Molecular Characterization of Bacteria Associated with Diarrhoea in Children (0-5) from some Selected Hospitals in Makurdi, Benue State

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Abstract- Molecular characterization of bacteria associated with diarrhoea in children (0-5) years attending selected hospitals in Makurdi was carried out. A total of 377 stool samples were collected from children attending Federal Medical Centre, General Hospital North Bank and Bishop Murray Memorial Hospital, High level Makurdi. Standard methods involving microbiological, biochemical and molecular analysis using PCR were employed in characterizing bacteria associated with diarrhoea in children (0-5) years. Out of the 377 stool samples examined for diarrhoea associated with bacteria, 51 (13.7%) were positive for *Escherichia coli, Shigella* and *Salmonella* species. Out of these 51, 40 (78.4) were *E. coli,* 8 (15.7%) *Shigella* and 3 (5.9%) were *Salmonella* respectively. Out of 51 positive for bacteria associated with diarrhoea in children, 17 (11.4%) were from Male and 34 (13.7%) from Female. Infection rate was highest 23 (29.5%) among children between the ages of 13 – 24 months and the least 2 (2.6%) between 40 – 60 months. Amplification result using PCR confirmed *Enterohaemorragic E. coli, Shigella dysentriae* and *Salmonella typhi.* Exclusive breast feeding increases immunity in children, therefore breastfeeding should be recommended for children of breast feeding age, so that incidence of diarrhoea infection can be brought to the bearest minimum.

Index Terms- Diarrhoea, Children, Bacteria, Molecular characterization

I. INTRODUCTION

Diarrhoea is a disease condition of having at least three loose or liquid bowel movements each day (James *et al.,* 2003). It often lasts for a few days leading to dehydration as a result of fluid and electrolyte loss (Sinclair *et al.,* 2013). The Signs of dehydration often begin with loss of normal skin stretches irritable behavior and progress as a decrease in urination, loss of skin color, a fast heart rate, and decrease in responsiveness as it increases in severity (Addy *et al.*, 2004). *Enterotoxigenic E. coli, Salmonella paratyphi, Shigella species* and virus appeared to be the most common etiological agents but Certain circumstances are associated with and especially high incidence of acute diarrhoea disease (WHO, 2017). However, the causes of approximately 40 % of the cases are still unknown (UNICEF, 2010). Diarrhoea is a major cause of childhood disease in the developing world, global mortality estimates from diarrhoea and its complications range from 1.5 to 5.1 million deaths per year for children under the age of five (Nataro and Kaper, 1998). The most common cause of diarrhoea is through infection of the intestines with a virus, bacteria or a parasite- a condition known as gastroenteritis. The infections are often acquired through consumption of food or water that has been contaminated with faeces, or through direct contact with an infected person (WHO, 2017). There are three types of diarrhoea which include; short duration watery diarrhoea, short duration bloody diarrhoea and persistent diarrhoea (which lasts for more than two weeks) (Agho *et al.*, 2011). The short duration watery diarrhoea may be as a result of infection by cholera. It is rare in developed world. If blood is present it is also referred to as dysentery (Agho *et al.*, 2011). Although at least 25 different bacteria and protozoa can cause an identical clinical symptoms of gastroenteritis whereas over 75% of gastroenteritis cases are caused by viruses. Viral gastroenteritis is one of the most common infectious diseases worldwide, causing significant morbidity and mortality in children. Four major viral pathogens are associated with gastroenteritis; three of them are RNA viruses which includes Rotavirus, Norovirus, and Astrovirus and one DNA virus which include enteric Adenovirus. (Akinyemi *et al.,* 2011).

II. MATERIALS AND METHODS

Determination of Sample Size

Samples size was determined by the Raosoft online calculator using accepted standard error of 5% at 95% confidence interval (Raosoft Sample Size Calculator, 2014). For this study, a minimum of 301 children were required; however, sample size of 377 children was used for the study.

