The Antibiogram of Pyocyanin-Producing Laboratory and Environmental *Pseudomonas aeruginosa* Isolates

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ABSTRACT

The high incidence of *Pseudomonas*–borne infections and difficulty associated with treating these infections is very worrisome. *Pseudomonas* has often been implicated in wound and hospital borne infections. Nevertheless, it's an organisms of pharmaceutical importance, producing a metabolic product with potentials for development of newer medicines. The study evaluated the antibiotic susceptibility profile of *P. aeruginosa* isolates that produced pyocyanin. Twelve *P. aeruginosa* were isolated from soil and laboratory samples and identified using selected cultural and biochemical tests. Pure isolates were subjected to antibiotic profiling using the Kirby-Bauer disk diffusion method. The isolates were fermented using formulated broth medium containing some selected salts for enhanced pyocyanin production. Fermentation products were subjected to centrifugation and the supernatant filtered using appropriate Whatman filter paper. All the isolates showed a positive reaction to oxidase, citrate and catalase tests whereas, a negative reaction was observed for indole test. Their biochemical reaction in combination with their microscopic features confirmed these isolates to be *Pseudomonas aeruginosa*. A 100 % (12/12) level of resistance was recorded for all the isolates against ceftazidime, cefuroxime, augmentin and nitrofurantoin while 99.9 % (11/12) resistance was recorded against cefixime. Other antibiotics tested such as gentamicin, ofloxacin and ciprofloxacin were active [100 % (12/12)] against all the test isolates with varying inhibition zones recorded. All the isolates that were at least multidrug resistant also produced pyocyanin a virulence factor. This explains high prevalence of *Pseudomonas aeruginosa* and prolonged infections.

Keywords: Pyocyanin, *Pseudomonas aeruginosa*, Multidrug-resistance, Pan-resistance, Sensitivity, Antibiotics, Fermentation.

Introduction

Pseudomonas aeruginosa, a species and member of the Genus *Pseudomonas*, is a

Gram negative bacterium that has been identified to be pathogenic as well as being of Pharmaceutical interest. The organism is capable of producing bioactive compounds (Adejumo *et al*., 2021) some of which can be developed into newer antimicrobial agents. It has been implicated in urinary tract infection and wound infection as multidrug-resistant and extended spectrum beta-lactamase producers and also important producers of the bioactive compound phenazine (El-Fouly *et al*., 2014; Patric and Tarek, 2018).

When cultured on cetrimide agar or nutrient agar supplemented with glycerol indicates, it is capable of producing pyocyanin, a diffusible green extra-cellular pigment, observed around the colonies de Araújo Jácome *et al.*, (2012).

P. aeruginosa has been implicated in several infections such as conjunctivitis, sore throat, wound infection, urinary tract infection and nosocomial infections (Oli *et al.*, 2017; Parul *et al*., 2019), causing delay in wound healing processes as a result of its ability to accumulate several virulent factors including production of pyocyanin. Production of pyocyanin has been observed to be one of the several virulence factors expressed by disease-causing *Pseudomonas aeruginosa* isolates de Araújo Jácome *et al.*, (2012). This virulence factor reported to cause tissue damage is due to the formation of reactive hydroxyl radicals and superoxides and favor's the pathogenesis of *P. aeruginosa* by providing protection against the action of antibiotics. Other virulent factors linked to the pathogenicity of *P. aeruginosa* includes enzymes: alkaline protease and elastase, lipopolysaccharide (LPs), pili etc. Consequently, elimination of *P. aeruginosa* in patients with infections is very difficult because of its resistance to a variety of antibiotics which is partly due to pyocyanin production de Araújo Jácome *et al*., (2012).

Its intrinsic virulent characteristics such as biofilm production, quorum sensing and pyocyanin production are all linked to its biosynthetic potentials and are responsible for its adaptive survival abilities in unfamiliar environments and conditions (Khan *et al*., 2008, Shen *et al*., 2006; Anusree *et al.*, 2015). Due to the debilitating physiological effects of pyocyanin-producing *Pseudomonas species* in wounds, pyocyanin has been proposed to be the species' most important virulence factor amongst others (Hall *et al.*, 2016). Therefore, culture and sensitivity test is very important and encouraged in distinguishing the trends in sensitivity of *P. aeruginosa* to commonly used antibiotics and important for choosing the right antibiotic.

