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Prevalence of Pathotypes of Diarrheagenic *Escherichia Coli* among Diarrhea Patients in Kwali, Federal Capital Territory, Nigeria

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Abstract

Background: Diarrheagenic *Escherichia coli* are important pathogens causing diarrhea in humans. Detection of these pathogens is difficult due to lack of rapid diagnostic tools in most health care facilities and this has led to poor diagnosis of the disease. The objective of this study was to detect diarrheagenic *Escherichia coli* and to determine the prevalence of the various pathotypes in patients with diarrhea in Kwali, Federal Capital Territory, Nigeria.

Methods: One hundred *Escherichia coli* isolates from stool samples of diarrhea patients attending the General Hospital Kwali were analyzed using Multiplex Polymerase Chain Reaction test with specific primers for the detection of five pathotypes of diarrheagenic *Escherichia coli.* Agarose Gel Electrophoresis and Auto Radiography were used to identify the various pathotypes and determine their prevalence.

Results: Diarrheagenic *Escherichia coli* were detected in 27(27.0%) isolates and the prevalence of the pathotypes was: Enteropathogenic *Escherichia coli;* 8(29.6%), Enterotoxigenic *Escherichia coli;* 3(11.1%), Enteroaggregative *Escherichia coli;* 13(48.2%) and Enteroinvasive *Escherichia coli;* 3(11.1%). The prevalence was highest in the age group less than five years; 12(44.4%).

Conclusion: The prevalence of diarrheagenic *Escherichia coli* was high in Kwali. There is need for epidemiologic and public health interventions such as water, sanitation and hygiene programs to drive down the high prevalence of diarrheagenic *Escherichia coli* disease in Kwali. Government should also intervene in providing and setting up rapid diagnostic tools in health care facilities nationwide.

Keywords: Diarheagenic *Escherichia coli*, multiplex polymerase chain reaction, pathotypes, prevalence, Kwali.

Introduction

Diarrheagenic *Escherichia coli* (DEC) are important pathogens causing a wide variety of gastrointestinal diseases like acute watery diarrhea, persistent diarrhea and dysentery.[1] Diarrheal diseases represent a public health burden for most developing countries.[2,3,4] In Nigeria, more than a third of the hospital beds for children are occupied by patients with diarrhea.[5] The high rates of child mortality attributable to diarrhea are in sub-saharan Africa and south east Asia.[6,7]

DEC strains are based on the specific virulence factors and phenotypic traits and are divided into the following pathotypes: Enteropathogenic *E. coli* (EPEC), vero

toxin-producing/shiga toxin-producing *E. coli* (VTEC/STEC) which include its well-known sub group enterohaemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC).[6,8,9,10,11,12] Detection of EPEC is based on the presence of *E. coli* adherence factor plasmid carrying *bfp* operon, encoding the type IV bundle-forming pilus (BFP) and *per* Operons. A transcriptional activator called plasmid encoded regulator (*per*) is the basis of typical and atypical classification of EPEC strains.[13] Diagnosis of ETEC is based on the production of heat-labile enterotoxins(*LT)* and or heat-stable enterotoxins(*ST)* which are virulence genes of the pathotype.[14]

The EHEC genome contains the same locus of enterocyte effacement as the EPEC and the intimate attachment of EHEC to host cells occurs through interaction between an adhesion called *intimin* (*eaeA*) and translocated intimin receptor (*Tir*). The detection is based on *eaeA* and entero hemolysin (*hly*) genes.[15,16] Diagnosis of EIEC is based on the invasion-associated locus (*ial*) of the invasion plasmid found in the strain.[17]The cryptic 1-Kb probe known as "*CVD 432* " or aggregative probe (AA) is used for the detection of EAEC which is a DNA fragment of the strain.[18] Polymerase Chain Reaction (PCR) offers a fast and reliable method for detection of pathotypes of diarrheagenic *E. coli* which, similar to immunoassay tests, can be used directly with stool samples as well as isolated colonies. Depending on the primers used, PCR test can distinguish between all virulence genes of the pathogen.[15]
Despite several public health intervention strategies by the government, studies show that resource poor communities continue to report cases of DEC infections in Nigeria with high prevalence.[6,9,19,20] This may be due to several non-specific factors that facilitate the continuous transmission of the disease such as behavioral, demographic and environmental factors.[20,21,22] The mode of transmission of DEC infection are by drinking contaminated water, eating uncooked food that is contaminated by the pathogens and through contact with an infected person.[6,8,22,23]Persons living in resource poor communities with poor living standards are at high risk of acquiring DEC infections.[21,22,24]

