Incidence and molecular characterization of multidrug resistance in Gram-negative bacteria of clinical importance from pharmaceutical wastewaters in South-western Nigeria

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Abstract

Background: The occurrence of antimicrobial resistant bacteria in the environment presents a major threat to public health because it reduces the effectiveness of antimicrobial treatment.

Aims: The study was set out to molecularly characterize Gram-negative bacteria with multidrug resistance and resistance determinants from pharmaceutical wastewaters in Nigeria.

Materials and Methods: Susceptibility of the bacterial isolates to 25 antibiotics belonging to 10 categories was tested using the disc diffusion method and Vitek 2. Screening for AmpC, Extended Spectrum Beta-lactamase and carbapenemase production was done by Polymerase Chain Reaction and sequencing.

Results: Ninety-seven Gram-negative bacteria, comprising 27 Enterobacteria and 70 nonfermenter bacterial isolates were detected. Antibiotic resistance observed was highest (70.1%) for sulfamethoxazole / trimethoprim and multidrug resistance was revealed in 17 bacterial strains (Klebsiella pneumoniae [7], Enterobacter gergoviae [3], Sphingomonas paucimobilis [1], Empedobacter brevis [1], Chryseobacterium indologenes [1], Pseudomonas aeruginosa [1], Burkholderia cenocepacia [1], Burkholderia cepacia and Stenotrophomonas maltophilia [1]). Extended Spectrum Beta-lactamase (CTX-M-15, SHV-12, SHV-2) was positive for 6 K. pneumoniae strains; there were neither AmpC detected nor the production of carbapenamase in all isolates tested.

Discussion: The study confirmed the presence of multidrug resistant Gram-negative bacteria with resistance determinants in wastewaters from pharmaceutical industries in Nigeria. Compounds of the wastewater may directly select or co-select these multidrug resistance strains.

Conclusion: The output of drug resistant bacteria into the environment is a potential risk to public health and may facilitate the spread of resistant genes.

KEYWORDS
ESBL, Gram-negative bacteria, multidrug resistance, Nigeria, pharmaceutical wastewater
INTRODUCTION

Antibiotic-resistant bacteria have emerged due to the selective pressure of antimicrobial use in humans and animals. Water plays an important role in the dissemination of these organisms in the environment (Amaya et al., 2012). This is due to its dynamic nature as it serves as reservoirs, gathering and dispersing bacteria carrying antibiotic-resistance elements; these are mostly encountered in municipal and industrial wastewaters as well as reclaimed waters, thereby posing public health risks. Resistance to antimicrobial agents in clinically relevant Gram-negative bacteria is an increasingly important problem, which in the last few years has spread from the hospital setting to the community (Ghai & Ghai, 2018; Martínez-Martínez & Calvo, 2010). The emergence of antimicrobial-resistant bacteria presents a major threat to public health because it reduces the effectiveness of antimicrobial treatment, leading to increased morbidity, mortality, and healthcare expenditure (Lymo, Buza, Subbiah, Smith, & Call, 2016). Studies have shown the prevalence of antibiotic-resistant pathogens in wastewaters discharged into water bodies (Guardabassi, Petersen, Olsen, & Dalsgaard, 1998; Pruden et al., 2006; Quach-Cu, Herrera-Lynch, Marciniak, & Adams, 2018; Reinthaler et al., 2003), leading to the challenges in antimicrobial therapy. A few authors, in their antibiotic-resistance comparative review using wastewaters from a hospital and a pharmaceutical plant, disclosed that the wastewater from the pharmaceutical plant had increased prevalence of antibiotic resistance among Acinetobacter species (Guardabassi et al., 1998). Recently, a study in Nigeria (Obayiuwana et al., 2018) identified antibiotic-resistant strains, such as Acinetobacter sp., Klebsiella pneumoniae, Proteus mirabilis, Enterobacter sp., and Bacterioides sp. with majority of isolates harboring resistance genes of the sulfonamide-resistant dihydropteroate synthase of Gram-negative bacteria (sul1, sul2) and intI1 genes from pharmaceutical wastewaters. Also Tahran et al. (2017) identified different genera of bacteria from pharmaceutical wastewaters in Tunisia, and these include Citrobacter, Acinetobacter, Pseudomonas, Delftia, Shewanella, and Rheinheimera; however, they suggested that most of these can be utilized as good candidates for bioremediation, thus useful for biotechnological applications. Although previously isolated genus: Exiguobacterium, Dechloromonas, Haliscomenobacter and Morganella from pharmaceutical wastewaters were also recorded; with some of them having 100% antibiotic resistance to amoxicillin and 6 patterns of multidrug resistance among 11 antibiotics tested, and these could have potential in spreading multidrug resistance as well as the associated public health implications (Tahran et al., 2015).

It is noteworthy to say that some of the environmental isolates from the wastewaters observed by these authors could have some relevance in bacterial pathogenesis as a result of the dissemination of resistance genes being transferred to their pathogenic counterparts. In a very recent study in press (University of Cambridge, England, 2019), drug-resistant Enterococcus faecium was isolated from a municipal wastewater plant and when a genomic comparison was done with E. faecium from bloodstream isolates of infected patients, results revealed two major lineages with ampicillin-resistant bacteria in clade A1 and A2 and vancomycin-resistant bacteria exclusive to clade A1, displaying a relatedness of environmental isolates to those that cause serious human diseases. There have been reports on the extensive detection of antibiotic-resistance bacteria (ARB) and antibiotic-resistance genes (ARG) in wastewaters (Bouki et al., 2013), highlighting the need for further research studies in this area. Therefore, the aim of the present study was to screen for antibiotic-resistant Gram-negative bacteria of clinical importance in pharmaceutical wastewaters in Nigeria and characterize multidrug-resistant (MDR) strains with resistance determinants.

