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Biochemical Characteristics and Antibiograms of Methicillin-Resistant Staphylococcus aureus and Multi-Drug Resistant Pseudomonas aeruginosa Isolated from Skin Wounds of Patients in a Tertiary-Level Hospital in Enugu, Nigeria

C. F. Kalu[1], V. C. Okore[1], F. C. Kenechukwu[2], D. C. Odimegwu[1]

[1]Department of Pharmaceutical Microbiology and Biotechnology, University of Nigeria, Nsukka, Enugu State, Nigeria;
[2]Department of Pharmaceutics, University of Nigeria, Nsukka, Enugu State, Nigeria.

Email: flora.okore.178618@unn.edu.ng

Abstract:
Infections by antibiotic-resistant pathogens are known to be the most challenging factor that can impair wound healing. Early detection and effective diagnosis contribute to appropriate therapeutic interventions, which significantly lead to successful treatment outcomes in non-healing wounds. The aim of this study was to identify and evaluate resistance patterns as well as characteristics of Methicillin Resistant Staphylococcus aureus (MRSA) and Multi-drug Resistant Pseudomonas aeruginosa (MDR-PA) isolated from skin wounds of patients in the National Orthopaedic Hospital, Enugu, Nigeria. Cultural and biochemical characterizations of the isolates were, therefore, carried out. Results of the biochemical tests confirmed the presence of Staphylococcus aureus and Pseudomonas aeruginosa isolates. Antibiotic disk diffusion method was adopted for antimicrobial susceptibility tests (ASTs) on the isolates. Based on inhibition zone diameters obtained, various degrees of resistance (50 – 100 %) as well as susceptibility (40 – 70 %) of the organisms were recorded against the test antibiotics. This showed that there was high prevalence of MRSA and MDR-PA strains among the wound pathogens in that part of the country. From the findings, it could be inferred that the occurrence of multi-drug resistant pathogens would contribute significantly to poor clinical responses to wound treatments with antibiotics.

Keywords: Antimicrobial resistance, methicillin resistant Staphylococcus aureus (MRSA), multi-drug resistant Pseudomonas aeruginosa (MDR-PA), skin wounds healing.

I. INTRODUCTION

Generally, wounds provide warm, moist, and nutritive environments for the proliferation of infectious microorganisms. Wound infections are among the leading causes of increased morbidity, mortality, prolonged hospitalization and economic burden for patients [1, 2]. The ultimate goals of wound treatment are to eradicate microbial infection, alleviate discomfort, reduce overall wound healing time and ensure restoration of tissue to pre-wound state [3].

Studies have successfully identified the most common resistant strains of bacterial pathogens responsible for wound infections [4 – 8]. Mostly, Gram-positive and Gram-negative bacteria, especially Streptococcus pyogenes, Escherichia coli, Klebsiella spp., Proteus spp., Pseudomonas spp., Acinetobacter spp., as well as methicillin-resistant strains of Staphylococcus aureus, coagulase-negative strains of staphylococci, Enterococcus spp., vancomycin-resistant strains of enterococci, Serratia marcescens, Enterobacter spp., Bacteroides spp. have been isolated from wounds [9, 10]. Some of these pathogenic bacteria form antibiotic-resistant biofilms, which may delay the healing rate of infected wounds [11].

A study by Garba et al. [12] illustrated the antibiotic susceptibility profile of bacterial isolates from infected wounds of diabetic patients in a specialist hospital in Jos, Nigeria. In the study, six bacterial isolates (Escherichia coli, Proteus mirabilis, Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, and Klebsiella pneumonia) were identified. The most prevalent bacteria were Pseudomonas aeruginosa and Staphylococcus aureus with high levels of resistance to multiple antibiotics. Rijal et al. [13] also carried out a study to ascertain the types of pathogens in infected wounds, and their susceptibility patterns in a tertiary hospital. About 65 % of the isolates was identified as bacteria out of which 57.6 % was Gram-positive species, largely Staphylococcus aureus. Among the isolated Gram-negative bacteria were Pseudomonas aeruginosa (5.26 %) and Acinetobacter spp (2.39 %). Results from the susceptibility study showed high rates of multi-drug resistance among the isolated bacteria. Trojan et al. [14] have reported that methicillin-resistant Staphylococcus aureus and some Gram-negative multi-drug resistant bacteria are increasingly associated with wound infections. Characterization of 165 of clinical wound isolates collected from three hospitals in Sokoto, Nigeria, revealed that 32 (19.4 %) were Pseudomonas aeruginosa, out of which 14 (8.5 %) were multi-drug resistant [15].