A previous study detected DEC from diarrhea stools of children with diarrhea from resource poor communities within the Federal Capital Territory Nigeria.[9] There is also evidence that diarrheal diseases can be caused by multiple etiologic microorganisms including *Salmonella* Spp., *Shigella* Spp.,*Campylobacter jejunum* and in some cases, vibrio cholerae 01.[18,25,26] This calls for more research in detection of DEC and determining the prevalence of the various pathotypes causing diarrhea in these communities, so as to provide timely epidemiologic and public health information for effective prevention and control strategies.

There is currently no vaccine against DEC infection. The success of any epidemiologic and public health measure to control the disease depends on the timely detection of the pathogen and the determination of the distribution of the various pathotypes within a high risk community.[13,14] This study identified the various pathotypes of DEC and the prevalence among patients with diarrhea in Kwali being a high risk community. The study has also provided baseline data for reference in the Federal Capital Territory Nigeria for effective epidemiologic and public health intervention on DEC disease

such as Water and Sanitation Hygiene (WASH) programs to drive down the high prevalence of DEC disease in Kwali.

Methodology

Study area and the healthcare facility

Kwali Area Council is one of the six councils in the Federal Capital Territory Nigeria. There are several resource poor communities spread across Kwali. These communities lack access to improved water and sanitation conditions and there is wide spread practice of open defecation amongst residents.[27] The health care facility used in this study was General Hospital Kwali which is the only government owned hospital rendering secondary health care services in the entire area council.

Study population and case definition

The study population was patients with diarrhea who attended the outpatient clinic of General Hospital Kwali and were resident in Kwali. Samples used in this study were stools (watery, loosed with mucus, with or without blood) from diarrhea patients who had more than three episodes of stooling in the preceding 24 hours. The respondents were included from all diarrheal cases who met the criteria for the case definition.

Sample size determination

The total sample size for the study was one hundred samples of diarrheal stools, collected from one hundred diarrhea patients (cases), calculated assuming a power of 80% at an alpha level of 0.05 using Open Epi Version 3.

Sampling technique

Medical laboratory officers of the hospital and the principal investigator identified cases from stool samples using the case definition. Only diarrheal cases who had more than three episodes of stooling in the last 24 hours were included in the study.

Data collection and analysis

An adapted structured questionnaire from a previous study was used to collect data which captured the demographic, behavioral and environmental characteristics of the respondents.[21] Samples were collected during the study period from January to August, 2018.

All data was analyzed using STATA 15 statistical software (Stata Corp, 4905 Lake way drive station, Texas 77845 USA).[20] Descriptive statistics were generated from the quantitative data and Chi square used to test for association between the qualitative variables. A p-value < 0.05 was considered statistically significant.

Storage and transportation of isolates

Escherichia coli isolates were inoculated onto nutrient agar slants in cryo vials and placed in a container with ice and transported to the molecular laboratory for DNA extraction, multiplex PCR test, agarose gel electrophoresis and auto radiography.

