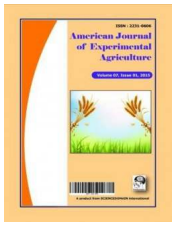

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Shiga Toxin Producing *Escherichia coli* (STEC) in Food Producing Animals from Trinidad and Tobago

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Authors' contributions

This work was carried out in collaboration between all authors. Authors FD and PEA designed the study, wrote the protocol and managed the analyses of the study. Authors MDF and CM wrote the first draft of the manuscript. Author PEA reviewed the experimental design and all drafts of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To determine the occurrence of Shiga toxin producing *Escherichia coli* (STEC) in faecal samples from representative food-producing bovine animals in Trinidad and Tobago.
Study Design: This was a prospective cross sectional observational laboratory based study.
Place and Duration of Study: Bovine faecal samples were collected from selected food animal farms located in the twin island of Trinidad and Tobago and processed at the Microbiology laboratories at the Veterinary School, Faculty of Medical Sciences, The University of the West Indies Campus, from March to May 2014.
Materials and Methods: 205 cattle faecal samples collected from 12 animal farms across Trinidad & Tobago were analyzed for *E. coli* bacteria. Using conventional and molecular microbiology laboratory techniques, 160 recovered *E. coli* isolates from these samples were then screened for possession of the intimin (*eae*), Shiga toxin 1 (stx_1) and Shiga toxin 2 (stx_2) genes.
Results: Shiga toxin producing *E. coli* (STEC) genes were detected in 9.4% (15/160) of *E. coli*

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isolates analyzed by molecular methods. Overall, 1.3% of the isolates were positive for the intimin (*eae*) gene; 6.6% for stx_1 and 7.3% for stx_2 toxins genes. All detected STEC positive isolates, however did not belong to any of the most known O serogroups associated with Shiga toxin genes; and were also all negative for Locus of Enterocyte Effacement (LEE) genes (*esp*A, *tir* and *esc*N).
Conclusion: There is low occurrence of *E. coli* producing stx_1 and stx_2 (STEC) genes in Trinidad and Tobago. STEC in the country is not associated with the seven most common serogroups or the LEE genes. This information has never been reported in Trinidad and Tobago before and therefore present a novel contribution to the epidemiology of STEC in the country and Caribbean region.

Keywords: Trinidad and Tobago; PCR; shiga toxin; Escherichia coli; STEC; Bovine.

1. INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) were first discovered in 1977 [1], and to date, they have proved to be potentially lethal zoonotic pathogens of significant concern for public health. This came to light after an outbreak was first reported in the USA in 1982 [2]. STEC in food producing animals such as cattle is a significant concern for public health because of its ability to be transmitted to humans through food and water contaminated with faeces from cattle [3,4]. STEC produce minor health problems in young cattle but it can produce a broad spectrum of clinical diseases in humans [5] since this organism is strongly associated with hemolytic uremic syndrome (HUS), a leading cause of acute renal failure in children [6].

Cattle are regarded as the natural reservoir of STEC [5], however other ruminant species (sheep, goats), wild and domestic animals (deer, cats, dogs and rodents) may act as other major reservoirs; while birds, swine, feral pigs, chickens and horses are considered spill-over hosts [7-11]. Human infection is diverse in its incidence and occurs usually through food, particularly inadequately cooked ground beef [12]. In the United States of America and North Wales there have been documented cases of direct transmission from calves to humans [13] through the faeco-oral route. Besides beef consumption, working or camping in rural areas and visiting farms have been reported or considered risk factors for acquiring STEC infections [7,14].

STEC O157 has been shown to be an important cause of illness in the United States [15]. And in Mexico, surveillance studies have reported recovery of non-O157 STEC strains in a large proportion of ready-to-eat meals, confirming that non-O157 serotypes are potential sources of infection in humans [16].

The presence of STEC in water, milk and bovine faecal sources have been established in rural north Trinidad by other researchers [17-19] and even in marine life or environments in the country [20], but no further studies have been carried out to characterize the virulent genes of *E. coli* isolated from farm animals. As stated above, the documented presence of STEC in milk, ground water and ready to eat items produced in Trinidad and Tobago [17-19] in conjunction with the pathogenic capacity and the propensity to cause an outbreak, warrants the determination of their prevalence and genetic characteristics. Also, several prevalence estimates, ranging from 0.1% to 62% of STEC in cattle have been reported in several countries [21-23] and also in North and South American countries, namely the USA, Mexico, Argentina, Brazil, Colombia, Peru and Venezuela [4,5,15,16,24-29], neighbours to Trinidad and Tobago but there is paucity of such estimates or information in this country.

