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Antibiotic Resistance Patterns of *Staphylococcus aureus* Isolated from Nostrils of Healthy Human Subjects in a Southeastern Nigeria Locality

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Abstract

Background. Antibiotic resistant bacteria have been a source of ever-increasing therapeutic problem with profound health and socioeconomic pressures. Hence, continued surveillance for bacteria susceptibility pattern is useful to determine the existing and future challenges of effective therapy.

Aim. This study was carried out to evaluate the resistance patterns of some community isolates of *Staphylococcus aureus* to some conventional antibiotics within a locality in southeastern Nigeria.

Material and methods. Preliminary characterizations of the plasmid DNA were also carried out.

Results. Here we show the results obtained, the resistances of the isolates to the antibiotics were recorded in the following order: Flucloxacillin > Cotrimoxazole > Cephalexin > Chloramphenicol > Tetracycline > Minocycline ~ Clindamycin > Gentamicin ~ Erythromycin > Amoxicillin-Clavulanic acid ~ Cloxacillin > Amoxicillin. The MIC results showed that the isolates were highly susceptible to Clindamycin but resistant to Tetracycline. Clindamycin was equally shown to exert a relatively higher bactericidal activity among five other antibiotics tested. Preliminary characterization of eight (8) isolated bacteria plasmids from eight resistant bacteria isolates gave mobilities (distances travelled) ranging from 3- 5 mm. These plasmids may be responsible for their observed high level of drug resistance. Early antibiotics susceptibility surveillance exercises therefore helps ascertain and generate a good framework for effective management and control of resistant and multiple-drug resistant strains.

Conclusion. The outcome of such surveillance exercises would both find usefulness in shaping the existing antibiotics prescription policies in order to achieve therapeutic endpoint and also help to slow down or prevent the emergence of multiple drug-resistant strains.

Introduction

Staphylococcus aureus is the cause of a wide range of pyogenic infections, though also a commensal of human skin and nares. It has emerged over the past several decades as a leading cause of hospital – and community – acquired infections (1). *S. aureus* has been found to be the most frequently isolated pathogen causing bloodstream infections, skin and soft tissue infections, and pneumonia (2-4). Staphylococcal infection leads to a worsening of some already existing superficial infections. Infection ranges from such superficial infection to deep infection as septicaemia, making *S. aureus* an important subject of consistent studies (5). Infection rate from *S. aureus* is high and the recent increased recognition of community acquired infections has important clinical and pharmacological implications for the health care provider (6). In recent

years, many isolates of *S. aureus* have evolved resistance to both synthetic and traditional antimicrobial chemotherapy and their prevalence outside the hospital is of potential epidemiological threat (7-8). Resistance to commonly available and affordable antibiotics poses a major concern in the management of bacterial infections, especially in resource poor countries (9). Imprudent practices in the use of antibiotics in human medicine and for prophylaxis in animal husbandry contribute significantly to the emergence of multidrug resistant (MDR) strains. In several studies worldwide, *Staphylococcus aureus* from normal flora seem to constitute an important reserviour of antimicrobial resistance gene (10) which can be transferred to other microbial pathogens thus propagating the resistance traits among microbial populations. The prevalence of antibiotic – resistant Staphylococci at various skin sites in both healthy and hospitalized patients has received considerable attention because of the role of these organisms as nosocomial pathogens especially in immune-compromised host. Thus surveillance studies and monitoring of antibiotic resistance in *Staphylococcus aureus* isolated from the nostril of human subjects is clearly important as data obtained from these exercises may be used to devise mechanisms for the appropriate use of antibiotics in chemotherapeutics as well as help to stem the emergence and subsequent spread of drug resistance among bacteria populations. Moreover, beneficial retrospective studies on multi-drug resistance must put the available conventional antibiotics in the area into consideration.

Based on this we embarked on this study to determine the resistance patterns of *Staphylococcus aureus* to conventional antibiotics. This paper therefore reports the prevalence of community-acquired MDR *S. aureus* in Nsukka metropolis, Southeastern Nigeria.

