Multiple Antibiotic Resistance Indices of *Shigella flexneri* and *Salmonella enterica* Associated with Diarrhoea in Children (0-5 Years) From Selected Hospitals in Kaduna Metropolis, Nigeria

[1*]Alhaji, A.I., [1]Mulade, G. L., [1]Ajibade, G. A., Haroun, A. A. and [2]Benjamin, O.I.
[1]Department of Biological Sciences, Faculty of Science, Nigerian Defence Academy Kaduna.
[2]Nigerian Defence Academy Hospital, Kaduna.
*Corresponding author: aialhaji@nda.edu.ng

Abstract: Diarrhoea diseases remain the second leading cause of death among children under five years globally. Nearly one in every five child deaths are due to diarrhoea, further compounded by antimicrobial resistance. As a result, better understanding of childhood diarrhoea occurrence can perhaps help reduce associated morbidity and mortality rates. This study was conducted to determine the multiple antibiotic resistance indices of *Shigella flexineri* and *Salmonella enterica* from diarrhoeic children less than five years from selected hospitals in Kaduna. A total of 264 stool samples were collected from children attending selected hospitals in the Kaduna metropolis. Standard methods involving microbiological, biochemical and molecular analysis using PCR and 16S rRNA molecular characterizations were employed in identifying bacteria associated with diarrhoea in children (0-5) years. Out of the 264 stools examined, a total of 162 (61.4%) were from males, while 102 (38.6%) were from females. The highest incidence was observed in children of 1-24 months of age and the least in children of 4-5 years of age. A total of 97 stool samples (36.7%) were positive for *Shigella* and *Salmonella* species, out of which 97, 60(22.7%) were *Shigella* and 37(14%) *Salmonella*. The multiple antimicrobial resistance index of these isolates revealed that 100% of the isolates had a MAR index of 0.5 and above, and showed significant resistance against Augmentin, Amoxacillin, Ampicillin-Cloxacillin (Ampiclox) Erythromycin and Gentamycin. The least resistance was observed against imipenem (45%). The high Multiple Antibiotic Resistance index of the isolates indicates previous exposure to antibiotics and the development of resistance to commonly prescribed antibiotics.

Keywords: Diarrhoea, *Shigella flexneri, Salmonella enterica*, Multiple Antibiotic Resistance (MARI).

INTRODUCTION

Acute diarrheal disease is still a serious public health issue resulting in medical consultations, mortality, and morbidity all throughout the world, particularly in low- and middle-income nations. (Thiam *et al.* 2017). Furthermore, diarrhoea disease is one of the most serious threats to children's health, with 1.7 billion episodes and 525,000 deaths worldwide each year in children under the age of five, according to recent figures. (WHO 2019).

Shigella and *Salmonella* species are among the most common pathogens recovered from diarrhoea patients. *Shigella* spp. was the second leading cause of diarrhoeal death among individuals of all ages in 2016, accounting for 212,438 deaths, and about 13.2% of all diarrhoea deaths. (Khalil *et al.* 2018). The diarrheal diseases are usually characterized by blood, cramping, abdominal pain, fever, nausea, and vomiting (CDC 2018). In Nigeria, the morbidity linked with *Salmonella* infections continues to rise, with some cases resulting in death. Since the beginning of the 1990s, *Salmonella* strains resistant to a variety of antibiotics, including first-line drugs for human treatment, such as ampicillin, chloramphenicol, and cotrimoxazole; and third-generation cephalosporins, have evolved, posing a severe public health danger (Umair and Siddiqui 2020).

Resistance of pathogenic organisms to antibiotics is an increasing problem in the treatment of most microbial infections. The rapid dissemination of drug-resistant bacteria has been identified as a global problem that seriously complicates the treatment of human infections (Fair and Tor 2014). However, some of the factors reported contributing to this increase, including high use of antimicrobial agents in humans and animals resulting in pressure for selection of resistant bacteria, the capacity of bacteria to disseminate antimicrobial resistance genes to other bacteria mainly by mobile genetic structures, and the facility of dissemination of resistant bacteria in different ecosystems.

