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Prevalence of *Escherichia coli* Pathotypes among Children with Diarrhoea in Zaria, Nigeria

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Authors' contributions

This work was carried out in collaboration between all authors. Author AS designed the study, wrote the protocol, managed the experimental processes and wrote the first draft of the study. Authors JAO, YKEI, and HWI supervised all the experimental processes and contributed to the first draft of the study. Authors JAO, JCI and AN managed the literature searches and contributed to the first draft of the study. All authors read and approved the final manuscript.

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ABSTRACT

Diarrhoea is one of the foremost public health problems worldwide especially among Children under five years in developing countries. Only few studies have investigated the epidemiology and virulence of *Escherichia coli* pathotypes in South-Eastern and South-Western Nigeria leaving the Northern part of the country unstudied. In this study, a total of 100 isolates of *E. coli* (45%) were obtained from the stool of 222 diarrhoea patients who were children below five (5) years attending Ahmadu Bello University Teaching Hospital, Shika, and Institute of Child Health, Banzazzau; an annex of Ahmadu Bello University Teaching Hospital, Shika-Zaria, Nigeria. The isolation and

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biochemical identification of the *E. coli* isolates were performed using standard microbiological procedures. A multiplex polymerase chain reaction (PCR) technique was used to differentiate the five (5) major diarrhoeagenic *Escherichia coli* pathotypes (EHEC, ETEC, EPEC, EIEC and EAEC) in one reaction condition, by using different diarrhoeagenic *E. coli* primers for different virulent genes found in *E. coli*. From the result obtained, only one (1) percent of the isolates was found to harbor the virulence gene out of the 100 *E. coli* isolated from the diarrhoea stools of children employed in this study.

Keywords: Enteroaggregative E. coli; Escherichia coli; diarrhoeagenic E. coli; multiplex polymerase chain reaction; prevalence.

1. INTRODUCTION

Escherichia coli (E. coli), documented as the most-studied bacterium, colonizes the gastrointestinal tract of most warm-blooded animals within hours or a few days after birth. The bacterium is ingested in foods or water or obtained directly from other individuals handling the infant [1]. *E. coli* is easily cultured in the clinical laboratory, but the identification of the different pathogenic genotypes requires virulence gene detection methods.

Based on pathogenesis, clinical manifestations and presence of specific virulence factors, there are five major diarrhoeagenic *E. coli* groups/pathotypes [2].

Enterohaemorrhagic *E. coli* (EHEC) strains are implicated in food-borne diseases principally due to ingestion of uncooked minced meat, raw milk or through ingestion foods contaminated with cattle faeces. These strains produce verotoxins 1 and 2 (Shiga-like-toxin 1 (stx1) and Shiga-like toxin 2 (stx2)) and variants thereof. They are involved in episodes of diarrhoea with complications. Serotype O157:H7 is the prototype of increasing importance and is associated with hemorrhagic colitis, bloody diarrhoea and hemolytic uremic syndrome (HUS) in about 10% of patients [3].

Enteropathogenic *E. coli* (EPEC) strains are a significant cause of infant diarrhoea in developing nations. EPEC were historically recognized on the basis of serotypes such as O55:H6 and O127:H6. EPEC (a non invasive and non toxin producing) is an established etiological agent of human infantile diarrhoea. Its pathogenicity (localized adhesion) destabilises intestinal epithelial cell function to produce distinctive “attaching and effacing” (A/E) lesions. EPEC cause a watery diarrhoea that may contain mucus but typically does not have blood in it. Vomiting, fever, malaise and dehydration are the symptoms, which may last for several days, resulting to chronic EPEC disease [1].

Enterotoxigenic *E. coli* (ETEC) produce toxins which are heat-labile (LT) and/or heat-stable (STa and STb) that also cause diarrhoea in humans and animals [4]. ETEC cause watery diarrhoea that can be mild in nature or in some instances can be a severe, cholera-like illness where rapid dehydration can be life-threatening. In endemic areas of ETEC-mediated diarrhoea, infants and children under the age of 5 are the most commonly affected. ETEC exposure in endemic areas is one of the most common causes of traveler's diarrhoea.

Enteroinvasive *E. coli* (EIEC) cause a broad spectrum of human’s diseases. They are biochemically, genetically and pathogenetically closely related to *Shigella* spp., both characteristically cause an invasive inflammatory colitis, but either may also elicit a watery diarrhoea syndrome indistinguishable from that caused by other *E. coli* pathogens. The pathogenesis of disease caused by EIEC and *Shigella* involves cellular invasion and spread, and requires specific chromosomal and plasmid borne virulence genes [5].