Materials and Methods

Microorganisms

Community strains of *Staphylococcus aureus* were isolated from nostrils of 100 healthy human subjects within Nsukka metropolis, Enugu State (having obtained their informed consent, and ethical approval) using sterile swab sticks. The population comprised of 60 female and 40 male undergraduate students, all aged between 18 and 26 years. Samples were collected between July and August 2008 while isolation and identification of the bacterial isolates were performed according to standard bacteriological techniques previously established (11-12). Thereafter all the *S. aureus* isolates were stored in agar slants at 4^0C until used for further studies. All study activities were conducted at the Pharmaceutical Microbiology unit of the Department of Pharmaceutics, University of Nigeria, Nsukka.

Culture Media and Reagents

The culture media used in the study include, Nutrient broth (Oxoid, England), Mannitol salt agar (Oxoid, England) Nutrient agar (Fluka Spain) and Peptone water. Gram Staining reagents, buffer solution, Tris-ethylenediamine tetra- acetic acid sodium sulfate (TENS), sodium acetate, Ethidium bromide and Bromo – phenol blue were all analar grade reagents.

Antibiotic Sensitivity Discs

The following antibiotics used were obtained from ABTEK, India: Amoxicillin – Clavulanic acid (AUG) 30 μg, Amoxicillin (AMX) 25 μg, Erythromycin (ERY) 5 μg, Gentamicin (GEN) 10 μg, Cotrimoxazole (COT) 25 μg, chloramphenicol (CHL) 30 μg, Cephalexin (CLX) 30 μg, Clindamycin (DAL) 2 μg, Flucloxacillin (FLX) 5 μg and Minocycline 30 μg. The following drugs were also used: Gentamicin (80 mg/ml) (Gentalek) Yugoslavia, Clindamycin (150 mg) (Dalacin CTM) Pfizer USA, Flucloxacillin (Floxapen 250 mg) Beecham England, Tetracycline (Tetraclin® 250 mg) Greenfield Pharm. India.

Antibiotic Sensitivity Test

Antibiotic sensitivity of the isolates was determined using previously established procedure (13). Briefly, the isolates were cultured in nutrient broth at 37^0C for 24 h. Two (2) loopfuls of the suspension of each isolate were inoculated into 20 ml of sterile molten agar in 10 cm diameter Petri dishes and mixed. The plates were allowed to set and the antibiotic Sensitivity disc (ABTEK, India) containing Amoxicllin – Clavulanate (AUG) 30 μg, Amoxicillin (AMX) 25 μg, Erythromycin (ERY) 5 μg, Gentamicin (GEN) 10 μg, Cotrimoxazole (COT) 25 μg, chloramphenicol (CHL) 30 μg, Cephalexin (CLX) 30 μg, Clindamycin (DAL) 2 μg, Flucloxacillin (FLX) 5 μg and Minocycline 30 μg.were aseptically placed on their surfaces. The plates were incubated at 37^0C for 24 h and the resultant inhibition zone diameters (IZDs) were measured and recorded.

Determination of minimum inhibitory concentration (MIC)

The antibiotics, Gentamicin, Clindamycin, Tetracycline, Cephalexin and Flucloxacillin were used for this assay. Standard protocols employing agar dilution

method were used for this assay (13). Briefly, stock solution of each antibiotic was made with distilled water. Five serial dilutions (2-fold) of each stock solution were done. Exactly 1 ml from each serial dilution was incorporated into 10 ml of molten nutrient agar and allowed to solidify. Each of the solidified plate was divided into nine sections and labeled. One loopful of each suspension of the test organisms was streaked on the plates according to their numbering. The MIC of each antibiotic for each organism was recorded after overnight incubation at 37^{0}C as the lowest concentration yielding no growth or a barely visible haze.

Determination of Minimum Biocidal Concentration (MBC)

This is an extension of the MIC Procedure, since the agar plates showing no growth in the MIC tests were used for this test. Discs were cut from each agar plate and transferred into corresponding container of fresh nutrient medium (13) and incubated at 37^{0}C for 48 h. Microbial growth or death were ascertained via turbidity of the medium. The minimal concentration of the antibiotic that produced total cell death is the MBC.

