

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

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: 160 cattle faecal samples collected from 12 animal farms across Trinidad & Tobago were screened for *E. coli* harbouring the intimin (*eae*), Shiga toxin 1 (*stx₁*) and Shiga toxin 2 (*stx₂*) genes using conventional and molecular microbiology laboratory techniques.

Results: Fifteen of the bovine faecal samples (9.4%) yielded *E. coli* that were positive for STEC. Overall, 1.3% of the isolates were positive for the intimin (*eae*) gene; 6.6% for *stx₁* and 7.3% for *stx₂* toxins genes. All the positive isolates, however did not belong to any of the known seven O serogroups associated with Shiga toxin genes and the majority of the strains were negative for Locus of Enterocyte Effacement (LEE) genes (*espA*, *tir* and *escN*).

Conclusion: There is low occurrence of *E. coli* producing *stx₁* and *stx₂* (STEC) genes in Trinidad and Tobago. STEC in the country is not associated with the seven most common serogroups or the LEE Shiga toxin genes. This information has never been reported in Trinidad and Tobago before and therefore ushers 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 potential lethal zoonotic pathogens and significant public health concerns. The public health concern of STEC came to light after its 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 contaminated food and water with faeces from cattle [3, 4]. The STEC organism does not produce many health problems in cattle but it can produce a broad spectrum of clinical diseases in humans [5] where they are 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].

Shiga toxin-producing *Escherichia coli* (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, suggesting that non-O157 could be a potential source 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 the 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. MATERIAL 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. Both the farms and the regional corporation areas were selected from a pool of the list to avoid bias. Each farm was visited once and during the visit, a faecal specimen was collected from the animal present on site. Approximately 10 – 20g 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 faecal pat was stirred and collected using a sterile wooden spatula or tongue depressor and gloves. Samples were placed into labeled, dry, sterile containers or whirlPak bags. Symptomatic and juvenile 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.

2.2 STEC isolation method from the 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 12g of the faecal materials and homogenized for about half an hour. This was then plated onto sorbitol-MacConkey (SMAC) agar plates and incubated for 18 – 20 hours at 37°C. Suspected pure colonies for *E. coli* were sub cultured on MacConkey and blood agar plates. Colonies were confirmed as *E. coli* if they were lactose-fermenting colonies on MacConkey agar plates, and biochemical tests including, non-hydrogen sulphide production, positive indole and motility (SIM) test, positive beta-glucuronidase and methyl red tests, and negative result for citrate tests according to International Standard Organization laboratory protocols [ISO 16654 :2001]. Pure colonies from the blood agar were stored in brain heart infusion (BHI) with 5% glycerol in -20°C until further molecular tests.

2.3 Detection of the virulent genes – *stx*₁, *stx*₂, *eae*, *tir*, by colony Polymerase Chain Reaction

Escherichia coli isolates 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) method 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 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. Reactions with the master mix have sufficient density for

direct loading onto agarose gels and did not require loading dyes. The PCR cycling program was performed using a Techne Flexigene Thermal-cycler (American Laboratory Trading East Lyme, CT). Primers used in this study are displayed in Table 1 and were all purchased from Sigma (St Louis, MO, USA). Samples were subjected to 25 PCR cycles, each consisting of an initial denaturation of 5 min at 94°C, then 25 cycles of 1 min of denaturation at 94 °C; 1 min of annealing at 52°C and 1 min of elongation at 72°C and a final extension time of 10 min at 72° C. Amplicons (5 µl) were resolved on 1% agarose gel in Tris-Acetate-EDTA (TAE) buffer alongside a 100bp ladder (New England Biolabs, Ipswich, MA) and viewed on a UV trans-illuminator after staining with Gelred™ dye (Biotium Inc., Hayward, CA). Also, samples positive for *stx*₁ or *stx*₂ were also tested for *escN*, *espA* and *tir* to confirm the 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°C; 1 min of annealing at 52°C and 1 min of elongation at 72°C. PCR reaction mixtures were resolved on 1% agarose gels stained with Gelred™ dye and viewed on a UV trans-illuminator. Also using method as previously described [32, 33], the genes coding the several Shiga toxin positive strains were also analyzed to determine if they belong 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°C. PCR reaction mixtures were resolved on 1% agarose gels stained with Gelred™ dye and viewed on a UV trans-illuminator.

Table 1: Target genes and primer sequence and amplicons used for detecting STEC in food producing animals from Trinidad and Tobago, 2014.