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Polymicrobial conditions of infected wounds often make wound treatment difficult in clinical settings. With early identification of invading pathogens and in vitro antimicrobial susceptibility tests, effective guides to efficacious antimicrobial therapies can be established [16]. Studies have been conducted in various parts of the world to identify bacterial profiles in pyogenic wound infections [12 – 15]. In Eastern Nigeria, however, there is limited epidemiological data on wound pathogens, particularly the multi-drug resistant bacterial strains as well as resistance profiles of the organisms. Such data, if available, could aid the selection of appropriate and effective antibacterial therapies for patients. Thus, the aim of this work is to carry out a preliminary study on the prevalence and antibiotic resistance profiles of S. aureus and P. aeruginosa isolated from skin wounds of patients in the Eastern part of Nigeria, using the National Orthopedic Hospital, Enugu (NOHE), as a pilot centre. The susceptibility of the pathogens to some commonly used antibiotics in wound management will equally be established. Findings of this study could provide a clue towards the choice of antibiotics in treatment and control of multi-drug resistance amongst wound pathogens in the health facility and its environs.

II. MATERIALS AND METHODS

When Materials

The following materials were used in this study: mannitol salt agar (TM Media, India), MacConkey media (HiMedia Laboratories Pvt. Ltd, India), Mueller-Hinton agar (TM Media, India), cetrimide agar (TM Media, India), nutrient agar (TM Media, India), oxacillin discs (Oxoid, Basingstoke, UK), standard antibiotic discs (HiMedia Laboratories Pvt. Ltd, India) consisting of tetracycline (TE) 30 µg, amoxicillin/clavulanic acid (AMC) (20/10 µg), ciprofloxacin (CIP) 10 µg, clindamycin (CD) 2 µg, co-trimoxazole (sulphamethoxazole/trimethoprim) (COT) (23.75/1.25 µg), cloxacilin (COX) 1 µg, erythromycin (E) 15 µg, cefalexin (CN) 10 µg, ceftriaxone (CTR) 30 µg, gentanicin (GEN) 10 µg, levofloxacin (LE) 5 µg, netilmicin sulphate (NET) 30 µg, and ofloxacin (OF) 5 µg. Other materials included: distilled water supplied by the Water Resource Mgt Lab of the University of Nigeria, Nsukka. Clinical isolates of methicillin resistant Staphylococcus aureus and multi-drug resistant Pseudomonas aeruginosa were collected from NOHE, Nigeria.

Collection of Clinical Isolates

Ethical approval (No: 2022/05/0016) was obtained from the Management of National Orthopaedic Hospital, Enugu prior to the study. Informed consent was also obtained from every patient who participated in this study. A total of 194 swab samples were collected from skin wounds of patients admitted in the hospital between May and September 2022. The swab sticks were gently placed at the centre of each wound area and rotated for about 10 seconds. Samples were collected every morning prior to wound dressing. The clinical specimens were immediately transported to the laboratory and cultured on nutrient agar.

Isolation and Characterization of S. Aureus

A solution of mannitol-salt agar was prepared according to the manufacturer's direction, and sterilized at 121 °C for 15 minutes. Using a sterile pipette, 20 ml of the sterilized agar was introduced in Petri dishes and allowed to solidify at room temperature. Sterile water was added into the clinical culture which was shaken for about 30 seconds to disperse isolates into a suspension. Using a sterile wire loop, a loopful of the bacterial suspension was collected and gently dropped on one quadrant of the agar plate to form primary inoculum. The primary inoculum was streaked over the agar surface using a sterile wire loop. The plates were incubated at 37 °C for 48 hours. From physical observation, presence of yellow or golden yellow colonies were presumptively taken as S. aureus. In order to confirm the presence of S. aureus, selected colonies were subjected to Gram staining and standard biochemical tests.

