

Original Research Article

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 randomly selected food animal farms located in the twin island of Trinidad and processed at the Microbiology laboratories at the Faculty of Medical Sciences, The University of the West Indies Campus, from March to May 2014

Materials and Methods: 160 cattle faecal samples randomly collected from April to May 2014 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 genes associated with STEC. Overall, 1.3% of the isolates were positive for the intimin gene; 11% for STEC; 6.6% for Stx1 and 7.3% for Stx2 toxins. 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) in Trinidad and Tobago. STEC in the country is not associated with the seven most common serotypes and non of the *E. coli* isolated harbored LEE Shiga toxin genes. This information has never been reported in Trinidad and Tobago before and therefore ushers a new epidemiology of STEC in the country and Caribbean region.

Keywords: Trinidad and Tobago, PCR, PFGE, Shiga toxin, *Escherichia coli*, STEC

1. INTRODUCTION

Diarrheal diseases are a major cause of morbidity, with infection rates ranging from two to 12 cases per person per year, mainly in developing countries [1]. It has also been estimated that in children under 5 years of age there are 12,600 deaths each day caused by diarrheal illnesses in Asia, Africa, and Latin America [1]. Non-pathogenic *E. coli* may be a staple constituent of the natural flora in the lower gastrointestinal tract of humans, however Shiga toxin producing *E. coli* (STEC) is zoonotic in nature, found primarily in the intestine of ruminants [2]. Human infection is diverse in its incidence and occurs usually through food, particularly inadequately cooked ground beef [3]. In the United States of America and North Wales there have been documented cases of direct transmission from calves to humans [4] through the faeco-oral route. Cattle are regarded as the natural reservoir of STEC, however other ruminant species such as sheep, goats, and deer may also act as reservoirs; while birds, swine, dogs and horses are considered spill-over hosts [5]. Asymptomatic colonization is also possible due to the absence of the Shiga toxin receptor globotriaosylceramide-3 (Gb3) in the intestinal vasculature of reservoir hosts [6].

The antigenic structure of *E. coli* plays a significant part in epidemiology and identification. The H, O and K (EAC) antigens are the major components used in serologic typing. The genes which are necessary to establish attaching and effacing lesions are coded for on a pathogenicity island (PAI) labelled the Locus of Enterocyte Effacement (LEE). LEE contains virulence genes acquired by horizontal gene transfer [7].

The intimin gene receives the designation *eae* for *E. coli*'s attaching and effacing while the translocated intimin receptor was named Tir [7].

The presence of STEC in water, cow and bovine milk sources have been established in rural north Trinidad by other researchers [8, 9, 10] but no further studies have been done to characterize the virulent genes of *E. coli* isolated from farm animals. The documented presence of STEC in milk, ground water and ready to eat items produced in Trinidad and Tobago in conjunction with the pathogenic capacity and the propensity to cause an epidemic warrants the determination of their prevalence and characteristics.

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 characterize their virulent genes.

2. MATERIAL AND METHODS

A prospective cross sectional and observational study was carried out over three months, March to May 2014 at twelve farms in five Regional Corporations of the country. A hundred and sixty faecal samples were collected from randomly selected adult cattle from farms across these Regional Corporation in Trinidad and Tobago. Samples were immediately transported to the laboratory on ice cooler for processing. Faecal samples from juveniles, bovine animals with symptoms of diarrhoea and those animals being treated or recently treated with antibiotics were excluded from the study. The faecal samples were processed for bacterial organisms using standard microbiological methods [11]. *Escherichia coli* isolates from positive samples were further characterized using molecular methods for intimin, Stx I or Stx II.

2.1 Colony Polymerase Chain Reaction

Escherichia coli isolates were screened for the intimin (*eae*), Shiga toxin 1 (StxI) and Shiga toxin2 (StxII) genes through the use of colony Polymerase Chain Reaction (PCR) amplification as described in literature [12]. All primers used in this study were purchased from Sigma (St Louis, MO, USA). 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). 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).

2.2 Detection of Shiga toxin 1 and 2 (StxI, StxII) by PCR

All isolates positive for the *eae* gene were further subjected to colony PCR to establish the presence of the Stx-I and Stx-II genes as previously described [12, 13]. 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. Samples positive for Stx1 or Stx2 were screened for *escN*, *espA* and *tir* to confirm absence of LLE pathogenicity island and intimin polymorphism. The primers used were as follows: *tir* (*Rc*) GCAGGCTTATTCTCTACCG; *tir* (*Fc*) GGAGTCAAAACGCGTT; *espA* ATATATGAGCTCTCTTCCGAGCGTGAGA and *espA*4 TATATGAGCTCGGCTGTTAATGCGACGC [12, 13]

