Asian Journal of Pharmaceutical and Health Sciences
www.ajphs.com

Detection of mutations in *gyrA* gene that codes for point mutation in Floroquinolone resistant *Salmonella enterica* serotypes isolated from a hospital in south east Nigeria.

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ARTICLE HISTORY

Received: 06.07.2012

Accepted: 08.08.2012

Available online: 10.11.2012

Keywords:

Quinolone,GyrA, ParC, *Salmonella enterica serovar*, mutation.

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ABSTRACT

A total of 55 stool samples were collected, out of which 25 clinical isolates of *Salmonella enterica* serotypes from the Hospital in the South east Nigeria were investigated on for β-lactamase enzyme production and the presence of mutation in the gyrase A gene, an enzyme that codes for point mutation and resistant in quinolone, using phenotypic and molecular methods. The presence of β-lactamase was revealed using Nitrocefine sticks and Double disc Synergy Test was used to detect ESBL'S positive Isolates. Analysis reveals that all the *S.enterica* had somatic and flagellated antigenic properties, out of which 13(52%) of the isolates were ESBL's positive, while 20(80%) of *S.enterica* serovars produced β-lactamase enzyme and 5(20%) were negative for β-lactamase. The polymerase chain reaction revealed the absence of double mutation in parC gene and also the presence of point mutation in gyr A gene in the DNA chromosome of 9(36%) of the isolates. From this study Levofloxacine and Ceftriaxone still retains the potency as the drug of choice for Salmonellosis as ciprofloxacin had increased MIC's of 3.125µg/ml. This is first report of detection of mutation in Gyr A enzyme in *S.enterica* , that codes for point mutation in the Quinolone Resistant Determining Region(QRDR'S) in Southeast Nigeria.

INTRODUCTION

Typhoid fever is a systemic infection caused by *Salmonella enterica* serotype *typhi*. This is a highly adapted human specific pathogen and possesses remarkable mechanism for persistence in host [1]. Most of the disease burden occurs in developing countries due to poor sanitary conditions [2]. Antimicrobial drug resistance has become increasingly common in *S.enterica*, which can complicate therapy [3]. Formerly classified as separate species, DNA hybridization studies have now shown that all pathogenic Salmonellae belong to a single species, *Salmonella enterica* which is subdivided into 7 subspecies (subsp). S. *enterica* subsp. *enterica* has over 2000 serovars which can cause disease in humans [4]. According to [5], the development of *S. typhi* strains that are resistant to antibiotics such as Co-trimoxazole, chloramphenicol and penicillins, historically used to treat *S. typhi* infection, has forced physicians to prescribe fluoroquinolones or third-generation cephalosporins. Fluoroquinolones are now recommended by most authorities for the treatment of typhoid fever. Unfortunately, resistance to first-generation fluoroquinolones is widespread in many parts of Asia **[4]** and Africa. According to [5], the data from their study shows that neither quinolone resistance nor third-generation cephalosporin resistance has emerged in Egypt. However, extrapolation from data in the literature suggests that quinolone resistance is likely to develop unless the use of this class drugs is restricted [5].

Quinolones act by binding to complexes that form between DNA and gyrase or topoisomerase and formation of this quinolone-enzyme-DNA complex that contains broken DNA, inhibits DNA synthesis. DNA gyrase is composed of two *GyrA* and two *GyrB* subunits,encoded by gyrA and gyrB [6,18] and topoisomerase by two ParC and two *ParE* subunits, encoded by parC and parE Type. Important mechanisms for quinolone resistance are mutations accumulating in the genes encoding DNA gyrase and topoisomerase *gyrA, gyrB, parC* and *parE* [6,7]. Fluoroquinolone resistance in *S. enterica* is usually mediated by at least one mutation in a DNA topoisomerase gene. At the moment the emergence of resistant strains to two major second

line drugs like ciprofloxacin and ceftriaxone is posing a major problem [8,9], especially in the Southeast part of Nigeria, as no documented report has been seen and for the fact that the *Salmonella* spp. has developed resistant to the first-line drug such as Chloramphenicol and Cotrimoxazole or Septrin. For these reason, this research was designed to find current drugs of choice for the treatment of typhoid fever caused by *S.enterica serovars* and possible resistant factors responsible for drug resistance in Nigeria, especially in the Southeast region where much data concerning its treatment and the possible drugs of choice have not really been visible or published.

