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Multidrug resistant verocytotoxin-producing *Escherichia coli* O157:H7 in the faeces of diarrhoeic and non-diarrhoeic dogs in Abeokuta, Nigeria

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ABSTRACT

Verocytotoxin-producing *Escherichia coli* (VTEC) O157:H7 is a predominant cause of haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) in humans. To assess the role of dogs as a possible source of transmission of VTEC O157:H7 to humans, the faeces of diarrhoeic (31) and non-diarrhoeic (63) dogs were examined for the presence of the organism. *Escherichia coli* O157:H7 was isolated from 22 (23.4%) out of 94 samples examined. The organism was detected in 5 (16.1%) out of 31 diarrhoeic faeces and 17 (26.9%) out of 63 non-diarrhoeic faeces, but the difference was not statistically significant (P>0.05). All the *E. coli* O157:H7 isolates produced one or both of verocytotoxin 1 and 2 (VT1 and VT2). Verocytotoxin 1 (VT1) was detected in 10 (45.5%) out of 22 isolates, VT2 in 8 (36.4%), while both toxin types were detected in four (18.2%) isolates. Sixteen (72.7%) out of 22 isolates were resistant to at least three antimicrobials from different classes, while 18 distinct antimicrobial resistance patterns were observed among the isolates. The isolates showed resistance to ampicillin (86.4%), chloramphenicol (36.4%), ciprofloxacin (4.5%), gentamicin (18.2%), kanamycin (68.2%), nalidixic acid (22.7%), neomycin (40.9%), norfloxacin (9.1%), streptomycin (63.6%), sulphamethoxazole/trimethoprim (63.6%) and tetracycline (77.3%). The present study showed that diarrhoeic and non-diarrhoeic dogs may serve as potential sources of multi-drug resistant VTEC O157:H7 transmissible to humans.

Key words: dogs, *E. coli* O157:H7, faeces, multi-drug resistance, verocytotoxin

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Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 is the dominant verocytotoxin producing *E. coli* strain that is known to be associated with both outbreak and sporadic cases of human diseases ranging from uncomplicated diarrhoea to haemorrhagic colitis, characterized by passage of bloody stool and abdominal cramps, with little or no fever (PATON and PATON, 1998; RANGEL et al., 2005; SUGIYAMA et al., 2005). In 5 to 10% of cases, infection may lead to haemolytic uraemic syndrome (HUS) defined by a triad of features of acute renal failure, thrombocytopaenia and microangiopathic haemolytic uraemia, which is the leading cause of acute renal failure in children and the elderly (PATON and PATON, 1998; TZIPORI et al., 2004).

Asymptomatic animals, especially ruminants, are the reservoir host of *E. coli* O157:H7. The organism is resident in the gastrointestinal tract of carrier animals and is shed intermittently in the faeces. The organism has been detected in the faeces of apparently healthy cattle, sheep, goats, pigs, dogs, bats and avian species (BEUTIN et al., 1999; DIPINETO et al., 2006; OJO et al., 2010a; OJO et al., 2010b; ITALIA et al., 2012). Transmission of *E. coli* O157:H7 to humans occurs through consumption of contaminated food substances or by contact with infected persons, carrier animals and contaminated environments (RILEY et al., 1983; PARRY and SALMON, 1998; CRUMP et al., 2003).

Production of verocytotoxins (VT), also called shiga toxins (ST), is the major determinant of the virulence of *E. coli* O157:H7 (PATON and PATON, 1998). Two major types of VT (VT1 and VT2) have been recognized (PATON and PATON, 1998). The two toxins are genetically and immunologically distinct, with only about 55 to 60% genetic and amino acid sequence relatedness (LEE et al., 2007). However, their mechanism of action is essentially the same. They inhibit cellular protein synthesis, leading to the death of affected cells (PATON and PATON, 1998). The toxins have a profound effect on the endothelial cells of blood vessels, thus causing endothelial damage (PATON and PATON, 1998). The toxins affect many organs of the body and contribute significantly to the clinical manifestation of *E. coli* O157:H7 infections (PATON and PATON, 1998; TZIPORI et al., 2004).

Although there are several reports on the occurrence of *E. coli* O157:H7 in food animals (ZSCHÖCK et al., 2000; OJO et al., 2010b), there is paucity of information on the possible roles of companion animals in the zoonotic transmission of *E. coli* O157:H7 to humans. Dogs are important human companions and the close contact that exists between humans and dogs may facilitate exchange of pathogens between them. In order to assess the role of dogs as possible reservoirs of *E. coli* O157:H7, the present study investigated the prevalence of verocytotoxin producing *E. coli* O157:H7 in the faeces of diarrhoeic and non-diarrhoeic dogs, and determined the antimicrobial susceptibility of the isolates.

