



Molecular Epidemiology of Shiga Toxin-Producing *Escherichia coli* Isolated from School Children in Ondo State, Nigeria

A. K. Onifade¹ and M. A. Oladoja^{1*}

¹Department of Microbiology, Federal University of Technology, PMB 704, Akure, Ondo state, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author AKO designed the study and supervised the work. Author MAO carried out the laboratory work, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches as well as analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Molecular techniques were used for studying the epidemiology of *Escherichia coli* from five major towns in Ondo state, namely; Akure and Ondo, Owo, Okitipupa and Ikare-Akoko. In total, 807 urine and faecal samples of apparently healthy primary school pupils were gathered between December 2012 and March 2013. Withal, 206 *E. coli* strains were isolated and characterized by biochemical tests. Polymerase chain reaction (PCR) assay was performed to determine the presence of the genes-encoding virulence factors. A total of 22 (12 male, 10 female) isolates out of 28 subjected to PCR amplification were found to possess only *stx1* virulence gene associated with Enterohaemorrhagic *E. coli* strains. PCR assay was demonstrated to be a useful technique for the epidemiological work of *E. coli* where this organism is a major cause of infection.

Keywords: Epidemiology; *Escherichia coli*; shiga toxin; urine; faecal; PCR.

*Corresponding author: E-mail: marymartin055@gmail.com;

1. INTRODUCTION

Molecular epidemiology is a branch of medical science that focuses on the contribution of potential genetic and environmental risk factors, which are identified at the molecular level, to the aetiology, distribution and prevention of diseases within families and across populations. This area of medical science improves our understanding of the pathogenesis of diseases by identifying specific pathways and molecular factors which influence the risk of contracting the disease [1].

Escherichia coli is a very adaptive enteric Gram-negative bacillus, which is easily recognized as non-invasive commensal. Most strains of *E. coli* live as commensals; many perhaps are opportunistic pathogens of human and even animals [2]. Certain *E. coli* isolates have been demonstrated to produce a toxin, which was initially called verotoxin because of its distinct effect on vero-cells. This family of toxins was recently called Shiga toxins (*stx*), owing to its close relation to the *stx* of *Shigella dysenteriae* type 1. Enterohaemorrhagic *E. coli* (EHEC) is the main group of verotoxigenic strains which has emerged as the leading cause of haemorrhagic colitis and haemolytic uremic syndrome (HUS) in man.

EHEC can be communicated in the first place through the ingestion of faecal contaminated foods, particularly undercooked beef. Nevertheless, a big number of outbreaks of EHEC have also been linked with ingestion of contaminated drinking water or contact with recreational water [3]. Infection with Shiga toxin (*stx*)-producing *Escherichia coli* (STEC) can ensue in a spectrum of examples; ranging from asymptomatic carriage to uncomplicated diarrhoea, haemolytic uraemic syndrome (HUS), bloody diarrhoea, haemolytic anaemia, thrombocytopenia, and acute renal failure. High fatality and morbidity rates have been reported for HUS, which could occur from infection with STEC strains. The pathogenesis of STEC had been observed to be linked with several bacterial virulence genes. More or less of these virulence factors include the intimin protein, two Shiga toxins called *stx1* and *stx2* and the plasmid-encoded protein known as haemolysin [4].

Information on the distribution of virulence genes of STEC strains isolated from children is rare in Ondo state. Thus, this work was designed to determine the molecular epidemiology of Shiga

toxin-producing *Escherichia coli* isolated from school children in five major towns of Ondo State, Nigeria.

2. MATERIALS AND METHODS

2.1 Collection of Samples

A total of 807 urine and faecal samples of apparently healthy children were collected from five major towns in Ondo state, Nigeria between December, 2012 and March, 2013. The towns include Akure, Owo, Ondo, Okitipupa and Ikare-Akoko, covering the three senatorial districts of the state. Samples were collected inside well-covered and sterile universal bottles, kept under cold condition and then transferred to the laboratory within 2 h of collection for culturing. Questionnaire containing information about age (2-15 years), gender, and health history was filled for each pupil.