DNA extraction

Three colonies of *E. coli* isolates from a fresh overnight culture on MacConkey agar was suspended in 100µl lysis buffer (InstaGene Matrix, Biorad) in a 1.5ml eppendorf tube, vortexed for 15 minutes and incubated for 1 hour at 56°C. It was then mixed by vortexing and incubated at 95°C for 1 hour, mixed again by vortexing then centrifuged at 13,200 rpm for 5 minutes. DNA suspension was vortexed and centrifuged at 13,200 rpm for 5 minutes and was stored at –20°C.[8]

Multiplex PCR procedures

The multiplex PCR assays were standardized for the detection of the following virulence markers: *eaeA* for the structural gene of *intimin* of EPEC, *bfpA*

for the structural gene of the bundle forming pilus of EPEC, *hlyA* for the plasmid encoded enterohemolysin of EHEC, *eltB* and *Stla* for the enterotoxins of ETEC, *ial* for the invasion associated locus of the invasion plasmid found in EIEC, and *CVD432* for the nucleotide sequence of EAEC fragment. The reaction mixture containing optimized protocol was carried out with a 50µl mixture containing 10mM of Tris – Hcl (pH8.3): 50mM of KCl, 20mM of $MgCl_2$ and 2mM concentration of each deoxynucleoside triphosphate (Inqaba Biotec. Ltd,Pretoria SA), 0.1 µl of 5U of Ampli Taq Gold DNA Polymerase (Inqaba Biotec. Ltd,Pretoria SA), 5 µl of the DNA template and 0.4 µl of each primer (Inqaba Biotec. Ltd,Pretoria SA) in a total volume of 50 µl.[12] The sequence of the primers, target genes and amplicon sizes of target genes are summarized in Table 1. The amplification was carried out in Eppendorf's master cycler pro (Eppendorf Ltd,Chennai India) with an initial denaturation temperature of 95^{O}C for 1 minute followed by 35 cycles of denaturation temperature at 94^{O}C for 1 minute, annealing temperature of 55^{O}C for 1 minute, and extension at 72^{O}C for 2 minutes and a final extension at 72^{O}C for 5 minutes.[12]

PCR controls: *E. coli* ATCC 35401, ATCC 43890, ATCC 43899, ATCC 43887, 97r were used as positive controls while *E. coli* ATCC 11775 was used as negative control.

Agarose gel electrophoresis of PCR product

Molecular biology grade agarose (1%w/v) was dissolved by heating in a microwave in Trisburate buffer (Gibco life tech, paisley UK).[20] Ethidium bromide stains (inqaba Biotec. Ltd,Pretoria SA) was added to the melted and cooled agarose solution. Each 100ml of 1% agarose was poured into the gel tray to set and a 10 µl of PCR reaction mixture was loaded onto each well.[12] 5 µl of the molecular marker consisting of 100-1000 bP ladder from Inqaba Biotec. Ltd, South Africa was used. Electrophoresis was performed at 80V for 75 minutes. The electrophoresed gels were visualized under UV light and images photographed in the transilluminator, bands observed and results interpreted accordingly (Fig. 1 and Fig. 2).

Ethical consideration

Ethical clearance for the study was obtained from the Health Research Ethics Committee of the Health and Human Services Secretariat of Federal Capital Territory Administration (FHREC/20/170/106). Informed consent was obtained from each patient after thoroughly explaining the study to them. In the case of children, assent was sought and obtained from the parents or care givers. All information received was treated as confidential and all study participants were given the option of declining participation in the study or opting out of it at any time in the course of the study without suffering any penalty.

Table 1 Primer sequence and amplicon size of target genes

Primer name	Target gene	Primer sequence (5'-3')	Amplicon size (bp)
ELTBF	*Elt*	TCTCTATGTGCATACGGAGC	322
ELTBR		CCATACTGATTGCCGCAAT	
ESTAF	*Stla*	GCTAAACCAGTAGAGGTCTTCAA	170
ESTAR		CCCGGTACAGAGCAGGATTACAA	
VTIF	*hlyA*	GCATCATCAAGCGTACGTTCC	534
VTIR		AATGAGCCAAGCTGGTTAAGCT	
EAEAF	*eaeA*	CACACGAATAAACTGACTAAAAG	229
EAEAR		AAAAACGCTGACCCGCACCTAAT	
LALF	*Ial*	CTGGTAGGTATGGTGAGG	320
LALR		CCAGGCCAACAATTATTTCC	
BEPAF	*bfpA*	TTCTTGGTGCTTGCGTGTCTTTT	450
BEPAR		TTTTGTTTGTTGTATCTTTGTAA	
PCVDF	*CVD432*	CTGGCGAAAGACTGTATCAT	630
PCVDR		CAATGTATAGAAATCCGCTGTT	