Materials and methods

Sample collection. Using sterile swabs, faecal samples were collected directly from the rectum of diarrhoeic (31) and non-diarrhoeic (63) dogs attending two veterinary hospitals in Abeokuta, Nigeria. Dogs visiting the clinics for various reasons were sampled with consideration for the presence or absence of diarrhoea in every case. Samples were collected from the patients before commencement of treatment. The samples were properly labelled and transported to the laboratory with ice-packs for microbiological analysis.

Isolation and identification of Escherichia coli O157:H7. Each sample was pre-enriched by inoculating a faecal swab into 9 mL of sterile Tryptone Soya Broth (TSB) (CM0129, Oxoid®, Basingstoke, UK) in universal bottles and incubated at 37 ^{O}C for 4 hours. One millilitre of the pre-enrichment culture was then transferred into 9 mL of modified Tryptone Soya Broth (mTSB) (CM0989, Oxoid®, Basingstoke, UK) supplemented with vancomycin (8 µg/mL), cefsulodin (10 µg/mL) and cefixime (0.05 µg/mL) (VCC selective supplement SR0190, Oxoid®, Basingstoke, UK) for the selective enrichment required for the isolation of *E. coli* O157:H7. The selective enrichment cultures were incubated at 37 ^{O}C for 18 to 24 hours. A loopful of mTSB selective enrichment culture was inoculated onto sorbitol MacConkey agar (SMAC) with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG) (CM0981, Oxoid®), into which cefixime tellurite supplement (SR0172, Oxoid®, Basingstoke, UK) was incorporated. The SMAC-BCIG agar plates were incubated at 37 ^{O}C for 18-24 hours. After incubation, the plates were examined for bacterial growth. Pale yellow colonies on SMAC-BCIG plates were selected for further identification tests. Selected colonies were tested for oxidase and catalase production. The isolates were also characterized biochemically, using a commercially available biochemical test kit for the identification of Gram-negative, oxidase negative bacilli (Microbact GNB 24E, Oxoid®, Basingstoke, UK) and the results were interpreted using the accompanying computer software package (Oxoid Microbact® 2000 version 2.03). Isolates identified as *E. coli* were identified serologically using an *E. coli* O157 latex test kit (DR0620M, Oxoid®, Basingstoke, UK) and *E. coli* H7 antiserum (Difco®, USA) by a slide agglutination test.

Antimicrobial susceptibility testing. Isolates identified as *E. coli* O157:H7 were tested for susceptibility to 11 antimicrobial agents including ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), neomycin (30 µg), norfloxacin (10 µg), streptomycin (10 µg), sulphamethoxazole/trimethoprim (23.75/1.25 µg) and tetracycline (30 µg). Susceptibility was determined by the standard Kirby-Bauer disk diffusion method (ANONYM., 2012a) and the result interpreted in accordance with the recommended guidelines (ANONYM., 2012b). Resistance to neomycin was determined using the breakpoint reported by SAYAH et al.

(2005) because the Clinical and Laboratory Standards Institute guidelines (ANONYM., 2012b) do not provide a breakpoint for *E. coli* susceptibility to neomycin.

Detection of verocytotoxin production by E. coli O157:H7 isolates. Production of verocytotoxin 1 and 2 by *E. coli* O157:H7 isolates was detected by the reverse passive latex agglutination test, using a VTEC-RPLA toxin detection kit (Oxoid®, TD0960). *Escherichia coli* O157:H7 isolates were inoculated onto Brain Heart Infusion Agar slopes (BHIA, Oxoid®, CM 0375) and incubated at 37 ^{O}C for 18 hours. Verocytotoxin was extracted from the bacterial culture by transferring part of the culture with a wire-loop into 1 mL of 0.85% sodium chloride solution containing polymyxin B, at a concentration of 5000 units per millilitre. The bacterial suspension was incubated for 30 minutes at 37 ^{O}C with periodic shaking at 5 minute intervals. After incubation, the suspension was filtered using a low protein binding membrane filter (Corning®, Germany) with 0.2 µm pore size. The filtrate was collected and used for toxin detection assay by reversed passive latex agglutination.

