academicJournals

Vol. 7(17), pp. 1703-1707, 23 April, 2013
DOI: 10.5897/AJMR12.1269
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Occurrence of non-O157 Shiga toxin-producing *Escherichia coli* in healthy cattle and goats and distribution of virulence genes among isolates

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Accepted 29 March, 2013

Shiga toxin-producing *Escherichia coli* (STEC) are receiving more attention mainly because they are zoonotic and food borne in nature. The objectives of the present study were to determine the occurrence of non-O157 Shiga toxin producing *E. coli* in cattle and goats and distribution of the virulence genes in the isolates. The overall isolation rates of non-O157 STEC was 15.27% (7/87) in cattle and 8.14% (22/144) in goats. Four serogroups namely O8, O26, O103 and O128 were detected. Of these serogroups, O8, O26 and O103 were common in both, cattle and goats, whereas O128 was carried by goats only. Using Duopath Verotoxin (DV) test, 52.63% of sero-positive *E. coli* isolates from cattle were positive for Stx1 shiga toxin whereas none of the isolates was positive for Stx2. Similarly, 42.9% (3/7) of sero-positive *E. coli* isolates from goats produced Stx1 and 14.3% (1/7) were positive for both, Stx_1 and Stx_2. The study on virulence genes showed that stx_1 was commonly distributed among non-O157 STEC isolates of cattle (57.9%) and goats (57.1%).

Key words: Non-O157 STEC, cattle, goats, virulence genes.

INTRODUCTION

Shiga toxin-producing *E. coli* (STEC), also known as Verotoxin-producing *E. coli* (VTEC) are recognized as important food-borne pathogens (Armstrong et al., 1996). Shiga toxin-producing *E. coli* has been associated as a causative agent in several human diseases which may range from mild diarrhea to severe and life-threatening conditions, such as hemolytic-uremic syndrome (Paton and Paton, 2003). Serotype O157:H7 has been named as the most common STEC serotype linked to clinical diseases in humans, however, serotypes other than O157 (non-O157) are increasingly recognized as important cause of human morbidity and mortality (Bettelheim, 2007). Among non-O157 STEC strains, serogroups O8, O26, O91, O103, O111, O128 and O145 are frequently isolated from human beings (Bettelheim, 2007; Fairbrother and Nadeau, 2006; Oporto et al., 2008).

The pathogenic capacity of STEC resides in two potent cytotoxins, Stx1 and Stx2, encoded by stx_1 and stx_2 genes (Paton and Paton, 2003). Studies have shown that *E. coli* strains possessing stx_2 are potentially more virulent than strains carrying stx_1 (Todd et al., 1999; Tesh et al., 1993). Other virulence factors, such as intimin (*eae)* and enterohaemolysin (*hlyA*), may enhance the pathogenicity of organisms but are not required for strains to cause severe disease (Paton and Paton, 2003).

Cattle are considered to be the primary reservoir of both O157 and non-O157 STEC bacteria (Fairbrother and Nadeau, 2006). Cattle harbor STEC without suffering from any pathological symptoms (Blanco et al., 1997).

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Wild animals have also been reported to harbor STEC (Fairbrother and Nadeau, 2006; Sanchez et al., 2009).

Among ASEAN countries, there are reports from Vietnam, Thailand and Malaysia on the presence of non-O157 STEC (Apun et al., 2006; Vu-Khac and Cornick, 2008; Suthienkul et al., 1990; Leelaporn et al., 2003). In Malaysia, both O157 and non-O157 STEC have been recovered from beef (Apun et al., 2006; Radu et al., 1998) and patients with diarrhea (Son et al., 1996). However, there is a lack of data on the presence of non-O157 STEC in farm animals. Therefore, the objective of the present study was to determine the occurrence of non-O157 STEC in cattle and goats and to detect the presence of virulence genes in the isolates.

MATERIALS AND METHODS

Samples collection

Recto-anal mucosal swab (RAMS) samples were collected from beef cattle (n=144) in seven farms and goats (n= 87) in four farms around Selangor, Negri Sembilan and Johor states in Malaysia during July to December, 2008. All the animals sampled were apparently healthy except in one cattle farm, where the herd suffered mild diarrhea due to parasitic infection.

