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MOLECULAR CHARACTERISATION OF *SHIGELLA SPECIES* FROM DIARRHEAL STOOLS OF HIV PATIENTS IN MAIDUGURI, NORTH EAST NIGERIA.

Microbiology

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ABSTRACT

Despite high morbidity and mortality due to *Shigellosis* especially in developing countries, unarguably due to poor personal hygiene and sanitary conditions. There is dearth of literature on molecular characterization of *Shigella species*. In this prospective cross sectional study, Seven hundred diarrheal stool samples were analyzed. Participants consisted of 300 HIV positive and 400 HIV negative patients. The samples were consecutively collected from consenting patients that presented for care in the four main health facilities in Maiduguri, Borno state, northeastern Nigeria. Molecular detection of highly conserved 16s rRNA gene for identifying genetic properties were performed by polymerase chain reaction (PCR). A total of 56 (8.0%) *Shigella species,* composed of 21 (7.0%) from HIV positive and 35 (8.7%) were isolated by conventional assay and 16s rRNA PCR. Molecular characterization of the 56 isolates based on more sensitive Multiplex PCR platform indicates only 32 isolates as *Shigella species*. They consisted of 14(45.2%) *S. flexneri*, 12(38.7%) *S. dysenteriae* and 5 (16.1%) *S. sonnei*. *S. flexneri* was commoner among HIV negative patients. There was no variation in the composition of the isolates based on gender or defined age group.

KEYWORDS

Shigella species, Molecular characterization, Northeastern Nigeria.

INTRODUCTION

Diarrheal diseases constitute a global public health threat as it is estimated to be responsible for thousands of deaths worldwide, especially in children (WHO, 2003). Its prevalence is disproportionately high in developing countries, mostly tropical and subtropical parts of the world where living standards is low characterized by poor personal hygiene, poor sewage and sanitary facilities and weak of health infrastructure (Asghar *et al* 2002; Ekwenye and Kazi, 2007). Since the advent of human immunodeficiency virus (HIV), cases in adults are increasingly recognized (Amadi, 2001; Huebner 1993). Inadequate host immunity appears to play an important role in the development of systemic shigellosis among HIV patients. Other risk factors for diarrheal diseases besides HIV include malnutrition in children, diabetes, and malignancy (Amadi 2001; Dronda 1998).

Shigellosis is a common cause of diarrhea in sub Saharan Africa including Nigeria (Ashgar *et al* 2001; Iwalokun 2001). Despite the high incidence of *shigellosis,* there is limited data on molecular characterization of *Shigella species* among general population including HIV patients.

MATERIALS AND METHODS

SAMPLE COLLECTION

Diarrheal stool samples were collected from participants that included HIV positive and HIV negative patients that presented for care at the four main Hospitals in Maiduguri from 2nd January – 30th December 2015. Sterilized sample container was used to collect the stool samples. The samples were processed on the same day immediately on receipt of the samples.

CULTURAL ISOLATION OF THE ORGANISMS:

Fecal specimens were processed according to published protocol (Cowman 2004). One lapful of fecal sample was streaked on MacConkey Lactose Agar (MLA) and Xylose Lysine Deoxycholate Agar (XLD) and incubated at 37^{0}C for 24 hours, the MLA plates were then observed for the presence of concave and colorless colonies were considered for further identification. Similarly, the XLD plates showing the presence of translucent or red colored colonies were considered for further identification. The colonies showing the desired morphology and color were again re-streaked on the same media to obtain a pure culture.

BIOCHEMICAL IDENTIFICATION OF THE POSITIVE COLONIES

Three characteristic colonies from the plating media was picked and inoculate into KIA as follows: stab the butt and then streak the slant with a zig-zag configuration and incubate at 37^{0}C overnight and then examine the following morning for reactions in the KIA tubes. Tubes suspicious for *Shigella* were seen to have an acid (yellow) butt and an alkaline (red) slant and no production of gas (no bubbles or cracks in the agar) and hydrogen sulfide (no black along the stabbed line). Other biochemical test, viz; MP, VP, Citrate, urease and motility were also conducted by inoculating putative isolates.