Enteroaggregative *E. coli* (EAEC) are pathogens associated with persistent diarrhoea in the developing world and have been implicated recently in the developed world as causes of both outbreaks and sporadic diarrhoea among AIDS patients. Enteroaggregative *E. coli* (EAEC) are a heterogeneous group of bacteria that display a wide array of virulence factors [6]. EAEC disease, as described by human volunteers, is a watery diarrhoea that occurs in some cases with abdominal cramps, but no fever and there is no invasion of the bloodstream [1].

2. METHODOLOGY

2.1 Study Population

The samples for this study were collected from Ahmadu Bello University Teaching Hospital, Zaria (including Institute of Child Health, Banzazzau), after ethical clearance (Ref. No.: ABUTH/HREC/TRG/36). The samples were collected for a period of six months (November, 2013 to April, 2014) from children (both male and female) with incidence of diarrhoea under the age of five (5) years, after obtaining informed consent from their parents or their guardians/attendants.

2.2 Inclusion Criteria

a. The study was limited to children of 0-5 years of age in Zaria within the study period
b. Only diarrhoea stool samples were analysed in the study.
c. Only samples from patients attending Ahmadu Bello University Teaching Hospital, Shika-Zaria were used.

2.3 Exclusion Criteria

a. Samples outside Ahmadu Bello University Teaching Hospital, Shika-Zaria were not used.
b. Non diarrheic stool samples were not analysed.
c. Diarrhoeic patients above five (5) years were not included.

2.4 Sample Collection and Treatment

The stool samples were cultured within 2-4 hours of collection. About one gram of stool sample was suspended in sterile nutrient broth and incubated at 37ºC for 24 hours. The concentrated isolates from the nutrient broth were then used to inoculate MacConkey agar plate and incubated at 37ºC for 24 hours. Colonies that showed typical characteristics of *E. coli* morphology were subcultured into eosin-methylene blue agar and incubated at 37ºC for 24 hours. The colonies that showed green metallic sheen, were further subjected to biochemical tests for confirmation.

2.5 Biochemical Tests

The biochemical tests that were used to identify the *E. coli* isolates are Indole test, Methyl red test, Voges-Proskauer test, Citrate utilization test and finally confirmed using Eijkmann test (fermentation of lactose and gas production at 44.5ºC for 24-48 hours).

2.6 Molecular Characterization of the *E. coli* Pathotypes

Molecular assay was carried out at Molecular Diagnostic Laboratory, Veterinary Teaching Hospital, Ahmadu Bello University, Zaria, Nigeria.

2.6.1 Genomic DNA extraction

All isolates were inoculated into 5 ml Luria-Bertani liquid medium and incubated for 24 hrs at 37ºC. DNA extraction was carried out using the ZR Fungal/Bacterial DNA MiniPrep™ ZRD6005 (Zymo research, CA, USA).

About 1.5 ml of the bacterial cell suspension was added into a microcentrifuge tube and centrifuged at 10,000 x g for 1 minute. It was then decanted and 400 µl of lysis buffer was added to the pellets. This was incubated at 70ºC for 30 mins and then centrifuged at 10,000 x g for 1 minute. The pellets were then transferred into a Zymo-Spin™ IIC Column in a new collection tube and centrifuged at 10,000 x g for 1 minute. Then 200 µl DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC Column in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute. This was followed by adding 500 µl Fungal/Bacterial DNA Wash Buffer to the Zymo-Spin™ IIC Column and centrifuge at 10,000 x *g* for 1 minute. The Zymo-Spin™ IIC Column was transfered to a clean 1.5 ml microcentrifuge tube and added 100 µl DNA Elution Buffer directly to the column matrix, and centrifuged at 10,000 x *g* for 30 seconds to elute the DNA. This was then used as template for the PCR.

