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Resistant Plasmid Profile Analysis of *Shigella spp* Isolated from Stool Samples of School Children from Selected Communities in Odeda Local Government, Ogun State

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Abstract: Shigellosis is a worldwide health concern especially in developing countries with poor sanitation, lack of personal hygiene and use of contaminated water supplies especially for young children. The emergence of multidrug-resistant *Shigella* strains incidence imply that shigellosis is an unsolved global health problem causing diarrhoea. This study therefore was carried out to determine the resistant plasmids of multidrug resistant serotypes of *Shigella* species isolated from stool samples of school children among selected communities in Odeda Local government with their biodata. A total of 10 *Shigella spp* isolates were obtained from stool samples collected from school children. Antibiotics susceptibility was performed and multi-drug resistant isolates were selected for plasmid profiling. Plasmid profiling of multi-drug resistant *Shigella* isolates was done by alkaline lysis method. Molecular characterization for identification of the bacterial isolates was carried out using 16S rRNA gene sequencing method. Data obtained were analyzed using One-way Analysis of Variance (ANOVA). Somatic serotyping characterized the isolates to be *Shigella flexneri* (2.02%), *Shigella boydii* (1.2%) and *Shigella sonnei* (0.81%). Plasmid profile analysis showed detectable plasmids with estimated sizes between 100bp to 1200bp. Genomic characterization revealed the isolates belonging to *Shigella sonnei strain* M-X2D, *Shigella flexneri strain* MHW4.1 and *Shigella boydii* strain 3052-94. This study confirmed the emergence of multidrug resistant R-plasmids among *Shigella spp* causing diarrhoea amongst school children in Abeokuta environs, Nigeria.

Keywords: Antimicrobial Resistance, *Shigella spp*, Resistant Plasmids, Diarrhoea

1. Introduction

Shigellosis is a worldwide health concern especially in developing countries with poor sanitation, lack of personal hygiene and use of contaminated water supplies [1]. Malnutrition and the lack of appropriate medical intervention contribute to the high mortality rate, especially for young children. Despite global success in the reduction of all cause and diarrhoea-specific mortality in the past 30 years, diarrhoea remains the second leading cause of death due to infections among children fewer than five years of age worldwide [2]. It is estimated that diarrhoea accounted for 9·9% of the 6·9 million deaths among children under 5 in 2011 [3, 4], and it has been reported that the prevalence rate of diarrhoea in Nigeria is 18.8%; which is a menace in sub-Sahara Africa [5].

The U.S. Centers for Disease Control and Prevention [6-8] has described antibiotic resistance as "one of the world's most pressing health problems", because "the number of bacteria resistant to antibiotics has increased in the last decade and many bacterial infections are becoming resistant to the most commonly prescribed antibiotic treatments [9]. The emergence of multidrug-resistant Shigella stains and a continuous high disease incidence imply that shigellosis is an

unsolved global health problem [1]. Since it is an acute intestinal infection, the symptoms can range from mild watery diarrhoea to severe inflammatory bacillary dysentery characterized by strong abdominal cramps, fever, and stools containing blood and mucus.

Multiple drug resistance isolates causing diarrhoea has serious implications for the empiric therapy against pathogenic isolates and for the possible co-selection of antimicrobial resistant mediated by multi-drug resistant Plasmids. *Shigella spp* from clinical isolates are known to harbor plasmids of different molecular sizes; it has been widely reported that bacteria harbor antibiotic resistant genes which can be horizontally transferred to other bacteria. The progressive increase in antibiotic resistance among enteric pathogens in developing countries has been reported [10] which might be due to environmental factors, geographic differences or different patterns of antibiotic usage [11].

In Nigeria, though cases of Shigellosis (bacillary dysentery) due to Shigella species have been reported, to this very moment, there are few reports in Africa and Nigeria on molecular characterization of Shigella among school children with Shigellosis (bacillary dysentery) especially within rural and crowded communities. Therefore, it is imperative to to determine the resistant plasmids of multidrug resistant serotypes of *Shigella* species isolated from stool samples of school children among selected communities in Odeda Local government with their biodata, and its genetic characteristics.

2. Methodology

2.1. Sample Collection

A total of 248 stool samples were collected from the pupils. The samples were collected into sterile, transparent, wide mouthed bottles. The name, age and sex of the pupils were properly labeled on the universal bottles. After collection, the samples were transported to the laboratory using ice packs as transport medium and processing was done within 2 hours of collection. The pupils' biodata was obtained by administering a semi- structured questionnaire.