PCR IDENTIFICATION

For PCR Amplification, DNA template was obtained using manufacturer's instruction of promega kits.

- *Shigella species* were identified using specific primers of each species. The sequences of the primers are shown in Table 1.
- The PCR reaction were performed in a final volume of 20µL USING 3µL DNA solution, 10µL master mix (Amplicon, Brighton, UK).
- Amplification was carried out in a Thermo cycler (Nexus Master cycler).

PCR Conditions were as follows:

- Initial denaturation at 95^{o}C for 5 min, followed by 30 cycles.
- Further denaturation at 95^{o}C for 45 seconds, annealing at 56^{o}C for 35 seconds and extension at 72^{o}C for 5 seconds. Final extension at 72^{o}C for 5 minutes.

Table 1. Species name and sequences of primer.

Species	Primer sequences	Repeat size (BP)
S. dysenteriae	F-TCTGATGTCACTCTTGCGAGT R- GAATCCGGTACCCGTAAGGT	248
S. sonnei	F- AATGCCGTAAGGAATGCAAG R- CTTGAAGGAGATTCGCTGCT	503
S. flexneri	F- TTTATGGCTTCTTTGTCGGC R- CTGCGTGATCCGACCATG	537

RESULTS

Of the 700 diarrheal stool samples obtained from patients from the three specialist hospitals in Maiduguri Metropolis (University of Maiduguri Teaching Hospital, UMTH (300 samples), State Specialist Hospital (100 samples), Umaru Shehu Ultra Modern Hospital (200 samples) and Mohammed Shuwa Hospital (100 samples), the *Shigella* species isolation rate was 56 (8%). The *Shigella* isolation rate of 21 (7%) among 300 HIV patients was incomparable to 35 (8.7%) from 400 samples from HIV negative patients, z = 0.68, p =0.49, 95% CI (-2.59 – 5.99). The distribution of *Shigella species* among participants based on HIV status was similar with the exception of *S. flexneri* that was higher among HIV negative cohort (4% vs 2.3%, Z =2.42, P = 0.016; 95% CI (9.16 – 70.83) as presented in Table 2. Stratification of the *Shigella* isolates based on gender and defined age groups are as presented in Table 3.

Table 2. Distribution of isolated *Shigella* species based on HIV status

Sample source	No of samples	Positive samples no(%)	*S. dysenteriae* no(%)	*S. flexneri* no(%)	*S. sonnei* no(%)
HIV (+ve)	300	21(7.0)	9(3.0)	7(2.3)	5(1.7)
HIV (-ve)	400	35(8.7)	11(2.8)	16(4.0)	8(2.0)
Total	700	56(8.0)	20(2.9)	23(3.2)	13(1.9)

Table 3: Age and sex distribution of *Shigella* sero group isolated by conventional method

Age group (yrs)	Total	No. of Positive Male			Total	No. of Positive Female		
		Serogroup				Serogroup		
		S. dysenteriae	*S. flexneri*	*S. sonnei*		*S. dysenteriae*	*S. flexneri*	*S. sonnei*
0 – 9	6	2	2	2	7	2	3	2
10 – 19	3	1	1	1	3	1	1	1
20 – 29	6	3	3	0	5	2	3	0
30 – 39	4	2	2	0	3	2	0	1
40 – 49	6	2	2	2	7	3	2	2
> 50	4	0	2	2	2	0	2	0
Total	29	10	12	7	27	10	11	6

