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DETECTION AND MOLECULAR CHARACTERIZATION OF SHIGA TOXIN *ESCHERICHIA COLI* AMONG PREGNANT WOMEN ATTENDING ANTENATAL CLINICS IN SELECTED HOSPITALS WITHIN KADUNA METROPLOLIS, NIGERIA.

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ABSTRACT

Shiga toxin producing Escherichia coli was investigated among pregnant women attending antenatal clinics in selected hospitals within Kaduna Metropolis. A total of 400 urine samples were collected and analysed for Shiga toxin E.coli . E.coli strain was isolated using Eosin methylene blue agar, sorbitol Mac Conkey agar (CT-SMAC) and preserved on Nutirent agar slant. The isolates were identified base on cultural characteristics. Molecular identification using Polymerase Chain Reaction (PCR) with stx1,stx2 and uidA primer was carried out. Out of the 400 samples collected, 85 (21.3%) were positive for Escherichia coli. While two (2) out of these isolates were confirmed to be Escherichia coli O157:H7.

Introduction:

Urinary tract infections (UTI) represent one of the most common diseases encountered in medical practice today (Aiyegoro *et al.*, 2007). U.T.I is a predominant type of bacterial infection among pregnant women (Nowicki, *et al.*, 2002). Hormonal and physiological changes in bladder volume and tone, may promote infection in pregnant women (Nowicki *et al*., 2002). About 150 million individuals have been reported to be affected by UTIs annually worldwide (Ntonifor and Ajayi, 2007).

Escherichia coli (E.coli) is one of the most versatile micro organism that rapidly

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Colonizes the gastro intestinal tract of the newborns (Kaper et al., 2004). Although *E.coli* usually represent an important commensal of the normal intestinal microbiota, other variants that are able to cause infections exist (Kaper et al., 2004; Croxen et al., 2013).

Nevertheless, cases of urinary tract infection (UTI) where EHEC isolates have been isolated from the corresponding urine sample have been reported. The first report of EHEC urine isolate was released in 1994 (9). Although the frequency of UTI caused by EHEC seems to be low.

Escherichia coli O157:H7 is a pathogenic serotype of *Escherichia coli* which colonize the gastrointestinal tract and cause a condition known as heamorragic colitis or bloody diarrhea (Chakroborty, 2017). *Escherichia coli* O157:H7 produces Shiga toxin type 2 (Stx2) or both toxins and its variants (Kalender and Kilic, 2016). The capacity of *E.coli* O157:H7 to produce Shiga toxins (stx) makes them of particular concern because stx has been linked to the development of haemolytic uraemic syndrome (HUS) that can lead to kidney failure (Krystle *et al*., 2011). The shiga toxin-producing *Escherichia coli* (STEC) are also called Verocytotoxin producing *E.coli* (VTEC) (AZucena *et al*., 2007). STEC strain that cause human infection belong to a large number of O:H Serotypes such as O26:H11, O103:H2. O111:h8, O145:H28 and O157:H7.

However, O157:H7 have been more frequently isolated from human with severe haemorrhagic colitis and haemolytic uraemic syndrome (Sewlikar and D'sooka, 2017). Infection caused by *Escherichia coli* O157:H7 have been a significant public health problem world-wide causing human diseases including diarrhea, haemorrhagic colitic (HC), haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenia porpura (Smith *et al*., 2003). The pathogenicity of *E.coli* O157:H7 is mostly attributed to its ability to produce the shiga toxins (stx1 and stx2) and the presence of the intimin (eae) gene, which is essential for adherence to the intestinal

epithelium via attaching and effacing mechanism (Vallance and Finlay, 2000).

MATERIALS AND METHODS

Study Area

This study was carried out in some selected hospitals within Kaduna metropolis which includes: Barau Dikko Teaching Hospital, 44 Nigerian Army Reference Hospital Tudun Wada, Yusuf Dantsoho Memorial Hospital Tudun Wada, General Hospital Kawo, General Hospital Sabon and Gwamna Awan General Hospital Nassarawa, Nigeria.

