Isolation of a multidrug-resistant *Escherichia coli* pathotype Stx2:Cnf1:Cnf2:Eae as a potential cause of hemorrhagic diarrhea and secondary septicemia in a dog

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Running title: MDR *Escherichia coli* causing canine diarrhea and septicemia

Abstract. *Escherichia coli* is a member of the family *Enterobacteriaceae* and is a commensal in the intestine of many animals as well as humans. Most strains are of low virulence. A dog developed vomiting and hemorrhagic diarrhea after surgery and died despite treatment. Postmortem examination revealed hemorrhagic gastroenteritis and colitis. A multidrug-resistant *E. coli*, with virulence factors Shiga-toxin–producing gene, stx2, *eae* gene, and cytotoxic necrotic factors CNF-1 and CNF-2, was isolated from internal organs. *E. coli* can easily acquire new genes for virulence factors and antimicrobial resistance as demonstrated by this isolate with characteristics of both enterohemorrhagic *E. coli* and necrotoxigenic *E. coli*. In addition, the isolate was resistant to all beta-lactam antibiotics tested, as well as to enrofloxacin by a disk diffusion methodology. Broth-based minimum inhibitory concentration analysis confirmed resistance to amoxicillin (>32 μg/mL), enrofloxacin (>32 μg/mL), fosfomycin (>128 μg/mL), and neomycin (>32 μg/mL). The discovery of such strains is a cause for concern given that *E. coli* can be shared by companion animals and their human owners.

Keywords: antimicrobial resistance; canine; *Escherichia coli*; virulence factors.

Escherichia coli is a member of the family *Enterobacteriaceae* and is a commensal in the intestine of many animals as well as humans. It is commonly found in the environment in soil and water. Infection may occur directly via contact with an infected human or animal, or indirectly through contaminated drinking water, food, or environment.[11,12,17]

Although most strains are of low virulence and only cause infection when a predisposing factor compromises the natural immunity of an animal, *E. coli* can easily acquire new combinations of genes for virulence factors and antimicrobial resistance (AMR).[12] As a feature of acquired virulence factors, some *E. coli* strains cause primary intestinal and extra-intestinal infections.[11,12,17]

E. coli strains typically possess virulence factors in various numbers and combinations. The pathotype of an isolate is determined by the virulence factors present and the type of infection caused by the strain. Necrotoxigenic *E. coli* (NTEC) strains have been implicated in extra-intestinal infections such as septicemia.[11] Extra-intestinal pathogenic *E. coli* in dogs tend to possess genes that produce virulence factors distinct from commensals and strains that cause intestinal infections.[17] Strains that cause intestinal infections may be classified as enterotoxigenic (ETEC) or attaching-and-effacing *E. coli* (AEEC). AEEC are further divided into enteropathogenic (EPEC) and enterohemorrhagic *E. coli* (EHEC). Shiga-toxin production is characteristic of these strains of *E. coli.*[11,13] Several strains of Shiga-toxin–producing *E. coli* (STEC) have been implicated in human hemolytic-uremic syndrome and hemorrhagic colitis.[12]

AMR in *E. coli* isolated from companion animals is a reason for concern because similar pathogenic strains can colonize both humans and animals, providing an opportunity for rapid spread of resistant strains.[14,15] The zoonotic potential of such strains of *E. coli* is highlighted by

several reports of the potential for dogs to harbor antimicrobial-resistant *E. coli* strains with virulence factors similar to strains isolated from humans.[1,4,9,19]

We describe here the virulence factors and the AMR profile associated with an *E. coli* strain isolated from a fatal case of canine enteric infection and septicemia.

A healthy 5-y-old, spayed female Fox Terrier was presented for surgical removal of a small ventral abdominal cutaneous mass by the local general veterinary practitioner. The surgical procedure was uneventful. Approximately 6 h post-surgery, the patient developed profuse hemorrhagic diarrhea and vomiting. She was given intravenous fluid therapy (Ringer lactate), intravenous maropitant citrate (Cerenia; Zoetis-Pfizer), and hyoscine butylbromide (Buscopan; Boehringer Ingelheim), as well as oral metronidazole suspension. Despite treatment, the patient died ~12 h later and was submitted for postmortem examination at the Section of Pathology, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria (South Africa).