METHODS

Wastewater sampling

Wastewater samples were collected from discharge points from 10 pharmaceutical industries located in South-western Nigeria. The Global Positioning System (GPS) readings were taken for every sample location. The industries are producers of antibiotics, such as ciprofloxacin and cotrimoxazole, analgesic agents, cough syrups, and a few other healthcare products. During and after the manufacturing process, the generated wastewaters are discharged directly (without pretreatment) into local rivers and lakes where human activities take place. The untreated wastewater samples were collected randomly (n = 30) from 10 pharmaceutical industries sampled from different sampling points along the wastewaters discharge channels from each pharmaceutical industry. These were preserved on ice packs contained in a flask and then taken immediately to the laboratory for routine microbiological analysis. Samples were processed immediately or stored at 4°C until use. Visit to each pharmaceutical industry for the collection of samples was done three times during the period of sampling. The sampling period was for 18 months, May 2011 to November 2012.

Isolation and characterization of Gram-negative bacteria

Bacteria were isolated on nutrient agar plates (Talaro, 2009), and subsequent screening for Gram-negative bacteria was done by the Gram-staining technique (Kola et al., 2012). Further characterization was carried out with the lactose fermentation test, oxidase test, and catalase test (Forbes et al., 2015; John et al., 2009).

Identification of Gram-negative bacteria

Gram-negative isolates were further identified using an automated method with Vitek 2 (Biomerieux Inc.). The Vitek card GN (Ref.) 21341 was used made up of 60 ingredients including sugars (Kola et al., 2012). In addition, specific PCRs for the identification of K. pneumoniae sensu stricto (Bialek-Davenet et al., 2014) and target for RecA gene sequencing for the identification of Burkholderia cepacia complex (Cesarini et al., 2009) were performed.
2.4 | Antimicrobial susceptibility testing

2.4.1 | Antimicrobial agents

Antimicrobial susceptibility testing was performed using the automated system Vitek2 including antibiotics (BioMerieux Inc.) with cards AST-N223 (Ref. 413110) and AST-N248 (Ref. 413397). For the disk diffusion method, BD sensidisc (Dickenson and Company) antibiotic disks with amoxicillin-clavulanate (AMC30), levofloxacin (LEV5), and colistin (CL10) were used.

2.4.2 | Determination of minimum inhibitory concentrations (MIC) and detection of extended spectrum beta-lactamase (ESBL)

Minimum inhibitory concentrations (MICs) are considered the “gold standard” for determining the antimicrobial susceptibilities of bacteria. Minimum inhibitory concentration is defined as the lowest concentration (mg/L) of an antibiotic that showed 100% inhibition of bacterial growth. All bacterial isolates were tested for their antimicrobial susceptibilities using the Vitek 2 system with cards AST-N223 (Ref. 413110) and AST-N248 (Ref 413397) for automatic determination of MICs, and following the EUCAST (European Committee on Antimicrobial Susceptibility Testing, 2013) and the CLSI (Clinical and Laboratory Standards Institute, 2012) guidelines and their corresponding MIC interpretation standards/clinical breakpoints. This Vitek MIC determination is based on cards with antibiotic substances in wells containing various concentrations of different antibiotics. For each antibiotic tested, turbidity was measured at regular intervals. For extended spectrum beta-lactamase (ESBL) confirmation, the proportional reduction of growth in wells containing a cephalosporin combined with clavulanic acid was then compared with that achieved by the cephalosporin alone. The result was interpreted as ESBL-positive or ESBL-negative through a computerized automated expert system (AES). Three control strains (Escherichia coli ATCC 25922, K. pneumoniae ATCC BAA-1705, and Pseudomonas aeruginosa ATCC 27853) were used to validate the measurement. Overnight cultures were introduced into tubes containing 2.5 ml inhalation solution (0.45% sodium chloride) with the aid of an inoculating loop and then further procedure applies as previously described (Kola et al., 2012).

2.4.3 | Disk diffusion method (Kirby Bauer method)

Bacterial isolates were subjected to the disk diffusion method (John et al., 2009) to determine the zones of inhibition and further confirm their susceptibility to antibiotics. For interpretation of results (susceptibility/intermediate resistance/resistance), clinical breakpoints were used.

2.4.4 | Multidrug-resistant (MDR) Gram-negative bacteria (MRGN)

Multidrug resistance was defined as the nonsusceptibility of an isolate to at least 1 agent in ≥3 antimicrobial categories; however, lists of antimicrobial agents were developed for each organism or organism group, as proposed harmonized templates that could be used by clinical, reference, and public health microbiology laboratories (Magiorakos et al., 2012).