Collection of urine samples

A total of 400 samples of urine was collected with the assistance of the laboratory staff of the selected Hospitals and processed as follows:
Midstream urine was collected into a 20ml calibrated sterile screw-capped universal container distributed to the pregnant women. The specimens were labeled appropriately and transported to the laboratory for analysis.

Sterilization of Media and Materials

The media used were Nutrient Agar (NA), MacConkey agar and Eosin Methylene Blue agar. All glass wares were washed with detergent and rinsed with water, then allowed to dry. The glass wares were sterilized in a hot air oven at 160^{o}C for 2hours. Media were prepared according to manufacturer's specification and sterilized by autoclaving at 121^{o}C for 15 minutes.

Isolation of *E. coli*

The urine samples were streaked on MacConkey agar and they were incubated at 37^{o}C for 24 hours. The suspected colonies which were smooth pinkish colonies were picked and subcultured on Eosine methylene blue agar using a flamed wireloop and it was incubated at 37^{o}C for 24 hours.
The plates with the positive growth having the greenish metallic sheen were further sub cultured into fresh plate of sorbitol Mac Conkey (CT-

SMAC). The plates were incubated at 37°c for 24 hours. Nutrient agar medium was prepared to preserve pure colonies. The nutrient agar slant was preserved in the refrigerator for further use.

Molecular Identification of *E. coli* O157:H7 using Polymere Chain Reaction

DNA Extraction using Phenol/Chloreform Protocol

Phenol/Chloroform/Isoamyl Alcholic Extraction

200µl of overnight broth culture of the isolation was placed a tube and was labeled tube one, equal volume of phenol/chloroform/isolation (pcl) was added and the solution was vortex vigorously for one minute. The solution was centeifuged at 4000rpm for five minutes; approximately 180µl of the top aqueous solution (supernatant) was transferred into a second tube and labeled tube two. About 200µl of elution buffer was added to tube one and was vortexes for the one minutes and centrifuge at 4000rpm for five minutes, top aqueous solution (supernatant) was transfer to the tube.

Chloroform Back Extraction

Equal volume of phenol/chloroform/isoamyl (pcl) was added to the second tube and was vortexes vigorously for the one minutes, the solution was centrifuge at 4000rpm from five minutes, and the top aqueous solution (supernatant) was transfer to another tube and labeled tube three.

Ethanol Precipitation

1µl of 7.5M of ammonium acetate was added to get a final concentration of 2.5M, 1µl of glycogen and 2.5mi of 100% ethanol was added and the solution was properly mixed, the solution was incubated at 20ºC overnight and was centrifuged for 20 minutes in a 4ºC centrifuge at 8000rpm and the supernatant was decanted carefully without disturbing the pellet. The pellet was washed by adding 300µl of 80% ethanol, vortex three times and centrifuge for 15 minutes in a 4ºC centrifuge at 8000rpm, the supernatant was decant without disturbing the pellet. The pellet was washed again by adding 300µl of 80% ethanol and vortexed three times and then centrifuged for 15 minutes in a 4ºC centrifuge at 8000rpm, the supernatant

was decanted without disturbing the pellet. The tube was centrifuge on a table top centrifuged to draw residual ethanol to the top ,the ethanol was removed using a p20 pipette, the pellet was air dried for 1-2 minutes and were re-suspended in appropriate volume of elution buffer.

Procedure for Polymerase Chain Reaction

The operation of polymerase chain reaction involves several cycles. There are three steps in one amplification cycles. They are: denaturtion (melting), annealing and polymerization (extention).

Table 1: Primer sequences and predicted lengths of PCR amplification products

Primer	Direction	Primer Sequence (5'-3)	Fragment size (bases)	Reference
stx 1	Forward Reverse	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	614	Gannon et al., 1992
stx 2	Forward Reverse	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779	Gannon et al., 1992

Denaturation (melting of target DNA)

The target DNA containing sequence (between 100 and 5,000 bases) to be amplified will be heat denatured 95ºC for the 15 minutes to separate its complementary strands. This process is called melting of target DNA.

Primer Annealing

The second step is the annealing of two oilgonucleotide primers to the denated DNA strainds. Since sequence of each oilgonucleotide primer is compulsory to 3 end of single stranded template, the primers will (hybridizes) each template. The primers will then be added in excess and the temperature will be lowered to about 68ºC for 60 minutes. The primers will form hydrogen bond i.e anneal to the DNA on both sides of the DNA sequence.