Sample Collection

A total number of 377 faecal specimens were collected from patients attending three different hospitals in Makurdi, namely Federal Medical Centre, Bishop Murray hospital and General Hospital North Bank. About 1 gram of the fecal sample was transferred into sterile 9ml Cary Blair medium under aseptic technique. The faecal samples were transported to the University of Agriculture Microbiology laboratory of Federal University of

Agriculture for further bacteriological analysis (Cheesbrough, 2006).

Microbiological Analysis

All the media used was prepared according to manufacturer's standard

Prepared media was first warmed to room temperature ($30\pm^0$C) and the agar surface to dry before inoculating.

The specimen was inoculated and streaked on SSA, EMB as soon as possible after collection, except on TCBS. All specimens on swab to be cultured were rolled over a small area of the agar surface and the plates incubated aerobically at 37ºC for 24 hours.

One gram (1g) of the faecal sample was inoculated into 10ml of alkaline peptone water and incubated at 37ºC for 24 hours, thereafter; it was sub cultured onto TCBS agar and further incubated for 24 hours (Tankeshwar, 2016).

Bacterial Isolation

The inoculated plates were incubated at 37 ° C for 48 hours. The plates were observed for the peculiar growth characteristics of *Salmonella*, *Shigella, E.coli* and *Vibro cholerae* and result recorded. Distinct colony with the growth characteristics of interest were sub-cultured into nutrient agar for biochemical and molecular characterization (Cheesbrough, 2006).

Biochemical Characterization of the Isolates

The isolated bacteria were subjected to the following biochemical analysis like citrate, oxidase, catalase, indole, methyl red test, Voges Proskauer test, motility test, hydrogen sulphide, starch hydrolysis and sugar fermentation test using methods of Cheesbrough, (2006).

Molecular Analysis
Bacterial DNA Extraction for PCR Assay

Genomic DNA was isolated as described by Arciola *et al.* (2001). Nutrient broth was used, the bacterial cultures were inoculated into the broth in the tubes and allowed for growth at 37 ºC overnight in orbital shaker. The tubes were then vortexed and 100 µl of the cultures were taken into Eppendorf tubes. They were centrifuged at 10,000 rpm for 5 minutes and the supernatant was removed, the pellet was washed using distilled water and centrifuged and the supernatant discarded again. The pellets were

re suspended in the lysis buffer 50mM Tris HCL P^H (8.5), 1mM EDTA, 0.5% SDS (sodium dodecyl surphate), 20 cg/ml proteinase K. The tubes were centrifuged at 1000rpm for 10 minutes, then 100 cl of the supernatant were transferred to a new 1.5ml Eppendorf tube, 3.0 µl of the supernatants was used in PCR reaction. Genomic DNAs were ready and stored at -20 º C.

DNA Amplification of target genes

Procedure for running the PCR for the detection of target genes involved the use of two sets of primers (Forward and Reverse oligonucleotides) which were mixed in the folowjng way: Two micro liters (2 µL) of water was added to the negative control tube, then 2 µL of sample (template DNA) was added to the sample tube and all the lids were closed. The procedure was completed by adding the positive control DNA and the lid also closed. The tubes were placed into the PCR automated themocycler (biometra) and programmed as presented in Table 1.

Preparation of the Agarose Gel

Agarose solution (1.5%) was prepared by dissolving 1.5g agarose powder in 100ml of 0.5X TBE buffer, boiled in a water bath, and cooled to 40-50 ºC. The agarose solution was poured into the set-up gel tray. The gel was left to solidify for 15-30 minutes. A staining bath containing a final concentration of 5 µg/mL ethidium bromide was prepared according to the method of PEN (2006). This stain served as an intercalating agent binding to double strand DNA and fluoresced when illuminated with UV light. Intact DNA usually appeared as a distinct band.

Electrophoresis procedure

The gel (comb) was put into the electrophoresis unit and refilled with buffer. The 5 µL of each PCR sample was mixed with approximately 2 µL loading dye and was loaded into the wells of the gel along with a molecular marker. The lid of the unit was replaced and the gel was run by starting the electrophoresis process. The lid of the unit was removed after a complete run of 30 minutes at 100 volts and the gel was placed in a staining-bath for about 30 minutes, removed, rinsed and the gel visualized for bands using the UV-transiluminator. They were observed for the presence of the specific bands of test samples corresponding to that of the control.