2.4.5 | Modified Hodge test (MHT) for the phenotypic detection of carbapenemases

Phenotypic detection of the production of carbapenemase was performed using the modified Hodge test (MHT). Bacterial isolates with resistance to carbapenem (ertapenem, imipenem, and/or meropenem) were tested for carbapenemase production. This method was done by establishing a McFarland standard of 0.5 of bacterial solution of a carbapenem-sensitive E. coli (ATCC 25922) that has already been introduced into 2.5 ml sterile NaCl with a sterile cotton swab, and measuring with the densiChek. Then, 0.5 ml of the suspension was further dissolved in 4.5 ml of NaCl to attain a ratio of 1:10. The suspension was further seeded on 2 plates of Mueller-Hinton agar. Sterile disks of ertapenem, imipenem, and meropenem (from BD sensidisc) antibiotics were carefully placed each in a triangular pattern on each plate, and this was done 15 min after seeding the plate. One control strain (carbapenemase-producer) was introduced into the seeded plates by making lines 2 mm away from the points where the 3 antibiotic disks were placed, making sure the lines do not intercept one another. The same procedure was carried out for the test strain on the second plate incubating the plates aerobically at 37°C for 18–24 hr. Positive results for carbapenemase producers were shown by the heart shape deformation of the inhibition zone, while negative results retained the usual round shape (Kothari et al., 2013).

2.5 | Genotypic characterization of resistance determinants

2.5.1 | Screening for presence of beta-lactamase encoding genes

All isolates with resistance to third-generation cephalosporins and/or carbapenem were screened for presence of ESBL encoding genes (blaTEM, blaSHV, blaCTX-M), plasmid-mediated AmpC genes (blaCMY, blaDHA, blaACC, blaFOX, blaMOX, blaIMP), and carbapenemase genes (blaOXA-48, blaKPC, blaVIM, blaIMP, blaNDM, blaOXA) by PCR using appropriate primers (Table S1) as described (Gröbner et al., 2009; Perez-Perez & Hanson, 2002; Pfeifer, Matten, & Rabsch, 2009).

2.5.2 | Preparation of DNA

The preparation of DNA for PCR gene amplification was carried out by introducing colonies of cell materials of overnight bacterial culture of ESBL-positive, resistance to third-generation cephalosporins and carbapenem resistance, plated on Luria Bertani (LB) agar in 200 µl of phosphate-buffered saline (PBS). Further procedure applies as previously described (Kola et al., 2012).
2.5.3 | Detection of ESBL encoding genes by PCR using a multiplex primer

The mixture for ESBL-MP-PCR reaction (25 µl) contained: 12.5 µl Mastermix (DreamTaq PCR (2x) Thermo Fisher Scientific), 0.3–0.8 µl each of the forward and reverse primers (TEM universal primers 0.8 µl; SHV-MP-primers 0.4 µl; CTX-M gesamt, CTX-M-14-B, CTX-M-21 and CTX-M-14-B and CTX-M-21, CTX-M universal primers 0.4 µl, CTX-M-9-MP primers 0.3 µl), 19.2 µl of aqua bidest, and 2 µl of DNA supernatant. The mix was amplified in a T300 thermocycler with the PCR conditions of 30 cycles of initial denaturation for 2 min at 95°C, denaturation for 30 s at 95°C, annealing for 30 s at 55°C, extension at 30 s at 72°C, and final extension for 4 min at 72°C. Then, 5 µl of each PCR product was transferred into 15 µl Eppendorf tubes and 1 µl of either the forward or reverse primers were introduced separately into 2 different tubes containing the PCR product as described (Pfeifer et al., 2009). Analysis of the resulting gene sequences was done with the use of basic local alignment search tool (BLAST; www.ncbi.nlm.nih.gov) to identify and confirm the amplified genes.

2.5.4 | Screening for carbapenemase genes

Screening for carbapenemase genes, bla_{OXA-48}, bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{NDM}, bla_{GUM}, was carried out with PCR amplification using appropriate primers (Table S1), and PCR products were resolved on gel electrophoresis as described (Kola et al., 2012; Pfeifer et al., 2009). For the sequencing of PCR-amplified genes, 14 µl of each PCR product was transferred into 15 µl Eppendorf tubes and 1 µl of either the forward or reverse primers were introduced separately into 2 different tubes containing the PCR product as described (Pfeifer et al., 2009). Then, further sequencing procedure was performed by a company (Mwg eurofins, Germany). Analysis of the resulting gene sequences was done using a multiplex primer method.

2.6 | Statistical analysis

Descriptive Charts from Microsoft Excel package (2010 version) from windows 8.1 operating system were used to analyze the data on susceptibility profile.

3 | RESULTS

3.1 | Isolation and characterization of Gram-negative bacteria

Gram-negative bacteria identified from wastewater samples were 97, and cellular morphology revealed that they were all bacilli (rod-shaped bacteria).

3.2 | Identification of Gram-negative bacteria with Vitek 2

Analysis of Gram-negative bacterial diversity on the various pharmaceutical industries wastewater samples using different identification methods revealed presence of 49 detectable bacterial spp., belonging to the Enterobacteriaceae family and the nonfermenters (Table S2). These include the following: K. pneumoniae (n = 7), from two industries, P. mirabilis (n = 7) from two industries, Enterobacter cloacae complex (n = 6) from four industries, Enterobacter gergoviae (n = 3) from one industry, Serratia marcescens (n = 2) from two industries, Alcaligenes faecalis (n = 5) from two industries, Providencia stuartii (n = 1) from one industry, Pantoea sp. (n = 1) from one industry, Cronobacter sakazakii (n = 1) from one industry, Myroides spp. (n = 2) from two industries, Sphingomonas paucimobilis (n = 1) from one industry, Weeksella virosa (n = 1) from one industry, Empedobacter brevis (n = 1) from one industry, Chryseobacterium indologenes (n = 1) from one industry, P. aeruginosa (n = 7) from three industries, B. cepacia (n = 1) from one industry, Burkholderia cenocepacia (n = 1) from one industry, and Stenotrophomonas maltophilia (n = 1) from one industry. Furthermore, by species-specific PCRs, we identified 7 K. pneumoniae as well, while with RecA gene sequencing, one B. cepacia and B. cenocepacia isolates were also identified (Table 1).

