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ASIO Journal of Medical & Health Sciences Research (ASIO-JMHSR)

Volume 2, Issue 1; 2017: 19-24

DETECTION OF DRUG RESISTANCE DETERMINANTS IN SALMONELLA ENTERICA ISOLATED FROM STOOL SAMPLES IN A TERTIARRY HEALTH CARE CENTER

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ARTICLE INFO

Research Article History

Article Received: 08/02/2017
Article Published: 31/03/2017

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ABSTRACT

Enteric fever is a multi-systemic disease caused by members of the group Salmonella enterica. This disease is posing a serious threat to public health as it is linked to unsanitary practices and personal hygiene techniques. This study was screened for Salmonella enterica using conventional and serological techniques. Two hundred and twelve (212) stool samples were collected from patients attending a Federal Medical center. The samples were screened for Salmonella using conventional cultural, serological and molecular methods. Six (6) isolates of Salmonella were identified. Standard disc diffusion method was used to determine the antibiotic resistance pattern of the isolates. Salmonella isolates shows high percentage of resistance against antibiotics with Gentamicin (100%), Nitrofurantoin (100%), Augumentin (83.3%), Cotrimoxazole, (83.3%) but sensitive to Ceftazidime (66.7%) and ofloxacin (83.3%). Others show varying percentage of resistance and susceptibility. Multiplex PCR technique was used in detecting resistance genes for the following standard typhoid fever drugs, ampicillin, chloramphenicol and ciprofloxacin. PCR amplification technique was used for amplification and detection of various drug resistance related genes found in the Salmonella isolates. The following genes were detected; tem (291bp) in 3 isolates, catP (636bp) in 2 isolates and gyrA (313 and 234bp) in all 6 isolates. This study confirmed the need for multistep diagnosis of enteric fever, so as to provide appropriate and effective treatment.

Keywords: Antibiotics Resistance Genes, Enteric Fever, Multidrug resistance, Salmonella, Typhoid Fever.

1.0. INTRODUCTION

Salmonella species are members of *Enterbateriaceae* family and have been divided into different subclasses. Only few are medically important like *Salmonella typhi, Salmonella paratyphi A, B and C* which causes Enteric fever. These organisms are noncapsulated, nonsporulating, facultative anaerobic bacilli, which have characteristic flagella, somatic and outer coat antigens [1]. Isolation and identification of *Salmonella* are very tedious and take several days before coming to the final conclusion. There has been great demand in terms of quick and sensitive detection of *Salmonella* from clinical settings and poultry in order to take timely therapeutic and prophylactic measures. Several PCR based assays have been developed for rapid detection of *Salmonella* sp [2,3]. Various serotype-specific PCR have also been developed for some common *serovars* to reduce time and cost in processing isolates by conventional serotyping which is very much labour intensive and time-consuming [4].

In the past few decades, emergence of antibiotic resistance among different species of bacteria is increasing [5]. Irrational use of antibiotics as growth promoters in poultry feeds and unprescribed taking of drugs are important factors that have favoured the selection of resistant bacteria in faecal microflora of man and poultry [6]. These resistant strains are easily passed to human through food chains resulting in serious consequences in terms of treatment failure and rapid outbreaks of resistant *Salmonellae*.

Doi: 10.2016-74831794; doi link: http://doi-ds.org/doilink/03.2017-18533989/

In many countries where sanitation is poor, typhoid and paratyphoid fevers, which are transmitted via the faecal-oral route, are major causes of gastric illness [7,8]. Sanitation and hygiene are difficult to implement in many developing countries. Unfortunately, the effectiveness of antimicrobial chemotherapy is also being eroded by the emergence of antibiotic resistance [9]. Non-typhoidal human *Salmonella* diarrhoea does not warrant antimicrobial therapy; however, there are occasions when the infections can lead to life-threatening systemic infections that require effective chemotherapy [10]. Increasing concern is the worldwide emergence of multidrug-resistant phenotypes among *Salmonella* serotypes, in particular *S. typhimurium and Salmonella typhi* [8,11]. In Africa and most other developing regions, multidrug resistance, particularly to commonly available antibiotics, remains a major challenge for the health system [8]. Inadequate sanitation to prevent strain dissemination and over-the-counter distribution of antimicrobials can exacerbate *Salmonella* infection.