Genes coding for resistance are frequently encoded on transferable plasmids that encode resistance genes. Multidrug-resistant (MDR) microorganisms arise when commensal or faecal isolates acquire such resistant genes. Multidrug-resistant (MDR) strains and strains that produce extended-spectrum-lactamases (ESBL) are also becoming more common in humans and animals (Zeighami *et al.* 2015).

Due to the high prevalence of multidrug resistance, there is a pressing need for broad-based, local antimicrobial resistance surveillance as well as the development of effective ways to reducing multidrug resistance in these organisms (Olayinka *et al.* 2004). The presence of plasmids containing one or more resistance genes, each encoding a single antibiotic resistance phenotype, is most commonly associated with multiple antibiotic resistance (MARI) in bacteria. (Nikaido 2009). Multiple antibiotic resistance indexing has proven to be a reliable and cost-effective method of tracing bacteria sources. The multiple antibiotic resistance index is determined as the ratio of the number of resistant antibiotics to which an organism is resistant to the total number of antibiotics to which the organism is exposed. (Mthembu, 2008). MAR index values greater than 0.2 indicate the high-risk source of contamination where antibiotics are often used.

This study was therefore aimed at investigating the antibiotic susceptibility pattern of diarrhoea associated *Salmonella enterica* and *Shigella flexneri*, and to determine the multiple resistance index of the isolates to commonly used antibiotics in the Kaduna metropolis.

MATERIALS AND METHODS

Study area and Study Population

The study was carried out in Kaduna metropolis, which is the capital of Kaduna state. It comprises of two local government areas: Kaduna South and Kaduna North and also extends to Chikun and Igabi Local Government areas. A cross-sectional study was conducted among children between the ages of 0-5 years, presenting with diarrhoea in selected hospitals in Kaduna metropolis. Ethical approval for the study was obtained from Kaduna State Ministry of Health, Kaduna. The sample size was determined in accordance with the formula of (Chioma *et al.,* 2019). A total of two hundred and sixty-four samples were collected from the diarrhoeic children for this study, where 21.1% was taken as the prevalence rate.

Sample Collection and Processing

Stool samples were collected from children under 5 years of age visiting the hospitals due to diarrhoea. Stool samples were collected using sterile stool containers and transferred to the microbiology laboratory immediately for laboratory analysis (Chioma *et al.* 2019).

Isolation and Characterization of *Salmonella* and *Shigella* spp.

Bacterial isolates were identified according to the standard microbiological procedures as described (Gillespie and Hawkey 2006), which includes the examination of specimens to detect, isolate and identify pathogens or their products using Microscopy, Culture techniques and Biochemical characteristics.

Isolation of *Salmonella* and *Shigella spp* from stool

Faecal samples were inoculated directly onto MacConkey Agar and *Salmonella-Shigella* (SS) Agar (Chioma *et al.* 2019). The plates were incubated at 37°C, respectively, for 18 - 24 h. All suspected isolates with growth characteristics of *Salmonella* and *Shigella spp.* were subjected to standard bacteriological and biochemical identification methods using Bergey's manual of systematic bacteriology.

Biochemical Characterization of Presumptive *Salmonella* and *Shigella* spp.

All isolates were subjected to the following biochemical tests: - Indole, methyl red, Voges-Proskauer, Citrate, catalase, coagulase, motility, sugar fermentation tests (glucose, lactose), mannitol salt agar, hydrogen sulphide production (H_2S), Urease reaction and blood agar.