3.3 | Antimicrobial susceptibility testing

The analyses of the profile of resistance to 25 antibiotics were observed in the Gram-negative bacterial isolates. The profile showed that overall percentage resistance ranges from 0% (ertapenem) to 70.1% (sulfamethoxazole/trimethoprim). Susceptibility was low for tigecycline (16.7%) and high for meropenem (94.8%; Table 2).
Furthermore, antimicrobial susceptibilities of Enterobacterial isolates \((n = 27)\) and the nonfermenter isolates \((n = 70)\) were analyzed independently. For the Enterobacteriaceae, the antibiotic-resistance ranged from 34.6% (cefotaxime and cefazidime) to 75% (ampicillin). And also, for the nonfermenters, the antibiotic-resistance ranged from 1.6% (levofloxacin) to 85.7% (sulfamethoxazole/trimethoprim; Figure 1). All seven \(K. pneumoniae\) isolates were resistant to ampicillin, piperacillin, ciprofloxacin, gentamicin, and sulfamethoxazole/trimethoprim but remained susceptible to carbapenems (ertapenem, imipenem, meropenem), amikacin, and colistin. Resistance to piperacillin/tazobactam, cefpodoxime, ceftaxime, and cefazidime was detected in six \(K. pneumoniae\) isolates, while four of them were resistant to tigecycline. \(P. mirabilis\) \((n = 7)\) were all resistant to tigecycline, but only one and four showed intermediate resistance to the ertapenem and imipenem, respectively, but all were susceptible to other antibiotics. The resistance profile for \(E. cloacae\) complex isolates \((n = 6)\) showed that all were resistant to ampicillin and cefpodoxime, while two were resistant to ciprofloxacin but all were susceptible to \(β\)-lactam and \(β\)-lactamase inhibitor combination (piperacillin/tazobactam), tigecycline, cephalosporins (cefotaxime, cefuroxime, ceftazidime), aminoglycosides (gentamicin), and carbapenems (ertapenem, imipenem, meropenem). The three \(E. gergoviae\) isolates were all resistant to piperacillin, piperacillin/tazobactam, cefotaxime, ciprofloxacin, and moxifloxacin, and two were resistant to gentamicin and trimethoprim/sulfamethoxazole. However, they were all susceptible to the carbapenems (ertapenem, imipenem, meropenem) and tigecycline. \(S. marcescens\) \((n = 2)\) isolates were both resistant to \(β\)-lactams (ampicillin), \(β\)-lactam, and \(β\)-lactamase inhibitor combination (ampicillin-sulbactam), cephalosporins (cefuroxime, cefuroxime-axetil), and both showed intermediate resistance to tigecycline. The only \(P. stuartii\) isolate was resistant to \(β\)-lactams (ampicillin, piperacillin), \(β\)-lactam, and \(β\)-lactamase inhibitor combination (ampicillin-sulbactam), cephalosporins (cefpodoxime), aminoglycosides (gentamicin), and tigecycline. \(P. aeruginosa\) isolates of this study were resistant to ampicillin, cefuroxime, and fosfomycin \((n = 7)\). \(B. cepacia\) group \((n = 2)\), \(S. maltophilia\) \((n = 1)\), \(S. paucimobilis\) \((n = 1)\), and \(E. brevis\) \((n = 1)\) isolates were all resistant/intermediate resistant to the carbapenems (imipenem and meropenem), respectively. The MIC values for the Vitek 2 breakpoint for resistance in these bacterial isolates range from ≤1 for gentamicin in \(E. cloacae\) strains to ≥320 for trimethoprim/sulfamethoxazole for all bacterial isolates; also for susceptibility, the range was from ≥0.2 for Amikacin in \(E. gergoviae\) strains to ≥20 trimethoprim/sulfamethoxazole in \(P. mirabilis\) strains.

### 3.4 Detection of ESBL production by Vitek 2

The screening for ESBL by Vitek 2 was positive for six \(K. pneumoniae\) isolates located at two geographical points, one from Ogun state and five from Lagos state as detected by the computerized AES.

### 3.5 MDR Gram-negative bacteria (MRGN)

MRGN was observed in isolates \((n = 17)\) from 6 pharmaceutical industries wastewater samples, at the 2 geographical locations sampled. These include the following: \(K. pneumoniae\) \((n = 7)\), \(E. gergoviae\) \((n = 3)\), \(E. brevis\) \((n = 1)\), \(S. paucimobilis\) \((n = 1)\), \(P. aeruginosa\) \((n = 1)\),
C. indologenes (n = 1), B. cenocepacia, B. cepacia (n = 1), and S. maltophilia (n = 1; Tables 1 and 3). All other isolates were either resistant to one single or more antibiotics of the same or different class, and some could also be referred to as multiple antibiotic resistances (MAR).