2.2 Isolation and Characterization of *E. coli*

Urine samples collected were streaked directly on Eosin methylene blue (EMB) agar plate using a sterile inoculating loop while faecal samples were first diluted with 5 ml of sterile distilled water, then a loop-full each was streaked on EMB agar plate. All inoculated plates were incubated at 37°C for 24 h. Colonies with greenish-black metallic sheen on EMB after incubation were suspected to be *E. coli* and were thereafter Gram-stained and viewed under an oil-immersion microscope. Also, all the suspected *E. coli* isolates were sub-cultured on MacConkey agar plates and incubated at 37°C for 24 h. The final pure colonies were characterized by biochemical tests such as citrate utilization test, urease test, H₂S production test, indole test, methyl red test, Voges-Proskauer test and sugar fermentation test [5].

2.3 DNA Extraction

Bacterial strains were grown overnight in Luria broth medium at 37°C. Ten millilitre (10 ml) of overnight culture of *E. coli* on Luria broth (LB) medium was pipetted into a 15 ml disposable polypropylene tube. The tube was capped tightly and centrifuged at 14,000 rpm for 10 minutes to pellet the cell debris. The supernatant was used as a template for PCR amplification [6].

2.4 PCR Detection of Virulence Genes in *E. coli* Strains

PCR technique was used for determining the presence of virulence genes: *bfp*, *eae*, *EAF*, *LT*, *stII*, *ipaH*, *virF*, *aafII*, *daaE*, *stx1*, *stx2*, *Ehly*, and *uidA*. For every gene of interest, a master mix was prepared containing: Taq polymerase (0.06 μ L), 10x buffer (2.5 μ L), dNTPs (0.25 μ L), $MgCl_2$ (0.75 μ L), and a primer (0.25 μ L each of both forward and reverse reactions). A 2 μ L of *E. coli* template DNA was added to the final PCR mixture before being subjected to PCR amplification under cycling conditions of: denaturation at 94°C, annealing at 55°C and strand elongation at 72°C. All the PCR products were electrophoresed on 1.5% agarose gels that were stained with ethidium bromide and examined under UV illumination [6].

2.5 Statistical Analyses

Data obtained are presented as mean \pm SE (standard error). The significance of difference between different treatment groups was tested using one-way analysis of variation (ANOVA) with the aid of Statistical Package for Social Sciences (SPSS, IL) version 17 at $P \geq 0.05$.

3. RESULTS

3.1 Isolation and Characterization of *E. coli* from Urine and Faecal Samples

The result of isolation of *E. coli* from urine and faecal samples collected from pupils aged 2-15 years in the five major towns in Ondo state, Nigeria is presented in Tables 1 and 2. From the result, two hundred and six (206) *E. coli* strains were isolated from both urine and faecal samples of pupils in five major towns in Ondo state. The result showed that, the number and percentage of *E. coli* isolated from urine is higher in female than in male pupils in Akure, Owo, Okitipupa and Ikare-Akoko, while for faecal samples, *E. coli* isolates were higher in females from two communities- Akure and Ikare-Akoko but varying results were found in other communities. The result of percentage *E. coli* isolated from the male faecal samples indicated that, Ondo location had the least percentage occurrence of *E. coli* with 20%, while above 40% was observed in other locations and Owo had the highest percentage (60.7%). From the result of *E. coli* isolated from female faecal samples, Ikare-Akoko had the highest percentage (76.5%) followed by Akure (70.3%) and Ondo (20%) was the least.

From the result of biochemical tests, *E. coli* showed positive reaction on the sugars used (i.e. Glucose, lactose, and sucrose), indole test, and methyl red test, but negative reaction was shown for hydrogen sulphide (H_2S) production, urease, citrate and Voges-Proskauer tests.