Results

Frequency of DEC pathotypes in Kwali

Diarrheagenic *E. coli* was detected in 27(27.0%) out of the one hundred samples analyzed. The highest frequency of detection was EAEC 13(48.2%), EPEC was the second highest with 8(29.6%) while EIEC and ETEC were both 3(11.1%). However, EHEC was not detected in this study (Table 2).

Frequency of DEC pathotypes according to gender

Diarrheagenic *E. coli* was more frequent in females; 56(59.3%) than in males; 44(40.7%). All pathotypes of DEC detected in this study were higher in females: EAEC; 7(53.8%), EPEC; 5(62.5%), EIEC; 2(66.7%) and ETEC; 2(66.7%) compared to males: EAEC; 6(46.6%), EPEC; 3(37.5%), EIEC; 1(33.3%) and ETEC 1(33.3%). There was a statistically significant association between the occurrence of DEC pathotypes and gender (Table 2).

Frequency of DEC pathotypes according to age

Diarrheagenic *E. coli* was detected in all age groups, with EAEC being the most frequent in children below 5 years; 8(61.5%). EIEC and ETEC were not detected in age groups greater than 15 years but were detected equally amongst three age groups {(< 5years – 33.3%), (5-9years – 33.3%) and (10–15years – 33.3%)}. There was a statistically significant association between occurrence of DEC pathotypes and age (Table 3).

Table 2 Association between DEC pathotypes and respondents' gender

Pathotypes	Female n (%)	Male n (%)	Total	*P* - value
EAEC	7(53.8)	6(46.6)	13(48.2)	0.048*
EIEC	2(66.7)	1(33.3)	3(11.1)	
EPEC	5(62.5)	3(37.5)	8(29.6)	
ETEC	2(66.7)	1(33.3)	3(11.1)	
Total	16(59.3)	11(40.7)	27(100.0)	

*P-value < 0.05 means statistically significant.

Table 3 Association between DEC pathotypes and respondents' age

Age group	EAEC n(%)	EIEC n(%)	EPEC n(%)	ETEC n(%)	Total (%)	*P*-value
< 5 yrs	8(61.5)	1(33.3)	2(25.0)	1(33.3)	12(44.4)	0.021*
5-9 yrs	3(23.1)	1(33.3)	2(25.0)	1(33.3)	7(25.9)	
10-15 yrs	1(7.7)	1(33.3)	3(37.5)	1(33.3)	6(22.2)	
>15 yrs	1(7.7)	0(0.0)	1(12.5)	0(0.0)	2(7.4)	

* P-value < 0.05 means statistically significant.