Antibiotic Susceptibility Testing

Antimicrobial resistance patterns of *Shigella* and *Salmonella spp.* isolates were determined by the standard disc diffusion method of Kirby-Bauer as described by the Clinical and Laboratory Standards Institute (2012). The bacteria isolates were screened for resistance against 10 antibiotics belonging to different families of antimicrobials (Mast Diagnostics, United Kingdom). These included penicillins [ampicillin (10 µg), amoxacillin (25 µg) and augmentin]; cephalosporins [ceftriazone (30 µg), ceftazidime (30 µg), cefoxitin (30 µg) and cefuroxime (30 µg)]; carbapenems; [imipenem (10 µg)]; aminoglycosides [gentamicin (10 µg)]; macrolides [erythromycin (20µl)]. Pure isolates previously grown on sterile nutrient agar were inoculated on sterile physiological-buffered saline (PBS) solution (0.85% NaCl) to make up a bacterial suspension with a density equivalent to 0.5 McFarland standards. Sterile cotton swab-stick (Copan, Italy) was stroke into the suspension and spread uniformly onto the entire surface of the Mueller Hinton agar plates. Relevant antibiotic discs were placed on the surface of the inoculated plates using a disc dispenser (Mast Diagnostics, UK) and were incubated at 37 °C for 18–24 hours.

Multiple Antimicrobial Resistance Index (MARI)

The isolates that were resistant to three or more than three classes of antibiotics were designated as multi-drug resistant (MDR) bacteria. The Multiple Antibiotic Resistance Index was calculated by using the formula as described by Mthembu *et al.* (2008) which is expressed as MAR index = a/b, where "a" represents the number of antibiotics to which an individual isolate is resistant to and "b" is the sum of antibiotics to which individual isolate was tested.

DNA Extraction

Bacteria isolates from pure colonies were placed into appropriately labelled Eppendorf tubes for DNA extraction. The DNA of the bacteria isolates were extracted using commercially available kits (Accu prep Genomic DNA extraction kit from Bioneer), following the manufacturer's instructions.

Amplification of 16S rRNA Gene using PCR (Accupower Hotstart PCR premix, Bioneer)

PCR amplification for the confirmation of *Shigella flexneri* and *Salmonella enterica* was performed on a thermal cycler (Thermal cycler PTC 100, MJ Research). The 2ul of the DNA as the template, 1µl each of the forward and reverse primer and 16µl of deionized water (nuclease-free water). The thermocycler was operated based on these conditions: pre denaturation at 95^{o}C for 5minutes; denaturation at 94^{o}C for 1 minute; annealing at 52^{o}C for 1 minute; extension at 72^{o}C for 1 minute for 25 cycles and a final extension at 72^{o}C for 5 minutes. GGACTACAGGGTATCTAAT (16S Primer Forward); AGAGTTTGATCCTGG (16S Primer reverse).

Agarose Gel Electrophoresis

Agarose gel (1.5% agarose in TAE (Tris-acetate-EDTA-buffer) containing 5ul ethidium bromide will be prepared. Then DNA ladder (100bp) was added separately in each gel well. Then the gel ran for at least an hour. DNA bands were visualized on an ultraviolet (UV) lightbox (Biorad). The amplified PCR product of the expected band size 789bp was confirmed by the Agarose gel electrophoresis system (Adegbite *et al.,* 2019).

Sequencing of PCR Products

The products of the DNA were be sequenced using a DNA sequence machine (ABI 3100). All the sequences were matched against nucleotide sequences present in GenBank using the BLAST of the NCBI program to identify the organism based on the most similar 16S rRNA gene.

Statistical Analysis

Results obtained from this study was recorded and analysed using Microsoft excel and statistical package for social sciences (SPSS) version 23.0 (California, USA). The analysis was carried out at a 95% level of confidence to verify the significance of the results obtained from the research.

RESULTS

A total of two hundred and sixty-four (264) stool specimens were collected from all four study areas and tested. Of the two hundred and sixty-four (264), one hundred and sixty-two (162) representing 61.4% were males, while one hundred and two (102) representing 38.6% were females (Table 1).