The objectives of this current study were to determine the occurrence of Shiga toxin producing *Escherichia coli* (STEC) in faecal samples from representative food-producing bovine animals from Trinidad and Tobago and also to characterize their virulent genes.

2. MATERIALS AND METHODS

2.1 Design and Sampling

This was a prospective cross sectional and observational study carried out over a three-month period, March to May 2014 at twelve farms in five regional districts of the country. To avoid bias, farms and regional corporation areas to be studied were selected from a pool of the list. The farms in this area are rural plants with traditional farming procedures. Each farm was visited once and during the visit, faecal specimens were collected from the animals present on site. Approximately 10 – 20 g of faecal materials were collected from healthy and dairy cattle that were observed defecating in their pens during the time of visit to the farms. The stools were stirred and collected using a sterile

wooden spatula or tongue depresssor and gloves. Samples were placed into labeled, dry, sterile containers or WhirlPak bags. Symptomatic and young cattle were excluded. Collected samples were transported to the laboratory on ice cooler/pack for processing at the Veterinary School diagnostic laboratory of the Faculty of Medical Sciences, The University of the West Indies at St. Augustine within four to six hours of collection. The distribution of number of faecal samples from each farm is depicted in Table 2.

2.2 Method of Isolation of *Escherichia coli* Isolates from 205 Bovine Fecal Samples

In the lab, minimal pre-enrichment was done which involved using a buffered peptone water at a ratio of 1:10 to 8 to 12 g of the faecal materials and homogenized for about half an hour. This was then plated onto Sorbitol-Mac Conkey (SMAC) and Blood agar plates and incubated for 18 – 20 h at 37℃. Bacterial growth was observed on SMAC plates as pink or creamy colonies while on the Blood agar plates the colonies were whitish, creamy, and some golden-yellow colours with zones of haemolysis in some. Gram stain and even oxidase tests were performed and further biochemical tests were set up and included triple sugar iron agar (TSIA), beta-glucuronidase, indole, motility, citrate and Methyl blue tests. Colonies were presumptuously confirmed as *E. coli* if they fulfilled these criteria: lactose fermenters (produced pink colored and mucoid colonies), negative hydrogen sulphide production, positive tests for indole, motility, beta-glucuronidase and methyl red tests, and negative results for citrate. None of these were screened for 0157:H7 because of lack of antiserum or latex reagents. However, pure colonies from the blood agar were stored in brain heart infusion (BHI) with 5% glycerol at -20℃ until further molecular tests. Quality control was achieved by use of *E. coli* (ATCC 25922) strain.

2.3 Detection of the Virulent Genes – *stx*$_1$, *stx*$_2$, *eae*, *esc*N, *esp*A and *tir* – by Colony PCR and Assessment of STEC Serogroup Genes by Multiplex PCR

E. coli isolates (n=160) were screened for several virulent markers including the *eae* gene (coding for the intimin adhesion), Shiga toxin 1 (*stx*$_1$) and Shiga toxin 2 (*stx*$_2$) genes using colony Polymerase Chain Reaction (PCR) as described in literature [30,31] with minimal modifications. Briefly, the reaction mixture comprised the following: 12.5 µl GoTaq® Green Master Mix (2X), 2 µl of each primer (forward and reverse), tiny portion of a colony as a source of template DNA, made to 25 µl by water nuclease-free (Promega, Madison, USA). The GoTaq® master mix contains two dyes (blue and yellow) that allow monitoring of progress during electrophoresis. The PCR was performed using a Techne Flexigene Thermal-cycler (American Laboratory Trading East Lyme, CT, USA). Samples were subjected to 25 PCR cycles, each consisting of an initial denaturation of 5 min at 94℃, then 25 cycles of 1 min of denaturation at 94℃; 1 min of annealing at 52℃ and 1 min of elongation at 72℃ and a final extension time of 10 min at 72℃. Amplicons (5 µl) were resolved on 1% agarose gel in Tris-Acetate-EDTA (TAE) buffer alongside a 100 bp ladder (New England Biolabs, Ipswich, MA) and viewed on a UV trans-illuminator after staining with Gelred™ dye (Biotium Inc., Hayward, CA). Samples positive for *stx*$_1$ or *stx*$_2$ were also tested for *esc*N, *esp*A and *tir* to confirm the presence of Locus of Enterocyte Effacement (LEE) pathogenicity island and intimin polymorphism. The isolates were subjected to 25 PCR cycles each consisting of 1 min of denaturation at 94℃; 1 min of annealing at 52℃ and 1 min of elongation at 72℃. PCR reaction mixtures were resolved on 1% agarose gels stained with Gelred™ dye and viewed on a UV trans-illuminator. Also, using methods as previously described [32,33], strains positive for the genes coding the Shiga toxins were also analyzed to determine if they belonged to one of seven O groups: 026, 045, 0103, 0111, 0121, 0145 and 0157. The primers used are also listed in Table 1. The isolates were subjected to 25 PCR cycles but with an annealing temperature of 50℃. PCR reaction mixtures were resolved as described above.