Primer Extension

(Polymerization) nucleotide triphosphate and a thermo-stable DNA polymerase were added to the reaction mixture. The DNA polymerase accelerated the polymerization process of the primers and therefore will extends the primer (68ºC) resulting in the synthesis of copies of target DNA sequence.

Agarose Gel Electrophoresis

One gram (1g) agarose powder (Bio Rad) was dissolved in 100 ml of Tris acetate-EDTA (TAE) (40mM Tris acetate, 2 mM EDTA, pH 8). The solution was boiled and allows cooling to 55ºC and five micro litter (5µl) of ethidium bromide added. Combs were inserted into plastic tray and molten agarose gel was poured into the plastic casting tray and allowed to cool and solidify. Ten micro litter (10µl) of each DNA was properly mixed with 2 µl of loading dye (Bromocrysol purple) and load into the agarose gel wells, already submerges in TAE inside the electrophosresis tank. One kb DNA ruler was loaded into the first well. The Gel was run 60 V for 1.5 h and the DNA bands viewed under UV trans-illuminator Gel Documentation machine, (Sambrook, 1989; Desmond, 2008).

RESULT

Figure 1 shows the prevalence of E coli and E. coli O157:H7 among pregnant in selected hospitals within Kaduna metropolis. Out of the 400 urine samples examined, eighty five(85) samples were positive for *Escherichia coli* while two(2) out of this eighty five were confirmed to be *Escherichia coli* O157:H7.

Fig 2 shows Gel electrophoresis of multiplex PCR product *E coli* O157:H7 showing a small chromosomal subunit. M= molecular ladder, lane 1-2 are positive control, lane 3= negative control, lane 4 is the band location of the organism at 614bp(stx1) and 779bp(stx2) respectively.

Fig 3 shows Gel electrophoresis of uidA expression in *Ecoli* O157:H7. Lane M= 100bp DNA ladder, lane 1-2 amplicon products of O157:H7 isolates, lane negative= Negative control