As seen in Table 2, one hundred and forty-seven (147) of the participants (representing 55.9%) were within the age range of 1 month to 2 years, while seventy-nine (79) of them (representing 29.9%) were within the age range of >2 years to 4 years with significant difference observed at p>0.05; and thirty-eight (38) of them (representing 14.2%) were within the age range of >4 years to 5 years of age, with no significant difference observed (p<0.05). The highest incidence was observed in children of 1-24 months of age and the least in children of 4-5 years of age. Table 3 shows the distribution of *Shigella and Salmonella* isolates in the stool samples, with respect to the hospital location from where the samples were obtained. There was a statistical difference observed in the prevalence obtained from hospitals studied at p > 0.05; Hospital A had the highest percentage prevalence of 45.5%, while Hospital B and Hospital C had a prevalence of 40%. The prevalence of *Shigella* and *Salmonella spp.* infection on patients in Hospital D was 23.5%.

Table 1: The Distribution of children with diarrhoea based on gender

Gender	Frequency
Male	162 (61.4%)
Female	102 (38.6%)

Table 2: Distribution of age ranges of the participants in all four study sites

Age range (years)	Frequency (%)
0-2	147 (55.9)
>2-4	79 (29.9)
>4-5	38 (14.2)

Table 3: Prevalence of *Shigella and Salmonella spp.* isolated from diarrhoeic stool samples according to hospital locations

Location	No. of Samples Collected	Samples Positive		Total (%)	X^2	p-value
		S. enterica	*S. flexneri*	All Sites		
Hospital A	88	11	29	40 (45.5)	12.00	0.213
Hospital B	60	10	14	24 (40)		
Hospital C	35	7	7	14 (40)		
Hospital D	81	9	10	19 (23.5)		
Total	264			97 (36.7)		

X^2=chi-square, p-value<0.05, (*) =statistically significant, (%) =Prevalence.

Table 4 show the antibiotic susceptibility pattern of *Salmonella spp.* isolated from diarrhoeic stool samples of children less than five years in Kaduna state. *Salmonella spp.* isolates showed maximum resistance (100%) to Cefuroxime, Augmentin, Amoxacillin, Ampicillin-Cloxacillin (Ampiclox) and Erythromycin, followed by Gentamycin (95%), Ceftriaxone (90%), Cefoxitin (90%) and Ceftazidime (85%), while the lowest

rate of resistance was observed against Table 5 show the antibiotic susceptibility pattern of *Shigella* spp. isolated from diarrhoeic stool samples of children less than five years in Kaduna state. *Shigella* spp. isolates showed maximum resistance (100%) to Ceftriaxone, Augmentin, Amoxacillin, Ampicillin-Cloxacillin and Erythromycin, followed by Ceftazidime, Cefoxitin, Imipenem (55%).

Cefuroxime, all showing 95% resistance, followed by Gentamycin (90%) while the least resistance was observed against Imipenem (45%). The isolates were classified as a multidrug-resistant when they proved resistant to at least one antimicrobial agent from three or more distinct classes.

Table 4: Antimicrobial Susceptibility Pattern of *Salmonella spp*, Isolated from Stool Sample of Diarrhoeic Children Less Than Five Years

		Sensitivity patterns (%)		
Antibiotic	**Disc Potency (µg)**	**S (%)**	**I (%)**	**Resistant**
Ceftriaxone	30	5	5	90
Ceftazidime	30	5	10	85
Cefoxitin	30	0	10	90
Cefuroxime	30	0	0	100
Augmentin	30	0	0	100
Amoxacillin	25	0	0	100
Ampicillin-Cloxacillin	10	0	0	100
Imipenem	10	20	25	55
Erythromycin	20	0	0	100
Gentamycin	10	0	5	95

R=Resistance, I=Index, S=Susceptible, %= percentage

Table 5: Antimicrobial Susceptibility Pattern of *Shigella spp*, Isolated from Stool Sample of Diarrhoeic Children Less Than Five Years

		Sensitivity patterns (%)		
Antibiotic	**Disc Potency (µg)**	**S (%)**	**I (%)**	**Resistant (%)**
Ceftriaxone	30	0	0	100
Ceftazidime	30	0	5	95
Cefoxitin	30	0	5	95
Cefuroxime	30	0	5	95
Augmentin	30	0	0	100
Amoxacillin	25	0	5	100
Ampicillin-Cloxacillin	10	0	0	100
Imipenem	10	30	25	45
Erythromycin	20	0	0	100
Gentamycin	10	5	5	90