Ethical clearance for the study was obtained from both State Ministry for Health & Education; Ogun State (Ref No: PL19/VOLIV/18) and was cleared by the Ethical Review Committee from College of Biosciences (COLBIOS), FUNAAB.

2.2. Microbiological Analysis

Stool samples were cultured within one hour of collection on to MacConkey agar, SSA agar, and Blood agar which were incubated aerobically overnight at 37ºC. Isolates were identified morphologically using the characteristics (size, shape, edge, and texture, degree of opacity, elevation and color) of each colony as described by Fawole and Osho, [12].

2.3. Biochemical Test

oxidation-fermentation tests, catalase, oxidase activity tests, Indole test, Gram's Stain, Methyl Red test, Voges Proskauer test, Citrate utilization test, Triple sugar iron test, Coagulase test, was carried out according to Cheesbrough, [13].

2.4. Antibiotics Susceptibility Test

Antibiotic sensitivity test was carried out on all the bacterial isolates using disc diffusion technique according to Clinical and Laboratory Standards Institute, [14]. Mueller Hinton's agar plates were inoculated with the standardized inoculums of the overnight pure bacteria culture. After inoculation, appropriate Gram negative antibiotic sensitivity discs were placed aseptically and incubated at 37°C for 24 hours; after incubation, the diameter of the zone of inhibition were measured and compared with zone diameter interpretative chart by Clinical and Laboratory Standard Institute, [14]. Ten different antibiotics with different disc concentration such as Gentamycin (GEN) 10µg/disc, Ceftriaxone (CRO) 30µg/disc, Cotrimoxazole (COT) 25µg/disc, Tetracycline (TET) 30µg/disc, Ciprofloxacin (CPX) 10µg/disc, Augmentin (AUG) 30µg/disc, Amoxicillin (AMX) 25µg/disc, Ofloxacin (OFL) 5µg/disc, Pefloxacin (PFX) 5µg/disc, Nitrofurantoin (NIT) 20µg/disc were used in this study.

2.5. Serological Analysis for Shigella

Isolates biochemically identified as *Shigella* were subjected to slide and tube agglutination test using polyvalent and monospecific somatic antisera according to the instruction of the manufacturer. The overnight pure culture of the different *Shigella* isolates was emulsified in a drop of 0.85% saline and mixed to form a smooth suspension on a clean dry tile. A drop of antisera was added to each suspension and mixed. The suspension was spread to cover the reaction area and rocked for one minute. Agglutination indicates positive reaction while no agglutination indicates negative reaction.

2.6. Plasmid Profiling

2.6.1. Plasmid DNA Extraction

Pure bacterial isolates were inoculated on Muller Hinton agar and incubated overnight. The grown culture was transferred into 1.5 ml micro centrifuge tube containing phosphate buffer saline (PBS) and centrifuged at 10,000 rpm for 30 seconds. The supernatant was decanted and the pellet cells were suspended in 200µl of solution A (100mM glucose, 50mM Tris Hydrochloride (PH 8.0), 10M EDTA) containing 10mg of lysozyme per ml and incubate for 30 minutes at 37°C. The freshly prepared 1% sodium dodecyl sulphate (400 µl) in 0.2N NaOH was added and mixed by inverting tubes. A 30% potassium acetate solution (300 µl) was added, mixed by vortexing and incubated on ice for 5 minutes and centrifuged at 5000rpm for 5 minutes. The supernatant was discarded and cells were extracted once with a phenol-chloroform mixture (1:1). Plasmid DNA was precipitated with absolute ethanol and 70% ethanol and

spinned repeatedly thrice. The tubes were opened and allowed to dry in an incubator. Plasmid DNA was dissolved with Trisacetic EDTA buffer [15].

2.6.2. Agarose Gel Electrophoresis

Agarose powder of 0.8 g was weighed and boiled in 100 mls of 0.5x TBE buffer (Trace Borate ethylene diamine tetra-acetic acid) until the solution became a clear gel. The agarose solution was allowed to cool to about 60ºC and 10 µl of ethidium bromide was added and mixed by swirling gently. It was then poured into electrophoresis tank containing the gel buffer with the comb in place to obtain a gel thickness of about 5 mm. The comb was then removed and 10 µl of the sample was mixed with 1µl of the loading dye and carefully loaded into each of the wells. The electrodes were connected to the power pack in such a way that the negative terminal was at the end where the sample has been loaded. The electrophoresis was allowed to run at 60 – 100 V until the loading dye has migrated about three- quarter of the electrodes. Electrodes are turned off and disconnected. The gel was observed on a UV- trans-illuminator [16].