3.2 Polymerase Chain Reaction of *E. coli* Isolated from Primary School Pupils in Ondo State

The result of polymerase chain reaction (PCR) amplification of *E. coli* isolates is presented in Table 3. Plates 1a and b showed the result of the *E. coli* isolates (n=28) on gel electrophoresis. Twenty-two (78.57%) out of 28 *E. coli* isolates subjected to uniplex PCR amplification were confirmed to possess *stx1* virulence gene, which is associated with Enterohaemorrhagic *E. coli* (EHEC) strains, while the remaining 6 (21.43%) isolates showed negative result for the gene. In all, 14 males and females each represented 68 and 138 isolates respectively. Of these, 16 (57%) belonged to faecal sample while the remaining 12 (43%) were isolated from urine.

4. DISCUSSION

This epidemiological survey is out to characterize a population of *E. coli* isolated from urine and faecal samples of school children in five major towns in Ondo State, Nigeria. It was found that a population of *E. coli* was present in the samples collected but sparingly diverse epidemiologically across the communities where sampling was carried out. Nevertheless, *E. coli* isolates were more in female than male urine and stool samples respectively. The investigation conducted by other workers showed that *E. coli* of different strains have been isolated from urine samples of patients suffering from cystitis and pyelonephritis [7] and from faecal samples of diarrhoeic and non-diarrhoeic patients [8,9].

From the statistics of samples collected and analyzed by [10] from South-Stockholm General Hospital and Rosen-Lund Hospital in Sweden following an outbreak of epidemic, more female patients suffered from *E. coli* infection most especially those with genes that code for TEM, OXA and CTX-M-1-type than male patients. Besides, a research conducted by [11] on both community and hospital care settings revealed that, there was a significant difference between the prevalence of *E. coli* in male (50/117) and female (100/133) cultures ($P < 0.001$). Additionally, the female anatomical configuration

also makes them to be more prone to urinary tract infection, due to the fact that the female urethra is much shorter and closer to the anus than in male. From these various points of view, it is evident that the female is more prone to urinary tract infection than male and this invariably showed the reason for a higher percentage of *E. coli* isolates in urine of females than males in this study. In the same vein, the study also showed that *E. coli* isolated from faecal samples were more in female (70/124)

than in male (55/107). *E. coli*, besides being a cause of diarrhoeae, it's also a microflora in the gastrointestinal tract (GIT), where its isolation may be possible irrespective of any outbreak of epidemic. The evidence of the incidence of *E. coli* isolates in female more than male faeces, vice versa, may not be too necessary because of its niche (GIT), unlike urinary tract where female anatomy might be reasonable. Thus, this study is in agreement with other works [12,13].

Table 1. *E. coli* isolates from urine and faecal samples of male and female pupils in Ondo state, Nigeria

Location		Urine	Stool
Akure	No. of male	60	37
	<i>E. coli</i> isolates from male (%)	05(8.3)	17(46.0)
	No. of female	71	37
	<i>E. coli</i> isolates from female (%)	31(43.7)	26(70.3)
Ondo	No. of male	30	05
	<i>E. coli</i> isolates from male (%)	03(9.7)	01 (20.0)
	No. of female	32	10
	<i>E. coli</i> isolates from female (%)	03(9.4)	02 (20.0)
Okitipupa	No. of male	82	28
	<i>E. coli</i> isolates from male (%)	02(2.4)	15(53.6)
	No. of female	66	41
	<i>E. coli</i> isolates from female (%)	15(22.7)	21(51.2)
Owo	No. of male	82	28
	<i>E. coli</i> isolates from male (%)	01(1.2)	17(60.7)
	No. of female	79	19
	<i>E. coli</i> isolates from female (%)	05(6.3)	08(42.1)
Ikare	No. of male	38	09
	<i>E. coli</i> isolates from male (%)	01(2.6)	05(55.6)
	No. of female	36	17
	<i>E. coli</i> isolates from female (%)	15(41.7)	13(76.5)