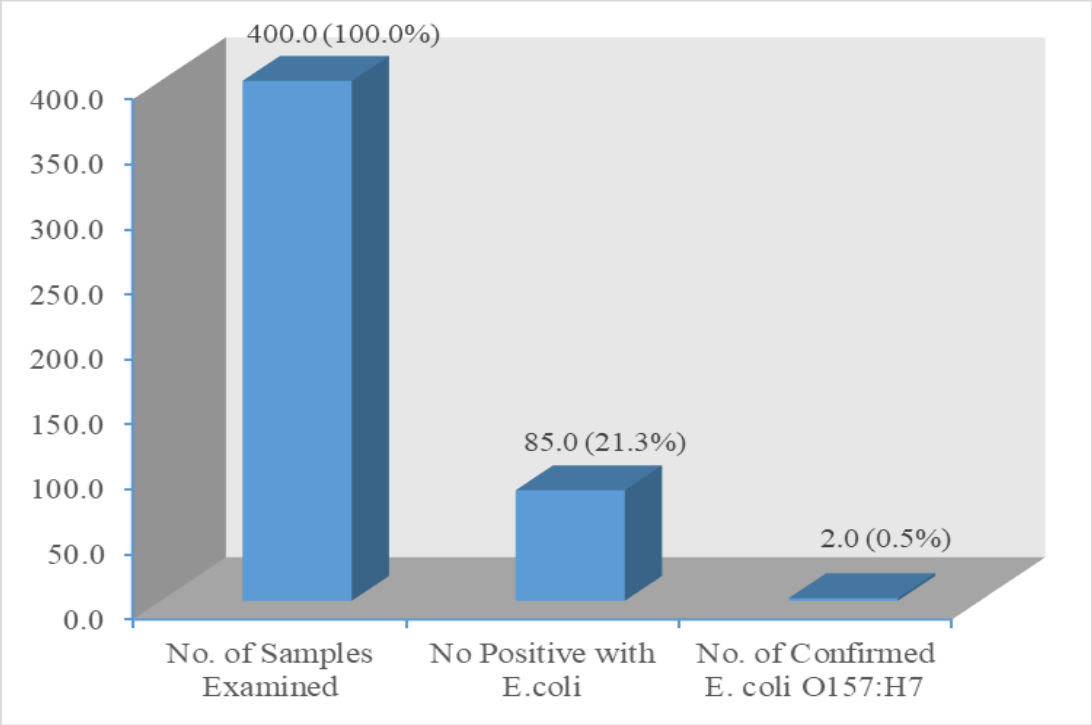

Figure 4.1: Prevalence of *Escherichia coli* and *Escherichia coli* O157:H7 among pregnant women with UTI in selected hospitals within Kaduna metropolis.

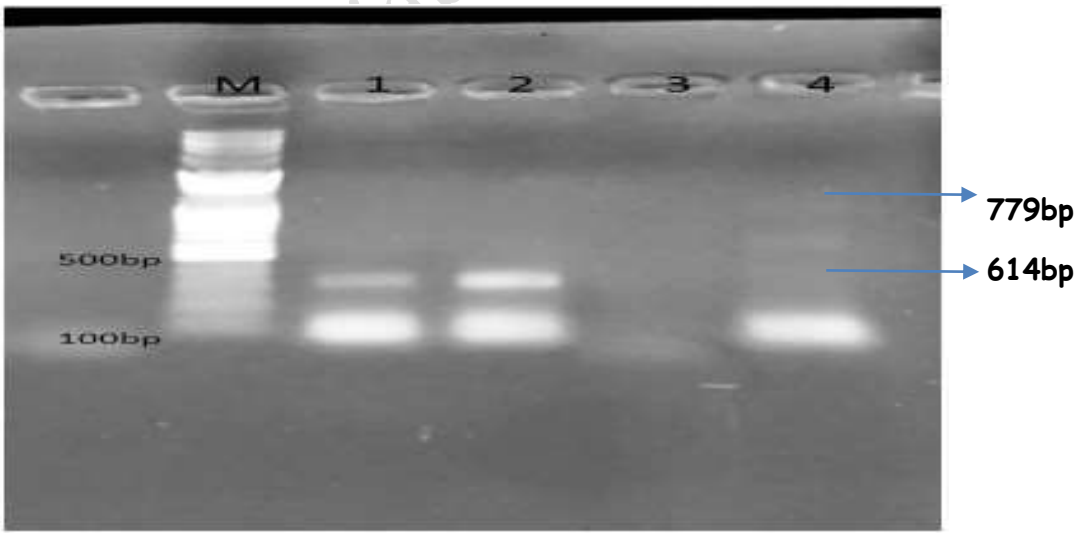

Plate I: Agarose Gel Electrophoresis of multiplex PCR products for detection of stx 1 (614 bp) and stx 2(779 bp)

Key: Lane M:100 bp; Lane 1 and 2= positive control; lane 3= negative control; lane 4 amplicon product of O157:H7 isolate