Plasmid Profile Studies Using Agarose gel Electrophoresis

Extraction of Plasmid DNA. Previously established protocols were employed for this study (14-16). Selected resistant isolates were grown in a 5 ml double strength Mueller Hinton broth for 72 h at 37^{0}C. The 72 h grown cultures were centrifuged in a micro centrifuge for 10 mins at 10,000 rpm to obtain pellets. The supernatant was gently decanted and the cell pellets were vortexed for 5 min. Thereafter, 300 μg of Tris EDTA (TE) buffer and 150 μL of 3.0 M sodium aqueous acetate was added at pH 5.2 and was vortexed for 3 mins to lyse the bacteria cell pellet. The samples were centrifuged again for 2 min in a microcentrifuge (Biofuge, Biotra Bio-trade Hecrus Sepatech Co. Ltd USA) and the supernatant was transferred to a fresh tube, mixed well with 0.9 ml of 100% ethanol which had been precooled to – 20^{0}C to precipitate the bacteria DNA. It was centrifuged again for 2 min and the supernatant was discarded. The pellet was rinsed twice with 1 ml of 70% ethanol and was dried under vacuum for 2 – 3 mins, after which it was resuspended in 20 - 40 μL of TE buffer for further use.

Preparation of Gel. A 1.0 g quantity of agarose was dissolved in 100 ml of Tris Borate EDTA buffer (TBE) to form 1.0% gel. The agarose solution was allowed to cool to a temperature of about 40^{0}C. Thereafter ethidium bromide was added and the mixture poured into a gel tray. This was allowed for 20 mins to solidity and the comb was carefully removed from the gel. The gel carrier was removed from the pouring tray and was placed in the gel electrophoresis box. A 250 ml TBE was used to fill the electrophoresis box until the gel was submerged.

Electrophoresis of the DNA Samples. Using micropipette, a 50 μL sample of DNA and 3 μL of loading dye (ethidium bromide) were added together and this was carefully mixed together by pipetting the solutions up and down (16). Each sample was loaded carefully into the gel wells, one sample per well and this was placed on the gel box at the negative charge end of the electrophoresis machine. Buffered water was added which sealed the agarose containing the sample DNA and acts as electrolyte by moving the current as well as the sample DNA towards the positive end for 2 hrs with a voltage of 63 V. Thereafter the agarose containing the sample DNA was removed and allowed to drain off. With the aid of UV light, UV certified safety glasses and camera, a picture showing size and movement of the sample DNA was taken to determine the mobility in millimeter using a known sample standard (16, 17).

Results

Samples were collected from the 100 human subjects within the Nsukka community thus representing a collection of wild type strains of *Staphylococcus aureus* available within the assessed commu-

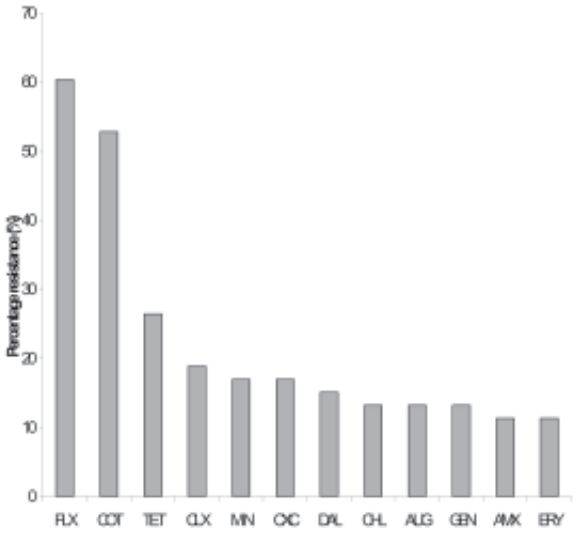

Figure 1: Antibiotic susceptibility rates among the S. aureus isolates. KEY: AUG = Amoxicillin – Clavulanic acid, AMX = Amoxicillin, TET = Tetracycline, ERY = Erythromycin, COT = Cotrimoxazole, CHL = Chloramphenicol, CLX = Cephalexin, CXC = Cloxacillin, DAL = Clindamycin, FLX = Flucloxacillin, MIN = Minocycline, GEN = Gentamycin.

nity. Fifty-three (53) isolates of *Staphylococcus aureus* were recovered from the human subjects. They were all isolated from the nostrils.