Table 1: Molecular characterization (PCR) Organization of Gene Primer-sequence

Organization	Gene	Primer	Primer-sequence	Bp fragment	Amplified temperature	Reference
E. coli	UidA	EC F	5"AAAACGGCACAAAAAGCAC-3"	147bp	65 ºC	Bej *et al.*, 1991
		EC R	ACGCGTGCTTACAGTCTTGCG			
E. coli	eae	EC F	TCAATGCAGTTCCGTTATCAGTT	482bp	58 ºC	Vidal *et al.*, 2005
		EC R	GTAAAGTCCGTTACCCCAACCTG	127bp repetitive		
Shigella	ERIC	ERIC F	ATGTAAGCTCCTGGGGATTCC		40 ºC	Versaloric *et al.*, 1991
		ERIC R	AAGTAAGTGACTGGGGTGAGCG			

Salmonella	InvA	InvA F	TCGTGACTCGCGTAAATGGCGATA	423bp	63 ℃	Freitas *et al.,* 2010
		InvA R	GCAGGCGCACGCCATAATCAATAA			
	ViaB	ViaB F	CACGCACCATCATTTCACCG	738bp	54 ℃	
		ViaB R	AACAGGCTGTAGCGATTTACG			

The molecular ladder used was 100bp to 1000bp.

Statistical Analysis

All data that were obtained from this study were entered into Microsoft Excel and analyzed using statistical package for social sciences (SPSS) version 19.0 statistical software.

Both descriptive and analytical statistics were employed. Comparison based on age, bacterial strains were performed using chi square test were used to analyze data at P<0.05 significant level (Guerra *et al.,* 2014).

III. RESULTS AND DISCUSSION

Table 2: Sex Distribution of Diarrhoea Children in Selected Hospitals in Makurdi

SEX	Number of stool sample Examined	Number positive (percentage)
Male	149	17 (11.4)
Female	248	34 (13.7)
Total	**377**	**51 (13.5)**

x^2= 70.153, DF=1. P<0.05

Table 3: Age Distribution of Diarrhoea Children in Selected Hospitals in Makurdi

Age group (Months)	Number of stool sample Examined	Number positive (percentage)
≤12	74	2 (2.7)
13-24	78	23 (29.5)
25-36	84	19 (22.6)
37-48	64	4 (6.3)
49-60	77	2 (2.6)
Total	**377**	**51 (13.5)**

x^2= 17.078, DF=4. P<0.05

Table 4: Molecular characterization of Diarrhoea causing Bacteria in Stool of Children Age 0-5 years in selected Hospital in Makurdi.