### TABLE 2
Number and percentage of antimicrobial susceptibility of gram-negative bacterial isolates from pharmaceutical wastewater samples

| Antimicrobial agent          | Number of tested isolates | Number of resistant bacteria (%) | Number of intermediate resistant bacteria (%) | Number of susceptible bacteria (%) |
|-----------------------------|---------------------------|---------------------------------|---------------------------------------------|-----------------------------------|
| Ampicillin (AMP)            | 43                        | 29 (67.4%)                      | ND                                          | 14 (32%)                         |
| Ampicillin/sulbactam (SAM)  | 29                        | 12 (41.4%)                      | ND                                          | 17 (58.6%)                       |
| Piperacillin (PIP)          | 97                        | 19 (19.6%)                      | 1 (1.0%)                                    | 77 (79.4%)                       |
| Piperacillin/tazobactam (TZP) | 97                       | 17 (17.5%)                      | 2 (2.1%)                                    | 78 (80.4%)                       |
| Cefuroxime (CXM)            | 43                        | 21 (48.8%)                      | ND                                          | 22 (51.2%)                       |
| Cefuroxime/axetil (ACE)     | 38                        | 13 (34.2%)                      | ND                                          | 15 (39.5%)                       |
| Cefpodoxime (CPD)           | 38                        | 21 (55.3%)                      | ND                                          | 17 (44.7%)                       |
| Cefotaxime (CTX)            | 42                        | 16 (38.1%)                      | ND                                          | 26 (61.9%)                       |
| Ceftazidime (CAZ)           | 97                        | 17 (17.5%)                      | ND                                          | 80 (82.5%)                       |
| Ertapenem (ERT)             | 28                        | ND                              | 3 (10.7%)                                   | 25 (89.3%)                       |
| Imipenem (IPM)              | 97                        | 6 (6.2%)                        | 13 (13.4%)                                  | 78 (80.4%)                       |
| Meropenem (MEM)             | 97                        | 3 (3.1%)                        | 2 (2.1%)                                    | 92 (94.8%)                       |
| Gentamicin (GEN)            | 97                        | 23 (23.7%)                      | 3 (3.1%)                                    | 71 (73.2%)                       |
| Ciprofloxacin (CIP)         | 97                        | 44 (45.4%)                      | 25 (27.8%)                                  | 28 (28.9%)                       |
| Moxifloxacin (MXF)          | 43                        | 20 (46.5%)                      | ND                                          | 23 (53.5%)                       |
| Levofloxacin (LEV)          | 75                        | 4 (5.3%)                        | 2 (2.6%)                                    | 69 (92.0%)                       |
| Tigecycline (TGC)           | 36                        | 19 (52.7%)                      | 11 (30.6%)                                  | 6 (16.7%)                        |
| Cefepime (FEP)              | 22                        | 7 (31.8%)                       | 3 (13.6%)                                   | 12 (54.5%)                       |
| Aztreonam (ATM)             | 22                        | 11 (50%)                        | 6 (27.3%)                                   | 5 (22.7%)                        |
| Amikacin (AMK)              | 22                        | 4 (18.2%)                       | 2 (9.1%)                                    | 16 (72.7%)                       |
| Tobramycin (TOB)            | 85                        | 15 (17.6%)                      | 1 (1.1%)                                    | 69 (81.2%)                       |
| Fosfomycin (FOS)            | 14                        | 9 (64.3%)                       | ND                                          | 5 (35.7%)                        |
| Colistin (COL)              | 80                        | 5 (6.3%)                        | ND                                          | 75 (93.7%)                       |
| Amoxicillin/Clavulanate (AMC)| 63                      | 10 (15.9%)                      | ND                                          | 53 (84.1%)                       |
| Sulfamethoxazole/Trimethoprim (SXT) | 97              | 68 (70.1%)                      | 1 (2.7%)                                    | 28 (28.9%)                       |

Abbreviation: ND, Not detected.

3.6 | MHT for the phenotypic screening of carbapenemases

Resistance to carbapenems could mean production of carbapenemase by the bacteria. All tested isolates that include B. cepacia, B. cenocepacia, S. maltophilia, S. paucimobilis, and E. brevis were resistant/intermediate resistant to imipenem and meropenem (Table 3); however, they were all negative for the production of carbapenemases by MHT, as observed by the inhibition zones that retained the round shape while the control strains indicated positive result (heart-shaped inhibition zones).

3.7 | Genotypic characterization of resistance determinants

3.7.1 | Screening for presence of beta-lactamase encoding genes

The presence of β-lactamase gene in K. pneumoniae strains was demonstrated by PCR amplification with ESBL multiplex primers. DNA sequencing of these genes revealed that 57.2% (n = 4) harbored the blaTEM-1 gene, 85.7% (n = 6) harbored the blaSHV genes, and 71.4% (n = 5) harbored blaCTX-M gene. The genes blaSHV encoding SHV-1, SHV-11 SHV-2, SHV-12, and SHV-28 were found in 66.7% (n = 4), 33.3% (n = 2), 16% (n = 1), 16% (n = 1), and 16% (n = 1) of the strains, respectively. The five strains encoding blaCTX-M also harbored blaSHV1, blaSHV28, and blaSHV2 genes, while three out of the five strains encoding blaCTX-M harbored blaTEM-1 gene as well (Table 3). ESBL genes detected in six K. pneumoniae isolates were blaCTX-M-15.
Screening for carbapenemase gene after PCR amplification and resolution of PCR product on gel electrophoresis revealed that there was no indication of *bla*<sub>OXA</sub>-48, *bla*<sub>KPC</sub>, *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>NDM</sub>, or *bla*<sub>GIM</sub> in all the MDR bacterial isolates with resistance to carbapenem when the primer pairs were used, as this also confirms the results obtained from the phenotypic screening with MHT earlier observed, for the absence of carbapenamase.