MATERIAL AND METHODS

A total of fifty-five stool samples were collected ,out of which twenty-five(25) *S.enterica serovars* were isolated and screened from the several units in the hospital (during July- September, 2010). The clinical human isolates of the *Salmonella enterica* obtained from the Routine Section of the Medical Microbiology Laboratory in the Southeast part of Nigeria were identified by cultural, biochemical characteristics and serotyping using slide agglutination test, to differentiate their antigenic properties. The antibiotic- sensitivity screening was carried out by the multidisc agar diffusion method on 20ml molten Muller Hilton Agar(MHA) using these antibiotics disc(Oxoid, India); Co-trimoxazole (Septrin) 30µg, Chloramphenicol 30µg, Ciprofloxacin 10µg, Amoxicillin 30µg,Streptomycin 30µg, Levofloxacin 5µg, Ceftriaxone 30µg, Cefotaxime 30µg, Ceftazidime 30µg, Amoxicillin/Clavulanic acid 30µg [10]. The tube dilution method of broth was used to determine the MIC of the test antibiotic against the clinical strains by the two fold serial broth dilution procedure [10, 11].

Serotyping using Slide Agglutination

The slide agglutination antigen kit (Lab-Care Diagnostics, India) was used for this analysis. The cultured colony on a nutrient agar medium were isolated and inoculated on the grease free slide. A drop of physiological saline solution was introduced and the contents mixed using a mixing stick. The results were observed macroscopically after one minute, for auto-agglutinating strains after rocking the slide for 3060 seconds. This procedure was repeated simultaneously with a drop of the antiserum (Lab-Care Diagnostics, India). The O antigen suspensions were tested first in order to detect if they were positive, before the monovalent O antisera were performed. A positive result shows that the isolated bacterium possesses the antigen corresponding to the antiserum. The same procedures were subsequently carried out on the H antigen suspension [5].

Detection of ESBL's using Double Disc Synergy Test (DDST).

The test inoculum (0.5 McFarland turbidity) was spread onto Mueller-Hinton agar (MHA;Oxoid, India) using a sterile cotton swab. A disc of augmentin (20 µg amoxycillin + 10 µg Clavunalic acid) was placed on the surface of MHA; then discs of ceftriaxone CRO (30 µg),Ceftazidime CAZ (30 µg) and Cefotaxime CTX (30 µg) were kept around it in such a way that each disc was at distance ranging between 16 and 20 mm from the augmentin disc (centre to centre). The plate was incubated at 37 °C overnight. Distances between the discs were required to be suitably adjusted for each strain in order to accurately detect the synergy. The organisms were considered to be producing ESBL when the zone of inhibition around any of the Broad-spectrum cephalosporin discs showed a clear-cut increase towards the augmentin disc.

DNA Extraction, Quantification and Purity test

1.5ml of an overnight broth culture was pipetted into eppendorf tubes and centrifuged at 5xg. The supernatant was discarded and sterile water was introduced into the tubes and the mixture was shaken vigorously by a vortexing machine and decanted. This process was repeated twice. Then the mixture was resuspended in 100ul sterile distilled water, vortexed and placed in a water block to boil for 10-20mins at 100^{o}C. After boiling, the mixture was then centrifuged for 10 mins and the purified chromosomal DNA pellets were transferred into a new tube and stored on ice for further analysis. The spectrophotometer lens (Nano drop ND1000) was cleaned with sterile distilled water and 0.2uL drop of the DNA sample was added on the lens, closed and then clicked to measure on the computer screen and the readings was observed. Purity level is known to be between 1.5 - 1.8 or 2uL.

PCR Amplification and Agarose gel Electrophoresis

The purified DNA template in PCR eppendorf tubes (2.0ul DNA) was mixed with the Master mix ready to load (Solis Biodyne with 7.5mM $MgCl_2$) containing all the required contents as stated by the manufactures descriptions except water and the primers for the amplification. The mixture of primers, DNA and the Master mix was vortexed to mix and then centrifuged before introducing it into the PCR machine (Eppendorf-Germany).Then the machine is adjusted to annealing temperature and switch to start. After amplification has completed, the PCR products were then taken for electrophoresis (CBS.Scientific company Inc.) on the agarose gel at 80-100volts and finally viewed on the UV light for visible amplified image of the genes. A DNA-marker of 100base pairs was used.