Target genes	Primer	Primer sequence (5' – 3')	Amplicon size (bp)	Ref
<i>stx</i> ₁	<i>stx</i> ₁ -F	TGTCGCATAGTGGAACCTCA	655	[30]
	<i>Stx</i> ₁ -R	TGCGCACTGAGAAGAAGAGA		
<i>stx</i> ₂	<i>stx</i> ₂ -F	CCATGACAACGGACAAGCAGTT	477	[30]
	<i>stx</i> ₂ -R	TGTCGCCAGTTATCTGACATTC		
<i>eae</i>	<i>eae</i> -F	CATTATGGAACGGCAGAGGT	375	[30]
	<i>eae</i> -R	ACGGATATCGAAGCCATTTG		
<i>ehxA</i>	<i>ehxA</i> -F	GCGAGCTAAGCAGCTTGAAT	199	[30]
	<i>ehxA</i> -R	CTGGAGGCTGCACTAACTCC		
<i>wzx</i> ₀₂₆	026F	AGGGTGCGAATGCCATATT	417	[31]
	026R	GACATAATGACATACCACGAGCA		
<i>wzx</i> ₀₄₅	045-F	GGGCTGTCCAGACAGTTTCAT	890	[31]
	045-R	TGTACTGCACCAATGCACCT		
<i>wzx</i> ₁₀₃	0103F	GCAGAAAATCAAGGTGATTACG	740	[31]
	0103R	GGTTAAAGCCATGCTCAACG		
<i>wzx</i> ₀₁₁₁	0111F	TGCATCTTCATTATCACACCAC	230	[31]
	0111R	ACCGCAAATGCGATAATAACA		
<i>wbq</i> ₀₁₂₁	0121F	TCAGCAGAGTGGAACCTAATTTTGT	587	[31]
	0121R	TGAGCACTAGATGAAAAGTATGGCT		
<i>wzx</i> ₀₁₄₅	0145F	TCAAGTGTTGGATTAAGAGGGATT	523	[31]
	0145R	CACTCGCGGACACAGTACC		
<i>rfb</i> ₀₁₅₇	<i>rfbE</i> -F	CAGGTGAAGGTGGAATGGTTGTC	296	[30]
	<i>rfbE</i> -R	TTAGAATTGAGACCATCCAATAAG		
<i>tir</i>	<i>tir</i> -Fc	GGCGACGGGTATTGTACAGG	479	[32]
	<i>tir</i> -Rc	GCAGGCTTATTCTCTACCGTACG		
<i>espA</i> ₁	Fc	CATGCCATGGATACATCAAATGCAACATCC		[33]
		CGGAATTCAGTTTACCAAGGGATATTGCTGAAATAG		

Ref = reference

2.5 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.

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 parts of Trinidad. A total of one hundred and sixty (160) faecal samples were collected from 12 selected pool of farms of healthy adult and dairy cattle farms across five regional districts (north, east, south-east, central and southwest) areas of Trinidad in the twin island of Trinidad and Tobago (Figure 1). Juveniles were excluded from sampling and analysis because most of the farm owners were uncertain about the age of their juveniles. 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, rate of shedding is variable. Depending on age, shedding in very little amounts occurs pre weaning or under 2 months and shedding at a much higher volume occurs post weaning after 2 – 6 months [35, 36]. Majority of the fecal specimens were 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 on Table 2. Specimens that yielded bacterial growth were confirmed as positive *E. coli* isolates if such organisms were lactose fermenting, positive on MacCkonkey media, sulphur negative, indole and motility positive on SIM tests and, citrate and oxidase negative.

The study showed an overall frequency rate of 9.4% (15/160) for the Shiga toxin-producing *E. coli*, and using Franz *et al* reported method and formula as above [34], true prevalence of the positive indices were obtained as follows: 1.3% possessing the intimin (*eae*) gene, 6.6% had stx_1 and 7.3% possessed stx_2 genes. The majority of toxin positive strains tested positive for both toxin genes. Samples from three farms produced STEC that were positive for both toxin genes 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*. Five samples yielded *E. coli* isolates which were positive for stx_1 , four were positive for stx_2 and one positive for *eae* in the Manzanilla area. Kernaham Farm 2 of the Manzanilla area was the only farm that produced isolates that tested positive for *eae* and the only farm to test positive for all three genes (stx_1 , stx_2 , *eae*) in question. This particular farm had few samples of which about half produced STEC, odds ratio = 2.35 (0.72<OR<8.12); RR = 2.20 (0.79<RR<6.10), and the difference between the positive samples and total number of cattle producing the faeces were not statistically significant, $p=0.1$. An eye has to be kept on this particular farm because of the greater number of food producing animals that produced STEC genes. Talparo area had three isolates positive for stx_1 and another three isolates positive for stx_2 , Tabaquite area farm produced no sample that yielded *E. coli* or its genes but in the Mayaro area there was a farm that produced an isolate that yielded only one stx_1 . Stx_1 and stx_2 were significantly correlated ($r = 0.99$, $p = .00$), 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. Stx_1 was present in 3/5 districts while stx_2 was present in 4/5 districts. Encountering farms that produced STEC that had stx_2 is very significant because as already reported in literature Stx_2 is 100-1000-fold more potent than Stx_1 [8]. The isolates positive for intimin (*eae*), stx_1 and stx_2 were negative for all the Locus of Enterocyte Effacement (LEE) genes (*escN*, *espA* and *tir*) and genes coding for these serogroups O26, O45, O103, O111, O121, O145 and O157.