### 3.7.2 | Screening for plasmid-mediated AmpC in MDR isolates

Screening for AmpC by resolution of PCR product on gel electrophoresis showed that there was no indication of *bla*<sub>OXA</sub>-48, *bla*<sub>KPC</sub>, *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>NDM</sub>, or *bla*<sub>GIM</sub> in all the MDR bacterial isolates with resistance to carbapenem when the primer pairs were used. However, there was a weak signal from two strains of *E. gergoviae* with the EBCM primer. DNA sequencing reaction and blasting revealed that they were outer protein membranes.

### 3.7.3 | Associated resistance in ESBL *K. pneumoniae*

Six *K. pneumoniae* strains harboring the ESBL genes have associated resistance in other classes of antibiotics. These ESBL-producing strains with resistance to the 3rd- and 4th-generation cephalosporins producing the enzymes TEM-1, SHV-1, SHV-2, SHV-11, SHV-12, SHV-28, and CTX-M-15 were also resistant to the fluoroquinolones (ciprofloxacin, moxifloxacin), the aminoglycosides (gentamicin, tobramycin) as well as the folate pathway inhibitors (trimethoprim/sulfamethoxazole).

### 4 | DISCUSSION

In order to analyze the bacterial contamination and presence of clinically relevant antibiotic-resistant bacteria, wastewaters from 10 pharmaceutical industries were investigated. The organisms isolated ranged from the Enterobacteriaceae to the nonfermenter group of organisms such as *Pseudomonas* spp., *Stenotrophomonas* sp., and *Burkholderia* spp., and these were in conformity with reports from other authors detecting similar organisms in wastewaters from the environments (Bouki et al., 2013; Chagas et al., 2011; De Boeck et al., 2012).

The antibiotic susceptibility patterns of the 97 Gram-negative isolates in this study showed that there were single, double, and multiple resistance phenotypes. The highest resistance rate was observed for sulfamethoxazole/trimethoprim (70.1%) especially in the nonfermenter bacterial strains. This is consistent with the view of Ruppe, Woerther, and Barbier (2015) on intrinsic resistance observed in nonfermenters. Most of the strains (80.4%–94.8%) were susceptible to imipenem and meropenem but broad resistance to cephalosporins was observed with the highest resistance rate found.
### TABLE 3: Characteristics of nonsusceptible MDR bacterial isolates to different antibiotic categories from pharmaceutical wastewater samples in Nigeria

| MRGN strain | PCllin | Ceph | Carb | AminG | PhosA | Gcycl | P+Bl | FloTpi | FloQ | CIP | Antibiotic-resistance pattern | B-lactamases | ESBLs |
|-------------|--------|------|------|-------|-------|-------|------|-------|------|-----|-----------------------------|---------------|--------|
| K. pneumoniae | R ≥ 128 | R ≥ 64 | S ≤ 0.25 | R ≥ 16 | R ≥ 16 | R = 8 | R ≥ 32 | R ≥ 320 | R ≥ 4 | AMP, CIP, MXF, GEN, SXT, SAM, PIP, TZP, CXM, ACE, CPD, CTX, CAZ | SHV-1 | CTX-M-15 |
| K. pneumoniae | R ≥ 128 | R ≥ 64 | S ≤ 0.25 | R ≥ 16 | R ≥ 16 | R = 8 | R ≥ 32 | R ≥ 320 | R ≥ 4 | AMP, CIP, MXF, GEN, SXT, SAM, PIP, TZP, CXM, ACE, CPD, CTX, CAZ | SHV-1 | CTX-M-15 |
| K. pneumoniae | R ≥ 128 | R ≥ 64 | S ≤ 0.25 | R ≥ 16 | R ≥ 16 | R = 8 | R ≥ 32 | R ≥ 320 | R ≥ 4 | AMP, CIP, MXF, GEN, TOB, SXT, SAM, PIP, TZP, CXM, CPD, CTX, CAZ, ATM, FEP, TGC | SHV-1 | CTX-M-15 |
| K. pneumoniae | R ≥ 128 | R ≥ 64 | S ≤ 0.25 | R ≥ 16 | R ≥ 16 | R = 8 | R ≥ 32 | R ≥ 320 | R ≥ 4 | AMP, CIP, MXF, GEN, SXT, SAM, PIP, TZP, CXM, ACE, CPD, CTX, CAZ, TGC | SHV-11, TEM-1 | SHV-12 |
| K. pneumoniae | R ≥ 128 | R ≥ 64 | S ≤ 0.25 | R ≥ 16 | R ≥ 16 | R = 8 | R ≥ 32 | R ≥ 320 | R ≥ 4 | AMP, CIP, MXF, GEN, SXT, SAM, PIP, TZP, CXM, ACE, CPD, CTX, CAZ, TGC | SHV-28, TEM-1 | CTX-M-15 |
| K. pneumoniae | R ≥ 128 | R ≥ 64 | S ≤ 0.25 | R ≥ 16 | R ≥ 16 | R = 8 | R ≥ 32 | R ≥ 320 | R ≥ 4 | AMP, CIP, MXF, GEN, TOB, SXT, SAM, PIP, TZP, CXM, CPD, CTX, CAZ, ATM, FEP, TGC | SHV-1, TEM-1 | CTX-M-15, SHV-2 |
| K. pneumoniae | R ≥ 128 | S ≤ 1 | S ≤ 0.25 | R ≥ 16 | R ≥ 16 | R = 8 | R ≥ 32 | R ≥ 320 | R ≥ 4 | AMP, SAM, PIP, CXM, ACE, GEN, CIP, MXF, TGC, SXT | ND | ND |
| E. gergoviae | R ≥ 128 | R ≥ 64 | S ≤ 0.25 | R ≥ 16 | S = 32 | I = 2 | R ≥ 32 | I ≥ 320 | R ≥ 4 | PIP, TZP, CTX, CAZ, GEN, CIP, MXF, FEP, ATM, TOB, SXT | ND | ND |
| E. gergoviae | R ≥ 128 | R ≥ 64 | S ≤ 0.25 | R ≥ 16 | R = 64 | I = 2 | R ≥ 32 | I ≥ 80 | R ≥ 4 | PIP, TZP, CTX, CAZ, CIP, MXF, FEP, ATM, FOS | ND | ND |
| E. gergoviae | R ≥ 128 | R ≥ 64 | S ≤ 0.25 | S ≤ 1 | S = 32 | I = 2 | R ≥ 32 | I ≥ 320 | R ≥ 4 | AMP, SAM, PIP, TZP, CXM, ACE, CPD, CTX, CAZ, GEN, CIP, MXF, SXT | ND | ND |
| S. paucimobilis | R ≥ 128 | S = 4 | R ≥ 16 | I = 4 | ND | ND | ND | ND | R ≥ 4 | PIP, TZP, CTX, IMP, CIP, MXF, ATM | ND | ND |
| E. brevis | R ≥ 128 | R ≥ 64 | R ≥ 16 | R ≥ 16 | ND | ND | ND | ND | R | S = 0.5 | PIP, TZP, CAZ, IMP, MEM, GEN, FEP, ATM, AMK, TOB, COL | ND | ND |
| P. aeruginosa | S ≤ 4 | S ≤ 1 | S = 1 | R ≥ 16 | R ≥ 256 | ND | R ≥ 32 | R ≥ 320 | S ≤ 0.25 | AMP, SAM, PIP, CXM, TZP, ACE, CPD, CTX, CAZ, GEN, TOB, FOS, SXT | ND | ND |
| S. maltophilia | R | R | R | S | ND | ND | R | ND | R | R | PIP, TZP, CAZ, IMP, MEM, CIP, LEV, SXT, AMC | ND | ND |
| B. cepacia | I = 64 | R ≥ 16 | R ≥ 16 | R ≥ 16 | ND | ND | ND | ND | R | R | AMP, TXP, CXM, CPD, CTX, CAZ, IMP, GEN, CIP, TGC, FEP, ATM, AMK, TOB, SXT | ND | ND |