2.7. Molecular Analysis

The molecular analysis for this study was carried out at International Institute of Tropical Agriculture (IITA) Ibadan. Bacteria genomic DNA was extracted from isolates using Qiagen DNA mini kit. (250) Cat No 51306 and was characterized using 16S rRNA gene sequencing methods such as: Polymerase Chain Reaction, Agarose gel electrophoresis, Sequencing and BLAST.

2.7.1. Bacterial Genomic DNA Extraction

Single colonies grown on medium were transferred to 1.5 ml of liquid medium and cultures were grown on a shaker for 48 h at 28 ºC. After this period, cultures were centrifuged at 4600x g for 5 min. The resulting pellets were re-suspended in 520 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Fifteen micro litres of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were added. The mixture was incubated for 1 hour at 37 ºC, then 100 µl of 5 M NaCl and 80 µl of a 10% CTAB solution in 0.7 M NaCl were added and mixed. The suspension was incubated for 10 min at 65 ºC and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifugation at 7200 x g for 20 min. The aqueous phase was transferred to a new tube, isopropanol (1: 0.6) was added and DNA was precipitated at –20 ºC for 16 h. DNA was collected by centrifugation at 7200 x g for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours and finally dissolved in 50 µl of TE buffer [17].

2.7.2. Polymerase Chain Reaction

PCR reaction cocktail consisted of 10 µl of 5x GoTaq colorless reaction, 3 µl of MgCl2, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each 27F 5'- AGA GTT TGA TCM TGG CTC AG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water, 8µl DNA template. PCR was carried out in a Gene Amp 9700 PCR System Thermal cycler (Applied Bio system Inc., USA) PCR profile at initial denaturation, 94°C for 5 min; 30 cycles, of 94°C for 30 s, 50°C for 60s and 72°C for 1 minute 30 seconds; and a final extension at 72°C for 10 minutes, and chill at 4°C.

2.7.3. Purification of Amplified Product

The PCR amplicons were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µl of Na acetate 3M and 240 µl of 95% ethanol were added to each about 40µl PCR amplified product in a new sterile 1.5 µl tube eppendorf, mix thoroughly by vortexing and kept at -20°C for at least 30 min. Centrifugation was for 10 min at 13000 g and 4°C followed by removal of supernatant (invert tube on trash once) after which the pellet were washed by adding 150 µl of 70% ethanol and mixed, then centrifuged for 15 min at 7500 g and 4°C. Again all supernatant was removed (invert tube on trash) and invert tube on paper tissue and left to dry in the fume hood at room temperature for 10-15 min It was then re-suspended with 20 µl of sterile distilled water and kept in -20°C prior to sequencing. The purified fragment was checked on a 1.5% Agarose gel ran on a voltage of 110V for about 1hr, to confirm the quality of the purified PCR product.

2.7.4. Quantity and Quality Check

Quantity of the amplified product was checked on a Nano-drop of model 2000 from thermo scientific to quantify the concentration of the amplified product and also determine purity by measuring at 260/280nm amount of proteins left in the DNA extract.

2.7.5. Agarose Gel Electrophoresis

Agarose powder of 0.8 g was weighed and boiled in 100 ml of 0.5x TBE buffer (Trace Borate ethylene diamine tetra acetic acid) until the solution became a clear gel. The agarose solution was allowed to cool to about 60ºC and 10µl of ethidium bromide was added and mixed by swirling gently. It was then poured into electrophoresis tank containing the gel buffer with the comb in place to obtain a gel thickness of about 5mm. The comb was then removed and 10µl of the sample was mixed with 1µl of the loading dye and carefully loaded into each of the wells. The electrodes were connected to the power pack in such a way that the negative terminal was at the end where the sample has been loaded. The electrophoresis was allowed to run at 60 - 100V until the loading dye has migrated about three- quarter of the electrodes. Electrodes are turned off and disconnected. The gel was observed on a UV- trans-illuminator [16].

2.7.6. Sequence Editing and Database Matching

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Bio-systems using manufacturers' manual while the sequencing kit used was that of Big Dye terminator v3.1 cycle sequencing kit. Bi-directional sequences obtained with forward and reverse primers were edited and aligned to generate a consensus

sequence using Bio Edit sequence Alignment Editor (version 7.1.9). Consensus sequences were then aligned with sequences deposited in the National Centre for Biotechnological Information (NCBI) gene bank by using the Basic Local Alignment search Tool (BLAST) to establish identities of the bacteria isolates [18].

