Mutations in the Quinolones Resistance Determining Regions of *gyrA* in Nosocomial *Staphylococcus aureus* and *Salmonella typhi*

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Abstract

In Nigeria, *Staphylococcus aureus* and *Salmonella typhi* are common causes of human infections and are also recognized as pathogens of public health significance. This study therefore, sought to determine the incidence and extent of fluoroquinolones resistance of *S. typhi* and *S. aureus* isolated from patients in Nigerian Defence Academy Hospital. A total of 60 samples obtained from patients with request for stool microscopy, culture and sensitivity, wound swabs, indoor air of surgical wards and swabbing of working benches were analyzed for the presence of *S. aureus* and *S. typhi*. The bacterial isolates were then subjected to antibiotic sensitivity testing using a modified Kirby-Bauer disk diffusion method. The antibiotic susceptibility patterns of *S. typhi* revealed that some of the isolates were resistant to two or more fluoroquinolones namely: ciprofloxacin, sparfloxacin, ofloxacin and pefloxacin. *S. aureus* on the other hand, also showed resistance to fluoroquinolones. The isolates that showed resistance to more fluoroquinolones were taken for molecular analysis. The genomic DNA was extracted and amplified using specific primer for *gyrA* by PCR, visualized using agarose gel electrophoresis and then sequenced. The amplicon sizes were 251bp respectively for each of the isolates. The detection of resistant pattern responsible for fluoroquinolones resistance showed that mutation had occurred. Mutation in nucleotide sequence was detected in *gyrA* gene of the fluoroquinolone resistant strains.

Keywords: Agarose Gel Electrophoresis, Fluoroquinolones, *gyrA* Gene, Mutation, *S. aureus*, *S. typhi.*

Introduction

Health care facilities such as hospitals, rehabilitation units and outpatient departments are places where people seek attention from their medical problems. Nosocomial infection is a global problem, affecting 6% of hospitalized patients and in some clinical services such as intensive care units, up to 5% of the patients are infected (Madigan *et al*., 2000). Over two million nosocomial infections are usually recorded each year worldwide and only few organisms

cause majority of the infections at several sites (Lark, 2001).

The most frequent infections are from surgical wounds, blood, urinary and gastro-intestinal tract infections. Factors that enhance the spread of nosocomial infections include weak immunity and the increasing variety of medical and surgical routes of infection (for example, patients with open wounds or tube in their body) as well as transmission of drug-resistant micro-organisms among crowded hospital populations where poor infection control practices exist (Amita *et al*., 2003). Reports have implicated *Staphylococcus aureus, Escherichia coli* and *Salmonella typhi* as the causative agents of hospital acquired infections (Lark, 2001). These organisms can grow at different temperatures and pH conditions in the hospital environment, and in addition, are able to exploit varieties of carbon and energy sources. These characteristics explain the ability of these pathogens to survive for a reasonable time in either dry or moist conditions in the hospital environment, causing infection (Beaudin *et al*., 2004).

Salmonella typhi on the other hand, causes typhoid fever (Mirza *et al*., 2006). The human specific pathogen has evolved some mechanisms for persistence in its host that ensure its survival and transmission (Parry *et al*., 2005). The global estimates for typhoid fever are 2.1 million episodes annually with 216 000 deaths (Crump *et al*., 2004).

Human beings are the carriers of *S. typhi* and *S. paratyphi* without any environmental reservoirs (Mirza *et al*., 2006). In third world countries, great reliance is placed on antibiotic chemotherapy in the treatment of typhoid fever because of the difficulties in preventing typhoid fever due to inadequate public health awareness.

Efforts to control diseases caused by nosocomial *S. aureus* and *S. typhi* using antibiotics have resulted in increased resistant strains of these organisms (Levy, 1998). Therefore, in order to effectively treat infections caused by these two pathogens, culture and antibiotic sensitivity tests should first be conducted. Once culture and sensitivity results confirm bacterial infection and sensitivity pattern, treatment may be modified (Paterson, 2000).