P. aeruginosa has also been identified as one of the most prevalent organisms associated with the hands and phones of health care workers and is documented as possible cause of nosocomial infections (Randle *et al*., 2006). Thus, *P. aeruginosa* is perceived as an opportunistic organism and linked to nosocomial infections (Parul *et al*., 2019).

Pyocyanin, a metabolic product of *P. aeruginosa*, is believed to be responsible for its virulent characteristics and ultimately its survival. The production of this compound as well as other physiological characteristic exhibited by *P. aeruginosa* is responsible for its resistance to different antibiotics, assuming a multidrug-resistant profile (Paula *et al*., 2012). Majority of treatments of infections caused by multidrug-resistant *P.*

aeruginosa involves the use of combination chemotherapy amongst other antibiotics such as carbapenems to which there is a high resistance index.

Considering the intrinsic antibiotic resistance abilities of *P. aeruginosa* isolates, the current study was carried out to assess the antibiotic susceptibility profiles of some *P. aeruginosa* isolates in relation to their pyocyanin production.

Materials and Methods

Sample collection

A total of twelve (7 laboratory and 5 soil isolates) isolates were screened for the production of pyocyanin.

Briefly, 100 g of each soil samples were collected aseptically (10 cm below) from each of the location sites using a disinfected spatula, dispensed into sterile 250 mL beakers and then covered with aluminum foil. The samples were immediately transferred to the laboratory and then processed using standard isolation techniques.

Culturing of Samples

Method reported by (Paul *et al*., 2017) with slight modifications was adopted. Here 0.005 g of each soil sample was weighed into a sterile petri dish and then, 20 mL of sterilized molten cetrimide agar (45 ^{o}C) was added. The petri dish was rotated gently to disperse the soil particles in the medium. Thereafter, the plates were incubated at 37 ^{o}C for 24 h. Emerging colonies observed to be pigmented were sub-cultured onto a freshly prepared cetrimide agar (CA) supplemented with glycerol in other to get pure isolates. Suspected *Pseudomonas* isolate were then subjected to identification processes such as microscopic examination and further characterized using selected biochemical tests.

Microbial identification and characterization

Bacteria isolates were identified through a combination of the colonial appearance (macroscopic) of each colony, Gram stain reaction (microscopy) and standard biochemical tests Cheesbrough, (2006). In other to observe their microscopic features, Gram staining reaction was carried out then selected biochemical test were also performed such as oxidase, citrate and catalase tests to confirm the identities of the isolates.

Antibiotic Susceptibility test

Isolates sensitivity to antibiotics was done using disc diffusion assay. The Kirby–Bauer disk diffusion method was adopted. Here, sterile nutrient broths were prepared and well-isolated colonies of each of the test organism growing on nutrient agar plate was inoculated into it. The broths were incubated at 37 ^{o}C until the culture equaled 0.5McFarland standard. A McFarland 0.5 turbidity standard corresponds to an inoculum of 1×10^{8}CFU/mL (Dalynn Biologicals, 2014). A sterile cotton swab was dipped in each of the inoculum suspension and the excess was removed by rotating the swab several times against the inside wall of the tube above the level of the fluid then inoculated on the surface of Mueller–Hinton agar (MHA). The surface of each of this plate was inoculated by streaking the swab over the surface. Streaking was repeated three times and each time the plate was rotated 60°. The

multiple antibiotic discs containing Ceftazidime (30 µg), Cefuroxime (30 µg), Gentamicin (10 µg), Cefixime (5 µg), Ofloxacin (5 µg), Amoxicillin + clavulanic acid (30 µg), Nitrofurantoin (200 µg), Ciprofloxacin (5 µg) were applied using a sterile forcep. To ensure complete contact of the discs with the agar surface, the discs were pressed down applying minimal pressure. The Plates were inverted and left on the work bench for 30 minutes to allow for pre-diffusion of antibiotics into the agar. Inoculated plates were inverted and incubated at 37 ^{o}C for 18 hr. After the incubation period, the diameter of zones of inhibition were measured and results interpreted. The susceptibility of each isolate to each antibiotic was shown by a clear zone of growth inhibition and this was measured using a meter rule in millimeters and the diameter of the zones of inhibition was then interpreted according to the standards of Clinical and Laboratory Standards Institute (2012).