Coagulase Test

Gram-positive organisms having the appearance of Staphylococcus aureus were sub-cultured on nutrient agar and incubated at 37 °C for 24 hours. Typical isolates (yellow or golden yellow colonies) were subjected to coagulase test. Diluted plasma was prepared by mixing 0.2 ml of plasma and 1.8 ml of normal saline to make a 2 ml volume. Three test tubes were labeled T (test), P (positive control) and N (negative control) respectively. A volume of 0.1 ml of the test organism suspension was placed in tube T. A volume of 0.1 ml suspension of standard Staphylococcus aureus ATCC 12600 was placed in tube P, and 0.1 ml of sterile normal saline was added to tube N. To each test tube, 0.5 ml of the diluted plasma was introduced and properly mixed. The tubes were incubated for 6 hours at 37 °C, and then observed for coagulation of the plasma.

Catalase Test

Two drops of 3 % hydrogen peroxide were placed on two clean, grease-free glass slides, labeled A and B, with B serving as control. Using a sterile wire loop, the test organism was transferred to slide A, while the standard S. aureus was placed on slide B. The glass slides were observed for the formation of gas bubbles. Presence of bubbles indicated a positive catalase reaction.

Multi-drug Resistance Test for S. Aureus

Fresh cultures of the isolates identified as Staphylococcus aureus were suspended in sterile normal saline and adjusted to 0.5 McFarland's standard. The bacterial suspension was then spread on Mueller-Hinton agar plates using a sterile bent glass rod and allowed to dry. Standard antibiotic discs (6 mm each) were placed on the surface of each agar. The plates were kept at room temperature for 30 minutes to allow for pre-diffusion of the antibiotics, and then incubated at 37 oC for 24 hours. Clear areas surrounding the discs, including areas covered by the discs, were measured and inhibition zone diameters were matched against sensitivity break points as specified by the Clinical and Laboratory Standards Institute [18].

Methicillin Resistance of S. Aureus (oxacillin test)

The isolates identified as S. aureus were further tested for oxacillin resistance. The isolates were suspended in 10 ml normal saline and adjusted to a concentration of 1 x 108 CFU/ml by comparing with 0.5 McFarland's standard. The bacterial suspension was spread uniformly on Mueller-Hinton agar medium using a sterile bent glass rod and allowed to dry.

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Oxacillin discs (6 mm) were placed on the agar surface. After a period of pre-diffusion, the plates were incubated at 37 °C for 48 hours. Diameters of inhibition zones, if any, were measured and noted.

Isolation and Characterization of P. Aeruginosa

Sterile water was introduced into the clinical culture and vigorously shaken to dislodge the isolates into a suspension. A loopful of the organism was collected and gently rubbed on one quadrant of cetrimide agar plate to provide the primary inoculums, which was then streaked on the agar surface. The plates were then incubated at 37 °C for 48 hours. Colonies with green pigments diffusing into the medium were subjected to Gram staining. Those that appeared as Gram-negative bacilli were indicative of P. aeruginosa. Further confirmation was carried out using the oxidase test.

Oxidase Test

A strip of Whatman's No. 1 filter paper was dipped in a freshly prepared solution of 1% tetramethyl-p-phenylene-diamine dihydrochloride and drained for about 30 seconds. The filter paper was then placed in a Petri dish. The test organisms were spread over the paper and observed for ten minutes. A dark purple colouration was indicative of positive oxidase test.

Multi-drug Resistance Test for P. Aeruginosa

The procedure for detecting multi-drug resistance of S. aureus was replicated for P. aeruginosa.

III. Results

A total of 194 swab samples were collected out of which 673 bacterial isolates were obtained. However, only strains of S. aureus and P. aeruginosa were of interest in this study. A total of 235 isolates (34 %) were identified as methicillin-resistant S. aureus while 126 isolates (18 %) were identified as multi-drug resistant P. aeruginosa. The cultural and biochemical characteristics of each organism are shown in Tables 1 and 2.