2.6.2 Polymerase chain reaction

A multiplex polymerase chain reaction (PCR) technique was used to differentiate the diarrhoeagenic *Escherichia coli* pathotypes as described by Persson et al. [7]. PCRs were performed in a total reaction volume of 25 µL containing 1× PCR buffer (50 mM Tris-HCl, 10 mM KCl, 5 mM $(NH4)_2SO4$, pH 8.3), 2.6 mM $MgCl_2$, 260 lM each of dATP, dCTP and dGTP, 520 lM dUTP, 0.15 U of UNG (Applied Biosystems, Foster City, CA, USA), 1.25 U of Taq polymerase (FastStart; Roche Diagnostics), and the 18 primers including 16S rDNA gene used as internal PCR control (Inqaba biotec,

Pretoria, South Africa) (Table 1). Template volumes were 7 µL when PCRs were performed with extracted DNA from the *E. coli.*

Amplification conditions include 94ºC for 6 min (initial template denaturation), 35 cycles at 94ºC for 50s (final denaturation), primer annealing at 57ºC for 40s, primer extension at 72ºC for 50s, and finally at 72ºC for 3 minutes (final extension).

2.6.3 Agarose gel electrophoresis

Amplicons were analysed by electrophoresis on agarose 1.5% w/v gels prepared under standard conditions, followed by staining with 20 µl of ethidium bromide and allowed to solidify. Thereafter, 25 µl of the sample (Amplicons) was then loaded onto the gel wells and allowed to run for 30 minutes at 120 v. Amplicons were visualized under a Trans-illuminator UV light of wavelength 302 nm. This was then photographed with a Polaroid camera and documented using gel electrophoresis documentation system.

3. RESULTS

A total of 100 isolates of *Escherichia coli* were recovered from 222 stool samples of Children under five (5) years of age within the period of the study. From the result obtained, only one (1) percent of the isolates was found to harbor the virulence gene out of the 100 *E. coli* isolated from the diarrhoea stools of children employed in this study. Plate 2, lane 14 showed a band which is interpreted to be antiaggregation protein (*aap*), a characteristic of enteroaggregative *E. coli* (EAEC) at a base pair of 232.