Table 2. *E. coli* isolates from different age groups and sexes per location

Location	Sex	Age group						Total
		2-3	4-5	6-7	8-9	10-12	13-15	
Akure	Male	0	1	9	7	7	0	24
	Female	1	5	15	24	10	0	55
	Total	1	6	24	31	17	0	79
Ondo	Male	0	0	3	0	0	1	4
	Female	0	1	2	1	0	1	5
	Total	0	1	5	1	0	2	9
Owo	Male	0	0	5	11	0	3	19
	Female	0	0	5	5	1	1	12
	Total	0	0	10	16	1	4	31
Okitipupa	Male	0	0	0	6	10	2	18
	Female	0	1	10	7	14	3	35
	Total	0	1	10	13	24	5	53
Ikare	Male	0	2	3	1	0	0	6
	Female	1	3	11	7	6	0	28
	Total	1	5	14	8	6	0	34
	Grand total	2	13	63	69	48	11	206

Table 3. PCR result of selected *E. coli* isolates among male and female pupils

Sample type	Male (PCR result)	Gene type	Sample type	Female (PCR result)	Gene type
Urine	Negative(-)	-	Urine	Negative(-)	-
Stool	Positive(+)	<i>stx 1</i>	Urine	Positive(+)	<i>stx 1</i>
Stool	Positive(+)	<i>stx 1</i>	Urine	Positive(+)	<i>stx 1</i>
Stool	Positive(+)	<i>stx 1</i>	Urine	Positive(+)	<i>stx 1</i>
Stool	Positive(+)	<i>stx 1</i>	Stool	Positive(+)	<i>stx 1</i>
Stool	Positive(+)	<i>stx 1</i>	Urine	Positive(+)	<i>stx 1</i>
Urine	Positive(+)	<i>stx 1</i>	Stool	Positive(+)	<i>stx 1</i>
Stool	Positive(+)	<i>stx 1</i>	Urine	Positive(+)	<i>stx 1</i>
Stool	Positive(+)	<i>stx 1</i>	Stool	Negative(-)	-
Stool	Negative(-)	-	Urine	Negative(-)	-
Stool	Positive(+)	<i>stx 1</i>	Stool	Positive(+)	<i>stx 1</i>
Urine	Positive(+)	<i>stx 1</i>	Urine	Negative(-)	-
Stool	Positive(+)	<i>stx 1</i>	Urine	Positive(+)	<i>stx 1</i>
Stool	Positive(+)	<i>stx 1</i>	Stool	Positive(+)	<i>stx 1</i>