Reference strains

STEC O111 (ECL 6611, The *E. coli* Laboratory, Saint-Hyacinthe, Canada) was used as a positive control whereas non-STEC (ATCC 11775) was used as negative control strain.

Enrichment of samples

Each RAMS sample was immediately put into 10 ml of modified EC broth (Oxoid, UK) supplemented with novobiocin 20 mg/L (Sigma-Aldrich, St Louis, USA). All samples were kept in a box containing ice and transported to the Veterinary Public Health Laboratory within a period of 4-6 h. Upon arrival at the laboratory, all samples were immediately incubated aerobically at 37°C for 24 h.

Plating of enriched samples

A loopful of each broth culture was streaked onto sorbitol MacConkey (SMAC) agar (Oxoid, UK) followed by incubation at 37°C for 24 h. From each SMAC agar plate pink colonies resembling with that of *E. coli* were subcultured onto Chromocult coliform agar (Merck, Germany). From Chromocult coliform agar, presumptive *E. coli* isolates were identified based on β-D-glucuronidase (GUD) reaction (dark blue to violet colored colonies) and subjected to standard biochemical tests according to Jang et al. (2004). Confirmed *E. coli* isolates were kept in nutrient agar slant (Merck, Germany) at room temperature until used.

Serogrouping of *E. coli* isolates

Biochemically confirmed *E. coli* isolates were subcultured onto nutrient agar (Merck, Germany) at 37°C for 24 h and serogrouped using selected O serogroups by applying slide agglutination test using monovalent antisera namely O8, O26, O91, O103, O111, O128, O145 and O157 (Denka Seiken, Japan) following the manufacturer's instructions.

Detection of Shiga toxin production

Colonies were examined for Stx1 and Stx2 production using Duopath® Verotoxins (DV) test (Merck, Germany) following the manufacturer's instruction.

Detection of virulence genes

Serogroups were further investigated for the presence of *stx*$_1$, *stx*$_2$, *eaeA* and *ehlyA* genes using multiplex Polymerase chain reaction (mPCR) assay as described by Fagan et al. (1999). Sero-positive *E. coli* isolates were cultured on SMAC agar and incubated aerobically for 24 h at 37°C. DNA from each isolate was extracted by InstaGene Matrix (Bio-Rad, Germany) according to the manufacturer's instruction. The PCR reaction was performed in a total volume of 50 µl containing 5 µl primer mix (0.2 µM of each primer), 25 µl multiplex PCR master mix (Qiagen, USA), 2 µl of DNA template and 18 µl of DNase/RNase free water. The assay was perform using thermalcycler (Bio-Rad, Germany) set with initial activation temperature as 95°C for 15 min followed by 35 cycles of 95°C for 20 s (denaturation), 58°C for 90 s (annealing), and 72°C for 90 s (extension). Amplified DNA fragments were then electrophoresed using 2% (w/v) agarose at 80 V for 90 min and stained with Gel-Red (Biotium, USA). The products on the gel were visualized using UV illumination (Bio-Rad, Germany). Primer sets used for mPCR are given in Table 1.

Statistical analysis

The agreement between mPCR and DV test was analyzed by inter-rater agreement test (Kappa's test) using SPSS version 20. The results for kappa (k) value were interpreted as poor (k≤0.20), fair (k= 0.21-0.40), moderate (k= 0.41-0.60), good (k=0.61-0.80) and very good (k=0.81-100).

RESULTS

Isolation and serogrouping

Overall, the Shiga toxin-producing *Escherichia coli (STEC)* isolation rate was 15.27% in cattle. Based on serogrouping, nineteen (13.19%) isolates were detected as non-O157 STEC whereas three isolates (2%) from one farm were found as O157. Three non-O157 serogroups obtained were: O8 (47.36%), O128 (36.84%) and O103 (15.78%) (Table 2).

Similarly, overall carriage of STEC in goats was recorded as 8.14% (7/87). The isolation rate of serogroups was as follows: O8 (28.57%), O26 (14.28%), O103 (14.28%) and O128 (42.55%). O157 was not detected in any goat samples. None of the single animal (cattle or goat) was found harboring more than one serogroup.