Pyocyanin assay

Pyocyanin production by isolated *Pseudomonas species* was carried out in formulated medium with some selected salts in specific quantities. Here, a final volume of 200 mL nutrient broth contained in a 1000 mL Erlynmeyer conical flask for each of the fermentation vessel was formulated with:

[formulation constituents: (K_2HPO_4 (1 g), KCl (0.5 g), yeast extract (0.3 g), glucose (8 g); nutrient broth (200 mL)]; then the pH was adjusted to 7.0 using 1 mL of [buffer solution: ($F_2SO_4.7H_2O$ (0.1 g), $ZnSO_4.7H_2O$ (0.4 g), $MnSO_4.7H_2O$ (0.4 g), Sterile distilled water (100 mL)], all of which were sterilized in the autoclave at 121°C, 15 psi for 15 min. Then, the media (fermentation broths) were cooled before the axenic *Pseudomonas* cultures were aseptically transferred into their respective fermentation flask and left for 21 days.

Extraction of Pyocyanin

After the period of fermentation of the Pseudomonas culture, the presence of pyocyanin was observed in the culture broth as a green colored pigment due after being homogenized. The bacteria were then removed by centrifugation (1000 rpm for 10 minutes) and filtration of the supernatant liquid through 0.45 µm filters (Hasset, 1992).

Pyocyanin was extracted from the broth with chloroform. The supernatant fermentation broth (40 mL), was collected in a sterile 100 mL conical flask and mixed with an equal volume of chloroform. The mixture was homogenized, resulting to the formation of two layers. The lower layer was the organic chloroform phase containing the blue pyocyanin, while the upper layer was the aqueous phase containing the culture material which was removed. The pyocyanin was re-extracted from the organic phase with 0.2 N Hydrochloric acid (20 mL) by mixing it together until a colour change from blue to red was observed. The pigment was passed into the aqueous phase, while the chloroform phase was discarded. Accordingly, 0.2 M borate-NaOH buffer (pH:10) was added dropwise to the extracted pyocyanin until the blue colour was observed again. The blue coloured pyocyanin was again extracted with chloroform. Purification of the pyocyanin was ensured by repeating this step two more times, until a clear blue solution of the pyocyanin in chloroform was obtained. The clear blue solutions of pyocyanin in chlor

oform were transferred into sterile petri dish es and left overnight to evaporate the chlorof orm (Sinem, *et al*, 2016).

The supernatant fermentation broth was tran sferred into clean sterile test tubes and mixed with an equal volume of chloroform to extra ct the pyocyanin. The aqueous phase was re moved while the pyocyanin was re-extracted with 0.2 N HCl (1 mL) until a colour change from blue to red was observed. Following thi s, the absorbance of the pigment solution wa s measured using a UV-VIS spectrophotome ter at 520 nm (Sinem *et al*, 2016).

Data analysis

All data are expressed as the mean ± SEM of three replicates of antibiotic susceptibility profile of isolated *Pseudomonas species* against the tested antibiotics. Data were analyzed using Statistical Package for Social Sciences (SPSS-20)**.**

RESULTS

The result as presented in Table 1 shows the number of *Pseudomonas* species isolated in this work, their morphological characteristics as well as specific reactions to selected biochemical tests. A total of twelve samples (three soil samples from generator house; two soil samples from a mechanic workshop and seven laboratory samples respectively were subjected to microbial analysis yielding three, two and seven pure isolates of *Pseudomonas* species from each of the samples respectively.

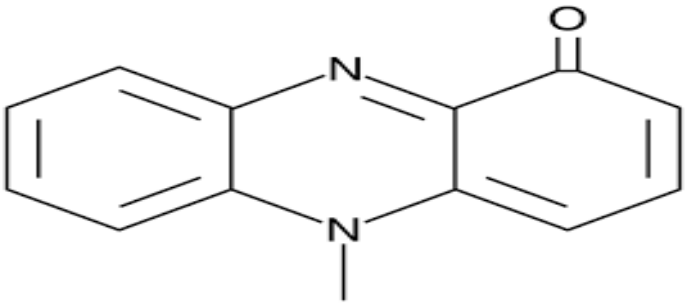
Twelve pure *Pseudomonas aeruginosa* isolates were confirmed [Generator house soil: 3 (25 %); Mechanic workshop soil: 2 (16.7 %); and laboratory clinical specimens: 7 (58.3 %)] by an aggregation of the results of their colonial morphology which appeared round and mucoid producing a yellow-green pigment. Also, their microscopic features appearing rod-like under the light microscope; and finally positive (+, +, +) responses to some selected biochemical tests such as oxidase, citrate, catalase while a negative (-) reaction was observed for indole test (-) for all the isolates thus, lead to their confirmation as *Pseudomonas aeruginosa*.