The Polymerase Chain Reaction (PCR) were performed under the following conditions with the (Solis biodyne 5x FIREPol) Master mix Ready to load. The thermo cycling condition for Gyr A were 30 cycles of 95^{O}C for 30 secs, 42^{O}C for1 min, 72^{O}C for 1 min, and 95^{O}C for 30 sec,(PCR timing 12.38 - 2.34hrs.).

GyrA- F (5'CGT TGG TGA CGT AAT CGG- 3') (oligo number;00123839_3), R(5'CCG TAC CGT CAT AGT TAT- 3') (oligo number;00123839_4), while ParC is F(5'CTA TGC GAT GTC AGA GCT GG-3') (oligo number;00123839_5) ,R(5'TAA CAG CAG CTC GGC GTA TT- 3') (oligo number; 00123839_6). while ParC is F(5'CTA TGC GAT GTC AGA GCT GG-3')(oligo number;00123839_5) ,R(5'TAA CAG CAG CTC GGC GTA TT-3')(oligo number; 00123839_6) from Fermenters Inc.. The resulting amplicon sizes were 251 and 260 base pairs for *gyrA* and *par C* respectively.

RESULTS

The results of the twenty five clinical isolates of *Salmonella enterica* obtained from stool sample from both male and female patients were analyzed adequately. The antibiotics resistant results shows that; Amoxicillin/Clavulanic acid 30µg (92%), Amoxicillin 30µg (76%), Chloramphenicol 30µg (68%), Streptomycin 30µg (68%), Ceftazidime 30µg(64%), Co-trimoxazole (Septrin) 30µg(64%). The susceptibility results revealed Levofloxacin 5ug (88%) to be the most susceptible of the antibiotics followed by, Ceftriaxone 30ug(88%), Cefotaxime 30ug(84%),and Ciprofloxacin 10ug(76%) by the *S. enterica* isolates. The results of the Phenotypic resistant and molecular characterization of the twenty-five are as shown (Table 1.).

Analysis reveals that thirteen 13(52%) of the isolates were

Table 1. Sepidemiological distribution and phenotypic resistance pattern of s. Enterica serovars. In relation to β-lactamase enzyme and esbl's production in south-east nigeria.

CODE S/NO	NUMBER OF ANTIBIOTICS	RESISTANCE PHENOTYPIC PATTERN	BETA LACTAMASE PRODUCTION	PRESENCE OF ESBL'S
O1.	4	AM,STR,CAZ,AMC	+ve	
O2.	2	CH,AMC	+ve	
O3.	2	CH,AMC	+ve	ESBL
O4.	3	SXT,CH,CAZ	-ve	
O5.	6	SXT,CH,AM,STR,CAZ,AMC	+ve	ESBL
O6.	4	LEV,CTX,CAZ,AMC	+ve	
O7.	3	SXT,CH,AMC	+ve	
O8.	2	SXT,CAZ	+ve	
O9.	7	SXT,CH,CPX,AM,STR,CAZ,AMC	+ve	ESBL
O10.	6	SXT,CH,CPX,AM,CAZ,AMC	+ve	ESBL
O11.	7	SXT,CH,CPX,AM,STR,CAZ,AMC	+ve	ESBL
O12.	7	SXT,CH,CPX,AM,STR,CAZ,AMC	+ve	ESBL
O13.	5	SXT,CH,AM,STR,AMC	+ve	
O14.	9	SXT,CH,AM,STR,LEV,CRO,CTX,CAZ,AMC	+ve	ESBL
O15.	5	SXT,CH,AM,STR,AMC	+ve	ESBL
O16.	6	SXT,CH,AM,STR,CAZ,AMC	+ve	ESBL
O17.	6	SXT,CH,AM,STR,CAZ,AMC	-ve	ESBL
O18.	6	SXT,CH,CPX,AM,STR,AMC	-ve	
O19.	6	SXT,CH,CPX,AM,STR,AMC	+ve	
O20.	7	AM,STR,LEV,CRO,CTX,CAZ,AMC	+ve	ESBL
O21.	4	SXT,AM,STR,AMC	+ve	
O22.	3	AM,STR,AMC	-ve	
O23.	4	AM,STR,CAZ,AMC	-ve	ESBL
O24.	6	AM,STR,CRO,CTX,CAZ,AMC	+ve	ESBL
O25	4	CH,AM,CAZ,AMC	+ve	