Shiga toxin production

Using DV test, 52.63% (10/19) of sero-positive *E. coli*

Table 1. Primer sequences and lengths of PCR amplification products (Fagan *et al.*, 1999).

Primer	Direction	Primer sequence (5'–3')	Fragment size (bp)
ehlyA	Forward	ACGATGTGGTTTATTCTGGA	165
	Reverse	CTTCACGTCACCATACATAT	
stx1	Forward	ACACTGGATVATCTCAGTGG	614
	Reverse	CTGAATCCCCCTCCATTATG	
stx2	Forward	CCATGACAACGGACAGCAGTT	779
	Reverse	CCTGTCAACTGAGCAGCACTTTG	
eaeA	Forward	GTGGCGAATACTGGCGAGACT	890
	Reverse	CCCCATTCTTTTTCATTGTCG	

isolates from cattle were positive for Stx_1 whereas none of the samples was positive for Stx2. Similarly, 42.9% (3/7) of sero-positive *E. coli* isolates from goats produced Stx1 and 14.3% (1/7) were positive for both, Stx1 and Stx2. All Shiga toxin positive isolates belonged to serogroups O8, O26, O103 and O128 (Table 2). All three O157 isolates from cattle were found to be negative for Shiga toxin production.

Detection of virulence genes by multiplex PCR

In cattle, it was 57.9% (11/19) of non-O157 *E. coli* and 66.6% (2/3) of O157 *E. coli,* whereas in goats, 57.1% (4/7) of non-O157 *E. coli* possessed the virulence genes (Table 2). Among the non-O157 STEC isolates from cattle, 57.9, 26.31, 26.31 and 31.6% were positive for *stx_1, stx_2, eaeA* and *ehlyA genes,* respectively. More so, one (5.2%) non-O157 *E. coli*, serogroup O128, contained all four virulence genes simultaneously, however two O157 STEC isolates possessed three genes, *stx_2*, *eaeA* and *ehlyA* together. In goats, 71.41% (5/7) of non-O157 *E. coli* were positive for the virulence genes and one (14.3%) non-O157 *E. coli*, belonged to serogroup O8, carried a complete set of all four genes.

DISCUSSION

In Malaysia, the occurrence of O157 STEC and non-O157 STEC in meat, meat products and hospitalized patients have been reported previously (Apan et al., 2006; Nazmul et al., 2008; Radu et al., 1998, 2001). However, to our knowledge, this was the first study carried out in farm animals in Malaysia on the prevalence of STEC and non-O157 STEC serogroups O8, O26, O91, O103, O111, O128 and O148.

Although a wide range of animal species have been found to harbor STEC, ruminants are considered as the most common source for transmission of both O157 and non-O157 STEC to humans (Nataro and Kaper, 1998). Worldwide, the prevalence rate of non-O157 STEC in cattle has been reported as 2.1 to 70.1% (Hussein and Bollinger, 2005) and 0.9 to 75% in goats(Orden et al., 2008; Wani et al., 2006; Zschock et al., 2000). Compared with the present study, lower isolation rates of non-O157 STEC in cattle were found in Spain, 4.1% (Blanco et al., 1997), France, 2.6% (Pradel et al., 2000), Brazil, 1.3% (Irino et al., 2005), Japan, 3.1-3.8% (Kijima-Tanaka et al., 2005; Miyao et al., 1998) and Hong Kong, 0.31% (Leung et al., 2001). None of the above mentioned serogroup was isolated in Canada (Wilson et al., 1992), India (Wani et al., 2006), Vietnam (Vu-Khac and Cornick, 2008) and Siri Lanka (Tokhi et al., 1993). However, a Japanese study gave a similar isolation rate of 6.2%(Kobayashi et al., 2001) and a study in Argentina revealed even a higher rate of isolation, 13.5% (Meichtri et al., 2004). The difference of prevalence values for STEC among studies are due to differences in sampling methods, sample size and detection methods used (Oporto et al., 2008).