3.1 Genomic DNA Extraction and PCR Analysis

The results of the genomic DNA extraction is as shown in plate 1 below

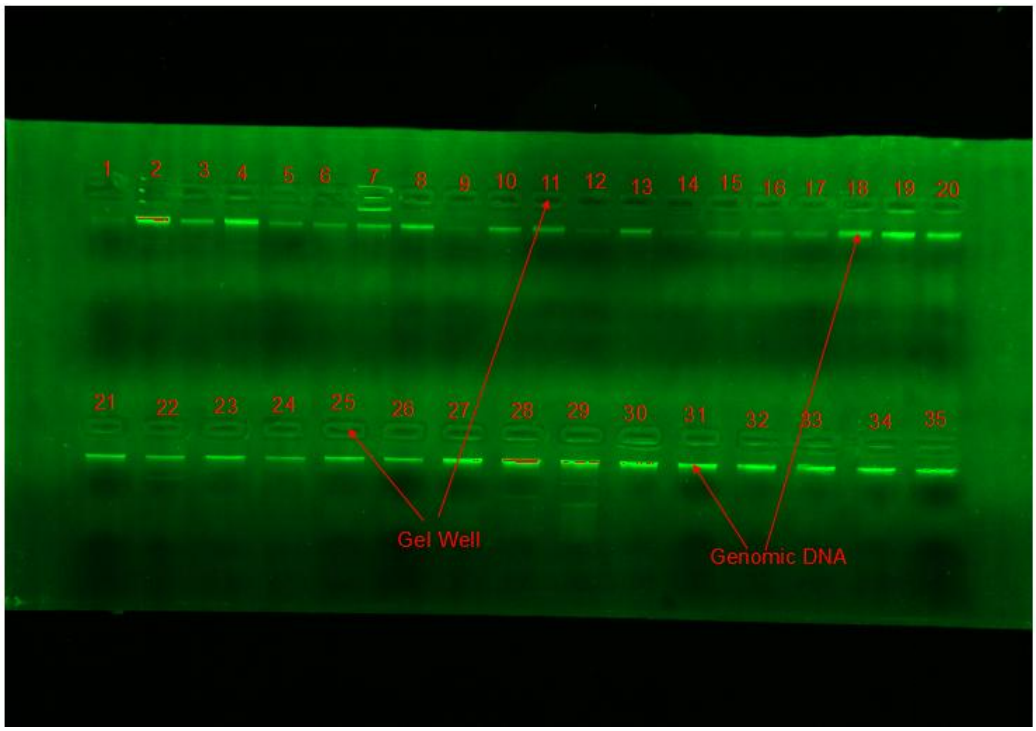

Plate 1. Genomic DNA on agarose gel (1.5%) electrophoresis

Key: Lane 1: Isolate 1; Lane 2: Isolate 2; Lane 3: Isolate 3; Lane 4: Isolate 5; Lane 5: Isolate 7; Lane 6: Isolate 8; Lane 7: Isolate 10; Lane 8: Isolate 12; Lane 9: Isolate 13; Lane 10: Isolate 17; Lane 11: Isolate 17**; Lane 12: Isolate 20; Lane 13: Isolate 22; Lane 14: Isolate 23; Lane 15: Isolate 26; Lane 16: Isolate 28*; Lane 17: Isolate 28**; Lane 18: Isolate 31; Lane 19: Isolate 33; Lane 20: Isolate 34; Lane 21: Isolate 35; Lane 22: Isolate 36; Lane 23: Isolate 37; Lane 24: Isolate 40; Lane 25: Isolate 41; Lane 26: Isolate 42; Lane 27: Isolate 44; Lane 28: Isolate 45; 29: Isolate 46; Lane 30: Isolate 50; Lane 31: Isolate 51; Lane 32: Isolate 52; Lane 33: Isolate 53; Lane 34: Isolate 58; Lane 35: Isolate 59*

The result of PCR analysis is as shown in plate 2 below

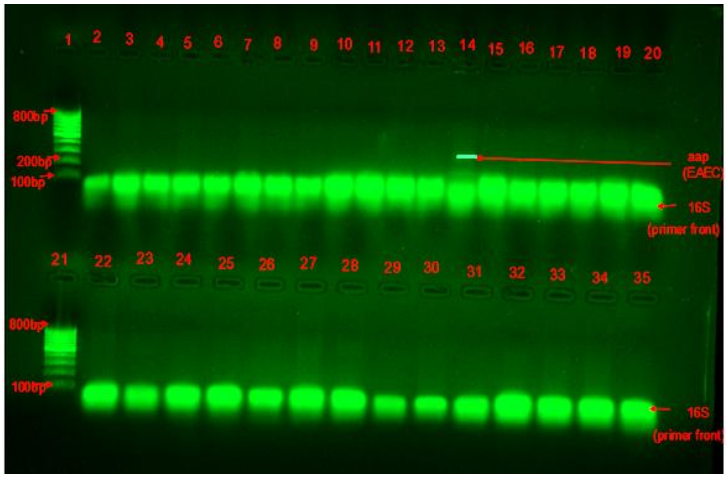

Plate 2. Multiplex PCR on agarose gel (1.5%) electrophoresis

Key: Lane 1: DNA ladder; Lane 2: Isolate 1; Lane 3: Isolate 2; Lane 4: Isolate 3; Lane 5: Isolate 5; Lane 6: Isolate 7; Lane 8: Isolate 10; Lane 9: Isolate 12; Lane 10: Isolate 13; Lane 11: Isolate 17; Lane 12: Isolate 17**; Lane 13: Isolate 20; Lane 14: Isolate 22; Lane 15: Isolate 23; Lane 16: Isolate 26; Lane 17: Isolate 28*; Lane 18: Isolate 28**; Lane 19: Isolate 31; Lane 20: Isolate 33; Lane 21: DNA ladder; Lane 22: Isolate 34; Lane 23: Isolate 35; Lane 24: Isolate 36; Lane 25: Isolate 37; Lane 26: Isolate 40; Lane 27: Isolate 41; Lane 28: Isolate 42; Lane 29: Isolate 44; Lane 30: Isolate 45; Lane 31: Isolate 46; Lane 32: Isolate 50; Lane 33: Isolate 51; Lane 34: Isolate 52 ; Lane 35: Isolate 53;*

4. DISCUSSION AND CONCLUSION

In this study, a total of 100 isolates of *E. coli* (45%) were obtained from the stool of 222 diarrhoea patients who were children below five (5) years attending Ahmadu Bello University Teaching Hospital, Shika, and Institute of Child Health, Banzazzau; an annex of Ahmadu Bello University Teaching Hospital, Shika-Zaria, Nigeria. The isolation and biochemical identification were performed using standard microbiological procedures. The low percentage of *E. coli* obtained (45%) from children with diarrhoea may be due to the fact that antimicrobial therapy previous to sample collection might have been given in some cases and it is a known fact that this can reduce the percentage of bacterial enteropathogens isolation [9].