TABLE 1: CULTURAL AND MICROSCOPIC CHARACTERISTICS OF ISOLATES

Characteristic	Appearance	Inference
Shape	Cocci Circular rod	*S. aureus* *P. aeruginosa*
Opacity	Opaque Transparent	*S. aureus* *P. aeruginosa*
Colour	Golden yellow Blue-green	*S. aureus* *P. aeruginosa*
Edge	Entire Entire	*S. aureus* *P. aeruginosa*
Arrangement	Clusters Single, pairs or short chains	*S. aureus* *P. aeruginosa*
Odour	Present Present	*S. aureus* *P. aeruginosa*

TABLE 2: BIOCHEMICAL CHARACTERISTICS OF ISOLATES

Test Reactions	Result	Inference
Gram's reaction	Purple colour Reddish-pink colour	*S. aureus* *P. aeruginosa*
Coagulase reaction	Presence of blood clots	*S. aureus*
Catalase reaction	Presence of gas bubbles	*S. aureus*
Oxidase reaction	Purple/blue colouration	*P. aeruginosa*

The antimicrobial susceptibility test (AST) results are presented in Figures 1 and 2. A large percentage of the two organisms of interest were resistant to all the antibiotics at various levels. It was obvious, however, that all isolates (100%) of S. aureus were resistant to cotrimoxazole, levofloxacin and oxacillin, as could be seen in Table 3. Generally, more isolates of S. aureus were resistant to all the antibiotics than the organisms that were sensitive. A similar pattern can be seen for P. aeruginosa (Table 4). In this study, significantly high level of resistance (80 %) to ceftriaxone was recorded among the P. aeruginosa isolates. Similarly, high occurrence of MDR-PA was observed against levofloxacin (80 %) and ofloxacin (80 %). From the percentage resistance profile, both isolates exhibited 50 – 60 % resistance to tetracycline, ciprofloxacin, clindamycin, and erythromycin.

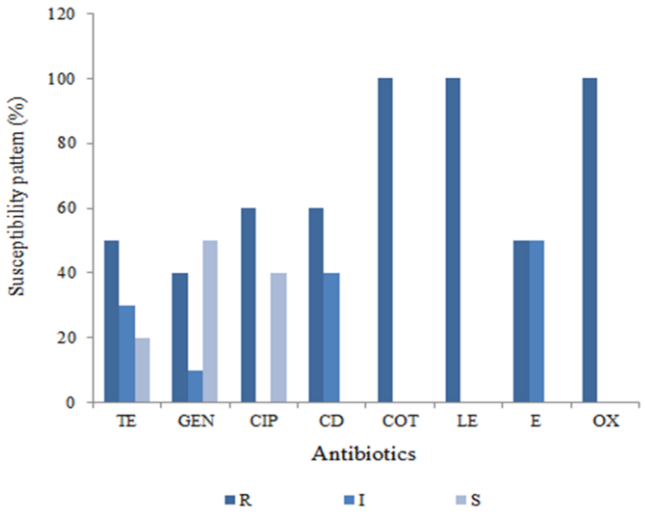

Figure 1: Percentage susceptibility chart for isolated S. aureus based on CLSI IZD break point standards. Key: R = Resistant, I = Intermediate, S = Sensitive, TE = tetracycline, GEN = gentamycin, CIP = ciprofloxacin, CD = clindamycin, COT = co-trimoxazole, LE = levofloxacin, E = erythromycin, OX = oxacillin.