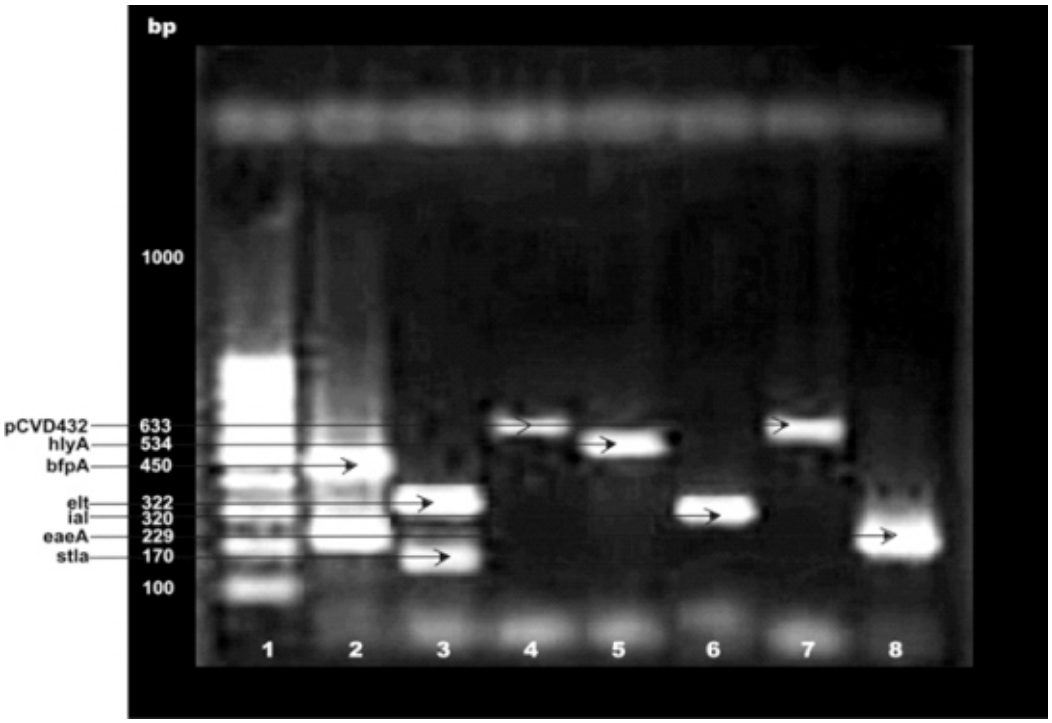

Plate 1: Agarose gel electrophoresis of 1% agarose of PCR product of virulence genes of diarrheagenic *E.coli*

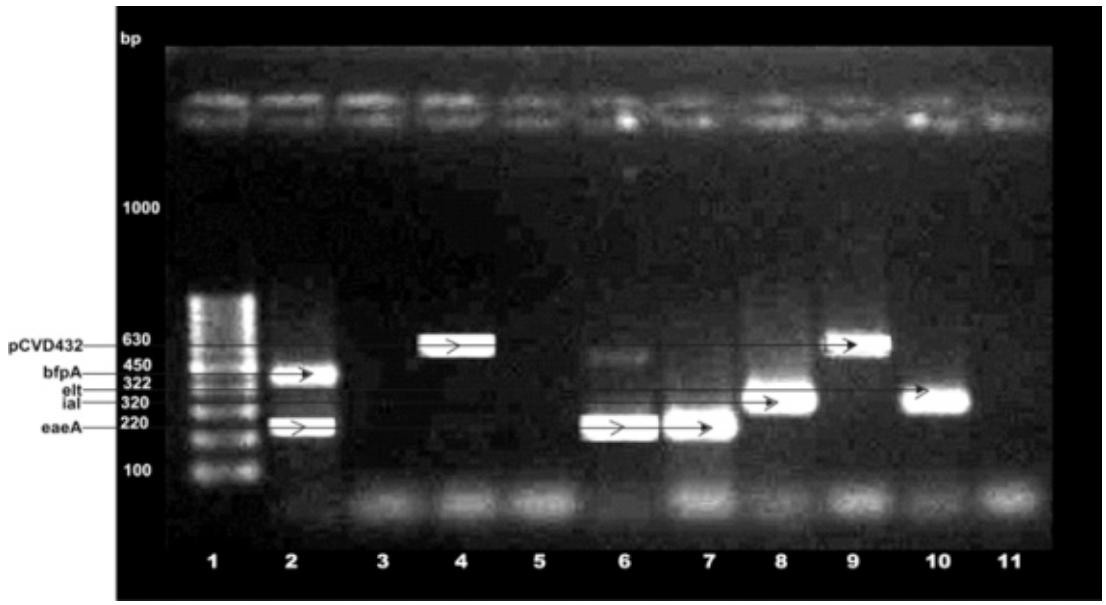

Plate 2: Agarose gel electrophoresis of 1% agarose of PCR controls of virulence genes of diarrheagenic *E.coli*