In their study, Karuiki *et al* [8] documented multidrug-resistant *S. typhimurium* as the predominant cause of community-acquired bacteraemic illness in both children and in adults. They observed non-typhoidal infection as well as multiple resistant isolates to commonly available antibiotics, including ampicillin, chloramphenicol, cotrimoxazole and tetracycline [8]. In developing countries such as Nigeria, the antibiotics most readily available for treatment of typhoid fever are ampicillin, chloramphenicol and Cotrimoxazole. The emergence of Multi-Drug Resistance (MDR) isolates to chloramphenicol. Ampicillin and Cotrimoxazole has greatly complicated disease management [12].

The aim of the study was to determine the antibiotic susceptibility patterns of isolates of *S. enterica* and detection of antibiotics resistance genes in *Salmonellae* in order to understand the rational use of antibiotics in Bayelsa state, Nigeria.

2. MATERIALS AND METHOD

Two hundred and twelve (212) stool samples were obtained between 2011 to 2013, from patients presenting with fever in a tertiary health care center in Yenagoa metropolis. The samples were screened for Salmonella enterica. The study identified six isolates using conventional laboratory techniques. Preliminary identification includes gram stain, test for motility, oxidase, indole urea and citrate test. Serotyping was done with respective monovalent antisera (Salmonella sero-group kit from Statens Serum Institute, Denmark) ranging from A-G and Vi antisera to confirm the serotype belonging to Salmonella enterica.

2.1. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was determined following the Kirby and Bauer disk diffusion method, in accordance with the clinical and laboratory standards institute (formerly National committee for clinical laboratory standards), using commercially available antimicrobial disks Abtec Biological Ltd [13]. Three to five colonies of the organism were inoculated into a tube containing tryptic soybroth and incubated overnight at 37°C. Standardization of the inocula was performed by diluting the broth cultures until turbidity matched the 0.5 McFarland standards. A sterile cotton swab was dipped into the standardized suspension, drained, and used for inoculating Mueller-Hinton agar, and antibiotic disks were placed on the agar using sterile forceps, gently pressed down to ensure contact. Antibiotics used were Ceftazidine (30µg), Cefuroxime (30µg), Gentamicin (10µg), Cefixime (5µg), Ofloxacin (5µg), Augumentin (30µg), Nitrofurantoin (300µg), Ciprofloxacin (5µg), Chloramphenicol (10µg), Ampicillin (25µg), Cotrimoxazole (25µg).

2.2. Amplification and Detection of catP, gyrA and tem Genes in *Salmonella enterica*

DNA Extraction was carried out on the samples using Bacteria DNA Preparation Kit (Jena Bioscience, Germany). The purity and concentration of the extracted DNA was evaluated using a NANODROP (ND 1000) Spectrophotometer (Thermo Scientific, USA). All the samples showed a DNA yield between 65 and 120ng, and the extracted DNA was optimally pure showing between 1.60 and 1.90.nm. A regular multiplex PCR was carried out using the primer pairs A (F) GCA CGA GTG GGT TAC ATC GA, A (R) GGT CCT CCG ATC GTT GTC AG, Cip (F) TAC CGT CAT AGT TAT CCA CGA, Cip (R) GTA CTT TAC GCC ATG AAC GT, ASRC(F) ATG GAG AAA AAA ATC ACT GG, ASRC (R) AAT TCA TTA AGC ATT CTG CCG AC and A nested multiplex PCR was also carried out using the primer pairs ASNC(F) CCG TTG ATA TAT CCC AAT GG, ASNC(R) CTG GTG AAA CTC ACC CAG GG, ASNT(F) GCT GGA TCT CAA CAG CGG TAA G, ASNT(R) CTG ACA ACG ATC GGA GGA CC, Cip (F) TAC CGT CAT AGT TAT CCA CGA, Cip (R) GTA CTT TAC GCC ATG AAC GT, ASNG(F)TGG GCA ATT TTC GCC AGA CGG, ASNG(R) ACT AGG CAA TGA CTG G. The PCR was performed in a 20µl reaction mixture containing 1X Hot FirePol Blend Master mix Buffer (Solis Biodyne, Estonia), 2.0 mM $MgCl_2$, 200µM of each deoxynucleoside triphosphates (dNTP) (Solis Biodyne), 20 pMol of each primer, 2 unit of Hot FIREPolDNA polymerase (Solis Biodyne), Enzyme, 2µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in an Eppendorf Vapo protected thermal cycler (Nexus Series, USA) for an initial denaturation of 95°C for 15 minutes followed by 35 amplification cycles of 30 seconds at 95°C; 30 seconds at 61°C, and 1 minute at 72°C. This was followed by a final extension step of 10 minutes at 72°C. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethidium bromide staining. 100bp DNA ladder (Solis Biodyne) was used as DNA molecular weight marker.