2.8. Data Analysis

Data was analyzed using Statistical Package for Social Sciences (SPSS) version 17.0 for windows (SPSS, Chicago IL and USA) into simple percentiles and test for significance. The level of significance was considered as $P < 0.05$.

3. Results

3.1. Percentage Antimicrobial Susceptibleness Profile

Table 1 depicts the percentage antimicrobial susceptibility profile of somatic antigenic positive *Shigella* strains (N=10), in which Five (5) isolates were confirmed to be *S. flexneri*, three (3) were given to be *S. boydii* and two (2) were *S. sonnei*. The antibiotic susceptibility revealed that the 10 *Shigella* isolates tested shows resistance to one or more antibiotics used. Resistance was seen to be higher in Augmentin, Nitrofurantoin and Amoxycillin at 100%, followed by Tetracycline and Cotrimoxazole at 90% resistance and 10% intermediate, with Gentamycin having 80% resistance. Susceptibility was seen to be higher in Ofloxacin, Pefloxacin and Ciprofloxacin at 80%.

Table 2 shows the Somatic Antigenic positive and negative *Shigella* strains in different age groups among the pupils**.** Out of 32 pupils studied in the Age group of 2-4, only 2 (20%) was positive for *Shigella spp*, while the remaining 30 was negative. Out of 82 pupils studied in the Age group of 5-7, 4 (40%) was positive for *Shigella spp,* while the remaining 78 was negative. Out of 80 pupils studied in the Age group of 8-10, 3 (30%) was positive for *Shigella spp*, while the remaining 77 was negative. Out of 54 pupils studied in the Age group of 10 and above, only 1 (10%) was positive for *Shigella spp* while the remaining 53 was negative. Age group 5-7 accounts for the highest percentage of positive *Shigella spp* (40%).

3.2. Plasmid Profiling and Molecular Identification

Table 3 shows the Molecular identification of *Shigella* isolates after the generated consensus sequences which was subjected to BLAST on the NCBI Database. Figure 1 shows the agarose gel electrophoretic picture of the plasmid profile analysis of the resistant *Shigella* isolates. Six of the isolates possessed plasmid bands while two had no plasmid bands. Isolate A possessed double plasmid bands at 1171bp and 1012bp, isolate E at 436bp and 936bp and isolate F at 436bp and 1012bp respectively. Isolates C, D and G possessed single plasmid bands at 1171bp, 1012bp and 946bp respectively. Isolates B and H possessed no plasmid bands. Lane M is the marker size.

Table 1. *Percentage antimicrobial susceptibility profile of somatic antigenic positive Shigella strains (N=10).*

Antibiotics	Susceptibility	Intermediate	Resistant
	Number (%)	Number (%)	Number (%)
Augmentin	0(0.0)	0(0.0)	10(100)
Ceftriaxone	3(30.0)	0(0.0)	7(70.0)
Nitrofurantoin	0(0.0)	0(0.0)	10(100)
Gentamycin	0(0.0)	2(20.0)	8(80.0)
Cotrimoxazole	0(0.0)	1(10.0)	9(90.0)
Ofloxacin	8(80.0)	1(10.0)	1(10.0)
Amoxicillin	0(0.0)	0(0.0)	10(100)
Ciprofloxacin	8(80.0)	1(10.0)	1(10.0)
Tetracycline	1(10.0)	0(0.0)	9(90.0)
Pefloxacin	8(80.0)	1(10.0)	1(10.0)

Table 2. *Somatic Antigenic positive and negative Shigella species in different age group among the pupils.*

Age group	Somatic antigenic positive *Shigella*		Somatic antigenic negative *Shigella*	
	Number N	Percentage %	Number N	Percentage %
2-4	2	20	30	12.6
5-7	4	40	78	32.8
8-10	3	30	77	32.3
10 and above	1	10	53	22.3
Total	10	100	238	100

P>0.05

Table 3. *Molecular characterization of Shigella serotypes found among the pupils.*

Source	Nucleotide base number	Gene Accession number	Percentage I Identity	Genomic identity
Faecal	1787	KJ806411.1	91%	*Shigella sonnei strain* M-X2D
Faecal	963	KJ620888.1	80%	*Shigella boydii strain* MHW4.1
Faecal	1518	AY696681.1	89%	*Shigella boydii strain* 3052-94

Genomic characterization reveals the isolates belonging to *Shigella sonnei strain* M-X2D, *Shigella boydii strain* MHW4.1 and *Shigella boydii strain* 3052-94.