Macroscopic examination revealed severe, diffuse, hemorrhagic gastroenteritis and colitis. Microscopic examination of sections of small and large intestine revealed multifocal enterocyte necrosis. Throughout the mucosa, but especially toward the villus tips, were extensive areas of hemorrhage, with severe congestion elsewhere. The lungs had moderate, diffuse, nonsuppurative interstitial pneumonia. Monomorphic colonies of short, gram-negative rods were scattered throughout the hepatic sinusoids and splenic sinuses.

Aerobic culture on Columbia agar with 5% horse blood (CBA; Thermo Fisher) and MacConkey agar without crystal violet (Thermo Fisher) yielded an isolate in pure, heavy growth from lung, liver, and spleen samples. Colonies were rough, gray, and non-hemolytic on CBA and

pink on MacConkey agar. Bacteria were gram-negative medium rods, catalase positive, oxidase negative, and indole positive. The isolate was confirmed as *E. coli* by API 10S (BioMérieux).

Antimicrobial susceptibility was determined by both disk diffusion and broth-based minimum inhibitory concentration (MIC) assays. The disk diffusion test was performed using Mueller–Hinton agar (Thermo Fisher) according to Clinical and Laboratory Standards Institute (CLSI) methods.[2] Interpretation parameters published by CLSI[3] were used, except for fosfomycin and colistin, for which parameters from the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[7] and Falagas and Kasiakou[8] were used, respectively. The antimicrobial disks (Oxoid) used were penicillin (10 IU), ampicillin (10 µg), amoxicillin–clavulanic acid (20/10 µg), cephalothin (30 µg), ceftiofur (30 µg), doxycycline (30 µg), enrofloxacin (30 µg), sulfonamide–trimethoprim (25 µg), fosfomycin (200 µg), and colistin (10 µg). The interpretation criteria for gentamicin were used to interpret the neomycin results.

MIC assays were performed according to the CLSI 2015 method,[2] and the breakpoints were from CLSI[3] and EUCAST.[7] The result was reported as the lowest concentration of antimicrobial for which no growth was detected.

The isolate was analyzed by an external laboratory for cytotoxic necrotizing factors (CNF-1 and CNF-2) as well as Shiga-toxin–producing genes (*stx1* and *stx2*) and intimin genes (*eae*; Table 1). Published PCR conditions were followed.[16,18]

The case history and postmortem examination results indicated that the *E. coli* under investigation was an EHEC strain. PCR analysis indicated that the isolate possessed genes for *stx2* and *eae*, characteristic of EHEC,[5,12] as well as *cnf1* and *cnf2* genes, which are characteristic of NTEC.[5]

Normally, Shiga-toxin–producing *E. coli* do not invade. Shiga-toxin is absorbed and distributed throughout the body via the blood stream.[12] The Shiga-toxins, Stx1 and Stx2, are cytotoxins encoded in bacteriophage genomes and transferred by transduction.[13] The toxins are protein synthesis inhibitors that cause cell death and necrosis. Stx2 is the most potent of the Shiga-toxins and is usually implicated in more serious infections.[12,13] In addition, EHEC strains are known to cause hemorrhagic enteritis.[6,13] Necrosis and hemorrhage were histologic lesions reported in our case.