3. RESULTS

The two hundred and twelve (212) stool samples screened, 6 isolates were obtained belonging to the *serovarenterica*.

3.1. Antimicrobial susceptibility testing

The bacterial susceptibility patterns to the antibiotics are presented in Table 1. The result shows the multi drug resistant (MDR) pattern of salmonella isolates in this study.

Doi: 10.2016-74831794; doi link: http://doi-ds.org/doilink/03.2017-18533989/

Table 1: Results showing the zones of inhibition of antibiotics agents against *Salmonella* enteric isolated measured in millimeter

Antibiotics	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6
Oflaxacin	0	16mm	13mm	13mm	16mm	14mm
Augmentin	0	9mm	0	0	0	0
Nitrofurantoin	21mm	20mm	25mm	25mm	21mm	23mm
Ciprofloxacin	0	18mm	0	0	0	17mm
Ceftazidime	9mm	20mm	29mm	29mm	26mm	22mm
Cefuroxime	0	17mm	20mm	20mm	0	21mm
Gentamicin	0	0	0	0	12mm	0
Cefixime	15mm	14mm	16mm	16mm	26mm	0
Chloramphenicol	18mm	21mm	10mm	10mm	21mm	0
Cotrimazole	11mm	0	16mm	16mm	9mm	0
Ampicillin	0	0	0	0	19mm	16mm

The antibiotic profile of *Salmonella* spp on the following antibiotics were presented; Ofloxacin (16.7%- resistance and 83.3% sensitive), Augmentin (83.3% resistance and 16.7% sensitive), Nitrofuratoin (100% resistant), Ciprofloxacin (66.7% resistance and 33.3% sensitive), Ceftazidine (33.3% resistance and 66.7% sensitive), Cefuroxime (50% resistant and 50% sensitive), Gentamicin (100% resistant), Cefixime (66.7% resistant and 33.3% sensitive), chloramphenicol (50% resistant and 50% sensitive), Cotrimazole (83.3% resistant and 16.7% sensitive) and ampicillin (50% resistant and 50% sensitive). The highest resistance was 100% for Gentamicin and Nitrofurantoin on the other hand highest susceptibility was observed for ofloxacin and Ceftazidine with values as 83.3% and 66.7% respectively. While the other antibiotics exhibited varying degrees of susceptibility and resistance.

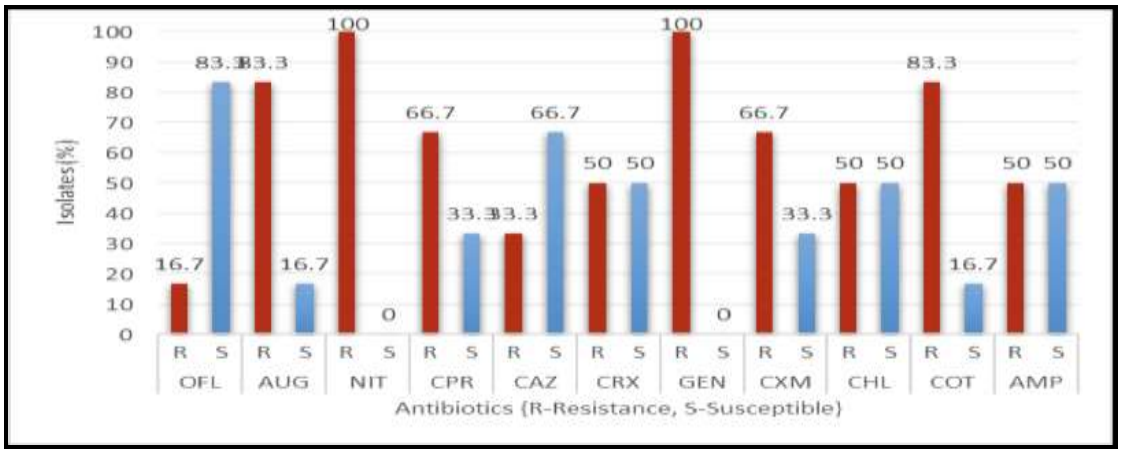

Figure 1: Results (in percentage) showing antibiotics resistance pattern of *Salmonelaenterica*