(Continues)
TABLE 3 (Continued)

| MIC (μg/ml) for susceptibility test in multidrug-resistant strains from antibiotic categories | B-lactamases | ESBLs |
|-----------------------------------|--------------|-------|
| MRGN strain | PClin | Ceph | Carb | AminG | PhosA | Gcyl | TGC | P+Bli | FoIPI | FloQ | CIP | Antibiotic-resistance pattern | B-lactamases | ESBLs |
| B. cenocepacia | S = 8 | S = 2 | R ≥ 16 | R ≥ 16 | ND | ND | ND | R ≥ 320 | R ≥ 4 | AMP, TZIP, CXM, CPD, CTX, IMP, GEN, CIP, MXFAMK, TOB, SXT | ND | ND |
| C. indologenes | R | R | S | R | ND | ND | ND | S | I | PIP, SAM, CAZ, CTX, GEN, FEP, AMK, | ND | ND |

Abbreviations: ACE, cefuroxime/axetil; AMC, amoxicillin/clavulanate; AminG, Aminoglycosides; AMK, amikacin; AMP, ampicillin; ATM, aztreonam; Carb, Carbapenem; CAZ, ceftazidime; Ceph, Cephalosporins; CIP, ciprofloxacin; COL, colistin; CPD, cefepoxide; CTX, cefotaxime; CXM, cefoxitin; ESBL, Extended spectrum β-lactamase; FEP, cefepime; FloQ, Fluoroquinolone; FoIPI, Folate pathway inhibitor; FOS, fosfomycin; Gcyl, Glycylcycline; GEN, gentamicin; I, Intermediate; IMP, imipenem; LEV, Levofloxacin; MEM, meropenem; MIC, Minimum inhibitory concentration; MRGN, Multidrug-resistant Gram-negative; MXF, moxifloxacin; ND, Not detected; P+BLi, Penicillins+b-lactamase inhibitors; PCIII, Penicillin; PhosA, Phosphonic acid; PIP, piperacillin; R, Resistance; S, Susceptible; SAM, ampicillin/subactam; SXT, trimethoprim/sulfamethoxazole; TGC, tigecycline; TOB, tobramycin; TZIP, tizobactam.

Bold, ESBL-producing K. pneumoniae and associated resistance to FloQ (CIP, MXF), AminG (GEN, TOB) and FoIPI (SXT).