2.4 Statistical Analysis

Farms were grouped by areas. Data for each sample from each farm was plotted into Statistical Program for Social Sciences Students (SPSS), Version 20. Prevalence was calculated as a ratio between the number of farms with positive samples and the total number of farms investigated as was done by Franz et al. [34]. True prevalence, *P*, was modelled with a beta distribution: *P*= (s+1)/ (n-s+1), with *s* representing the number of positive samples and *n* signifying the total number of farms or samples [34]. A bivariate correlation analysis was conducted to determine whether or not there was any significant relationship between *stx*$_1$ and *stx*$_2$ genes.

Table 1. Target genes, primer sequences and amplicon sizes used for detecting STEC in food producing animals from Trinidad and Tobago, 2014

Target genes	Primer	Primer sequence (5' – 3')	Amplicon size (bp)	Ref
stx$_1$	stx1-F	TGTCGCATAGTGGAACCTCA	655	[30]
	Stx1-R	TGCGCACTGAGAAGAAGAGA		
stx$_2$	stx2-F	CCATGACAACGGACAAGCAGTT	477	[30]
	stx2-R	TGTCGCCAGTTATCTGACATTC		
eae	eae-F	CATTATGGAACGGCAGAGGT	375	[30]
	eae-R	ACGGATATCGAAGCCATTTG		
*ehx*A	ehxA-F	GCGAGCTAAGCAGCTTGAAT	199	[30]
	ehxA-R	CTGGAGGCTGCACTAACTCC		
*wzx*026	026F	AGGGTGCGAATGCCATATT	417	[31]
	026R	GACATAATGACATACCACGAGCA		
*wzx*045	045-F	GGGCTGTCCAGACAGTTCAT	890	[31]
	045-R	TGTACTGCACCAATGCACCT		
*wzx*103	0103F	GCAGAAAATCAAGGTGATTACG	740	[31]
	0103R	GGTTAAAGCCATGCTCAACG		
*wzx*0111	0111F	TGCATCTTCATTATCACACCAC	230	[31]
	0111R	ACCGCAAATGCGATAATAACA		
*wbq*0121	0121F	TCAGCAGAGTGGAACTAATTTTGT	587	[31]
	0121R	TGAGCACTAGATGAAAAGTATGGCT		
*wzx*0145	0145F	TCAAGTGTTGGATTAAGAGGGATT	523	[31]
	0145R	CACTCGCGGACACAGTACC		
*rfb*0157	rfbE-F	CAGGTGAAGGTGGAATGGTTGTC	296	[30]
	rfbE-R	TTAGAATTGAGACCATCCAATAAG		
tir	tir-Fc	GGCGACGGGTATTGTACAGG	479	[32]
	tir-Rc	GCAGGCTTATTCTCTACCGTACG		
*esp*A1	Fc	CATGCCATGGATACATCAAATGCAACATCC		[33]
		CGGAATTCAGTTTACCAAGGGATATTGCTGAAATAG		

Ref = reference

3. RESULTS AND DISCUSSION

The present study investigated the prevalence of Shiga toxin producing *Escherichia coli* (STEC) in small farms within rural communities, located in the agricultural areas of several regions of Trinidad. A total of two hundred and five (205) faecal samples were collected from 12 selected pools of healthy adult and dairy cattle farms across five regional districts (north, east, southeast, central and southwest) areas of Trinidad in the twin island of Trinidad and Tobago (Fig. 1). Young cattle were excluded from sampling and analysis because most of the farm owners were uncertain about the age of these animals. So to preserve uniformity and present a more accurate picture of the prevalence in Trinidad and Tobago, they were excluded. Also as reported in literature, the rate of shedding is variable. Depending on age, shedding in very little amounts occurs preweaning or under 2 months of age, and shedding at a much higher volume occurs post weaning after 2 – 6 months [35,36]. One hundred and sixty (160) *E. coli* isolates were recovered from the 205 samples processed. Majority of these *E. coli* isolates were recovered from fecal specimens collected from the Aripo area (41.3%, 66/160), followed by Mayaro (20%, 33/160), Talparo (16.3%, 26/160), Manzanilla (14.4%, 23/160) and Tabaquite area (7.5%, 12/160), as summarized in Table 2.