Materials and Methods

Sampling Technique

A multi-staged sampling technique was employed for isolation of *S. typhi* while *S. aureus* was isolated from wound swabs, indoor air of surgical wards using pour plate technique and swabbing of working benches and door handles.

Molecular Characterization of Species of Bacteria

The bacterial species that were biochemically characterized were subjected to strain level identification using molecular approach. These involve extracting their DNA using standard protocols, amplifying the DNA using PCR and sequencing the gene of interest using next generation sequencing (Hakanen *et al*., 2001).

Detection of Mutation in gyrA Gene of the Bacterial Isolates

The genomic DNA of *Staphylococcus aureus* and *Salmonella typhi* that was extracted was subjected to PCR searching for *gyrA* gene. The PCR was operated based on the following conditions: pre-denaturation (94°C for 5 min), 30 cycles of denaturation (94°C for 30 sec), annealing temperature was 48°C for 30 sec, extension was 72°C for 30 sec and final extension was 72°C for 5 min using Perkin Elmer PCR machine. 20 reaction mix tube was used and the reaction volume was 20µl.

The PCR product was ran in 1.5% agarose gel electrophoresis stained with ethidium bromide and viewed under ultraviolet for visible amplified image of the genes. A DNA marker of 100 base pairs was used. The specific primers used to amplify the quinolones resistance determining region (QRDR) were designed from accession number sequences X78977 and M68936 as follows: *gyrA*- Forward reaction 5^1(CGT TGG TGA CGT AAT CGG)-3^1 Reverse 5^1(CCG TAC CGT CAT AGT TAT) $3^{1.}$

1.

Results

PCR Amplification of Genomic DNA (gyrA Gene) from *S. typhi* and *S. aureus* that was Resistant to Fluoroquinolone

Agarose gel electrophoresis pattern showing single PCR amplified products of *gyrA* from *S. typhi* and *S. aureus* with resulting amplicon size of 251 base pair bands for both *S. typhi* and *S. aureus.* A 100 base pairs molecular marker was used as shown in Figure