Doi: 10.2016-74831794; doi link: http://doi-ds.org/doilink/03.2017-18533989/

3.2. Amplification and Detection of Catp, Gyra and Tem Genes In*Salmonella Entericaserovar*

Out of the 6 isolates that were analyzed for the presence for catP, gyrA and temgene, regular multiplex PCR gave discrete bands of 636 bps for *catP*, 311 bps for *tem* and 313bps for*gyrA*. However, a problem was encountered regarding the amplification of *gyr*A and *tem* genes as the bands representing *tem* (311 bps) and *gyrA*(313 bps) genes overlapped and were indistinguishable. The presence of each of them was confirmed by omitting the other. As this PCR was followed by a nested multiplex PCR that would provide the final result, this overlapping was not a problem as primers could be designed to have discrete products in next stage. Results of the next stage were a nested multiplex PCR with separated bands for each gene. Different bands of 436 bps (*catP*gene), 313 and 234 bps (*gyrA*gene) and 291 bps (*tem* gene) were obtained. The results show*tem* (291bp) was seen in Is3 isolates, *catP*(436bp*)* was seen in Is2 and *gyrA*(313 and 234bp) was seen in all 6 isolates.

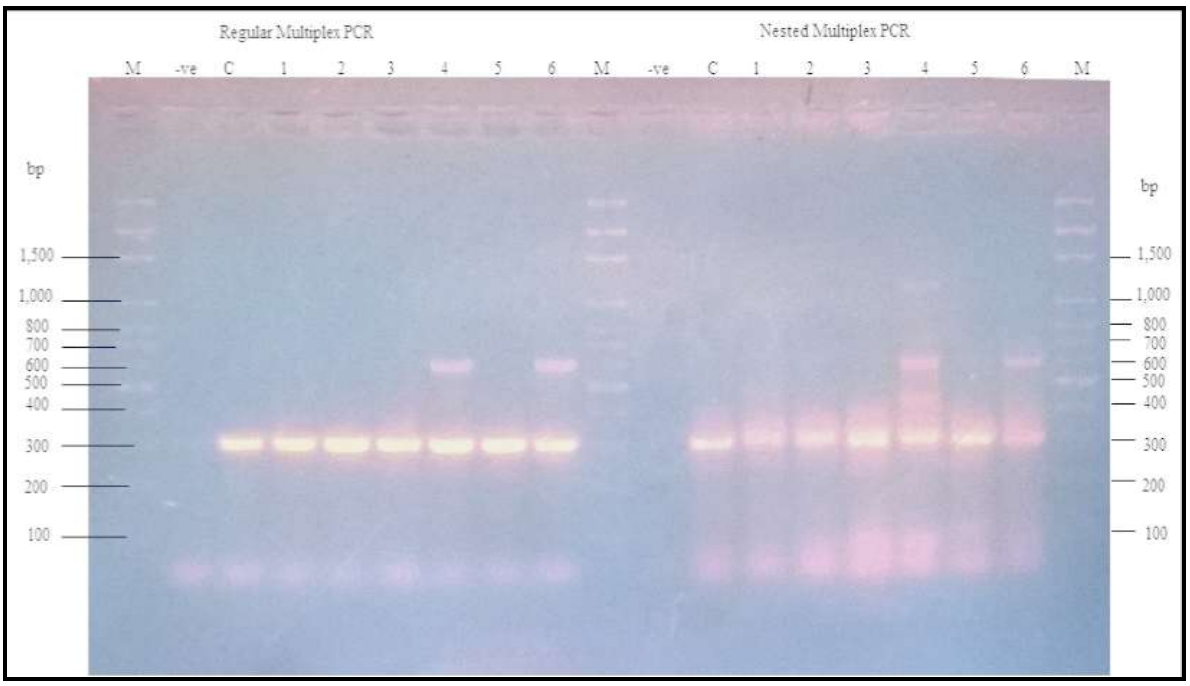

Figure 2; Multiplex PCR showing the amplicons of *catP, gyrA&tem* genes *(CatP; Chloramphenicol (436bp), gyrA; Ciprofloxacin (313 and 234bp), tem Ampicillin (291bp)*