2.3 Serotyping by PCR

Shiga toxin (Stx) positive strains were further analyzed to determine if they belong to one of seven O groups: 026, 045, 0103, 0111, 0121, 0145 and 0157. The isolates were subjected to 25 PCR cycles as previously described [14] 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. The following primers were used for serotyping *E. coli* strains harboring Stx genes: 0157F CAGGTGAAGGTGGAATGGTTGTC; 0157R TTAGAATTGAGACCATCCAATAA; 0121R TTCTGCATCACCAGTCCAGA; 0121F TCATTAGCGGTAGCGAAAGG; 0103R AAGCTCCCGAGCACGTATAA; 0103F TAAGTACGGGGGTGCTTTTT; 0103F TAAGTACGGGGGTGCTTTTT; 045R TGTACTGCACCAATGCACCT; 045F GGGCTGTCCAGACAGTTCAT; 0111R AACGCAAGACAAGGCAAAAC; 0111F CAAGAGTGCTCTGGGCTTCT; 026R AGCGCCTATTTCAGCAAAGA; 026F GGGGGTGGGTACTATATTGC; 0145R AACGAACACCATACACCTTGTCT and 0145F TGCTCGACTTTTACCATCAAC.

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 as was done by Franz *et al* [15]. 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 [15]. A bivariate correlation analysis was conducted to determine whether or not there was any significant relationship between stx1 and stx2.

3. RESULTS AND DISCUSSION

All positive *E. coli* isolates were sulphur negative, indole positive and motile and citrate negative. The majority of samples 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).

Fifteen (15) samples were positive for STEC and its associated genes. One was positive for *eae*, nine were positive for stx1 and ten positive for stx2. The isolate that tested positive for *eae* was further screened by PCR amplification and was positive for stx1 but negative for the stx2 gene as depicted on Figure 1

The study showed an overall frequency rate of 11% for the Shiga toxin-producing *E. coli*, with 1.3% possessing the intimin gene, 6.6% had Stx1 and 7.3% possessed Stx2 genes. The majority of toxin positive strains (3/5) tested positive for both toxin genes. Three farms were positive for both toxins. In the Aripo area there were three samples that yielded *E. coli* that were positive for Stx2 but none of the samples simultaneously tested positive for either Stx1 or *eae*. Five samples yielded *E. coli* isolates which were positive for stx1, four were positive for stx2 and one positive for *eae* in the Manzanilla area. Kernaham Farm 2 of the Manzanilla area was the only farm that yielded isolates that tested positive for *eae* and the only farm to test positive for all three genes in question. Talparo area had three isolates positive for stx1 and another three isolates positive for stx2, Tabaquite area farm produced no sample that yielded *E. coli* or its genes but Mayaro area farm had a farm that produced an isolate that yielded one stx1 only. Stx1 and stx2 were significantly correlated ($r = 0.99$, $P = .00$), indicating a strong positive relationship. About a quarter (27%, 4/15) of the isolates produced both stx1 and stx2. Both stx1 and stx2 were present in isolates recovered from 42.8% (3/7) of the farms that yielded positive tests for either gene. Stx1 was present in 3/5 districts while stx2 was present in 4/5 districts. The isolates positive for intimin, StxI and StxII were negative for all the LEE genes (*escN*, *espA* and *tir*) and all serotypes examined.

The findings of this research supported previous evidence that cattle are a major reservoir of Shigella toxin producing *E. coli* (STEC). Stx1 and stx2 were present in *E. coli* isolates recovered from 58% (7/12) of the farms surveyed and this gave an overall prevalence of 10.9%. This rate is very much higher than what was reported in Washington, where the STEC recovered from healthy heifers was 2.8% and 0.15% in healthy adult cows [16]. But our rate was similar to the rate from Germany that was 10.8% [17]. None of the *E. coli* strains harbouring Shiga toxin genes could be serotyped by a multiplex PCR for the seven commonly known STEC serogroups (O157, O145, O121, O111, O103, O45 and O26) implying that new STEC serotypes might be present in Trinidad. The seven O groups tested for are commonly associated with human infection [18] but not exhaustive, therefore a broader spectrum of testing may have yielded

positive results. Indeed, previous research has reported up to twenty-eight different serotypes associated with STEC [19].

In this 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* positive intimin negative occurrence with a high incidence of *exhA* presence [19]. Some studies consider *exhA*, a hemolysin, as the major virulence factor in intimin negative STEC [19, 20]. We recommend that *exhA* be screened for in subsequent 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 studies in Australia [21] 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 among bacterial genes [22]