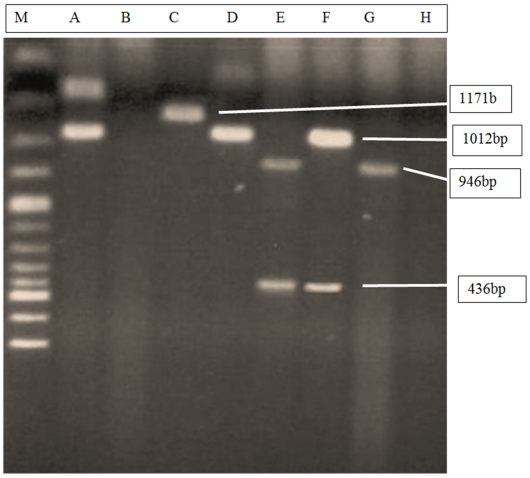

Figure 1. *Agarose gel (1.0%) electrophoresis of plasmid DNA isolated from resistant Shigella isolates.*

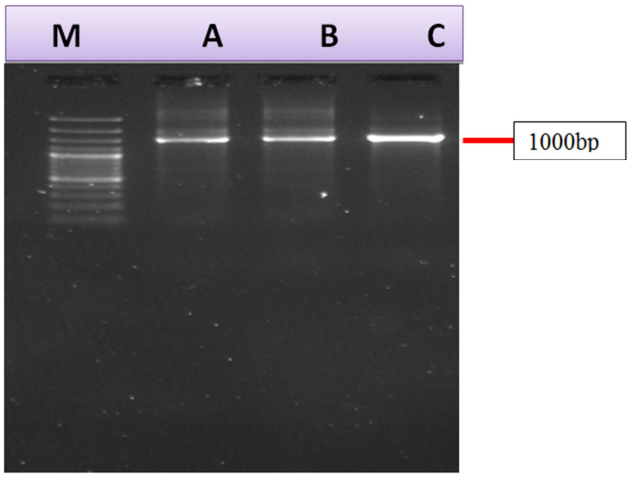

Figure 2. *The gel picture of electrophoresis used in separation of the amplified PCR products.*

4. Discussion

There were detectable plasmids in six (75%) of the eight multi - drug resistant *Shigella* isolates while two (25%) had no plasmid bands. Plasmid mediated resistance to various antimicrobial drugs have been demonstrated by various works including Isawumi *et al*., [19] in Ile-Ife, Ocean *et al*., [20] in Ayingba, Kogi state, Pazhani *et al*., [21, 22] in India, Ranjbar *et al*., [23] in Iran. Plasmids have been found to confer drug resistance to their host bacteria by various mating processes such as conjugation, transduction and transformation [24]. The plasmids obtained from the *Shigella* isolates had plasmid weight ranging from 436bp to 23.13bp. These plasmids weights were similar to plasmids reported in Ile-Ife, Osun state, Nigeria by Isawumi *et al,* [19] and Ocean *et al*, [20] in Ayingba, Kogi state.

The inability to detect plasmids in some of the isolates may be evident that some of the multi-drug resistance observed in this research, is not only being induced by plasmids but by some external factor as evidenced by the percentage antimicrobial susceptibleness profile. Genomic characterization revealed the isolates belonging to *Shigella sonnei* strain M-X2D, Shigella flexneri strain MHW4.1 and *Shigella boydii* strain 3052-94. This study confirmed the emergence of multidrug resistant R-plasmids among *Shigella spp* causing diarrhoea amongst school children in Abeokuta environs, Nigeria.

5. Conclusion

This study highlighted the emergence of multidrug resistant R-plasmids among *Shigella spp* causing diarrhoea in Selected Communities in Odeda Local Government; Abeokuta, Southwestern Nigeria. The uncontrolled use of antibiotics has contributed largely to this situation. Thus government should make considerable effort to establish an antibiotic policy for the country. It is therefore recommended that extending mandatory surveillance to include shigellosis not only at hospitals but in the community to gain a better understanding of plasmids mediated resistance *Shigella spp* be carried out. Monitoring of plasmids mediated resistance and antimicrobial susceptibility testing was necessary to avoid treatment failure in patients with diarrhoea.

Conflict of Interest

The authors declare that they have no competing interests.

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