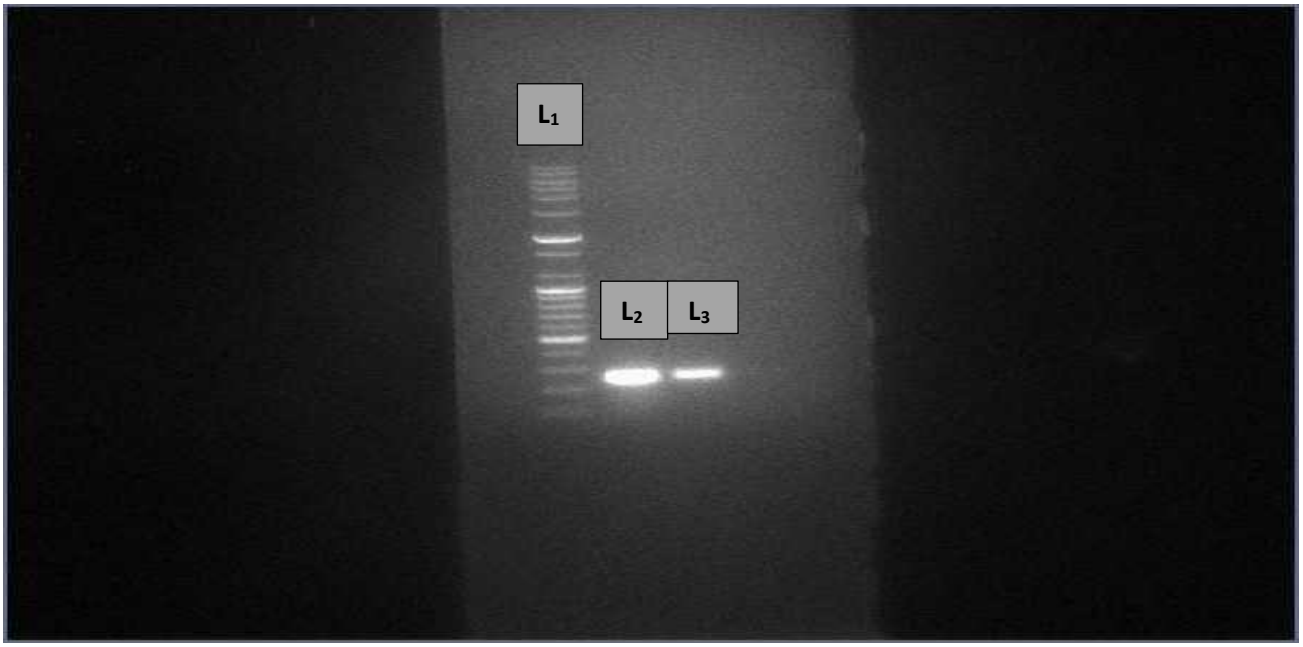


Figure 1: Agarose Gel Electrophoresis Pattern showing PCR Amplified Products of *gyrA* from *S. enterica serovar typhi* and *Staphylococcus aureus*.

KEY: L1 – DNA Marker, L2 – *Salmonella typhi*, L3 – *Staphylococcus aureus.*

DNA Sequencing

The PCR products containing the gene of interest were selected for DNA sequencing with the same primer sequence.

The polymerase chain reaction analysis shows that *S. enterica serovar typhi and S. aureus* possessed mutation in the *gyrA* gene. The outcome of the DNA sequence of the PCR product of *S. typhi* and *S. aureus gyrA* gene was given as shown below (Figure 2 & 3):

Salmonella typhi 1
TCAGTTCATGGGCAATTTTCGCCAGA
CGGATTTCCGTATAACGCATTGCGCC
GAGAGTCGCCGTCGATGGAACCGAA
GTTACCCCGGCCCTCTACCAGCATGT

AACCCAGGGGGAAGGGTGGCGCCAT ACTGACTATCCTGTCATAACCCGCAA. The outcome of the DNA sequence was blasted using the National Centre for Biotechnology Information database and the result was as follows:

Sequence ID: lcl|Query_32315Length: 563Number of Matches: 1
Related Information

Range 1: 239 to 392Graphics Next Match Previous Match
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
200 bits(108)	3e-56	139/154(90%)	2/154(1%)	Plus/Minus

```
Query  1    TCAGTTCATGGGCAATTTTCGCCAGACGGATTTCCGTATAACGCATTGC-GCCG-AGAGT  58
            ||||||||||||||||||||||||||||||||||||||||||||||||| |||| |||||
Sbjct  392  TCAGTTCATGGGCAATTTTCGCCAGACGGATTTCCGTATAACGCATTGCCGCCGCAGAGT  333

Query  59   CGCCGTCGATGGAACCGAAGTTACCCCGGCCCTCTACCAGCATGTAACCCAGGGGGAAGG  118
            |||||||||||||||||||||| | || |||||||||||||| ||| | ||| |
Sbjct  332  CGCCGTCGATGGAACCGAAGTTACCCTGACCGTCTACCAGCATGTAACGCAGCGAGAATG  273

Query  119  GTGGCGCCATACTGACTATCCTGTCATAACCCGC  152
             |  |||||||| ||| ||| |||||||| ||||
Sbjct  272  GCTGCGCCATACGGACGATCGTGTCATAAACCGC  239
```

Figure 2: Blast Sequence showing Mutation in *Salmonella typhi.*

From the above, there was removal of two bases in query one meaning that deletion mutation has taken place, and in query 59 and 119 there was exchange of single nucleotide for another meaning that substitution mutation has occurred as shown in Figure 2.