Using a micropipette, 25 µL of diluents (supplied with the toxin detection kit) was dispensed into 24 wells in three rows (eight wells in each row) of V-bottom microtitre plate. With 25 µL of the filtrate under test, a 1:2 serial dilution (double dilution) was made in each row from the first well to the seventh. The eighth well was left containing only the diluents. After this, 25 µL of latex coated VT1 rabbit antiserum was added to all the eight wells in the first row, latex VT2 to the wells in second row and control latex to the wells in third row. The contents of the wells were mixed by gentle agitation to avoid spillage. The microtitre plate was covered and the reaction allowed tocontinue undisturbed on a laboratory bench at room temperature for 20-24 hours. Each reaction well was then examined visually against a black background for agglutination. The degrees of agglutination in the wells were scored and interpreted according to the manufacturer's instruction.

Statistical analysis. Data were expressed in absolute numbers and percentages. Proportions were compared using Chi-square test at $P<0.05$ probability level, using the Statistical Package for Social Sciences (SPSS) version 16 software (ANONYM., 2007).

Results

Prevalence of E. coli O157:H7 in the faeces of dogs. Escherichia coli O157:H7 was isolated from 22 (23.4%) out of 94 samples examined. The prevalence of *E. coli* O157 in diarrhoeic dogs was 15.8% in puppies and 16.7% in adults. Among the non-diarrhoeic dogs, the prevalence was 27.3% in puppies and 26.8% in adult dogs (Table 1). Overall, 5 (16.1%) out of 31 diarrhoeic faeces and 17 (26.9%) out of 63 non-diarrhoeic faeces were positive for *E. coli* O157:H7. There was no significant difference ($P>0.05$) between the prevalence of *E. coli* O157:H7 in the faeces of diarrhoeic and non-diarrhoeic dogs. Also,

the observed differences in the prevalence of the organism in puppies and adult dogs were not statistically significant (P>0.05).

Verocytotoxin production by E. coli O157:H7. All the *E. coli* O157:H7 isolates produced one or both verocytotoxins. Verocytotoxin 1 was detected in 10 (45.5%) out of 22 isolates, VT2 was detected in 8 (36.4%), while both toxin types were detected in four (18.2%) (Table 2).

Table 1. Prevalence of *Escherichia coli* O157:H7 in the faeces of dogs in Abeokuta, Nigeria

Nature of sample	Number sampled	Number (%) positive
Diarrhoeic faeces		
Puppies	19	3 (15.8)
Adults	12	2 (16.7)
Subtotal	31	5 (16.1)
Non-diarrhoeic faeces		
Puppies	22	6 (27.3)
Adults	41	11 (26.8)
Subtotal	63	17 (26.9)
Overall total	94	22 (23.4)

Table 2. Verocytotoxin production in *Escherichia coli* O157:H7 isolates from the faeces of dogs in Abeokuta, Nigeria

	Number of isolate producing verocytotoxins		
Sources of isolate (number tested)	VT1	VT2	VT1/VT2
Diarrhoeic faeces of puppies (3)	1	1	1
Diarrhoeic faeces of adults dogs (2)	0	2	0
Subtotal (5)	**1**	**3**	**1**
Non-diarrhoeic faeces of puppies (6)	3	2	1
Non-diarrhoeic faeces of adults dogs (11)	6	3	2
Subtotal (17)	**9**	**5**	**3**
Overall total (22)	10	8	4

Antimicrobial resistance in E. coli O157:H7. The *E. coli* O157:H7 isolates showed resistance to ampicillin (86.4%), chloramphenicol (36.4%), ciprofloxacin (4.5%), gentamicin (18.2%), kanamycin (68.2%), nalidixic acid (22.7%), neomycin (40.9%), norfloxacin (9.1%), streptomycin (63.6%), sulphamethoxazole/trimethoprim (63.6%) and tetracycline (77.3%) (Table 3). Sixteen (72.7%) out of 22 isolates were resistant to at least three antimicrobials from different classes, while 18 distinct antimicrobial resistance patterns were observed among the isolates (Table 4).

Table 3. Antimicrobial resistance in *Escherichia coli* O157:H7 isolates from the faeces of dogs

Antimicrobial agent (disk concentration/μg)	Resistance breakpoint diameter (mm)	Number (%) of resistant isolates
Ampicillin (10)	≤13	19 (86.4)
Chloramphenicol (30)	≤12	8 (36.4)
Ciprofloxacin (5)	≤15	1 (4.5)
Gentamicin (10)	≤12	4 (18.2)
Kanamycin (30)	≤13	15 (68.2)
Nalidixic acid (30)	≤13	5 (22.7)
Neomycin (30)	≤12	9 (40.9)
Norfloxacin (10)	≤12	2 (9.1)
Streptomycin (10)	≤11	14 (63.6)
Sulphamethoxazole/ Trimethoprim (23.75/1.25)	≤10	14 (63.6)
Tetracycline	≤11	17 (77.3)