Discussion

Four pathotypes of diarrheagenic *E. coli* were detected in this study. However, EHEC was not detected which tends to align with previous studies.[19,24,28,29] This may be due to the pathogen's inability to survive stress conditions, such as the low pH levels found in the gastrointestinal tract contributing to its very high infectious dose .[30] This study showed that the prevalence of EAEC was the highest in Kwali compared to other pathotypes of DEC and this is in agreement with findings from previous studies which reported high prevalence of EAEC.[10,11,12,31,32] This is largely due to the reason that EAEC is endemic in sub-Saharan African.[32] Also, it was observed that the prevalence of EPEC was higher than that of EIEC and ETEC . This is in conformity with what previous studies documented in Nigeria and India.[11,12,19,20] These studies showed a high occurrence of EPEC particularly in children and this concurred with the findings in this study. This is also largely due to the reasons that EPEC is endemic in sub Saharan Africa and in south east Asia.[11,12,19,20] The inadequacies in implementation of Water and Sanitation Hygiene(WASH) programs and the several resource poor communities in these parts of the world may have made the pathogen endemic. [32]

Detection of EIEC was low in this study compared to EAEC and EPEC, and this is in agreement with previous studies that showed low prevalence of EIEC when compared to other pathotypes.[8,9,19,20] Acquisition of the invasive plasmid (PINV) encoding the ability to invade host tissues is probably the single most important event that has given rise to the evolution of both *Shigella* and EIEC from non-pathogenic *E. coli* .[33] In this study, ETEC was not detected in age groups greater than 15 years and it is in contrast with findings from a previous research which reported prevalence of ETEC in age groups greater than 15 years, although, their study showed that samples were collected only from adults with diarrhea in southwestern Nigeria.[31] The prevalence of EAEC ,EPEC , EIEC , and ETEC were

higher in females compared to males. Previous research has documented that the female gender is a risk factor associated with DEC infection.[22] This may be due to domestic exposure to contaminated water and food.

Children below 5 years were the most infected by DEC compared to other age groups in this study and there was a statistically significant association between frequency of detection of DEC pathotypes and age. This reflects the combined effects of declining levels of maternally acquired antibodies, the lack of active immunity in infants, the ingestion of food and water contaminated with the pathogens and the direct contact with human faeces. All diarrheal cases included in this study had *E. coli* in their stool samples and this is in agreement with previous studies conducted elsewhere.[8,9,10,12,19,20,34,35] This confirms the fact that *E. coli* is a common bacteria isolated from human stool.[28]

This study has documented the prevalence and distribution of various pathotypes of diarrheagenic *E. coli* in Kwali, as baseline data for reference in the Federal Capital Territory, Nigeria. A limitation of the study, however, is that DAEC was not included among the pathotypes of DEC analyzed and this was due to the unavailability of DAEC primers in the course of preparation for this research. This was mitigated by the use of verotoxin-producing *E. coli* primers of which DAEC is a well-known subgroup.

Conclusion

The prevalence of DEC in Kwali, Federal Capital Territory Nigeria is high and this is largely due to the low living standards in Kwali. The EAEC and EPEC were the most prevalent pathotypes of DEC while EHEC pathotype was not detected in this study. Further studies on detection and prevalence of DEC pathotypes from diarrhea stools should be carried out in other area councils of the Federal Capital Territory to compare with findings from Kwali. There is need for epidemiologic and public health intervention such as Water and Sanitation Hygiene (WASH) programs to drive down the high prevalence of the disease in Kwali.There is also need for government intervention in providing and setting up rapid diagnostic tools to diagnose DEC infection in health care facilities nationwide.

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