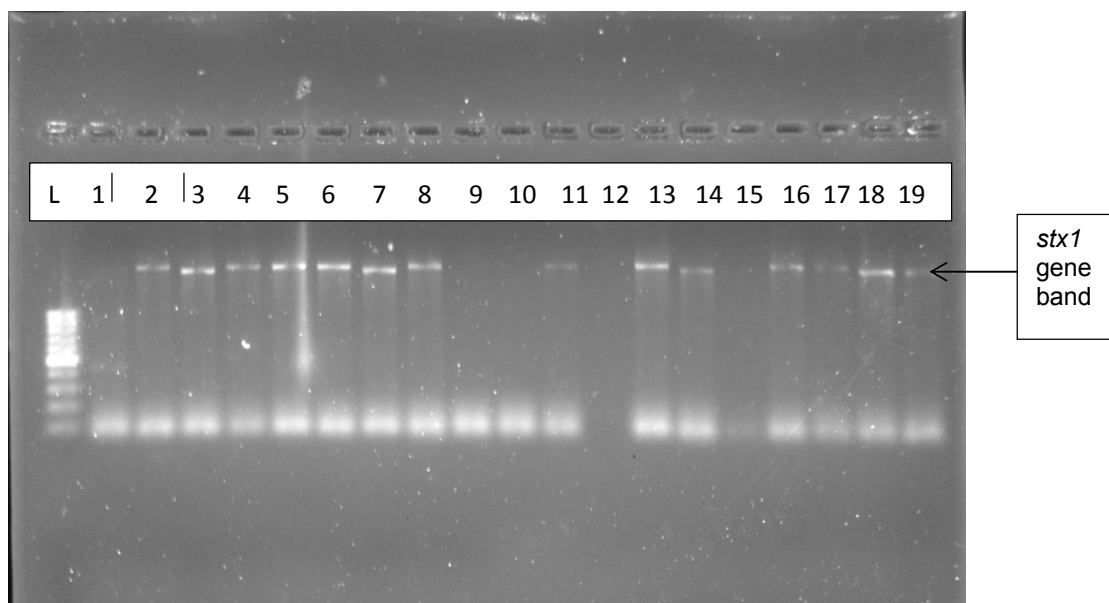


Plate 1a. Result of Gel Electrophoresis showing *E. coli* strains carrying *stx1* gene (Lanes 2-8,11,13,14,16-19); *E. coli* strains showing no band for *stx1* gene (Lanes 1,9,10,15)
L=Lambda (100 bp), Lanes 1 to 14= *E. coli* isolated from female pupils, Lanes 15 to 19= *E. coli* isolated from male pupils

The incidence of *E. coli* carrying shiga toxin gene (*stx1*) in these pupils may be associated with the nature of food and water they take. Most foodstuffs and water may be grossly polluted due to an array of sources, including domestic and/or wild animals, malfunctioning septic systems, industrial outlets, combined sewer overflows, and wastewater effluents, as well as ineffectively controlled treatment stages and, to an extent, the persistence of bacteria after disinfection [14,15]. In general, the less sanitized environment has

largely rendered our intakes unfit, thereby increasing the prevalence of this toxin.

The *stx1* gene of Shiga toxin happens to be a major virulence factor of Enterohaemorrhagic *E. coli* (EHEC) strains detected in this study and it is known for producing life-threatening disease in humans with two key clinical microbiological observations: Haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) [16,17]. However, in recent times, Shiga toxin-producing *E. coli* strains have been implicated in the

aetiology of urinary tract infection (UTI). [18] reported two incidences of urinary tract infection caused by STEC in elderly women, though both strains belonged to the O157:H7 serotype and carried genes associated with severe illness. Besides, urine samples' data from the work done by [19,20], demonstrated that *E. coli* O157:H7 were isolated (2.3%) and (1.5%) respectively from urine. In addition, three EHEC isolates obtained from the urine of hospitalized patients suffering from UTIs were geno- and phenotypically characterized according to [21]. These isolates carried typical EHEC virulence markers and one of them exhibited a localized adherence (LA)-like pattern on T24 urinary bladder epithelial cells. *E. coli* O157:H7 serotype was also isolated from urine samples of adult males and females suffering from severe UTI in Nigeria. From the report, a very high prevalence of this *E. coli* serotype (46.4%) among tested patients was observed; their study cited that renal diseases associated with severe UTI were mainly caused by *E. coli* O157:H7 [22]. These and other works [13] showed that STEC strains are implicated in UTI and in support of this study.

Enterohaemorrhagic *E. coli* strains are also known to be involved in diarrhoea in both male and female. From our result, *stx1* virulent gene was identified and this was consistent with what other researchers have got. Results from other researchers have shown that Shiga toxin-producing *E. coli* causes diarrhoea. It was

observed that the genes encoding Shiga toxins and intimin were the most commonly detected virulence factors isolated from diarrhoeic and non-diarrhoeic stools of infants [13,4,23]. Although samples in this present study were collected from non-diarrhoeic children, but there might be an occurrence of watery diarrhoea in the population studied, now or later [24].