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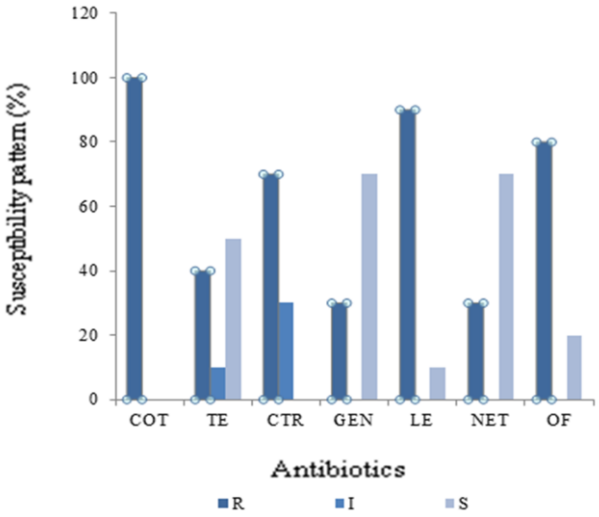

Figure 2: Percentage susceptibility chart for isolated P. aeruginosa based on CLSI IZD break point standards. Key: R = Resistant, I = Intermediate, S = Sensitive, COT = co-trimoxazole, TE = tetracycline, CTR = Ceftriaxone, GEN = gentamycin, LE = letilmicin, NET = netilmicin sulphate, OF = ofloxacin.

TABLE 3: PERCENTAGE (%) SUSCEPTIBILITY DISTRIBUTION OF ISOLATED S. AUREUS

Antibiotic	S	I	R
Tetracycline 30 µg	20	30	50
Gentamycin 10 µg	50	10	40
Ciprofloxacin 5 µg	40	0	60
Clindamycin 2 µg	40	0	60
Co-trimoxazole 25 µg	0	0	100
Levofloxacin 5 µg	0	0	100
Erythromycin 15 µg	0	50	50
Oxacillin 1 µg	0	0	100

Key: R = Resistant, I = Intermediate, S = Sensitive

TABLE 4: PERCENTAGE (%) SUSCEPTIBILITY DISTRIBUTION OF ISOLATED P. AERUGINOSA

Antibiotic	S	I	R
Cotrimoxazole 25 µg	0	0	100
Tetracycline 30 µg	50	10	40
Ceftriaxone 30 µg	0	30	70
Gentamicin 10 µg	70	0	30
Levofloxacin 5 µg	10	0	90
Netilmicin sulphate 30 µg	70	0	30
Ofloxacin 5 µg	20	0	80

Key: R = Resistant, I = Intermediate, S = Sensitive

IV. DISCUSSION

Grape-like clusters of cocci have conventionally been suggestive of S. aureus. The golden yellow appearance of the colonies is attributed to carotenoid pigment produced by S. aureus [19]. On the other hand, rod shaped organisms with striking blue-green colouration are indicative of P. aeruginosa. The characteristic blue-green colour associated with P. aeruginosa has been shown to be pyocyanin (N-methyl-1-hydroxyphenazine), which is a water-soluble phenazine pigment [20]. Unpleasant odour was perceived in both cultures and this is common with wound pathogens. The metabolic activities of these organisms produce volatile compounds with offensive odour which is often one of the distinguishing features [19].

In Gram's test, the primary stain, gentian violet, penetrated cell walls of S. aureus isolates, thereby, giving off a purple coloration. This is normally attributed to the thicker and denser peptidoglycan layer in the cell wall of Gram-positive organisms [21]. It is believed that iodine, which acts as a mordant, firmly binds the primary dye to microbial cells, thereby preventing its diffusion out of the cell wall during decolourization. On the other hand, P. aeruginosa loses the purple colour once the primary stain is washed off. It only becomes visible when the counter stain, safranin dye, is added. A likely explanation to this is that Gram-negative bacteria have cell walls with thinner layers of peptidoglycan (which make up only 10 % of the entire cell wall) and high lipid content [21]. Upon completing the Gram's test, S. aureus retained purple colour while P. aeruginosa were identified with the characteristic reddish-pink appearance.

Formation of clots in the T tube was indicative of coagulase-positive S. aureus. As expected, the P tube showed positive result while the N tube showed no clots due to the absence of S. aureus. This biochemical test identifies bacteria that have the intrinsic ability to produce coagulase enzymes. The enzymes are known to cause coagulation of blood plasma by converting fibrinogen to fibrin [22]. Gas bubbles were observed from the catalase test which indicates presence of catalase positive S. aureus. The enzyme plays a significant role in breaking down hydrogen peroxide, a potent oxidizing agent that gives off oxygen in the form of gas bubbles [22]. The presence of purple/blue colouration appearing within 5 – 10 seconds of an oxidative reaction between the test organisms and tetramethyl-p-phenylene diamine dihydrochloride solution was a confirmation of P. aeruginosa. The oxidase test is based on secretion of cytochrome oxidase enzymes by the organism [22]. Organisms that contain cytochrome C are normally associated with secretion of the intracellular oxidase enzymes [23].