R=Resistance, I=Index, S=Susceptible, %= percentage

The multiple antimicrobial resistance indices of the isolates were found to be above the acceptable 0.2 threshold value. *Salmonella enterica* were resistant to at least 5 of the 10 antibiotics, screened having a MAR index of at least 0.5 while *Shigella flexneri* were resistant to at least 7 of the 10 antibiotics screened having a MAR index of at least 0.7 (Table 6). *Shigella flexneri* presented the highest multidrug resistance, showing a MAR index of 1.00.

Based on 16S rRNA gene sequencing, bacterial species were taxonomically confirmed as *Shigella flexneri* and *Salmonella enterica* as shown in Plate 1.

Table 6: Multiple Antibiotic Resistance (MAR) Indices of *Salmonella* and *Shigella* Isolates

MAR Index	No. of Isolates (%)	
	Salmonella enterica	*Shigella flexneri*
0.1	0	0
0.2	0	0
0.3	0	0
0.4	0	0
0.5	10	0
0.6	10	0
0.7	15	20
0.8	30	60
0.9	35	15
1.0	0	5

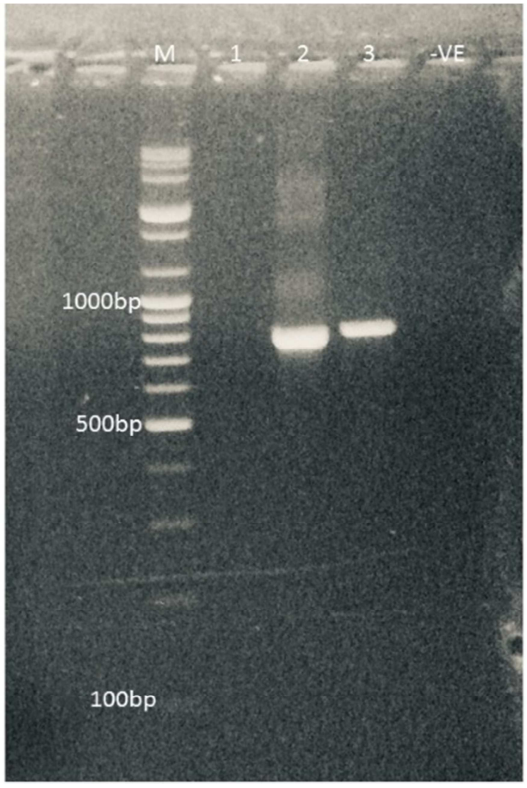

Plate 1: Agarose gel electropherogram after PCR amplification of 16S rRNA gene from diarrhoeic stool isolates. (M) Molecular Marker of 100bp; (2) *Shigella flexneri*; (3) *Salmonella enterica* (-ve) Negative control with no visible band.

DISCUSSION

Two hundred and sixty-four stool specimens collected from diarrheal patients were examined to determine the prevalence of *Shigella* and *Salmonella* isolates. Of the specimen examined, only 97 were positive for *Shigella* and *Salmonella* isolates giving a prevalence of 36.7%. The prevalence obtained from this research is higher compared to data from other researchers which showed 18.1%, 17.45% and 8.6% prevalence reported in a recent study in Ethiopia by (Teshome *et al.* 2019; Ameya *et. al.* 2018; Abera *et al.* 2021).

Similar reports of 27.3% *Shigella* cases followed by 17.7% *Salmonella* cases were also recorded in Indonesia (Tjaniadi *et. al.* 2003) and 8.6% by Muhammad *et. al.* (2021) in a study conducted in Bangladesh. A higher prevalence of 54% *Salmonella* and 30% *Shigella* was obtained in a study conducted in Egypt (Wafsy *et. al.* 2000).