All confirmed 160 *E. coli* isolates were subjected to molecular analysis and results showed an overall frequency rate of 9.4% (15/160) positive STEC genes. Using method and formula reported by Franz et al. [34], true prevalence of the positive indices were obtained as follows: 1.3% had the intimin (*eae*) gene, and 6.6% had *stx*$_1$ gene and 7.3% possessed *stx*$_2$ gene. Majority of the STEC positive strains tested positive for both Shiga toxin genes. Samples from three farms produced STEC that were positive for both *stx*$_1$ and *stx*$_2$. In the Aripo area there were three samples that yielded *E. coli* that were positive for *stx*$_2$ gene but none of the samples simultaneously tested positive for either

stx$_1$ or *eae*. In the Manzanilla area, five samples yielded *E. coli* isolates which were positive for *stx*$_1$, four were positive for *stx*$_2$ and one positive for *eae*. The Kernaham Farm 2 in Manzanilla area was the only farm whose *E. coli* isolate tested positive for *eae,* and also the only farm where positive results for all three genes (*stx*$_1$, *stx*$_2$, *eae*) were obtained. Few samples were received from this particular farm and yet half of them produced STEC positive isolates. Although the difference between the positive samples and total number of faecal specimens was not statistically significant (p=0.1), an eye has to be kept on this particular farm because of the potential of being a source of STEC isolates into the food chain market. Talparo area had three isolates positive for *stx*$_1$ and another three isolates positive for *stx*$_2$. Tabaquite farms recorded no sample that was positive for STEC genes while in one farm located in Mayaro, an *E. coli* isolate positive for *stx*$_1$ was recovered from one of the animal's specimen.

Overall, the *stx*$_1$ and *stx*$_2$ genes were present in *E. coli* isolates recovered from 58% (7/12) of the farms surveyed, and *stx*$_1$ and *stx*$_2$ were significantly correlated (r =0.99, p = 0.00), indicating a strong positive relationship. About a quarter (27%, 4/15) of the isolates produced both *stx*$_1$ and *stx*$_2$. Both *stx*$_1$ and *stx*$_2$ were present in isolates recovered from 42.8% (3/7) of the farms that yielded positive tests for either gene. The *stx*$_1$ gene was present in 3/5 districts while *stx*$_2$ was present in 4/5 districts. The finding of farms that produced STEC that had Stx2 is very significant because as already reported in literature Stx2 is 100-1000-fold more potent than Stx1 [8]. The isolates positive for intimin (*eae*), *stx*$_1$ and *stx*$_2$ were negative for all the Locus of Enterocyte Effacement (LEE) genes (*esc*N, *esp*A and *tir)* and genes coding for the serogroups 026, 045, 0103, 0111, 0121, 0145 and 0157.

Table 2. Distribution of 160 *Escherichia coli* isolates from 205 bovine faecal samples recovered from animal farms in Trinidad and Tobago, in 2014 (%)

Farm	Location	NS	NEC
London farm	Aripo	13	10 (6.2)
Aripo govt. farm	Aripo	52	46 (28.9)
Ogiste farm	Aripo	12	10 (6.2)
Kernaham 1	Manzanilla	8	5 (3.1)
Kernaham 2	Manzanilla	14	11 (6.9)
Kernaham 3	Manzanilla	5	2 (1.2)
Kernaham 4	Manzanilla	10	5 (3.1)
Maffiking farm	Talparo	22	19 (11.9)
Bristol farm	Talparo	10	7 (4.4)
Tabaquite farm 1	Tabaquite	10	2 (1.2)
Tabaquite farm 2	Tabaquite	14	10 (6.2)
Ortoire farm	Mayaro	35	33 (20.6)
Total	12	205	160 (100)

NS = total number of specimens collected from each farm; NEC = number of Escherichia coli isolates recovered and subjected to molecular analysis

Table 3. Distribution of Shiga toxin producing *Escherichia coli* (STEC) genes from 160 *Escherichia coli* isolates recovered from bovine faecal samples from animal farms in Trinidad and Tobago, in 2014

Farm	Location	STEC	*stx*$_1$	*stx*$_2$	*eae*	*p*-value
London farm	Aripo	0	0	0	0	
Aripo govt. farm	Aripo	2	1	1	0	<0.000
Ogiste farm	Aripo	1	0	1	0	
Kernaham 1	Manzanilla	0	0	0	0	
Kernaham 2	Manzanilla	5	4	4	1	0.1
Kernaham 3	Manzanilla	0	0	0	0	
Kernaham 4	Manzanilla	1	1	0	0	
Maffiking farm	Talparo	4	2	3	0	0.001
Bristol farm	Talparo	1	1	0	0	
Tabaquite farm 1	Tabaquite	0	0	0	0	
Tabaquite farm 2	Tabaquite	0	0	0	0	
Ortoire farm	Mayaro	1	0	1	0	
Total	12	15	9	10	1	