The isolate investigated possessed the *eae* gene. Shiga-toxin–producing *E. coli* often have additional virulence factors.[12] Intimin, the protein encoded by the *eae* gene, is the most significant of these.[12] The *eae* gene is part of the chromosomal pathogenicity island LEE (locus for enterocyte effacement).[12] Intimin is responsible for adhesion to the intestinal epithelium, as well as the attaching-and-effacing lesion.[12]

The cyclomodulin, cytonecrotic factor (CNF) causes necrosis of the mucosa of the intestine and is usually associated with NTEC.[17] CNF-1 is part of a pathogenicity island, whereas CNF-2 is encoded on a plasmid.[6] Experimental data indicate that these cytotoxic factors may lead to toxemia and interfere with the formation of the enterocyte cytoskeleton. As result of the damage, the enterocyte becomes phagocytic, ingesting bacteria, and allowing bacterial multiplication and transcytosis.[5] CNF-1 may also efface microvilli.[5] In our case, both CNF-1 and CNF-2 were present. These factors would have contributed to the enterocyte necrosis seen microscopically and the invasive nature of this particular *E. coli*.

Our isolate proved to be multidrug resistant[10], with resistance to penicillin, penicillin with β-lactamase inhibitors, cephalosporins, and fluoroquinolones. Susceptibility to doxycycline was intermediate (Table 2). The MIC of selected antimicrobials for the isolate was determined (Table

3). Interpretive criteria for ampicillin were used for amoxicillin; those for gentamicin were used for neomycin, and those for tetracycline were used for chlortetracycline and doxycycline. Resistance was detected to amoxicillin (>32 μg/mL), enrofloxacin (>32 μg/mL), fosfomycin (>128 μg/mL), and neomycin (>32 μg/mL). In contrast to the disk diffusion results, the isolate proved to be resistant to fosfomycin and neomycin in the MIC test. In addition, the MIC test indicated that the isolate was still sensitive to doxycycline.

Virulence factors of 2 pathotypes, EHEC and NTEC, combined in this single strain of *E. coli* resulted in a new and virulent pathotype (Stx2:Cnf1:Cnf2:Eae) that appeared responsible for the fatal outcome. In addition, the isolate was multidrug resistant. The discovery of strains such as this is a cause for concern because *E. coli* can easily be transferred from companion animals to their human owners and vice versa.

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References

1. Bentancor A, et al. Profile of Shiga toxin-producing *Escherichia coli* strains isolated from dogs and cats and genetic relationships with isolates from cattle, meat and humans. Vet Microbiol 2012;156:336–342.

2. Clinical and Laboratory Standards Institute (CLSI). Standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard. 4th ed. CLSI, 2013. CLSI document VET01-A4.

3. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; CLSI supplement. 3rd ed. CLSI, 2015. CLSI document VET01S.

4. de Almeida PMP, et al. Characterization of atypical enteropathogenic *Escherichia coli* (aEPEC) isolated from dogs. Vet Microbiol 2012;158:420–424.

5. De Rycke J, et al. Necrotoxic *Escherichia coli* (NTEC): two emerging categories of human and animal pathogens. Vet Res 1999;30:221–233.

6. DebRoy C, Maddox CW. Identification of virulence attributes of gastrointestinal *Escherichia coli* isolates of veterinary significance. Anim Health Res Rev 2001;2:129–140.

7. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters. Version 6.0. EUCAST, 2016. https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_6.0_Breakpoint_table.pdf

8. Falagas ME, Kasiakou SK. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. Clin Infect Dis 2005;40:1333–1341.

9. Kataoka Y, et al. A 3-year epidemiological surveillance of *Escherichia coli* O157:H7 in dogs and cats in Japan. J Vet Med Sci 2010;72:791–794.

10. Magiorakos A-P, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 2012;18:268–281.

11. Markey B, et al. Enterobacteriaceae. In: Clinical Veterinary Microbiology. 2nd ed. Mosby Elsevier, 2013:258–293.

12. Mora A, et al. Characteristics of the Shiga-toxin-producing enteroaggregative *Escherichia coli* O104:H4 German outbreak strain and of STEC strains isolated in Spain. Int Microbiol 2011;14:121–141.

13. Navarro-Garcia F. *Escherichia coli* O104:H4 pathogenesis: an enteroaggregative *E. coli*/Shiga toxin-producing *E. coli* explosive cocktail of high virulence. Microbiol Spectr 2014;2:2.6.10.