4. DISCUSSION

This study observed a high percentage of resistance of *Salmonella* enterica isolates to antibiotics such as Nitrofurantoin (100%), Gentamicin (100%), Augumentin (83.3%), Cotrimoxazole, (83.3%), Ciprofloxacin (66.7%) and Ampicillin (50%). Similar finding was also observed in a study by Adeshina *et al* [14] Nitrofurantoin had the highest percentage of Resistance (100%) and susceptibility by ofloxacin (83.3%). The ineffectiveness of ampicillin, chloramphenicol, gentamicin, ofloxacin, ceflaximide, polymixin B, carbencillin, and tetracycline against *Salmonella enterica* has been previously reported by Adachi *et al* [15] and Filioussis *et al* [16]. The varying pattern of microbial isolates among different studies emphasized the need for surveillance to evaluate and monitor periodically the changing pattern of the microflora especially in a hospital setting [17]. The emergence and continual increase in the multiple drug resistance, early determination of drug resistance pattern along with timely diagnosis of typhoid fever has become a matter of vital importance. Multidrug resistance is directly linked to severity of enteric fever. The association has been ascribed to incorrect initial treatment and thus further progression of the disease and later presentation to hospital [11,18]. The frequency of resistance in *Salmonella enterica* has increased dramatically, presumably due to the extensive use of antimicrobial agents which has also led to the emergence of multidrug-resistant (MDR) strains. There has been an increasing concern about the prevalence of MDR *S. typhi* strains that are insusceptible to chloramphenicol, ampicillin, and trimethoprim [19, 20]. In a multivariate analysis, drug resistance was associated independently with higher bacteremias. This suggested that the multidrug-resistant phenotype may be associated with virulence in *S. typhi* [21].

In this study, the results were largely comparable to global trends regarding efficacy of ampicillin and ciprofloxacin. Resistance to ampicillin was particularly notable. Just a single isolate was fully susceptible to ampicillin (TABLE 1). The presence of resistance genes regarding chloramphenicol and ampicillin (fig 1), at such a high level in local population indicates that these drugs are still largely used despite of their inefficacy.

Quinolones are broad-spectrum antimicrobial agents that have been used widely in clinical medicine. During 1990's, quinolones especially ciprofloxacin became the drug of choice in the treatment of MDR typhoid, but resistance to quinolones has increased remarkably in certain areas of the world recently [22, 23].

Doi: 10.2016-74831794; doi link: http://doi-ds.org/doilink/03.2017-18533989/

In this study, the results were largely comparable to global trends regarding efficacy of ampicillin and ciprofloxacin. The presence of this resistance genes for chloramphenicol and ampicillin (fig 2), at such a high level in local population indicates that these drugs are still largely used despite their inefficacy. Even though four isolate representing Isolate resistance to ciprofloxacin could be found it's still the choice of drug for the treatment of enteric fever because it is widely used.

Resistance to a number of antibiotics by *Salmonella enterica* has become a serious problem as shown in the results obtained, which records resistance to most of the test antibiotics. Similar resistance pattern had been reported by Doughari *et al* [24]. In an earlier study by Doughari *et al* [24], Ciprofloxacin was the most sensitive drug, but the findings in this study it was resistant. This could be due to development of resistance as a result of misuse of this antibiotic in disease conditions [25].

5. CONCLUSION/RECOMMENDATION

Consequently, upon findings from this study; there were MDR *Salmonella* isolates identified, therefore we recommend the reevaluation of the choice of drugs and the rational use of antibiotics in Nigeria for the treatment of typhoid fever. The results of this research also revealed that the antibiotic resistance in *Salmonella* is a significant problem and therefore recommend a continuous Surveillance and monitoring of antimicrobial resistance patterns within study location. The study also recommends more elaborate research into the different serogroups of *Salmonellae* with a view to understanding their epidemiology, modes of transmission, best approach for diagnosis, treatment and methods of control.

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Doi: 10.2016-74831794; doi link: http://doi-ds.org/doilink/03.2017-18533989/

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Doi: 10.2016-74831794; doi link: http://doi-ds.org/doilink/03.2017-18533989/