ESBL's positive, while 20(80%) of *S.enterica* serovars produced B-lactamase enzyme and five 5(20%) were negative for B-lactamase as shown PhenotypicaIly(Table 1.). Polymerase Chain Reaction (PCR) results shows that 9(36%) of the isolates analysed for mutation in the gyrase gene , had the mutant gyrA gene that codes for the point mutation in the quinolone resistant determining regions and none of the *S.enterica* had *par*C gene, known as topoisomerase iv(coding for double mutation) in *S.enterica*. Out of the seven *S.enterica* that has the mutation in gyrA gene, two of the isolates were resistant to ciprofloxacine and only one was resistant to Levofloxacine. Other isolates having mutations in their gyrA genes were still susceptible to the floroquinolones, thereby indicating that the floroquinolone are still potent despite the existent of the mutation in gyrA gene and that only a single mutation in gyr A gene is not sufficient enough for clinical resistance to floroquinolones as revealed in (12).

DISCUSSION

Antimicrobial drug resistance has become increasingly common in *S.enterica*, which can complicate therapy according to [13]. A single point mutation gyrA confers partial resistance to floroquinolones. If a second gyrA point mutation is added, the resistance increases somewhat. However, a mutation in parC added to a single gyrA mutation confers full *in vitro* resistance to first-generation fluoroquinolones. Based on the type and nature of the resistance found on the floroquinolone used in this study ,these analysis also reveals the region of resistance in the Gyrase DNA of the organism and possibly the position of the amino acids

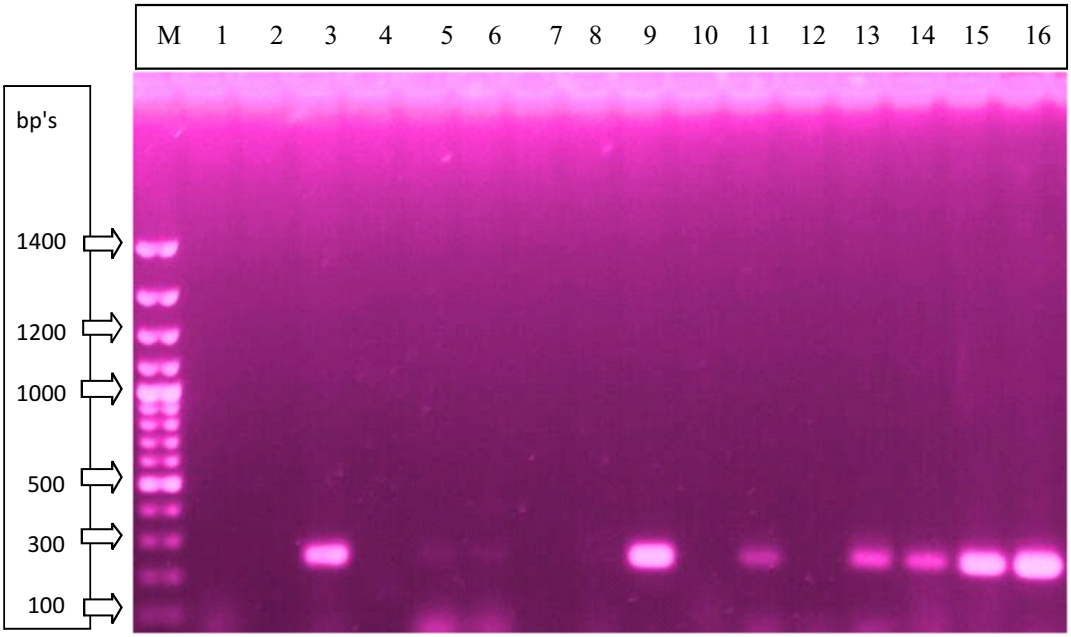

Fig.1: Agarose gel electrophoresis pattern showing single PCR amplification products of GyrA genes showing mutations in from S.enterica serotypes. Lane 1- 16 shows isolates from the hospitals. The amplicons on lane 3,5,6,9,11,13,14, 15 and 16 shows positive GyrA(point mutation) genes present in the DNA of S.enterica serotypes with amplicon size of 251bps . Lane M shows DNA molecular marker (100- bp ladder).