14. Osugui L, et al. Virulence genotypes, antibiotic resistance and the phylogenetic background of extraintestinal pathogenic *Escherichia coli* isolated from urinary tract infections of dogs and cats in Brazil. Vet Microbiol 2014;171:242–247.

15. Saputra S, et al. Antimicrobial resistance in clinical *Escherichia coli* isolated from companion animals in Australia. Vet Microbiol 2017;211:43–50.

16. Tóth I, et al. Production of cytolethal distending toxins by pathogenic *Escherichia coli* strains isolated from human and animal sources: establishment of the existence of a new *cdt* variant (type IV). J Clin Microbiol 2003;41:4285–4291.

17. Turchetto S, et al. Phenotypic features and phylogenetic background of extraintestinal hemolytic *Escherichia coli* responsible of mortality in puppies. Vet Microbiol 2015;179:126–130.

18. Vidal M, et al. Single multiplex PCR assay to identify simultaneously the six categories of diarrheagenic *Escherichia coli* associated with enteric infections. J Clin Microbiol 2005;43:5362–5365.

19. Wedley AL, et al. Carriage of antimicrobial resistant *Escherichia coli* in dogs: prevalence, associated risk factors and molecular characteristics. Vet Microbiol 2017;199:23–30.

Table 1. Primers used to detect *Escherichia coli* cytotoxic necrotizing factors, Shiga-toxin–producing and intimin genes.

Target gene	Primer sequence (5′–3′)	Product size (bp)	Reference
cnf1	For: GGGGGAAGTACAGAAGAATTA Rev: TTGCCGTCCACTCTCTCACCAGT	1,111	[16]
cnf2	For: TATCATACGGCAGGAGGAAGCACC Rev: GTCACAATAGACAATAATTTTCCG	1,240	[16]
stx1	For: CAGTTAATGTGGTGGCGAAGG Rev: CACCAGACAATGTAACCGCTG	348	[18]
stx2	For: ATCCTATTCCCGGGAGTTTACG Rev: GCGTCATCGTATACACAGGAGC	584	[18]
eae	For: TCAATGCAGTTCCGTTATCAGTT Rev: GTAAAGTCCGTTACCCCAACCTG	482	[18]

cnf1 = cytotoxic necrotizing factor 1; *cnf2* = cytotoxic necrotizing factor 2; *eae* = intimin gene; For = forward; Rev = reverse; *stx1* = Shiga-toxin–producing gene 1; *stx2* = Shiga-toxin–producing gene 2.

Table 2. Antimicrobial concentration of disks as well as results of the disk diffusion assay.

Antimicrobial	Concentration	Zone diameter (mm)	Interpretation
Amoxycillin–clavulanic acid	20/10 µg	0	Resistant
Ampicillin	10 µg	0	Resistant
Ceftiofur	30 µg	0	Resistant
Cephalothin	30 µg	29.7	Resistant
Colistin	10 µg	14.7	Sensitive
Doxycycline	30 µg	16.7	Intermediate
Enrofloxacin	30 µg	0	Resistant
Fosfomycin	200 µg	29.7	Sensitive
Gentamicin	10 µg	23.2	Sensitive
Penicillin	10 IU	0	Resistant
Sulfonamide–trimethoprim	25 µg	28.4	Sensitive

Table 3. MIC interpretation criteria[3] and results of broth-based MIC assays.

Antimicrobial	Interpretation criteria (μg/mL)			MIC (μg/mL)
	R	I	S	
Amoxicillin	≥1	0.5	≤0.25	>32
Chlortetracycline	≥16	8	≤4	4
Doxycycline	≥16	8	≤4	2
Enrofloxacin	≥4	1–2	≤0.5	>32
Fosfomycin	>32	—	≤32	>128
Neomycin	≥8	4	≤2	>32

I = intermediate; R = resistant; S = sensitive; — = no criteria.