The choice of AST is normally influenced by factors such as accuracy, sensitivity, time, cost, as well as ease of reproducibility. Antibiotics used for the ASTs are usually chosen based on their clinical relevance in the management of wound infections. The antibiotics are often chosen from the following classes: tetracyclines, β-lactams, quinolones, aminoglycosides, co-trimoxazole, macrolides and cephalosporins. Results generated by the in vitro susceptibility

tests were generally interpreted and reported as resistant, susceptible or intermediate to the action of a particular antibiotic. Clear areas surrounding the discs were measured and inhibition zone diameters are matched against break point standards specified by the Clinical and Laboratory Standards Institute (CLSI) [18]. Bacterial isolates showing resistance to three or more classes of antibiotics were characterized as multi-drug resistant (MDR) [18].

Isolates of interest in this study are notorious for developing resistance to commonly used antibiotics. This is evidenced in the 100 % antibiotic resistance to co-trimoxazole, levofloxacin and oxacillin by S. aureus. Beta-lactam antibiotics are known to exert their antimicrobial effect by inhibiting the synthesis of peptidoglycan which protects and confers rigidity to microbial cell wall. The inherent resistance to oxacillin is subject to cleavage of β-lactam ring by β-lactamase enzymes secreted by the S. aureus isolates [24]. Absence of inhibition zone to oxacillin is normally taken as proof of methicillin resistance in S. aureus. Resistance to oxacillin equally implies that the isolates are not susceptible to a large number of other β-lactams, including the cephalosporins. It is noteworthy that ceftriaxone, which is one of the most commonly prescribed 3rd generation cephalosporins, seems to be losing its efficacy to the MDR-PA, as could be seen in this study.

The aminoglycosides, quinolones and tetracylines are popular classes of antibiotics prescribed by clinicians in Nigerian hospitals [25]. Interestingly, in this study, both MRSA and MDR-PA isolates expressed varying degrees of susceptibility (40 – 70 %) to antibacterial agents in these classes. The relatively reduced degrees of resistance place these antibacterial agents above some others in the management of wound infections. It is, therefore, suggested that the use of these drugs be restricted to severe nosocomial infections to avoid further loss of sensitivity.

The relatively high prevalence of MRSA and MDR-PA recorded in this study is of public health concern. This trend could be attributed to lack of strict adherence to the standard rules of antibiotic usage. There is also the issue of antibiotic misuse associated with self-medication and unauthorized sharing of antibiotics among individuals that experience similar clinical symptoms [26], practices that are of major socio-medical concern.

V. Conclusion

In this study, high levels of resistance by S. aureus and P. aeruginosa isolated from skin wounds have been established. These findings imply that invasion of wounds by multi-drug resistant pathogens results in partial or complete failure of wound treatments. Some of the tested antibiotics had bacteriocidal effects on the pathogens which make them relevant options in wound treatment. In order to avert the possibility of further resistance, it is recommended that combination therapies involving these active agents be adopted in management of chronic wounds. It is believed that when these wound pathogens are exposed to antibacterial agents with diverse mechanisms of action, resistance would be considerably reduced.

Routine antibiotic susceptibility surveillance should be adopted as an essential step in monitoring and control of resistance. This process could ultimately contribute substantially to successful clinical outcomes. Data obtained from this study reveals the need for strict regulation and rational use of antibiotics for wound management in the Eastern part of Nigeria. Adopting these precautionary measures will help to avert misuse and further loss of antibacterial potency in antibiotics. In addition, strict adherence to policies and guidelines for the prescription and use of antibiotics in wound care should be implemented at all times.

VI. Conflict of Interest

No conflicts of interest were declared by the authors.

VII. References

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