Table 4. Antimicrobial resistance patterns of *Escherichia coli* O157:H7 the faeces of dogs

Resistance groups	Resistance patterns	Number and Source of isolates			
		Diarrhoeic faeces		Non-diarrheic faeces	
		Puppies	Adult dogs	Puppies	Adult dogs
1	Chl	-	-	-	1
2	AmpKan	-	-	-	2
3	AmpTet	-	1	-	1
4	AmpNeoTet	1	-	-	-
5	CipNalStr	-	-	-	1
6	AmpKanStrTet	-	-	1	-
7	ChlNorSulTet	-	-	-	1
8	AmpKanStrSulTet	-	-	1	1
9	AmpNeoStrSulTet	1	-	-	-
10	AmpChlKanNeoSul	-	1	-	-
11	AmpGenKanStrSulTet	-	-	-	1
12	AmpChlKanStrSulTet	-	-	1	1
13	AmpKanNalNeoStrSulTet	-	-	-	1
14	AmpGenKanNalNeoStrSulTet	-	-	-	1
15	AmpChlGenKanNeoStrSulTet	-	-	1	-
16	AmpChlKanNalNeoStrSulTet	-	-	1	-
17	AmpGenKanNeoNorStrSulTet	-	-	1	-
18	AmpChlKanNalNeoStrSulTet	1	-	-	-
Total		3	2	6	11

Discussion

In the present study, verocytotoxin-producing *E. coli* (VTEC) O157:H7 was detected in the faeces of diarrhoeic and non-diarrhoeic dogs. Moreover, there was no significant difference in the prevalence of the organism in the faeces of diarrhoeic and non-diarrhoeic dogs. Indeed, the prevalence of VTEC O157:H7 was slightly higher in non-diarrhoeic dogs than in diarrhoeic dogs, suggesting that VTEC O157:H7 may not be a primary cause of diarrhoea in dogs. A previous study has shown that oral administration of VTEC O157:H7, isolated from the clinical condition in humans, failed to induce either signs of intestinal colonization or invasion of internal organs in dogs (KUSUNOKI et al., 2004). However, *E. coli* O157:H7 was recovered from the faeces of inoculated dogs. Findings from the present study also suggest that the presence of diarrhoea did not increase the possibility of VTEC O157:H7 detection. In addition, age did not significantly influence the shedding of VTEC O157:H7 in dogs.

Verocytotoxin production was detected in all the *E. coli* O157:H7 isolates from dogs in this study. Previous studies have reported the detection of VTEC in dogs in both diarrhoeic and non-diarrhoeic dogs (HAMMERMUELLER et al., 1995; BEUTIN, 1999; SANCAK et al., 2004). In a review by BEUTIN (1999), the rate of VTEC detection was up to 12.3 in healthy dogs and 8.8 in diarrhoeic dogs. Similarly, HAMMERMUELLER et al. (1995) reported that 17.2 of diarrhoeic dogs were positive for VTEC while SANCAK et al. (2004) detected VTEC in the faeces of 28.1 of dogs. However, the VTEC strains were not identified by serotyping. The specific detection of VTEC O157:H7 in dogs as reported in the present study is higher than the 4.0% isolation rate in Argentina (BENTANCOR et al., 2007) and the 0.16% in Japan (KATAOKA et al., 2010). The higher prevalence of VTEC O157:H7 as observed in the present study might be due to the possibly higher exposure of the dogs examined in the present study to the organism. It has been suggested that dogs may acquire VTEC O157:H7 from ruminants, which are considered to be the principal reservoirs of the organism (HOGG et al., 2009). In the study area, dog-owners are in the habit of feeding their dogs undercooked beef, restaurant leftovers, as well as foetuses and offal obtained from slaughter cattle at abattoirs. These foods might be contaminated with *E. coli* O157:H7 (PRADEL et al., 2000; KHANNA et al., 2008; OJO et al., 2010b) and hence serve as vehicles for its transmission to dogs. Besides, dogs might wander from home and have direct access to slaughter-houses and meat markets where they scavenge for remnants of fresh meat (ADEYEMO, 2002) and thus may acquire *E. coli* O157:H7 in the process. Wandering dogs may also acquire the organism from a contaminated environment. In the study area, ruminants (cattle, sheep and goats) are reared under the extensive and semi-intensive system of management and are a common sight on the street. These animals litter the environment with their dung and this contributes to environmental *E. coli* O157:H7 contamination.