STEC = Shiga toxin producin E. coli isolates; stx$_1$ *= Shiga toxin 1 genes; stx*$_2$ *= Shiga toxin 2 genes; eae = intimin genes*

Fig. 1. Map showing sites of bovine fecal specimen collection from Trinidad and Tobago, 2014

Isolation and detection of STEC carriage in ruminants varies widely and differences may arise because of several factors including methodology used to identify the organism, sampling strategy, types of samples, enrichment procedures, immunomagnetic separation and choice of culture media [2-10]. Our analysis could not be devoid of such factors and shortcomings. We were not able to use methods for screening 0157:H7 such as antiserum or latex reagents that give rapid turn around time and sensitive results; although they're not fully specific. We used a SMAC media that can rapidly aid the detection of STEC including the ones producing 0157:H7. We also used beta-glucuronidase tests which do not detect the positive 0157 strains since this enzyme is not expressed by most 0157 STEC isolates. But despite the shortcomings in our analysis, the detection of STEC in healthy dairy and adult cattle in this study was confirmed by molecular analysis. Our detection of *E. coli* isolates positive for STEC gene in this analysis is in agreement with other reports in literature that supports the fact that cattle are natural reservoir of STEC [7]. The overall prevalence of 9.4% (15/160) among the faecal materials analyzed in the present study is very much higher than 2.8% reported in Washington where STEC was recovered from healthy heifers, or 1.25% recovered from different locations in Mexico [16]. However, our rate was similar to what was observed and reported in dairy cows in Argentina [24]. None of the *E. coli* strains harboring Shiga toxin genes was positive for the seven commonly known STEC serogroups (O157, O145, O121, O111, O103, O45 and O26) analyzed using multiplex PCR, implying that other STEC serotypes might be present in Trinidad and Tobago. The seven O groups tested are commonly associated with human infection [8] but they are by no means the only STEC groups causing human infections. Works by other researchers have reported up to twenty-eight different serogroups associated with STEC [37].

In this our analysis it was noted that only one isolate was positive for intimin and the remaining isolates were negative. Recent studies have shown a trend of *stx*+ eae- occurrence with a high incidence of *exh*A presence [37]. Some studies consider *exh*A, a gene encoding hemolysin toxin or protein, as the major virulence factor in intimin negative STEC [37,38]. We would therefore recommend that *exh*A be screened for in future studies on STEC in Trinidad and Tobago. With only one isolate testing positive for intimin, we can hypothesize that the majority of Shiga toxin producing *E. coli* are LEE-negative (*esp*A and *esc*N) and other studies [38] support this finding. However, it is unclear why the *eae* positive strain was negative for LEE genes since these are found on the same locus. We could not rule out the possibility

of polymorphism in the gene sequences as this is now a common phenomenon amongst bacterial genes [39].

Cattle are reservoirs for STEC and there are potentially several factors that can influence the incidence of Shiga toxin occurrences or lead to spread of infection from a continuous exposure to such animals. These factors include (a) poorly designed feeding troughs resulting in feed being contaminated with the faeces of wild or domestic animals; and (b) surface water and groundwater sources may be contaminated from effluent runoff from farms and urban areas [7]. The presence of all three genes (*stx1, stx2* and *eae*) was seen at only one farm, the Kernaham farm 2. This farm provided only 6.9% of all samples collected but contributed 33.3% of all positive samples. This was the highest rate if compared to all the other farms, although this difference was not statistically significant (p=0.1).

4. CONCLUSION

Obviously, further investigation is warranted, but our data suggests that the prevalence of *E. coli* positive for stx_1 and stx_2 is relatively low in Trinidad and Tobago. There were no strains harboring LEE genes. This study has revealed that STEC in Trinidad and Tobago is not associated with the seven most common serogroups. This information is reported for the first time in this country and shows novel data that represent an important contribution to the knowledge of the epidemiology of STEC in the Caribbean region. We recommend future research approaches should concentrate on identifying STEC serogroups present in Trinidad and Tobago and other pathogenic profiles including risk factors.

ETHICAL APPROVAL

The ethical approval for this study was granted by the Ethics Review Committee of the University of the West Indies, St. Augustine Campus. Oral and/or written permission was obtained from the Authority or Managers at the Farms.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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