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 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 most 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 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].
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 serotypes 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 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. 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 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 faecal stools were 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 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.

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 12 g of the faecal materials and homogenized for about half an hour. This was then plated onto Sorbitol-Mac Conkey (SMAC) agar plates and incubated for 18 – 20 h at 37°C. Suspected pure colonies for E. coli were subcultured on Mac Conkey and blood agar plates. Colonies were confirmed as E. coli if they were lactose positive on Mac Conkey agar plates, and biochemical tests yielded including negative hydrogen sulphide production, positive indole and motility tests in agar SIM, positive beta-glucuronidase and methyl red, and negative results 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 – stx1, stx2, eae, escN, espA and tir – by colony PCR and assessment of STEC serogroup genes by multiplex PCR
E. coli isolates were screened for several virulent markers including the eae gene (coding for the intimin adhesion), Shiga toxin 1 (stx1) and Shiga toxin 2 (stx2) 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 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. Reactions in 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 Tecne Flexigene Thermal-cycler (American Laboratory Trading East Lyme, CT,
USA). 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 100 bp 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 stx1 or stx2 were also tested for escN, espA 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°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 methods as previously described [32, 33],
strains positive for the genes coding the several 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°C. PCR reaction mixtures were resolved as described above.

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

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primer</th>
<th>Primer sequence (5’ – 3’)</th>
<th>Amplicon size (bp)</th>
<th>Ref</th>
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<td>stx1</td>
<td>stx1-F</td>
<td>TGTCGCATAGTGGAAACCTCA</td>
<td>655</td>
<td>[30]</td>
</tr>
<tr>
<td></td>
<td>Stx1-R</td>
<td>TGGCAGCTAGAAGAAGAAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx2</td>
<td>stx2-F</td>
<td>CCATGACACGGACAGCAGATT</td>
<td>477</td>
<td>[30]</td>
</tr>
<tr>
<td></td>
<td>stx2-R</td>
<td>TGGCGCCGATTCATGGCATTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eae</td>
<td>eae-F</td>
<td>CATTATGGAACGGCAGGAGTT</td>
<td>375</td>
<td>[30]</td>
</tr>
<tr>
<td></td>
<td>eae-R</td>
<td>ACGGATATGGAAGCCATTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ehxA</td>
<td>ehxA-F</td>
<td>GCGAGCTAAGCAGCTTGAAAT</td>
<td>199</td>
<td>[30]</td>
</tr>
<tr>
<td></td>
<td>ehxA-R</td>
<td>CTGGAGGCTGCACTAACTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wzx026</td>
<td>026F</td>
<td>AGGTTGCGATGCGATATTG</td>
<td>417</td>
<td>[31]</td>
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<tr>
<td></td>
<td>026R</td>
<td>GACATAATGACATACCGAGCA</td>
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<td>890</td>
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<td>045-R</td>
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<tr>
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<td>0103F</td>
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<td>740</td>
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<td>0103R</td>
<td>GGTTAAAGCGCATGCTACAG</td>
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<td>0111F</td>
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<td>0121F</td>
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<td>0145F</td>
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<td>523</td>
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<td>296</td>
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<td>rtbE-R</td>
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<tr>
<td>tir</td>
<td>tir-Fc</td>
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<td>[32]</td>
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<td></td>
<td>tir-Rc</td>
<td>GACGGCTTTATTTACCGTAGC</td>
<td></td>
<td></td>
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<tr>
<td>espA1</td>
<td>Fc</td>
<td>CATGCGCATGATACATCAAATGCACTCC</td>
<td>533</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCGAAATCAGTTTACCAAAGGATATTGAAATAG</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ref = reference</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3
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 \textit{Escherichia coli} (STEC) in small farms within rural communities, located in the agricultural areas of several regions of Trinidad. A total of one hundred and sixty (160) faecal samples were collected from 12 selected pools 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). 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]. 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 \textit{E. coli} isolates if such organisms were lactose fermenting positive on Mac Conkey 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 STEC, and using the method and formula reported by Franz et al. [34], true prevalence of the positive indices were obtained as follows: 1.3% had the intimin (\textit{eae}) gene, and 6.6% had $stx_1$, gene and 7.3% possessed $stx_2$ gene. The majority of $stx$ positive strains tested positive for both Shiga 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 \textit{E. coli} that were positive for $stx_2$ gene but none of the samples simultaneously tested positive for either $stx_1$ or \textit{eae}. In the Manzanilla area, five samples yielded \textit{E. coli} isolates which were positive for $stx_1$, four were positive for $stx_2$ and one positive for \textit{eae}. The Kernaham Farm 2 in Manzanilla area was the only farm that produced the isolate that tested positive for \textit{eae}, and the only farm where positive results for all three genes ($stx_1$, $stx_2$, \textit{eae}) were obtained. 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, $p=0.1$. A watchful 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 \textit{E. coli} or its genes but in the Mayaro area there was a farm that produced an isolate positive for $stx_1$.

Overall, the $stx_1$ and $stx_2$ genes were present in \textit{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 $stx_2$ 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 (\textit{eae}), $stx_1$ and $stx_2$ were negative for all the Locus of Enterocyte Effacement (LEE) genes (\textit{escN}, \textit{espA} and \textit{tir}) and genes coding for these serogroups 026, 045, 0103, 0111, 0121, 0145 and 0157.

Carriage of \textit{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 point to cattle are a natural reservoir of Shiga toxin producing E. coli STEC [7]. The overall prevalence of 9.4% (15/160) among the faecal materials analyzed in the present study is very much higher than the 2.8% reported in Washington where STEC was recovered from healthy heifers, and the 1.25% recovered from different locations in Mexico [16]. However, our rate was similar to the 9% prevalence observed 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 were 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, works by other authors 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 remaining isolates 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 stx1 and stx2 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 performed.

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 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 produced 33.3% of all positive samples, the most higher rate of any farm, although this was not statistically significant. The farms in this area are rural plants 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.

<table>
<thead>
<tr>
<th>Farm</th>
<th>Location</th>
<th>N</th>
<th>STEC</th>
<th>stx₁</th>
<th>stx₂</th>
<th>eae</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>London farm</td>
<td>Aripo</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aripo govt. farm</td>
<td>Aripo</td>
<td>46</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>≤0.000</td>
</tr>
<tr>
<td>Ogiste farm</td>
<td>Aripo</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Kernaham 1</td>
<td>Manzanilla</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Kernaham 2</td>
<td>Manzanilla</td>
<td>11</td>
<td>5</td>
<td>4</td>
<td>4</td>
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<td>0.1</td>
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<td>Manzanilla</td>
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<td>0</td>
<td>0</td>
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<td></td>
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<tr>
<td>Kernaham 4</td>
<td>Manzanilla</td>
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<td>1</td>
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<td>0</td>
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<tr>
<td>Maffiking farm</td>
<td>Talparo</td>
<td>19</td>
<td>4</td>
<td>2</td>
<td>3</td>
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<td>Bristol farm</td>
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<td>Tabaquite farm 1</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>Tabaquite farm 2</td>
<td>Tabaquite</td>
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<td>Ortoire farm</td>
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<td>1</td>
<td>0</td>
<td>1</td>
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<td></td>
<td>12</td>
<td>160</td>
<td>9</td>
<td>10</td>
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</table>

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 E. coli positive for stx1 and stx2 is relatively low in Trinidad and Tobago. There were 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 serogroups. The present 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 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.

REFERENCES