The *Shigella* genus specific PCR produced the expected amplified DNA band in all *Shigella* species strains tested. Specific band of 566 bp obtained from standard and isolates belonging to four *Shigella* species. Standard PCR assays also produced the expected fragments of 248bp, 503 bp, and 537 bp, for *S. dysenteriae, S. sonnei and S. flexneri*, respectively. The 16s rRNA PCR was used to identify and characterize the *Shigella* strain commonly found in the study area. All the isolates that show positive by serological test were confirmed by the 16s rRNA PCR corresponding to the size 556bp as shown in Figure 1(a). The multiplex PCR assay was optimised for simultaneuos detection and differentiation of three pathogenic *Shigella species* as depicted in Figure 1(b). A total of 31 out 56 positives sample that were confirmed by 16s rRNA shows positive by multiple PCR. Of the 31 isolates that were positive by multiplex PCR, 14 (45.2%) were *S. flexneri*, while *S. dysenteriae* and *S. sonnei* constituted 12(38.7%) and 05(16.1%) respectively. No *S. boydii* serogroup was detected as depicted in Table 4.

Table 4. Performance conventional assay and molecular method in detecting *Shigella* species

Source of sample	No of sample analysed	No of positive for *Shigella*		No. Positive sample for *Shigella* subgroup			
		Conventional methods	Multiplex PCR	16rRNA	Multiplex PCR		
					S. dysenteriae	*S. flexneri*	*S. sonnei*
HIV Positive patients	300	26	11	all positive	4	8	2
HIV negative patients	400	30	20		8	6	3
Total	700	56	31 (4.4%)	56 (8.0%)	12(38.7%)	14(45.2%)	05(16.1%)

Figure 1(a). Detection of *Shigella species* genes by using 16s ribosomal RNA, M: Marker- 100bp, 1-25: 566bp

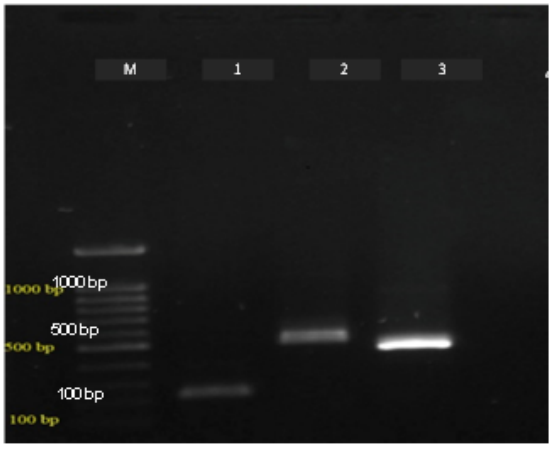

Figure 1(b). Detection of *shigella species* genes by multiplex PCR, M: Marker-100bp 1:S.dysenteriae-248 bp, 2:S. flexnerie-537 bp, 3: S.sonnei-503 bp, 4: Negative control.

The 56 positive isolates were subjected to antimicrobial susceptibility testing and were found to be (100%) sensitive to ofloxacin, ciprofloxacin and cefuroxime as presented in Figure 2.

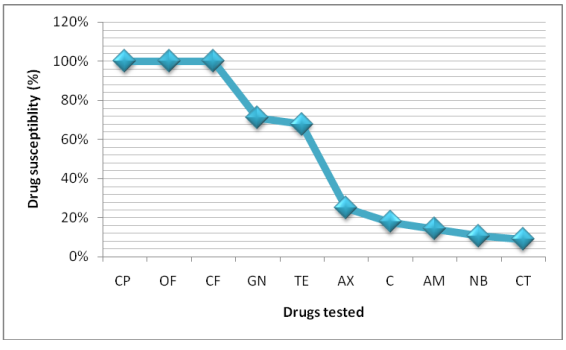

Figure 2. Susceptibility of the *Shigella* isolates to some antimicrobial drugs

Key: CP = Ciprofloxacin, OF = Ofloxacin,
CF = Cefuroxime, GN = Gentamycin,
TE = Tetracycline, AX = Amoxycillin,
C = Chloramphenicol, AM = Ampicillin,
NB = Nitrofurotoin, CT = Cotrimaxazole

DISCUSSION

Shigella is a significant cause of infectious diarrheal disease worldwide, especially in developing nations. Unfortunately, the

incidence of diarrheal diseases, an important surrogate of socioeconomic development is either static or increasing in most tropical and sub- tropical nations. The finding of *Shigella* isolation rate of 8.0% obtained in this report shows that shigellosis contributes significantly to the burden of diarrheal disease in our environment. Our finding is in tandem with similar study conducted in south western Nigeria and other developing countries (Abdulrasheed 2013; Huruy *et al* 2008). This is in contrast to reports from western countries that reported lower prevalence rates. Taking HIV status and gender into account, there was no observed variation of prevalence rate between HIV positive patients and HIV negative patients. The prevalence rates was also incomparable based on gender, this is in sharp contrast to previous studies that reported higher male preponderance (Khan *et al*., 1989 and Akinyemi, 2000).