Figure 1 shows the antibiotic percentage resistance profile among the tested isolates. From the Figure, the resistances of the isolates of *Staphylococcus aureus* to the antibiotics were in the following order: Flucloxacillin > Cotrimoxazole > Cloxacillin > Chloramphenicol > Tetracycline > Minocycline ~ Clindamycin > Gentamicin ~ Erythromycin> Amoxicillin- Clavulanic acid ~ Cloxacillin > Amoxicillin. Thus, the highest resistance (> 60%) was recorded for Flucloxacillin while the least resistance (< 20%) was recorded for Amoxicillin. It is interesting to note here that while very high resistance (least susceptibility) was displayed for Flucoxacillin, a penicillin, the least resistance (highest susceptibility) was displayed by Amoxicillin which is another penicillin.

Table 1: Results of Minimum Inhibitory Concentration (MIC) in µg/ml + SEM.

Sample Isolates	Clindamycin	Tetracycline	Flucloxacillin	Cephalexin	Gentamycin
03	100.00±0.00	100.00±50.0	25.00±0.00	100.00±0.00	50.00±0.00
13	3.125±1.563	100.00±0.00	12.50±0.00	12.50±3.125	25.00±0.00
31	50.00±12.50	100.00±0.00	25.00±0.00	25.00±6.25	100.00±0.00
34	3.125±0.00	3.125±1.563	25.00±6.25	6. 25±1.563	6.25±1.563
40	100.00±0.00	100.00±0.00	50.00±0.00	100.00±0.00	50.00±12.5
45	3.125±1.563	50.00±12.5	6.25±1.563	25.00±0.00	12.50±0.00
70	6.25±0.00	100.00±0.00	3.125±0.00	25.00±6.25	12.50±0.00
79	3.125±0.00	100.00±0.00	25.00±6.25	25.00±0.00	12.50±3.125
97	6.25±1.563	50.00±12.5	6.25±3.125	6.25±0.00	12.50±3.125

Table 1 shows the minimum inhibitory concentration (MIC) profile of the various isolates to the inhibitory activities of some representative antibiotics standards. The very least MICs values of 3.125 µg/ml were recorded by Clindamycin against isolates 13, 34, 45, and 79 respectively. Moderate MICs were recorded by Flucloxacillin, Cephalexin, and Gentamicin in the aforementioned order, while relatively more isolates (isolates 3, 13, 31, 40, 70, and 79 respectively) recorded highest MICs of 100 µg/ml for Tetracycline thus representing a lower susceptibility outcome.

Table 2: Results of Minimal biocidal concentration MBC (µg/ml) + SEM.

Sample Isolates	Clindamycin	Tetracycline	Flucloxacillin	Cephalexin	Gentamycin
03	100.00±0.00	100.00±50.00	50.00±0.00	100.00±0.00	50.00±0.00
13	6.25±1.563	100.00±0.00	12.50±0.00	50.00±12.50	50.00±0.00
31	50.00±12.50	100.00±0.00	100.00±0.00	50.00±12.5	100.00±0.00
34	12.50±0.00	12.50±3.125	25.00±6.25	12.50±3.125	25.00±6.25
40	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	50.00±12.5
45	12.50±3.125	100.00±50.00	12.50±3.125	100.00±0.00	25.00±0.00
70	100.00±0.00	50.00±0.00	100.00±0.00	25.00±6.25	25.00±0.00
79	6.25±0.00	100.00±0.00	100.00±50.00	100.00±0.00	25.00±6.25
97	12.50±3.125	100.00±50.00	6.25±1.563	6.25±0.00	25.00±6.25

The result of the Minimum Bactericidal Concentration (MBC) is presented in Table 2. Again, the more favourable activity of Clindamycin can be seen to reoccur here. This is followed by the microbiocidal effects recorded by Flucloxacillin, Cephalexin, Gentamicin, and Tetracycline.

Table 3: Profile of plasmids isolated from drug-resistant *S. aureus* strains.

S. aureus isolate	Mobility (mm) + SEM
03	4.0 ± 0.00
13	4.5 ± 0.00
31	5.0 ± 0.00
34	5.0 ± 0.00
40	3.0 ± 0.00
45	4.0 ± 0.00
70	4.0 ± 0.00
79	4.0 ± 0.00

Preliminary characterization of the resistance plasmids isolated from the resistant bacteria strains is shown in Table 3. Mobility values recorded ranges from 3.0 mm to 5.0 mm.