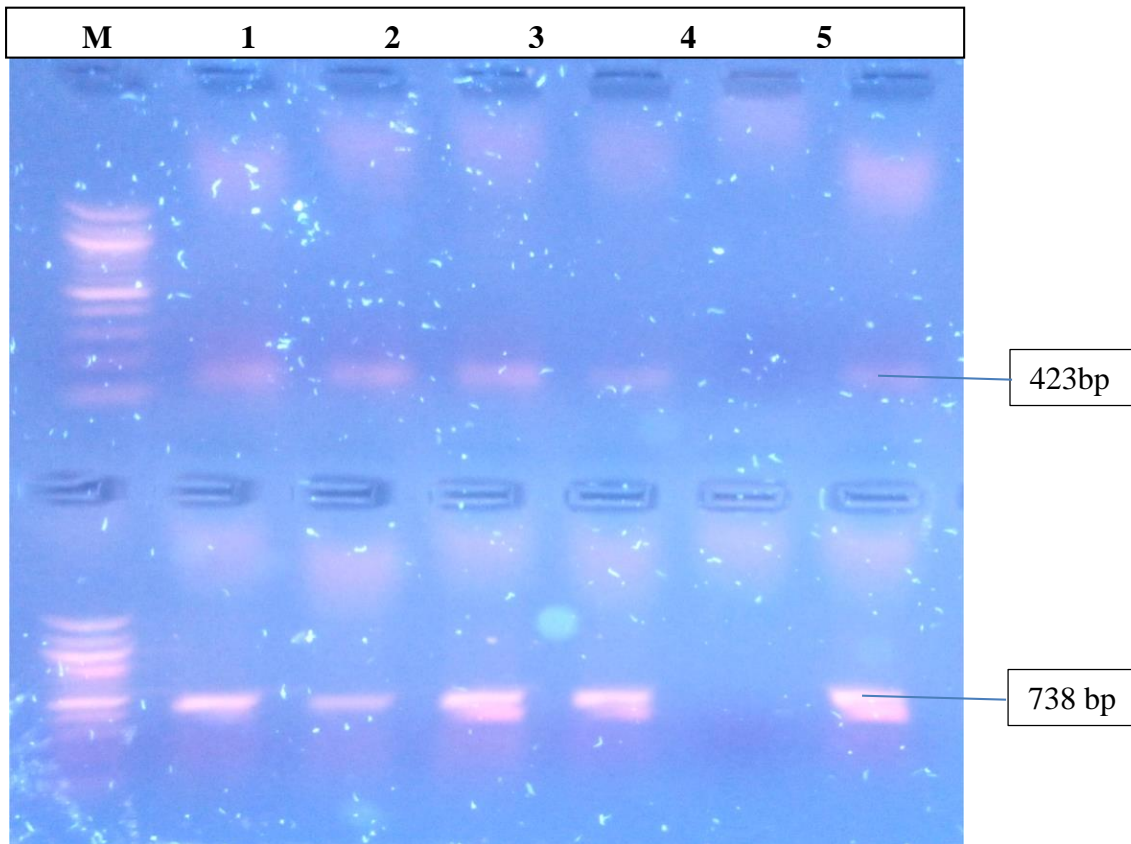| Species	Number of positive (%)		Number confirmed molecularly		Number not confirmed	Total (%)	
Enterohaemorrhagic E. coli	40	(10. 6)	40	(10. 6 %)	0	40	(10. 6 %)
Shigella dysentriae	8	(2.1)	8	(2.1 %)	0	8	(2.1 %)
Salmonella typhi	3	(0.8)	3	(0.8 %)	0	3	(0.8 %)
Total	**51**	**(13.5)**	**51**	**(13.5)**	**0**	**51**	**(13.5)**

Plate 1: Amplification of *Salmonella typhi*
Upper gel: InvA gene amplification yields 423 bp fragments to confirm *Salmonella* species
Lowe gel: ViaB gene amplification yields 738 bp fragment (lanes 8, 9, and 10) to confirm *Salmonella typhi* and distinguish typhoidal from non-typhoidal *Salmonella* species (lanes 2,-7, 11 and 13). Land 1 = 100 bp DNA size marker. Lane 12 = negative control.
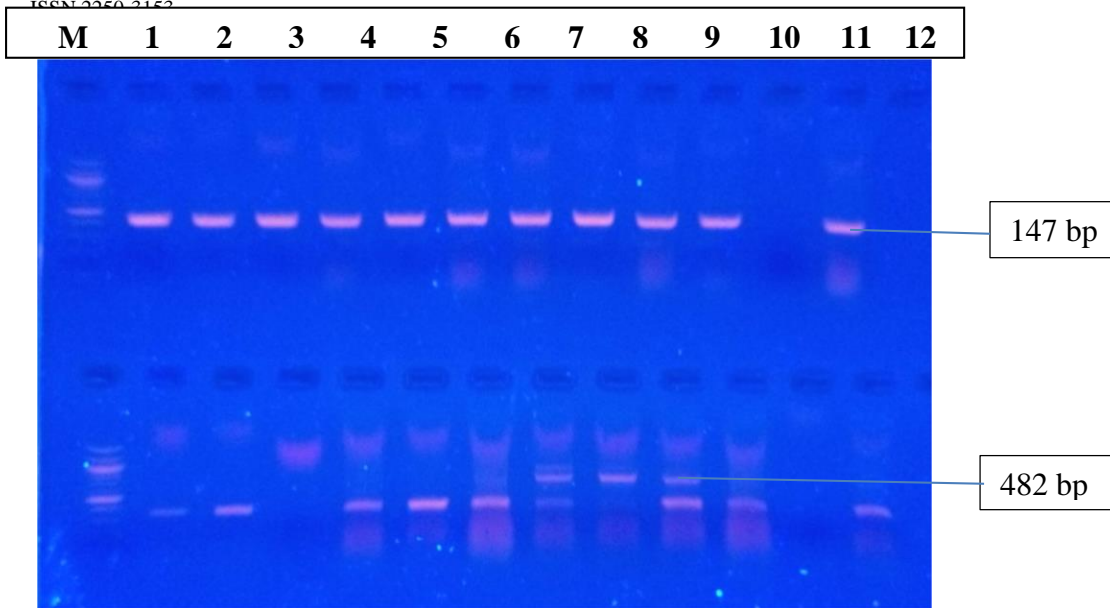
Plate 2: Amplification of Enteroheamoragic *E. coli*
Upper gel: uidA gene amplification generates 147 bp fragments to confirm *E. coli*
Lower gel: eae gene amplification generates 482 bp fragments to confirm enteroheamoragic*E. coli*