either at the Ser-83 or the Asp-87 codon ,might be the reason for the type of resistant. Out of the Nine *S.enterica* that has the mutation in gyrA gene two of the isolates were resistant to both ciprofloxacine and Levofloxacine. Other isolates having mutations in their gyrA genes were still susceptible to the floroquinolones, which still reveal that the floroquinolone are still potent despite the existent of the mutation in the gyrA gene and that only a single mutation in gyr A gene is not sufficient enough for clinical resistance to floroquinolones as revealed in [12]. This may be due to the fact that the mutation in the parC gene that codes for double mutation in the chromosomal DNA of the *S.enterica* were not present in the isolates studied, but the presence of the mutation in gyr A is a good marker indicating that floroquinolones should not be used or chosen for treating the respective infection either caused by *S.enterica* or otherwise. One of the *S.enterica* was found to be resistant to nine out of the ten antibiotics tested including levofloxacine with the exception of Ciprofloxacine which has increased MIC of 3.125µg/ml . According to [14], the pattern of *S. typhi* resistance is changing rapidly. Multi Drug Resistant *Salmonella Typhi* (MDRST) and strains resistant to ciprofloxacin and ceftriaxone are a major threat in developing world, most likely in the Southeast Nigeria where less documentation of patients data as regards to drug resistance are in the increase.

Clinically, these resistant strains may fail when treated with a second-generation fluoroquinolone such as ciprofloxacin. [15] In this study a third- generation floroquinolone was used and analysis shows that it had the highest susceptibility rate as that of Ceftriazone (88%), a third generation Cephalosporin. Infact Levofloxacine(88%) had higher rate of susceptibility than Ciprofloxacine(76%). This report correlates the work of (17), when they reported decreased sensitivity to ciprofloxacin and they suggested possible alternatives as ceftriaxone and cefotaxime, and reassured that organisms were fully susceptible to these drugs higher in both partially and fully resistant strains than in fully susceptible strains [4]. Also it may be surprising to know that Cefotaxime had a good susceptibility on *S.enterica* in this study, as 52% of the isolates were found to be extended spectrum β-lactamase.

CONCLUSION

According to [17], when looking for reasons for the rapidly increased of quinolone resistance in travelers' Salmonella isolates, three issues must be considered, such as; transferable resistance, mutational resistance, and clonal spread. In this study analysis revealed that apart from plasmid which may be transferable from one place or the other , gyr A, an enzyme that codes for point mutation in floroquinolones is one of the factors discovered to be the cause of resistance in *S,enterica* serovars isolated in this hospital. In this study, 12% of the isolates were at least resistant to two antibiotics out of the 10 antibiotics tested in this study, 16% of the isolates were resistant to 4 antibiotics, while 28% were resistant to 6 antibiotics, which mean that there are still multidrug resistant *Salmonella* Isolates in this region. Thus, either clonal spread or resistance due to mutations in chromosomal genes remains the potential mechanism accounting for the high level of reduced fluoroquinolone susceptibility in Southeast Nigeria, as also reported in South Asia by [16]. The emergence of mutation-based resistance may be fostered by selection pressure caused by the use of antimicrobial agents in either human medicine or agriculture. Accordingly, the alarming increase in floroquinolone resistance observed during the past few years among food borne pathogens has aroused speculation that this might be an effect of the use of quinolones in animal husbandry as reported by [18].Thus care should be taken in order not to be transmitting this resistant factors in our daily activities, either in the hospital or farming activities. It is also recommended that these drugs should be administered with utmost care and caution

to avoid further resistant. Furthermore prescriptions drugs should be restricted to only Medical personnel such as the Pharmacist, Physicians and other paramedical such as the Microbiologist and the Biochemist for appropriate dispensing. Finally, hand washing in the hospital or at home is also recommended as a useful, safe and aseptic technique to patients to prevent diseases such as Typhoid fever caused by *Salmonella enterica* species. [15]

ACKNOWLEDGEMENT

I am grateful to the management and staff of the Nigerian Institute of Medical Research (NIMR), Yaba Lagos, especially Dr S.I Smith and Fowora for the facilities provided for this work, and the authorities of the teaching hospitals for their assistances. Also my special thanks goes to the Management and staff of the Federal Medical centre Owerri for support.

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