All the isolated *Pseudomonas aeruginosa* isolates in this work showed similar colonial/cultural, microscopic and biochemical characteristics. Similarly, El-Fouly *et al*., (2014) reported the isolation of twenty *Pseudomonas* from soil, water and clinical specimens.

The antibiotic sensitivity profiles of all the isolates in this work revealed a degree of similarity in their susceptibility and resistance to the selected antibiotics tested (Table 1). A 100 % level of resistance was recorded by all the isolates against Ceftazidime, Cefuroxime, Augmentin and Nitrofuratoin while a 99.9 % resistance was recorded against Cefixime. Other antibiotics tested such as Gentamicin, Ofloxacin and Ciprofloxacin were active against all the test isolates with varying inhibition zones recorded. Also, the antibiotic profiles of the isolates were classified as being either multi-drug resistant (showing resistance to at least three different classes of antibiotics among the antibiotics tested) or pan-resistant (showing resistance to all the tested antibiotics).

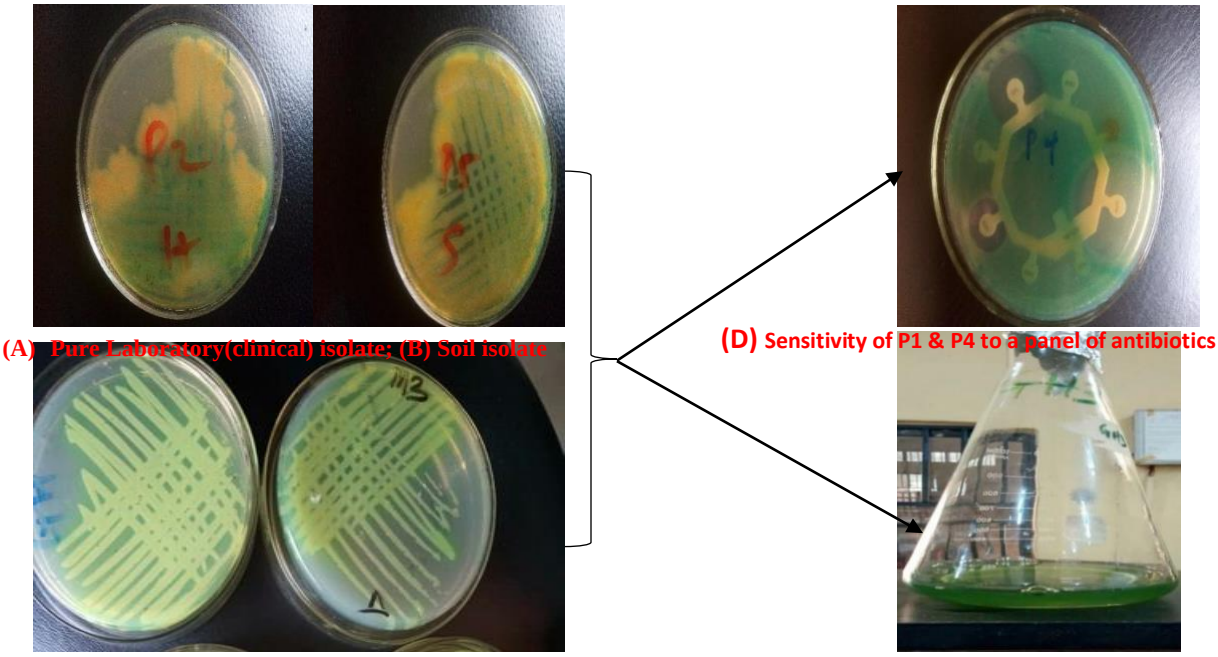
The isolated *Pseudomonas aeruginosa* species in this study were screened for pyocyanin production. Pure cultures were

grown on cetrimide agar thereafter transferred into 100 ml conical flask containing the formulated fermentation broth. All the isolates produced varying amounts of pyocyanin which was observed by the intensity of the blue colour (Figure 2 d-e).