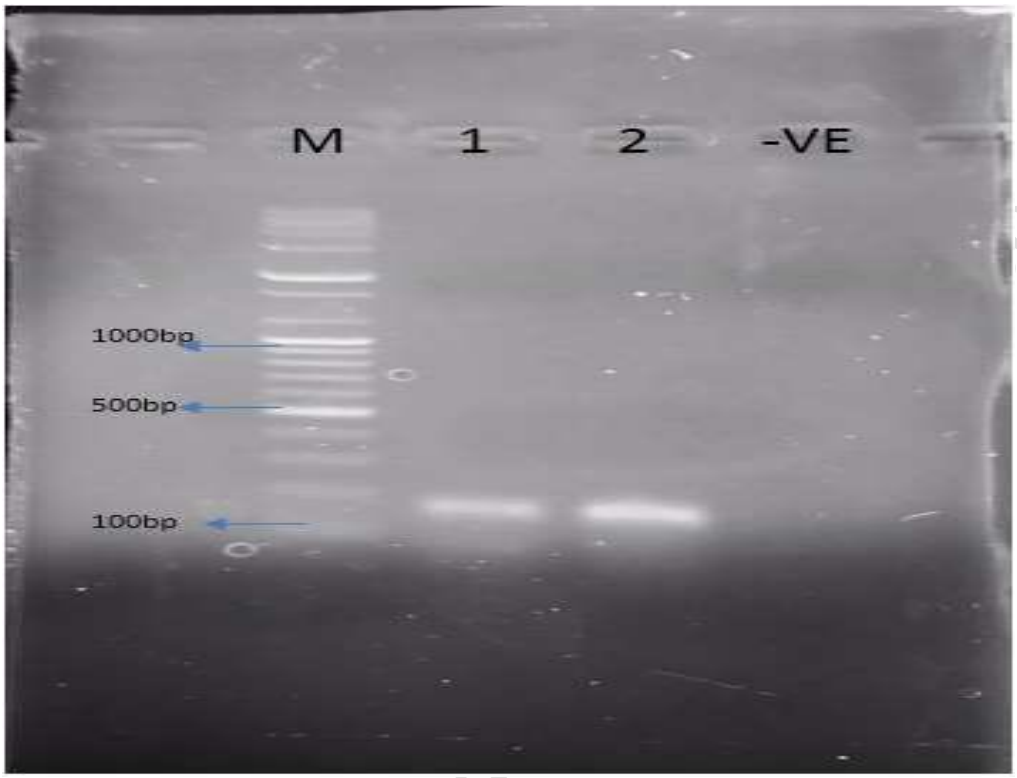

Plate II: Agarose Gel Electrophoresis of the extracted DNA.
Key: Lane M: 100 bp DNA ladder; lane 1-2 amplicon products of O157:H7 Isolates; Lane –ve = Negative control

DISCUSSION

Enterohemorrhagic Escherichia coli (EHEC) strain have occasionally been documented to be the causative agents of UTIs in human (Beutin *et al*., 1994; Gadea Mdel *et al*., 2012). *Escherichia coli* O157:H7 is the most studied strain among all other pathogenic strains of *E.coli* because it has been recognized as the leading cause of human food borne infection throughout the world with fatal complication such as haemolytic uremic syndrome that ends in renal failure.

The prevalence of *Escherichia coli* O157:H7 in this study was found to be 0.5% (2/400). The value obtained might be due to the sample size and also

the nature of the sample. This results agree with that of (Francisco *et al.*, 2014) where three EHEC isolates were obtained among hospital patients with symptomatic UTIs. The value was lower than 2% to 5% of cases obtained with *Escherichia coli* O157:H7 infection among children with Hemolytic uremic syndrome reported by Nazemi and Abbas in Iran (Hooman *et al.*, 2018).

Following DNA extraction using commercially available kits. Plate I shows the gel photo of the extracted DNA. This revealed the presence of expected total (genomics and plasmid) DNA bands. The Gel electrophoresis of E.coli O157:H7 showing small chromosomal submit. Lane M being a 100Bp molecular ladder, Lane 1 and 2 are the positive control Lane 3 is negative control, Lane 4 is the multiplex PCR products or amplicon for detection of stx1 and stx2 gene at 614bp and 779bp respectively.

Plate 2 also shows electrophoresis of uidA expression in *Ecoli* O157:H7. Lane M being a 100bp molecular ladder, Lane 1 and 2 are the amplicon product of the extracted DNA and –ve is the negative control.

CONCLUSION

Escherichia coli account for majority of urinary tract infections in young adults and pregnant women (Joshua *et al.*, *2006*). STEC are associated with a broad spectrum of human illness throughout the world, ranging from mild diarrhea to hemorrhagic colitis and hemolytic uremic syndrome e.t.c. Base on the result of this study, we can come to this conclusion that an E.coli bacterium is one of the most prevalent pathogenic factors found in urinary tract. In a few occasion shiga toxin *E.coli* infection comes with urinary tract infection.

RECOMMENDATION

1. The federal ministry of health should expand services for prevention of *Escherichia coli* infection and treatment to pregnant women.
2. More research should be carried out on shiga toxin *E.coli* infection in association with UTI as most research focuses on

shiga toxin *E.coli* infection in salad, vegetables, meat and diary products.

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