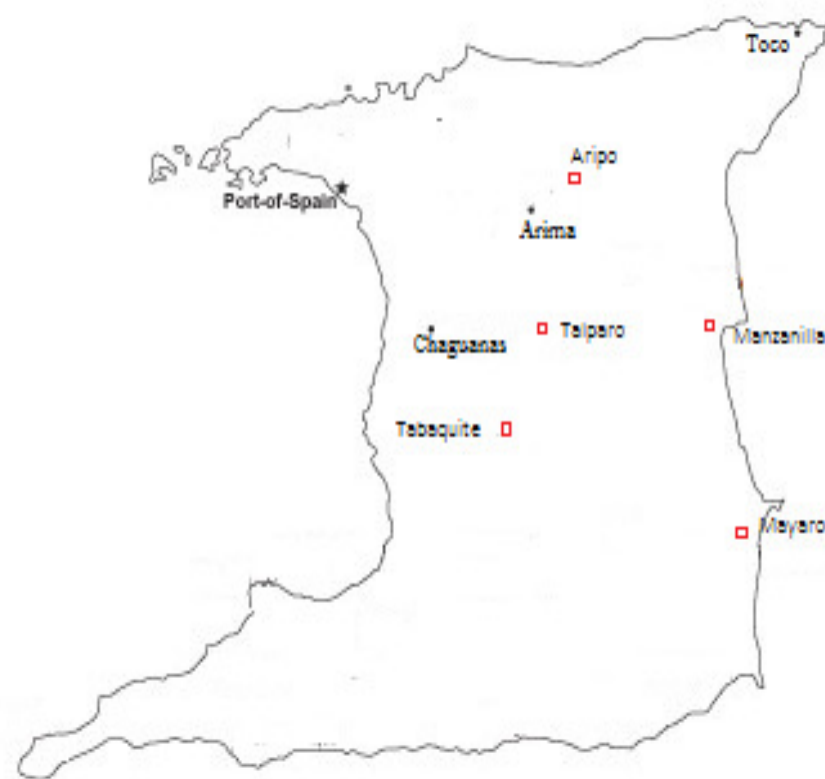
Statistically a strong correlation was seen between *stx1* and *stx2* as evidenced by the results of the bivariate correlation analysis. The occurrence of both toxins in the same strain is not unusual. The well-characterised EHEC strain EDL933, which caused multiple deaths in the USA possessed both toxins [22]. However, non-O157 strains are often found to harbour either of the toxins 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. In a study done by Gamage *et al* [23], 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 [24]. However, this was not relevant in this study since the quantification of toxins was not a part of this study.

March *et al.* used the latex agglutination test to detect *E. coli* O157:H7 since it has 100% sensitivity and specificity [25]. Though this test may be specific, it is laborious to prepare and complete with the interpretation of the results subjective. In our study, testing for verocytotoxicity was done with Vero cell monolayers on cultures positively identified as O157. Since this was the only strain tested, for it is possible that the extent of the presence of STEC was greatly understated and it is quite probable that pathogenic non O157 strains could have been overlooked. The use of PCR can then be implemented in the determination of serogroups as it is more efficient and less laborious than slide agglutination [25].

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 *stx* 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 [5]. 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 faecal specimen collection from Trinidad and Tobago

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Table showing distribution of bovine faecal samples and recovered *E. coli* genes from animal farms in Trinidad and Tobago.

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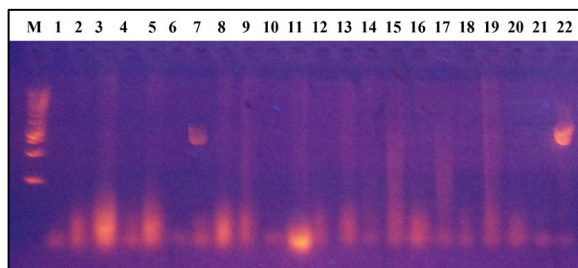
Farm	Location	N	P	stx1	stx2	eae
London farm	Aripo	10	0	0	0	0
Aripo govt. farm	Aripo	42	2	1	1	0
Ogiste farm	Aripo	10	1	0	1	0
Kernaham 1	Manzanilla	5	0	0	0	0
Kernaham 2	Manzanilla	11	5	4	4	1
Kernaham 3	Manzanilla	2	0	0	0	0
Kernaham 4	Manzanilla	5	1	1	0	0
Maffiking farm	Talparo	19	4	2	3	0
Bristol farm	Talparo	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		160	15	9	10	1

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194 N = total number of specimens collected; P = number positive specimens

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Fig 2: Gel electrophoresis image of Shiga toxin producing *E. coli* strain that is positive for intimin gene, Trinidad and Tobago



Lane M: marker; lane 7: positive isolate, lane 22: positive control

4. CONCLUSION

The prevalence of *stx1* and *stx2* is relatively low in Trinidad and Tobago. There are no strains harboring LEE Shiga toxin genes. This study has revealed that STEC in Trinidad and Tobago is not associated with the seven most common STEC serotypes. This information has never been reported in the country before and presents novel data that ushers a new epidemiology of STEC in the Caribbean region. Future research approaches should concentrate on identifying STEC serotypes present in Trinidad and Tobago and other pathogenic virulence profiles.

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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