IV.   DISCUSSION

Diarrhoea due to bacterial infections is one of the main causes of morbidity and mortality amongst infants and young children in most developing countries including Nigeria (Odetoyin *et al.*, 2015; Igbinosa and Beshiru, 2018). One of the important steps towards the control of diarrhoea is the identification and classification of the enteropathogens involved in diarrhoea disease in the country (Olowe *et al*., 2003). In this study, results shows that three bacterial species (*Enteroheamoragic Escherichia coli, Salmonella typhi, Shigella dysenteriae)* were isolated from diarrhoeic children. The prevalence of cases of diarrhoea in Makurdi, Nigeria with a potential bacterial pathogen detected was 13.53%. This was higher than 7.9% prevalence reported in China by Yu *et al*. (2018) but lower than the findings of this study. It is however in contrast with the work by Lubbert *et al*. (2016) who reported that the most common pathogenic bacteria associated with acute diarrhoea in children under 5 years of age were found to be *Salmonella* spp. However, it corresponds with Odetoyin *et al.* (2015) who reported toxin-producing *E. coli* to be (42, 16.7 %). This variation in prevalence might be attributed to differences in infrastructural and socio-economic indices. Although there are geographical difference in the spectrum of bacteria incriminated in childhood diarrhoea, Enterohaemoragic *Escherichia coli* and *Shigella dysenteriae* species were isolated at a high rate. Statistical analysis showed that *Escherichia was* significantly associated with diarrhoea in children younger than 3 years (*P*<0.05). There appear to be conflicting reports about the association of *Salmonella* species with diarrhoea. Conversely, the occurrence of *Salmonella* species in this study agrees with the findings from Abakaliki, south –eastern Nigeria (Ogbu *et al*., 2008). In addition, this report of Enteroheamorragic *Escherichia coli* species (78.4%) is in agreement with former reports from similar studies in China (Yin *et al*., 2018) which reported *E. coli* to be significantly associated with diarrhoea. Bacterial isolation

age - wise diminished between the ages of 0-12, 37- 46 and 47 -60 months but significantly high between 13-24 and 25-36 months and is in consonance with past reports from Abuja (Ifeanyi *et al*., 2009). Molecular characterization confirmed presence of enteroheamorragic *E.coli,* and *Salmonella typhi.*

V.   CONCLUSION

It was concluded from findings of this study that *Enteroheamorragic Escherichia coli, Salmonella typhi, Shigella dysenteriae* were associated with diarrhoea disease in children (0-5) from the study area. *Enteroheamorragic Escherichia coli* had the highest prevalence of occurrence. Children within 25- 36 months were mostly affected with diarrhea.

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