Multiplex PCR was performed to characterize the *E. coli* isolates into their respective pathotypes/diarrhoeagenic groups as described by Persson et al. [7]. From the result obtained, only one (1) percent of the isolates was found to harbor the virulence gene out of the 100 *E. coli*, isolated from the diarrhoea stools of children employed in this study. Plate 2, lane 14 showed a band which is interpreted to be antiaggregation protein (*aap*), a characteristic of enteroaggregative *E. coli* (EAEC) at a base pair of 232. The very low percentage (1%) prevalence of diarrhoeagenic *E. coli* obtained in this study is in close agreement with the study conducted and reported by Chigor et al. [10], who reported a prevalence of *E. coli* 0157: H7 in children with diarrhoea as 5.4% in Zaria, Nigeria. Also, Ogunsanya et al. and Olorunshola et al. [11,12], reported a prevalence of 5% EHEC 0157:H7 in humans, in Lagos, Nigeria. But it is in contrast with the study conducted by Nweze, who reported 19.6% prevalence of diarrhoeagenic *E. coli* in a study conducted in Southeastern Nigeria [13], an incidence higher than 40% has been reported in Bangladesh [14]. A lower incidence of about 30% has been reported in Jordan [15]. The very low (1%) prevalence of diarrhoeagenic *E. coli* obtained in this study might be attributed to encouragement of breast feeding and educating the mothers on proper personal hygiene by the dieticians both in the main Teaching Hospital and the Institute of Child Health. The subjects employed in this study may be infected by other pathogens other than diarrhoeagenic *E. coli* since there are different pathogens that can cause diarrhoea in children, including Rotavirus, *Salmonella* spp., *Shigella* spp., *Campylobacter jejuni*, *Entamoeba histolytica,* and *Giardia lamblia* [16,17].

Table 1. Gene targets, primer sequences and amplicon sizes for the multiplex PCR

Primer	Gene target	Virulence factor/gene	Sequence (5'-)	Pathotype	Amplicon size
StFh	Human estA	STIh	TTTCGCTCAGGATGCTAAACCAG	ETEC	151 bp
StRh			CAGGATTACAACACAATTCACAGCAGTA		
StFp	Porcine estA	STIp	CTTTCCCCTCTTTTAGTCAGTCAACTG	ETEC	160 bp
StRp			CAGGATTACAACAAAGTTCACAGCAG		
PS1	eltA	LTI	AAACCGGCTTTGTCAGATATGATGA	ETEC	479 bp
PS2			TGTGCTCAGATTCTGGGTCTCCT		
PS3	vtx1	VT1	GTTTGCAGTTGATGTCAGAGGGA	VTEC	260 bp
PS4			CAACGAATGGCGATTTATCTGC		
PS5	Vtx2	VT2	GCCTGTCGCCAGTTATCTGACA	VTEC	420 bp
PS6			GGAATGCAAATCAGTCGTCACTC		
PS7	eae	Intimin	GGYCAGCGTTTTTTCCTTCCTG	EPEC	377 bp
PS8			TCGTCACCARAGGAATCGGAG		
PS9	ipaH	IpaH	TTGACCGCCTTTCCGATACC	EIEC	647 bp
PS10			ATCCGCATCACCGCTCAGAC		
PS11	aap	antiaggregation protein	CTTTTCTGGCATCTTGGGT	EAEC	232 bp
PS12		(dispersin)	GTAACAACCCCTTTGGAAGT		
PS13	16SrDNA	16S rDNA	GGAGGCAGCAGTGGGGAATA	CTRL	1062 bp
PS14			TGACGGGCGGTGTGTACAAG		

These Primer sequences identifies only five (5) E. coli Pathotypes.

KEY: R = A or G, Y = C or T, CTRL=CONTROL. Adopted from Persson et al. and Nataro et al. [7,8]

Antimicrobial therapy previous to sample collection might have been given in some cases where it was recommended, and it is known that this can reduce the percentage of bacterial enteropathogens isolation [9]. Finally, the direct effect of other pathologies, such as malaria, cannot be discarded. It has been previously described that between 5% and 38% of malaria cases present diarrhoea as one of the main symptoms [18].

In conclusion, molecular characterization of the *E. coli* isolates showed that, only 1% of the isolates carried a virulence gene (antiaggregation protein/dispersin), a characteristic of enteroaggregative *E. coli*. This report opens the door to further studies addressed to analyze the specific epidemiology, resistance patterns, and virulence of diarrhoea-causing pathogens in Zaria, and the whole country in general.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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