Among the participants in the study, males were more affected with diarrhoea 162 (61.4%), compared to 102 (38.6%) who were females. This is in line with the work of Akinwumi *et al.* (2021), Begum and Ahmed (2013) and contrast to Ugboko *et al.* (2020) and Aye. *et al.* (2019) where they reported that female children were more infected (22.33%) than male children (18.33%). The reason for this difference may be that male children are more prone to infections than their female counterparts as reported by (Muenchhoff and Goulder 2014; WHO 2007). It's also possible that differences in disease incidence are due to environmental exposures that differ by gender and age. Boys have also been observed to be more active than girls at the age of the research, hence they could be exposed to more sources of infections than girls (Abdullahi *et al.* 2010). Boys are disproportionally afflicted by diarrheal sickness, and they are more likely to be hospitalized or die, according to epidemiologic data from the United States (Jarman *et al.* 2018).

Age group 0 – 2 had the highest number of samples collected which is 147 (59%) while age group 4 – 5 had the least number collected 38 (14.2%). The children aged 0 – 2 years were at high risk of developing diarrhoea compared to children aged 4-5. This follows the same trend with studies conducted in Ethiopia (Getachew *et al.* 2018; Melese *et al.*2019), India (Ahmed *et al.* 2008), Abuja (Ifeanyi *et al.* 2010), and Benue (Abdullahi *et al.* 2018). The high prevalence of diarrhoea at this age could be due to declining maternally acquired antibodies; bacterial infections tend to increase especially if the child's immunity is weakened or if they are malnourished (Alshehri *et al.* 2004), also, the introduction of weaning foods which may be unhygienic are introduced at this age and the children within this age group have not learnt the rudiment of adherence to aseptic or hygienic practice. The age range of 4 -5 years had the lowest frequency of bacterial isolates in this study. This is probably because of fewer tendencies to put contaminated objects into the mouth, this finding is consistent with that of Getachew *et al.* (2018). Most enteric pathogens stimulate at least partial immunity against repeated infection or illness, which helps to explain the declining incidence of disease in this age group (Melese *et al.* 2019).

In the present study, *Shigella* and *Salmonella* spp. demonstrated a much higher resistance rate against Ceftriaxone, Cefoxitin, Augmentin, Amoxacillin and Ampicillin-Cloxacillin (Ampiclox) (100%), this substantiates the fact that; these antibiotics used in treating a broader range of bacterial infections are the most administered antibiotic in children, thus suggesting an elevated use of this drug, and consequently resulting in high resistance rates and a severe threat to public health (Fair & Tor, 2014). An earlier study in Kaduna (Lawal 2017) also showed high resistance of *Shigella and Salmonella* to ampicillin, augmentin and gentamycin. These antibiotics are widely used to treat diarrhoea because of their low cost and ready availability (Fair and Tor 2014). The isolates were, however, most susceptible to imipenem (45%), indicating that imipenem is the most effective antimicrobial against the isolates.

Multiple antibiotic resistance index helps analyse health risks, as well as to check the extent of antibiotic resistance (Joseph *et al.* 2017). MAR index analysis has been used to differentiate isolates from different sources using antibiotics that are commonly used in the treatment of infectious cases. According to Thenmozhi *et al.* (2014), MARI values higher than 0.2 indicates existence from high risk contaminated sources with frequent use of antibiotics. It's

worrisome that 100% of the isolates collected in this study have a MAR index of 0.2 or above.

In principle, these findings reveal inappropriate use of antimicrobials in the region which poses a significant therapeutic setback and consequently, public health burden. Similarly high results of 0.63 MAR index were obtained in a study conducted in Oyo, southwest, Nigeria (Ayandele *et al.* 2019).

CONCLUSION

The prevalence (36.7%) of *Shigella* and *Salmonella* were high in the study area. The high prevalence and alarming rate of resistance to commonly prescribed antimicrobials are a serious public health problem in the study area. Continuous surveillance of antimicrobial susceptibility patterns should be done for choosing antimicrobials for empirical treatment. Imipenem can be a drug of choice for empirical treatment of infections caused by these pathogens. A significant number of the diarrhoeic isolates had a MAR index > 0.5 indicating previous exposure to antibiotics.

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