The outcome of the DNA sequence of the PCR product of *S. aureus gyrA* gene was given as shown below (Figure 3):

Staphylococcus aureus 1
TATAACGCATTGCTGCTGCCCATCTCCATCCATTGAACCAAGTACCTTGGCCATCAA CAAGCGATAACGATAACTGAATC
TTGAGCCATACGTACCATTGCTTCATAAATAGATGAGTCACCATGAGGTGATATTTA CCGATTACGTCACCAACG.

The result of the DNA sequence was blasted using the National Centre for Biotechnology Information database and the result was as follows:

Staphylococcus aureus strain N21OS gyrase subunit A (*gyrA*) gene, partial cds
Sequence ID: KX819742.1Length: 492Number of Matches: 1
Related Information
Range 1: 210 to 370GenBankGraphics Next Match Previous Match
Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
254 bits(137)	5e-64	154/161(96%)	6/161(3%)	Plus/Minus

Query 1 TATAACGCATTGCTGCTGC-CCATCTCCATCCATTGAACCAA-G-TACCTTGGCCATCAA 57
Sbjct 370 TATAACGCATTGCTGCTGCGCCATCTCCATCCATTGAACCAAAGTTACCTTGGCCATCAA 311

Query 58 CAAGCG-ATAACGATAACTGAA-TCTTGAGCCATACGTACCATTGCTTCATAAATAGATG 115
Sbjct 310 CAAGCGGATAACGATAACTGAAATCTTGAGCCATACGTACCATTGCTTCATAAATAGATG 251

Query 116 AGTCACCATGAGG-TGATATTTACCGATTACGTCACCAACG 155
Sbjct 250 AGTCACCATGAGGGTGATATTTACCCATTACGTCACCAACG 210.

Figure 3: Blast Sequence showing Regions of Mutation in *Staphylococcus aureus.*

From the above blast sequence, there was removal of three nucleotides in query 1 (deletion mutation), in query 58 there was removal of two nucleotides (deletion mutation) and finally in query 116 there was one exchange of nucleotide for another and removal of one nucleotide (Figure 3).

Discussion

Salmonella typhi isolates were obtained from stool samples and *S. aureus* from wound swab and swabbing of surgical wards. The presence of *S. typhi* in stool samples shows a carrier state among the patients. According to Singh (2001), carriers may shed *S. typhi* either through their stool or urine continuously or intermittently and the carrier state is usually the source of contamination. Carriers can serve as reservoirs or host for the dissemination of *S. typhi* causing public health problems.

The high yield of *S. aureus* from swabbing the surgical wards and wound swab is an indication that the wounds serve as a habitat for the organism (Prescott, 2002) and could therefore be one of the major sources of *S. aureus* infection. *S. aureus* is a common cause of surgical wound or ulcer infections (Donkor *et al.*, 2008). Most cases of wound infection are acquired either through the hands of a health care worker who is colonized with *S. aureus* or from the patients' own reservoir (Sheretz *et al.*, 1996).

The presence of multiple-drug resistant strains of *S. typhi* and *S. aureus* among the

isolates may be attributed to two main reasons. Firstly, wrong use of antibiotics arising from self-medication in suspected bacterial infections (Newman *et al.,* 2006). Self-medication prevents early reporting of patients to hospitals at the onset of disease symptoms, except where complications had occurred (Brusch *et al.,* 2010). Secondly, either clonal and/or extrachromosomal resistant genes may be potential mechanisms for the level of multiple-drug resistance as noticed in this study. The implication of the multiple drug resistance recorded in this study is that efficacy of the relatively cheap empirical therapy for *S. typhi* and *S. aureus* infections could be jeopardized.

There have been reports from different parts of the world about resistance pattern of clinical isolates (Saha *et al*., 1999). In this study, most of the isolates were found to be resistant to fluoroquinolones namely: Ciprofloxacin, Sparfloxacin, Ofloxacin and Pefloxacin. Some of the isolates were susceptible to Septrin, Levofloxacin and Amoxicillin.