Discussion

Bacteria isolates were recovered from the nostrils of humans. These strains should be expected to display the typical community-type acquired genetic susceptibility traits of the *S. aureus* microbial species since they are non-hospital strains and are relatively unexposed to wide array of antimicrobial agents associated with the hospital practice and environments. It is interesting to note (Figure 1) that while very high resistance (least susceptibility) was displayed for Flucoxacillin, a penicillin, the least resistance (highest susceptibility) was displayed by Amoxicillin which is another penicillin. Penicillins are known to exert their antimicrobial effect by inhibition of the synthesis of peptidoglycan, which is a heteropolymeric component of the cell wall, which provides a rigid mechanical stability by virtue of its highly cross-linked lattice wall structure (18-20), and the result of this inhibition is loss of bacteria cell rigidity and subsequent rupture or lysis of the bacteria cells (18). Hence it is very plausible to envisage quite uniform pattern of susceptibility by the test microorganisms to the members of the penicillins family, albeit with only slightly varying differences. Moreover, the inherent weakness associated with this antimicrobial class is resident in their β-lactam chemical ring nucleus which has been subject to attack by β-lactamase enzymes produced by certain microorganisms including some *S. aureus* strains (20, 21).

Therefore, if the observed reduced susceptibility

of the *S. aureus* strains to Flucloxacillin is due to the chemical disruptive activities of possible β-lactamase enzymes produced by the *S. aureus* strains, then, why this trend does not seem to be replicated with regards to Amoxicillin does appear to be very clear. This observation is further heightened when you consider the relatively poor activity of Amoxicillin-Clavulanic acid in comparison with Amoxicillin used alone. Clavulanic acid present in the Amoxicillin-Clavulanic acid complex is meant to afford protection to the β-lactam chemical ring nucleus present in the Amoxicillin, and this protection should be expected to enhance the activity of Amoxicillin. Hence the Amoxicillin-Clavulanic acid complex should demonstrate clearly significantly higher susceptibility rates over the Amoxicillin alone.

Instead, a reverse trend is rather recorded from the study thus suggesting that other mechanism(s) may be responsible for these inconsistencies. One likely explanation for this phenomenon may be related to permeability and absorption factors governing antibiotic transfer across the microbial cells. It is quite possible that the Amoxicillin-Clavulanic acid complex, which is a larger molecule than Amoxicillin, may experience greater difficulty in permeability and overall transport across the microbial cell wall/membrane barrier. Thus only relatively limited quantity may be available to exert an antimicrobial effect since antibiotics must first penetrate the bacteria cells before they can be mobilized to produce their antimicrobial effect. Secondly, varied and disproportionate structural hindrances introduced by molecular structural differences among these antibiotics may serve to modulate the compulsory pre-activity structure-activity-relationship (SAR) between the β-lactamase enzymes and these antibiotics thereby rationalizing the overall degradative effect of these enzymes, and the consequent activities of the antibiotics.

Again, from the percentage resistance profile results, the three (3) best agents showing relatively good susceptibility profile (Amoxicillin, Amoxicillin-Clavulanic acid (Augmentin), and Cephalexin) are all bactericidal agents that produce their antimicrobial effect through inhibition of bacteria cell wall synthesis. The other agents (Chloramphenicol, Tetracycline, Clindamycin, Minocycline, Gentamycin and Erythromycin) all show quite moderate susceptibilityresistance profile (< 40% > 20%) and they are known to exert their antimicrobial activities through other means of inhibition of bacteria protein synthesis (18). There appears therefore, a seeming correlation between the overall recorded antimicrobial activity and mode of action of the antibiotics used. It would generally appear from the results of this study that antimicrobial agents acting by inhibition of bacteria cell wall synthesis were more effective against the *S. aureus* strains except for the unusually high resistances recorded for Flucloxacillin and Cloxacillin. This anomaly as have been explained from the foregoing may be related to a combination of permeability/absorption factors and inherent degradative enzyme-antibiotics SAR-resolved antimicrobial property of the antibiotics associated with the Penicillins/β-lactam group. Nonetheless, this generally observed advantage of the Amoxicillin, Amoxicillin-Clavulanic acid (Augmentin), and Cephalexin over the other antimicrobial agents will be expected to find usefulness in clinical practice requiring the use of antibiotics in the management of infections and epidemics caused by *S. aureus strains*, and this again underscore the need to always carry out a pre-treatment antimicrobial susceptibility testing before embarking on antibiotics treatment of infections in clinical settings.