Limited data exist on molecular characterization of *Shigella species* from developing nations that bears the most burden of shigellosis. *Shigella* is a very fastidious microorganism that "dislikes" transport, and for which no enrichment medium exists (Shears, 1996). *Shigella species* are easily inactivated or killed by exposure to inappropriate transportation of specimens and prior antibiotic use (Thiem *et al.,* 2004). Isolation of *Shigella species* from faeces is associated with imperfect results due to low specificity and sensitivity of conventional culture methods. In the current study, we characterized the *Shigella* species using polymerase chain reaction (PCR); it is an improved, quick, and robust methods of straight detection of *Shigella* species from faecal samples. Several PCR protocols have been developed for direct detection of *Shigella species.* in faecal, food and environmental samples (Islam *et al* 1998; Lindqust 1999; Faruque *et al.,* 2002) to circumvent the flaws of conventional culture methods.

A specific PCR using 16s rRNA conserved region was evaluated for the rapid and specific detection of *Shigella* species. The results showed that this locus is an appropriate target for this purpose. Several previous studies have used standard PCR for rapid detection of bacterial pathogens such as *Shigella* species. However, multiplex PCR has the advantage of simultaneous detection and identification of several target genes in a single reaction (Zhao *et al*., 2013: Fazzeli H *et al*., 2013: Bhattacharyya *et al* 2013: Wei et al. 2013: Kumar *et al*., 2013: Dixit *et al*., 2011).

Many researchers evaluated two multiplex PCR assays for simultaneous detection of typical and atypical E. coli pathovars and *Shigella species* (Aranda *et al*, 2004). Their results showed that the multiplex PCR was a potentially valuable tool for rapid diagnosis of *Shigella species*. Thong *et al* 2005) designed a multiplex PCR assay for simultaneous detection of chromosomal-and plasmid encoded virulence genes (set1A, set1B, ial and ipaH) in *Shigella species*. Unlike our study, the limitation of their Multiplex PCR assay was its inability to differentiate *Shigella species* (Thong *et al*., 2005; Binet *et al*., 2014). Our results also showed that the multiplex PCR using four primers sets was able to detect *Shigella* species and to differentiate three species of *Shigella* simultaneously in a single reaction by the combinations of the different-size amplicons without any cross-reactivity. Generally molecular based method like PCR have a well-developed potential to overcome certain insufficiencies of the conventional method and is found to be sensitive and reproducible but its application in diagnostic field is difficult due to high cost and the requirement of skilled person (Rahn *et al*., 1992). The results of 16srRNA PCR do not discriminate between *Shigella* at the genus or species level, nor did they differentiate *Shigella* from closely related pathogens such as salmonella and other *enterobacteriaceae.* Multiplex PCR was able to identify and differentiate *Shigella* species into their serogroups based on their target genes in a single reaction.

CONCLUSION

Shigellosis is a common cause of diarrhea in our environment. Molecular characterization of *Shigella* species using using multiplex PCR indicates *S. flexneri* as the prodorminant strain. Isolation of Shigella species using the conventional culture methods and 16s rRNA PCR do not discriminate between *Shigella* at the enus or species level, nor did they differentiate *Shigella* from closely related pathogens such as salmonella and other *enterobacteriaceae.* However, this study suggests multiplex PCR can accurately detect *Shigella* strain. It can therefore be used for identification of *Shigella* species with high speed and appropriate sensitivity and specificity.

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