Thus, this trend of result supports the work of some authors when they stated that most reports from developing countries are showing MDRST strain and fluoroquinolones resistant strains (Saha *et al.,* 1999).

Many reports have been put forward by several authors (Brusch *et al*., 2010) that fluoroquinolones are highly effective against susceptible organism, yielding a better cure rate than Cephalosporin. They also stated that unfortunately, resistance to first generation fluoroquinolones is widespread in many parts of Asia (Brusch *et al*., 2010). Furthermore, they stated that in recent years, third generation Cephalosporins have been used in regions with high fluoroquinolones resistance rates, particularly in South Asia and Vietnam. It has been reported that fluoroquinolone resistance in *S. enterica* is usually mediated by at least one mutation in a DNA topoisomerase gene. However, in clinical human isolates of *Salmonella* spp., mutations are usually confined to *gyrA.* In this study, the mutations detected were found in *gyrA* genes in the chromosomes of the *S.enterica* serovars. This trend of results showed that there have been some forms of resistant mutants along the *S. enterica serovars'* family, and that the presence of mutations in *gyrA* gene of the isolates showed that the resistance to fluoroquinolone is evolving in an ominous direction as reported by Brusch *et al*. (2010).

gyrA mutation is a good marker indicating that fluoroquinolones should not be chosen for treating the respective infection (Randal *et al*., 2005).This implies that these patients in whom these isolates were recovered from, especially may have been on fluoroquinolone treatment for a long time (Hirose *et al*., 2002). Also reports have it that *S. typhi* most commonly develops fluoroquinolone resistance through specific mutations in *gyrA* and *parC*, which codes for the binding region of DNA gyrase and topoisomerase IV respectively (Brusch *et al*., 2010).

It has been stated that transferable resistant to quinolones is sometimes rare in bacteria in vivo, but clonal or resistance due to mutation in chromosomal gene remains the potential mechanisms accounting for high level of reduced fluoroquinolone susceptibility in Southeast Asia (Hakanen *et al*., 2001). These trends of results or reports are applicable to the analysis obtained in this study, as the mutations found were chromosomally mediated resistant gene.

In this study, the mutations that are responsible for the fluoroquinolone resistance in the *gyrA* of the *Salmonella enterica* serovars and *Staphylococcus aureus* were investigated and the sequences for the Quinolone Resistant Determining Region (QRDR) of the *gyrA* gene of the isolates which showed resistance to some fluoroquinolones were detected and analysis

revealed mutation. Sequence analysis also revealed that some of the positions of the nucleotides in the *gyrA* mutation were deleted and some others substituted.

This finding is in line with the work of Hirose *et al*. (2002) when they stated that, a single mutation at either the Ser-83 or the Asp-87 codon were found after sequencing the genes of *S. enterica serovar typhi* and *serovar paratyphi* A (Hirose *et al*., 2002). This indicates that *gyrA* mutations are of principal importance for the fluoroquinolone resistance of *serovars typhi* and *paratyphi* A among the *Salmonellae* (Hirose *et al*, 2002).

5. Conclusion

The emergence of antimicrobial resistance in any part of the world may have a global bearing and thus deserves universal attention. *Staphylococcus aureus* and *Salmonella typhi* were therefore characterized using conventional methods and confirmatory test. Their antibiotic susceptibility pattern was therefore determined using Kirby-Bauer method and the organisms showed high resistance to fluoroquinolones. Fluoroquinolones resistant trait of the organisms showed that mutation has taken place.

However, in this study, it could also be suggested that extensive use and abuse of fluoroquinolone in human diseases could be responsible for the rapidly increasing quinolone resistance of *Salmonella enterica* and *Staphylococcus aureus* in this part of Nigeria as observed. Therefore, prescription of these drugs should be done only by medical personnel and appropriate dispensing techniques should be adopted in every hospital to avoid under dosage or over dosage.

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Conflict of Interest

Authors have declared that no competing interests exist.

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