Blind treatment of infections with chemotherapeutic agents should be discouraged since this could lead to treatment failure with the possible risk of morbidity and mortality, as well as, a waste of economic resources. MIC results of antimicrobial agents normally represent useful pre-clinical quantitative analytical parameter that finds prospective application in pre-clinical and clinical settings. Apart from the practical utility of MIC values as a means of cutoff points demarcating between microbial species and strains on the basis of the antimicrobial susceptibility rate, the possession of lower MICs by an antimicrobial agent is quite suggestive of a higher inherent antimicrobial property (22). Additionally, MIC values must synchronize with pharmacokinetic plasma and tissue distribution of the antibiotic to ensure that adequate amounts of the antibiotic are made readily available at the sites of infection. It is therefore expected that lower MIC values would enhance this outcome as well as help to limit the clinical occurrence of unwanted drug side effects since smaller but effective doses of the antibiotics could then be administered to patients in accordance with pre-determined frequencies.

Consequently, considering the MIC results generated (Table 1), Clindamycin (followed by Flucloxacillin, Cephalexin, Gentamicin, and Tetracycline) seem to present as the agent of choice for a general non-specific clinical treatment of infections caused by the isolated *S. aureus* strains within the examined Nsukka community. This scheme may be extrapolated for other neighbouring communities within the Enugu State axis due to demographic relatedness of these localities. However, since the MIC results also showed some wide bacteria-strain-specific variations, we would suggest that antibiotic treatments options should be

rather tailored to confront the specific *S. aureus* strain involved on the basis of their favourable susceptibility profile to the specific antibiotic to be used as determined by a proper laboratory analytical procedure. Given the Minimum Bactericidal Concentration (MBC) (Table 2), although all the antibiotics caused bacteria cell death at concentration approaching 100 µg/ml this however is quite far from the actual serum concentration usually encountered at the doses employed in clinical practice, and even though it is true that that the prognosis of chemotherapeutic treatment of bacteria infections is a combination of the antimicrobial property of the antibiotics used and the overall immunological dynamics occurring within the host; the MBC results obtained still point to a very low susceptibility of a large proportion of the bacteria isolates to the antibiotics employed in the test. This development raises a cause for genuine concern in the future of infectious diseases control of bacteria origin.

Agarose gel electrophoresis was employed for the molecular characterization of the isolated plasmids from the bacteria strains. Thus, the relative profiles of the DNA fragments and plasmids were characterized on the basis of their comparative molecular weights and speed of travel through the electrophoretic agarose system (23). The presence of some plasmid DNA in the isolates corresponding to the reference standard DNA fragments suggests that their antimicrobial resistance is possibly plasmid-mediated and as such could be referred to as Resistance plasmids (R-factor). The isolated plasmids may be responsible for possibly mediating some or all of the expressed resistances of the microorganisms. Further studies including resistance gene curing and actual sequencing of the isolated plasmid genomes would be required to firmly establish the role of the isolated plasmids in the observed resistance patterns of the microorganisms. In bacteria, the acquisition of resistance may be due to chromosomal mutations or through plasmids that are often capable of transfer from one strain of organism to another, even across the species barrier.

The process of transfer and acquisition of resistance determinants among microorganisms is a natural, unstoppable phenomenon exacerbated by the abuse, overuse and misuse of antimicrobials in the treatment of human illness and in animal husbandry, aquaculture and agriculture agriculture (24-25). Moreover, the drugs to which the isolates were resistant to, are commonly used antibiotics in the studied environment, thus the observed effects recorded in this study must not be overlooked but should present a useful background for rational use and prescription of antimicrobial agents. This kind of study should also be carried out for different communities and geographical settings since the occurrence of antibiotic resistant strains can clearly vary across different environmental settings depending on a host of various factors prevalent in such environments that influence selection of development of antibiotic resistant strains.

In a conclusion, the high level of resistance among the isolates was found to be common with commonly used antibiotics. The MIC and MBC results showed that among the five antibiotics used, Clindamycin had the best antibacterial activity. The presence of plasmid DNA in the eight most resistant isolates may be responsible for their observed high antibiotic resistance. It is therefore recommended that a good antibiotics use policy put in place as well as ethical and rational prescription practices by clinicians, and every healthcare personnel involved in the use of antibiotics in clinical and non-clinical settings would help control and prevent the emergence of MDR microorganisms.

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