Studies had shown that Shiga toxin family with related structural and similar biological activity is composed of *stx1* which is essentially identical to the toxin of *Shigella dysenteriae* differing in a single amino acid and, *stx2* with less than 60% amino acid homology to *stx1* [25,26]. This indicates that there is possibility of gene transfer between Enterohaemorrhagic *E. coli* and *Shigella dysenteriae* strains [27]. Owing to these vital observations, and because man cannot exist without his environment, the interaction most times result into infection caused by these pathogens. It had also been observed that the presence of *stx1* and *stx2* alone may not be sufficient to cause disease in humans except when other virulent genes such as *eaeA*, *ehxA* or *saa* are present [16]. Thus, since the samples were collected from apparently healthy children, detection of *stx1* gene, which had been observed not capable of causing a disease condition alone, may further contribute to the fact that the *E. coli* strain isolated from these children were not carrying other genes.

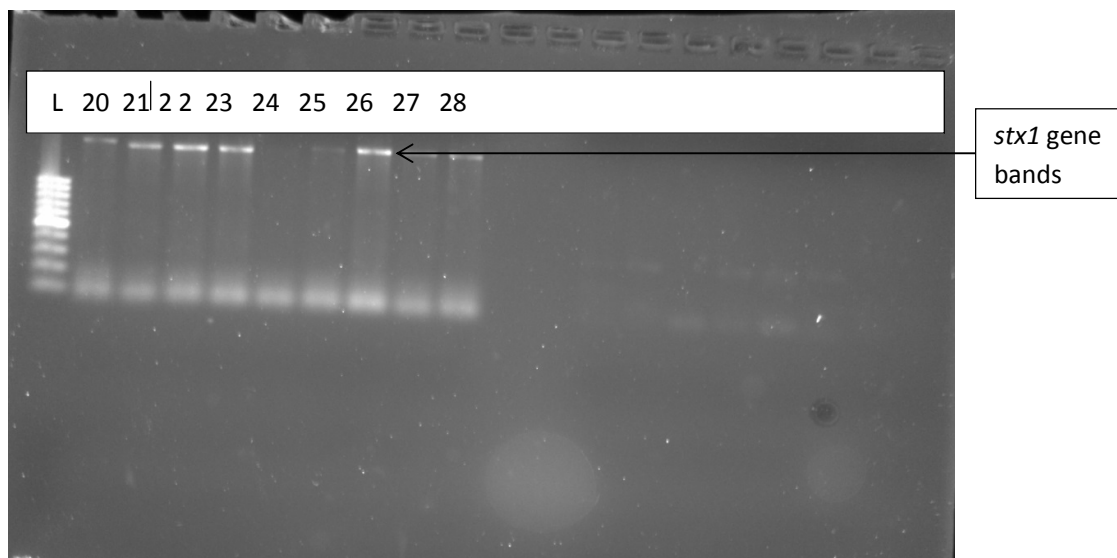


Plate 1b. Result of gel electrophoresis showing *E. coli* carrying *stx1* gene (Lanes 20,21,22,23,25,26,27 and 28). Sample carrying no *stx1* gene (Lane 24). L=Lambda (100bp)

5. CONCLUSION

In conclusion, there is a strong indication of the presence of *E. coli* in both urine and faecal samples collected from apparently healthy children in Ondo state. In the same vein, the molecular study of these isolates showed that, *stx1* gene was predominant among the isolated *E. coli*, which suggested that most of them belong to the Enterohemorrhagic *E. coli* group. The presence of the *stx1* gene alone has been proven to be incapable of causing an infection, and thus further strengthened the fact that the samples were collected from apparently healthy pupils.

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ETHICAL APPROVAL

The sampling was community-based and letter (To Whom It May Concern) was sent to the Universal Basic Education offices in all the Local Government Areas by the Department of Microbiology, Federal University of Technology, Akure to seek necessary consent and permission from the board. The consent of parents, Head teachers and pupils was also sought and granted before any sample was collected from the schools.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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