Pyocyanin: $C_{13}H_{11}N_2O$

Figure 1: Structure of pyocyanin





(F) Purified pyocyanin (organic phase) extracted with chloroform produced by isolates from soil. The variation in the color intensity may be due to the pyocyanin content.

Figure 2: Production of pyocyanin by *Pseudomons aeruginosa* isolates.

Table 1: Antibiotic sensitivity testing of the *Pseudomonas aeruginosa* isolates

Isolate code	Antibiotic disc concentration (µg) / Inhibition zone diameter (mm)								Antibiotic status
	CAZ	CRX	GEN	CXM	OFL	AUG	NIT	CPR	
P1L	0±0	0±0	15±0	0±0	19.5±0.7	0±0	0±0	30.5±0.7	MDR
P2L	0±0	0±0	14.5±0.7	0±0	21±0	0±0	0±0	30±0	MDR
P3L	0±0	0±0	8±0	0±0	0±0	0±0	0±0	0±0	MDR
P4L	0±0	0±0	15.5±0.7	0±0	21±1.4	0±0	0±0	31±0	MDR
P5L	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	PR
P6L	0±0	0±0	10±0	0±0	0±0	0±0	0±0	0±0	MDR
P7L	0±0	0±0	14±0±0	0±0	15.5±0.7	0±0	0±0	27±1.4	MDR
P8S	0±0	0±0	14.5±0.7	0±0	22±0	0±0	0±0	31±0	MDR
P9S	0±0	0±0	13	0±0	21.5±2.1	0±0	0±0	29.5±1.4	MDR
P10S	0±0	0±0	13±1.4	0±0	20.5±0.7	0±0	0±0	35.5±0.7	MDR
P11S	0±0	0±0	23±1.4	0±0	22.5±1.4	0±0	0±0	31.5±0.5	MDR
P12S	0±0	0±0	12±1.4	0±0	21±2.8	0±0	0±0	30±0	MDR

Key: PL: *Pseudomonas* species (laboratory isolate); PS: Soil derived-*Pseudomonas* aeruginosa;

CAZ: Ceftazidime (30 µg); **CRX:** Cefuroxime (30 µg); **GEN:** Gentamicin (10 µg); **CXM:** Cefixime (5 µg); **OFL:** Ofloxacin (5 µg); **AUG:** Augmentin (30 µg); **NIT:** Nitrofurantoin (300 µg); **CIP:** Ciprofloxacin (5 µg).

MDR: Multi-drug resistance (resistance to three or more different classes of antibiotics tested), PR: Pan-resistance (resistance to all the antibiotics tested)

DISCUSSION

The antibiotic profiles of the isolated *Pseudomonas aeruginosa* in this work revealed varying degree of sensitivity by the isolates to some commonly used antibiotics. From a broad perspective, the result of the antibiotic sensitivity as presented in (Table 1) showed that 11/12 (91.7 %) of the isolates were multi-drug resistant while 1/12 (8.3 %) was observed to be pan-resistant. In addition one antibiotic (1/12) was degraded by two isolates (P3L and P5L), two antibiotics (1/12) were degraded by one isolate (P6), five antibiotics (9/12) were degraded by nine isolates (P1L, P2L, P4L, P7L – P12S), and eight antibiotics (1/12) were degraded by one isolate (P5). The observed resistance demonstrated by the isolates in this study can be described to be either multi-drug resistance 11/12 (91.7 %) or pan resistance 1/12 (8.3 %). However, most of the isolates exhibited multi-drug resistance (MDR) (Table 1). Clear zones completely devoid of the organisms' pigment and cells were observed for all the active antibiotics (Figure 1D). Total resistance by the isolates was recorded against all the tested cephalosporins (ceftazidime, cefuroxime and cefixime) screened. Whereas, gentamicin 11/12 (91.7 %), ofloxacin and ciprofloxacin 10/12 (83.3 %) were observed to be the most active antibiotics screened. This is contrary to the study conducted by Paula *et al*., (2012) who recorded the highest level of resistance against gentamicin and ciprofloxacin amongst other antibiotics screened.