The presence of VTEC O157:H7 in dogs, as revealed by the present study, showed that dogs could be important as reservoirs of VTEC O157:H7 and may be potential sources of zoonotic transmission to humans. The close interaction that exists between dogs and humans may facilitate easy transfer of VTEC O157:H7 from dogs to humans. Outbreaks of clinical VTEC O157:H7 infections in humans have been linked to close association with asymptomatic carrier dogs (TREVENA et al., 1996; HOGG et al., 2009). Members of dog-keeping households, veterinarians and dog handlers are at risk of acquiring VTEC O157:H7 from dogs. However, dogs are not considered the true maintenance host of VTEC O157:H7 (HOGG et al., 2009). Following acquisition of VTEC O57:H7, dogs shed the organism transiently in faeces, but may pass the infection to humans and also contaminate the environment during the period of shedding.

The present study revealed a high rate of antimicrobial resistance among VTEC O157:H7 isolated from dogs. The majority of the isolates demonstrated multidrug resistance to three or more drugs from different antimicrobial classes. There was high resistance (above 60%) to first-line antimicrobials (ampicillin, tetracycline, sulphamethoxazole/ trimethoprim and streptomycin), commonly administered empirically in the treatment of bacterial infections in dogs. The rate of resistance to the new generation antimicrobials, such as ciprofloxacin and norfloxacin, was very low (below 10%). These resistant strains may cause refractory zoonotic infection in humans. The high rate of antimicrobial usage in small animal practice and the feeding of dogs with animal products containing high levels of antimicrobial residues may contribute to the emergence of antimicrobial resistance in bacteria recovered from dogs (PRESCOTT et al., 2002; DIPEOLU and ALONGE, 2002). The development of antimicrobial resistance in VTEC O157:H7 may also be due to the acquisition of conjugative resistance plasmids from other resistant bacteria micro-flora resident within the gastrointestinal tract of the host animal (ZHAO et al., 2001). The problem of antimicrobial resistance in the treatment of human infections has been associated with zoonotic transfer of resistant bacterial strains from animals to humans (BYWATER et al., 2004).

In conclusion, the present study showed that VTEC O157:H7 may not be an important aetiological agent of diarrhoea in dogs. However, diarrhoeic and non-diarrhoeic dogs of all ages may serve as potential sources of multi-drug resistant VTEC O157:H7 transmissible to humans. Adherence to the principles of personal hygiene may minimise human exposure to VTEC O157:H7 from dogs.

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SAŽETAK

Verotoksični sojevi bakterije *Escherichia coli* (VTEC) O157:H7 pretežito uzrokuju hemoragijski kolitis (HC) i hemolitičko-uremijski sindrom (HUS) u ljudi. Radi procjene uloge pasa kao mogućeg izvora prijenosa VTEC O157:H7 na ljude, pretraženi su uzorci njihova proljeva (31) i normalno formiranog fecesa (63) na prisutnost te bakterije. *Escherichia coli* O157:H7 bila je izdvojena iz 22 (23,4%) od 94 pretražena uzorka. Bila je dokazana u 5 (16,1%) od 31 uzorka proljeva i 17 (26,9%) od 63 uzorka normalno formiranog izmeta. Nije bila ustanovljena statistički značajna razlika (P>0,05). Svi izolati bakterije *E. coli* O157:H7 proizvodili su jedan ili oba verotoksina: 1 i 2 (VT1 i VT2). Verotoksin 1 bio je dokazan u 10 (45,5%) od 22 izolata, VT2 u osam (36,4%), dok su oba tipa toksina bila dokazana u četiri (18,2%) izolata. Šesnaest (72,7%) od 22 izolata bilo je otporno na najmanje tri antimikrobne tvari različitih skupina. Među izolatima je bilo ustanovljeno 18 različitih obrazaca otpornosti na antimikrobne tvari. Izolati su pokazivali otpornost na ampicilin (86,4%), kloramfenikol (36,4%), ciprofloksacin (4,5%), gentamicin (18,2%), kanamicin (68,2%), nalidiksičnu kiselinu (22,7%), neomicin (40,9%), norfloksacin (9,1%), streptomicin (63,6%), sulfametoksazol/trimetoprim (63,6%) i tetraciklin (77,3%). Istraživanje je pokazalo da psi s proljevom i bez proljeva mogu biti izvor multiplorezistentne VTEC O157:H7 za ljude.

Ključne riječi: pas, *E. coli* O157:H7, izmet, višestruka otpornost, verotoksin