13S01165-5	17A	23	*Escherichia coli*	*amp*C type 3
			13S01165-6	17A	23	*Escherichia coli*	*amp*C type 3
13S01166	1.96E+06	0.0010	13S01166-4	17B	23	*Escherichia coli*	*amp*C type 3
			13S01166-5	17B	23	*Escherichia coli*	*amp*C type 3
			13S01166-6	17B	23	*Escherichia coli*	CTX-M 14/18

13S01175	1.00E+02	0.0000	13S01175-3	6A	23	*Escherichia coli*	CTX-M 1 TEM 1b&1c
13S01176	2.13E+08	0.0334	13S01176-4	6B	23	*Escherichia coli*	SHV 12/129
			13S01176-5	6B	23	*Escherichia coli*	CTX-M 1 TEM 1b&1c
			13S01176-6	6B	23	*Escherichia coli*	CTX-M 14/18 TEM 1b
			13S01176-7	6B	23	*Escherichia coli*	CTX-M 14/18 TEM 1b
			13S01176-8	6B	23	*Escherichia coli*	TEM 1b&52StPaul
13S01188	4.90E+04	0.0001	13S01188-4	22A	24	*Escherichia coli*	CTX-M 65
			13S01188-5	22A	24	*Escherichia coli*	CTX-M 1 TEM 1b/104
			13S01188-6	22A	24	*Escherichia coli*	*amp*C type 3
14S00011	4.22E+05	0.0008	14S00011-4	9A	26	*Escherichia coli*	TEM 30var
			14S00011-5	9A	26	*Escherichia coli*	TEM 30var
			14S00011-6	9A	26	*Escherichia coli*	TEM 30var
14S00012	5.21E+07	0.0575	14S00012-4	9B	26	*Escherichia coli*	CTX-M 1
			14S00012-5	9B	26	*Escherichia coli*	CTX-M 1
			14S00012-6	9B	26	*Escherichia coli*	CTX-M 1
14S00013	1.00E+02	0.0000	14S00013-5	9C	26	*Escherichia coli*	CTX-M 1
14S00017	9.38E+06	0.0225	14S00017-4	11A	26	*Escherichia coli*	CTX-M 1
			14S00017-5	11A	26	*Escherichia coli*	CTX-M 15 TEM 1b&1varA
			14S00017-6	11A	26	*Escherichia coli*	CTX-M 15 TEM 1b&1varA
14S00018	1.00E+02	0.0000	14S00018-3	19A	26	*Escherichia coli*	CTX-M 1
			14S00018-6	19A	26	*Escherichia coli*	CTX-M 1/61
			14S00018-7	19A	26	*Escherichia coli*	CTX-M 1

Column CFU/g shows cfu/g faeces of non-wild-type Enterobacteriaceae with reduced susceptibility on MCC. Column F shows fraction of cfu/g faeces of non-wild-type Enterobacteriaceae with reduced susceptibility on MCC compared to total Enterobacteriaceae on MC

*Gene characterisation: AmpC types were assigned according to Mulvey *et al*.[7]