The isolation media, SMAC agar was used to isolate *E. coli* from cattle and goats. The primary purpose of SMAC agar is to differentiate between sorbitol fermenting and sorbitol non-fermenting *E. coli* which is the core feature of most O157 strains (March and Ratnam, 1986). As most non-O157 STEC are very similar to other *E. coli,* it is suggested to use common culture media for *E. coli* such as SMAC or MacConkey agar (Bettelheim, 2007).

PCR analysis of 19 STEC isolates from cattle and seven from goats showed various distribution patterns of virulence genes among the isolates with the toxigenic profile *stx_2*/*eaeA*/*ehlyA* significantly associated with *E. coli* O157 and non-O157 isolates. In this study, *stx_1* and *ehlyA* were the predominant genes identified from cattle and goats, respectively. Oporto et al. (2008) reported that *stx_2* was predominant in cattle, whereas in sheep the

Table 2. Distribution of virulence genes among STEC isolates**.**

Animal	Serogroup(s)	Number of isolates in serogroup (%)	Number of Shiga toxin producing *E. coli* (%)		Number of isolates positive for virulence genes (%)			
			Stx1	Stx1-Stx2	*stx1*	*stx2*	*eaeA*	*ehlyA*
Cattle	O8	9 (40.9)	5 (55.56)	0 (0.00)	6 (66.66)	4 (44.44)	0 (0.00)	3 (33.33)
	O103	3 (13.63)	2 (66.67)	0 (0.00)	2 (66.66)	0 (0.00)	2 (66.66)	2 (66.66)
	O128	7 (31.81)	3 (42.86)	0 (0.00)	3 (42.85)	1 (14.28)	3 (42.85)	1 (14.28)
	O157	3 (13.63)	0 (0.00)	0 (0.00)	0 (0.00)	2 (66.66)	2 (66.66)	2 (66.66)
	Total	22	10 (45.45)	0 (0.00)	11 (57.9)	5 (26.31)	5 (26.31)	6 (31.6)
Goats	O8	2 (28.57)	0 (0.00)	1 (50.00)	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)
	O26	1 (14.28)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (100)
	O103	1 (14.28)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
	O128	3 (42.85)	3 (100.00)	0 (0.00)	3 (100)	0 (0.00)	0 (0.00)	3 (100)
	Total	7	3 (42.86)	1(14.29)	4 (57.14)	1 (14.28)	1 (14.28)	5 (71.42)

combination stx_1/stx_2 was more frequently found. Blanco et al. (1997) described a different distribution of *stx* genes among STEC strains isolated from diarrheic and healthy cattle, with higher prevalence of stx_1 in diarrheic calves (81%), whereas stx_2 only, or both stx_1 and stx_2 were predominant among STEC strains from healthy cattle. Sanchez et al. (2009) also reported higher detection of stx_1 genes from non-O157 *E. coli* isolates from sheep.

Using DV test, it was found that all non-O157 STEC produced only Stx1 toxin except for one isolate from goat that produced both Stx1 and Stx2. However, multiplex PCR detected six isolates producing stx_2. Failure to produce Stx2 in DV test for stx_2 positive strains may possibly be due to the toxins produced by the bacteria which were below the detection limit of the test kit. Moreover, it was also reported that not all stx_1/stx_2 positive strains produce Stx_1/Stx_2 toxins (Bettelheim, 2007; Paton and Paton, 2003). Park et al. (2003) reported that DV test had 100% sensitivity resulting in no false positive when compared to primer EHEC assay (Meridian Bioscience, Ohio) as a gold standard. In the present study, detection of Stx1 was comparable using both assays, DV and mPCR, on the contrary Stx2 detection by DV test was poor compared to mPCR which was probably due to low level of toxin production thus was not detected by DV assay.

In conclusion, the present study demonstrated the prevalence of non-O157 in cattle and goats with distribution of virulence genes. These animals act as important reservoir for the pathogenic *E. coli* and can be a potential source for food contamination. The distribution of virulence factors among STEC strains from cattle and goats suggest that some of them represent a potential risk for human infections. Good management practices and control strategies at the production stage are crucial to avoid widespread distribution of the organisms.

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