Amongst the broad spectrum antibiotics screened, amoxicillin-clavulanic acid had no activity against any of the isolates; this may be due to a previous exposure of the laboratory isolates to this antibiotic resulting from antibiotic abuse by individuals who indulge in self-medications also, may be attributed to an intrinsic development of genetic resistance by all the isolates. Also, P8S-P12S isolates from the different soil samples observed to have resisted the effect of Amoxicillin-clavulanic acid may have done so through their inherent intrinsic resistance factors such as pyocyanin production amongst other virulence factors they exhibit which aids in their survival in the presence of antibiotics Paula *et al*., (2012).This is important and at the same time not a good event, as none of the soil isolates had had prior exposure to any antibiotic thus, provides insights towards making the gene(s) responsible for pyocyanin production a target for the action of antibiotics by developing antibiotics that can target and inhibit the mechanism by which pyocyanin is produced. The isolated *Pseudomonas aeruginosa* in this work demonstrated more resistance 5/12 (41.7 %) than susceptibility 3/12 (25 %) to the antibiotics screened with P5L 12/12 and P6 11/12 observed to be the most resistant isolates demonstrating resistance levels of 100 % (12/12) and 91.7 % (11/12),

respectively. The pattern of sensitivity demonstrated by most of the isolates was observed to be similar irrespective of the source of the organism. Table 1 shows that 91.7 (11/12) of the isolates were observed to be multidrug resistant demonstrating resistance to all the tested second generation cephalosporins. From this study, a higher level of resistance was recorded against the β-lactam (Penicillin and cephalosporins) antibiotics used whereas higher susceptibility rate to quinolones tested was recorded. In a previous study on the sensitivity pattern of some *P. aerugionosa* isolates, Josef *et al*., (2015) reported higher susceptibility and higher resistance by some isolates of *P. aeruginosa* to the tested cephalosporins and quinolonoles respectively. The observed resistance in this study may be linked to the low permeability of the cell wall of the isolates to the antibiotics tested in addition to efflux pump resistance mechanisms

In this study, we observed that all the isolates 11/12 (91.7%) that expressed multidrug resistance also produced pyocyanin. Similary, de Araújo Jácome *et al*., (2012) showed that isolates of P aeruginosa that produced pyocyanin also expressed multidrug resistance. Thus, the expression of a virulence factor such as pyocyanin production by the isolates in this study may be associated with their multidrug resistance and pan-resistance activities observed. Also, the amount of pyocyanin produced by each isolates was observed to correlates with the intensity of the blue-green color (Figure 2F) as there were varying color of pyocyanin produced by these isolates when observed visually.

Pyocyanin production by *Pseudomonas* is perceived to be a unique mechanism by which they evade the inhibitory effects of most antibiotics. Therefore, a distortion in this process of pyocyanin production simultaneously makes this organism susceptible to an antibiotic hitherto resistant to.

Furthermore, the poor sensitivity of the isolates to most of the antibiotics tested in this study as revealed by their antibiotic sensitivity profiles could be attributed to an intrinsic inactivation mechanism specific to *P. aeruginosa* isolates. This inactivation/resistance mechanism has also been linked to the pathway responsible for the biosynthesis of pyocyanin a virulence factor reported as one of several factors responsible for the establishment of infection Shannan *et al*., (2012).

Finally, pyocyanin has been reported to possess antimicrobial activities. El-fouly *et al*., (2014) reported the antibacterial activities of pyocyanin against some selected bacteria while a broad spectrum antimicrobial activities was observed by Hamad *et al.*, (2020).

CONCLUSION

In conclusion, the intrinsic property of *P. aeruginosa* being multidrug resistant in addition to a pan-resistant isolate was confirmed. This pose a great concern and calls for appropriate use of antibiotics .Also, our study showed pyocyanin production seem to be unique virulent factor, in that it confers some level of protection for the organisms against the effect of antibiotics at the same time exhibit antimicrobial activities. Therefore, mechanism(s) capable of

inactivating the biosynthesis of virulence factors such as pyocyanin in *P. aeruginosa* is required. This study, provides additional information on the significance of pyocyanin in the antibiotic susceptibility profile of *P. aeruginosa*, its intrinsic multidrug and a gradual change to pan-resistance status.

ACKNOWLEDGMENTS

The authors are grateful to the Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University Awka-Nigeria for providing some of the isolates used.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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