Reported carriage of *E. coli* O157:H7 in ruminants varies widely and differences may arise because of several factors – methodology used to identify the organism, sampling strategy, types of samples, enrichment procedures, immunomagnetic separation and choice of culture media [2 – 10]. The identification of STEC in healthy dairy and adult cattle in this study is in agreement with other reports in

literature that cattle are a natural reservoir of Shiga toxin producing *E. coli* (STEC) [7]. The *stx*₁ and *stx*₂ were present in *E. coli* isolates recovered from 58% (7/12) of the farms surveyed and this gave an overall prevalence of 9.4% (15/160) among the sampled faecal materials analyzed. This rate is very much higher than what was reported in Washington, where the STEC recovered from healthy heifers was 2.8% and 1.25% recovered from different locations in Mexico [16]. But our rate was similar to the prevalence observed in Argentina that was 9% [24]. None of the *E. coli* strains harboring Shiga toxin genes was positive for the serogroup analyzed using multiplex PCR for the seven commonly known STEC serogroups (O157, O145, O121, O111, O103, O45 and O26) implying that other STEC serotypes might be present in Trinidad and Tobago. The seven O groups tested for are commonly associated with human infection [8] but not exhaustive, therefore a greater number of faecal samples and a broader spectrum of testing could have yielded positive results. Indeed, work by others have reported up to twenty-eight different serogroups associated with STEC [37]. In this present study it was noted that only one isolate was positive for intimin and the rest were negative. Recent studies have shown a trend of *stx*⁺ *eae*-occurrence with a high incidence of *exhA* presence [37]. Some studies consider *exhA*, a gene encoding hemolysin toxin or protein, as the major virulence factor in intimin negative STEC [37, 38]. We would therefore recommend that *exhA* be screened for in future studies of STEC in Trinidad and Tobago. With only one isolate testing positive for intimin, it can be deduced that the majority of Shiga toxin producing *E. coli* are LEE-negative (*espA* and *escN*) 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]

Statistically a strong correlation was seen between the genes *stx*₁ and *stx*₂ as evidenced by the results of the bivariate correlation analysis. Further statistical analysis revealed that a comparison of the number of samples with the positives gave an odds ratio equal to 35.88(7.96<OR<220.34) and a relative risk =21.00 (<5.22<RR<84.41), the difference is statistically significant (p<0.000). The occurrence of both toxin genes in the same strain is not unusual. The well-characterized EHEC strain EDL933, which caused multiple deaths in the USA possessed both toxin genes [39]. However, non-O157 strains are often found to harbor either of the toxin genes and further analysis is therefore required to exclude this possibility. The presence of non-pathogenic bacteria can also influence the production of the Shiga toxin [40]. In a study done by Gamage *et al*, it was demonstrated that when a phage was added to phage-susceptible bacteria, a 40-fold increase in toxin production occurred as compared to a pure culture of lysogens, while its addition to phage resistant bacteria caused significant reduction in toxin production [40, 33]. However, this was not relevant in this study since the quantification of toxins was not a part of the study.

Cattle are a reservoir for STEC and can sustain an infection without continuous exposure. However, there are various factors that can potentially influence the incidence of shiga toxin occurrences by creating repetitive exposure such as poorly designed feeding troughs resulting in feed being contaminated with the faeces of wild or domestic animals. Surface water and groundwater sources may be contaminated from effluent runoff from farms and urban areas [7]. The presence of all three genes tested for was seen at only one farm, Kernaham farm 2. This farm provided only 6.9% of all samples collected but produced 33.3% of all positive samples, the most of any farm, although this was not statistically significant. The farms in this area are rural with traditional farming procedures.

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



Table 2: Distribution of shiga toxin producing *Escherichia coli* (STEC) genes from 160 bovine faecal samples recovered from animal farms in Trinidad and Tobago, 2014.

Farm	Location	N	STEC	stx ₁	stx ₂	eae	p-value
London farm	Aripo	10	0	0	0	0	
Aripo govt. farm	Aripo	46	2	1	1	0	<0.000
Ogiste farm	Aripo	10	1	0	1	0	
Kernaham 1	Manzanilla	5	0	0	0	0	
Kernaham 2	Manzanilla	11	5	4	4	1	0.1
Kernaham 3	Manzanilla	2	0	0	0	0	
Kernaham 4	Manzanilla	5	1	1	0	0	
Maffiking farm	Talparo	19	4	2	3	0	0.001
Bristol farm	Talparo	7	1	1	0	0	
Tabaquite farm 1	Tabaquite	2	0	0	0	0	
Tabaquite farm 2	Tabaquite	10	0	0	0	0	
Ortoire farm	Mayaro	33	1	0	1	0	
Total	12	160	15	9	10	1	

N = total number of specimens collected; **STEC** = number of specimens positive for STEC

4. CONCLUSION

Obviously, further investigation is warranted, but our data suggests that the prevalence of *stx*₁ and *stx*₂ is relatively low in Trinidad and Tobago. There are no strains harboring LEE Shiga toxin genes, and STEC in Trinidad and Tobago is not associated with the seven most common STEC serogroups. This information has never been reported in the country before and presents novel data that could usher a new contribution to 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 virulence profiles including risk factors.

CONSENT

Not applicable

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.

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