Sphingomonas paucimobilis is another opportunistic pathogen that rarely causes infection in humans because of its low virulence. A recent study reported by Pratama et al. (2016) and Saeb, David, and Saeb (2016) detected that 83% of the S. paucimobilis from the wastewater were resistant to carbapenems, ampicillin/subactams, and trimethoprim/sulfamethoxazole, which also correspond to our findings. Enterobacter aerogenes is a relatively rare human pathogen, and the detection of S. paucimobilis in the wastewater could be harboring resistance genes which may be transferred to their pathogenic counterparts. The resistance to cefepime, cefotaxime, and cefuroxime of S. paucimobilis has been reported in the wastewater in the Northern part of Nigeria. The study conducted by Olukosi (2017) detected only one isolate belonging to the 178 isolates from drinking water samples, which is the highest resistance rate among the non-β-lactam antibiotics.

K. pneumoniae isolates was also observed in wastewaters from the Democratic Republic of Congo (De Becker et al., 2012). In addition, K. pneumoniae isolates were also observed in wastewaters from the Democratic Republic of Congo (De Becker et al., 2012), although the resistance rates to amikacin and sulfamethoxazole/trimethoprim were lower. The resistance to cefepime, cefotaxime, and cefuroxime of S. paucimobilis has been reported in the wastewater in the Northern part of Nigeria. The study conducted by Olukosi (2017) detected only one isolate belonging to the 178 isolates from drinking water samples, which is the highest resistance rate among the non-β-lactam antibiotics.

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and to cefotaxime and amikacin (Cheong et al., 2008). As there are no definitive guidelines for antimicrobial therapy for this bacterial infection, treatment is done with individualized antibiotic therapy according to the in vitro susceptibility profile of clinical isolates (Özdemir et al., 2011; Pratama et al., 2016; Toh et al., 2011). Some studies recommended fluoroquinolones, third-generation cephalosporins, and carbapenems as excellent drugs of choice as initial therapy for *S. paucimobilis* infection (Hsueh et al., 1998; Maragakis et al., 2009; Morrison & Shulman, 1986; Ryan & Adley, 2010), but unfortunately in our study, this bacterium was resistant to these classes of antibiotics recommended, which begs the question: can an individual infected with this bacterium be successfully treated in a case of an emergency? This susceptibility profile of multidrug resistance in this bacterium poses new threat to the antibiotic therapy of this infection; however, Ryan and Adley (2010) suggested that therapy should be adjusted when the susceptibility results are available, or if the patient fails to respond to initial therapy. They also observed that no antibiotic-resistant mechanisms have yet been elucidated for *S. paucimobilis*.

*Empedobacter brevis* was previously known as *Flavobacterium breve* and the pathogenicity of the bacteria is rare and is usually limited to healthcare workers. However, no specific virulence factors have been identified for these species (Sharma et al., 2016). Although there is a first case report of neonatal bacteraemia and meningitis secondary to *E. brevis* and the infant responded well to cefoperazone-sulbactam, they also observed that all the previous six case reports have been published only in adults, with various manifestations. The organism was sensitive to tetracycline, tigecycline, and cefoperazone-sulbactam and was resistant to ciprofloxacin, colistin, amikacin, amoxyclav, ampicillin, cefazolin, cefepime, cefotaxime, ceftazidime, cefixime, ertapenem, gentamicin, imipenem, levofloxacin, meropenem, piperacillin/tazobactam, piperacillin, and tobramycin (Sharma et al., 2016). Our study also showed similar resistant patterns in this bacterium with resistance to piperacillin, piperacillin/tazobactam, ceftazidime, imipenem, meropenem, gentamicin, cefepime, aztreonam, amikacin, tobramycin, colistin except for the fluoroquinolones. Schroeder et al. (2015) reported a case of 60-year-old male infected with this organism and was treated with ciprofloxacin.

*Pseudomonas aeruginosa*, *B. cepacia* groups, and *S. maltophilia* that are recognized to be MDR in this study are also known to be opportunistic pathogens responsible for nosocomial infections. This study identified MDR *P. aeruginosa* with resistance to ampicillin, gentamicin, tobramycin, fosfomycin, piperacillin, ampicillin/sulbactam, piperacillin/tazobactam, cefuroxime, cefuroxime/axetil, cefpodoxime, cefotaxime, ceftazidime, and sulfamethoxazole. A study also isolated *P. aeruginosa* from hospital wastewaters in Nigeria, with MDR characteristics; the isolates were resistant to piperacillin, tetracycline, nitrofurantoin, ceftazidime, gentamicin, aztreonam, ciprofloxacin, and ofloxacin (Falodun et al., 2019). Another study by Odjadare et al. (2012) recorded multiple antibiotic resistance in *Pseudomonas* spp from municipal wastewater treatment facility in South Africa, recording resistance to penicillins, rifampin, sulfamethoxazole, and cephalosporins. These studies are comparable to our findings as well, indicating concerns for risk assessment in the wastewaters.

Two *Burkholderia* strains, *B. cepacia* and *B. cenocepacia* from the wastewaters belonging to genomovar I and III, respectively, showed different resistance patterns. This is consistent with previous reports describing high levels of variability in environmental *B. cepacia* complex strains, with high degrees of phenotypic similarity between clinical and environmental strains of *B. cepacia* complex (Araque-Caldeson et al., 2008; Meinkow, Zaborina, & Dhiman, 2000; Wang, Fast, Valentine, & Benkovic, 1999). The MIC values obtained for imipenem, tobramycin, amikacin, ceftazidime, ciprofloxacin, piperacillin, and piperacillin/tazobactam ranging from 0.5 to 128 (µg/ml) of the environmental isolates of *B. cepacia* complex from this study were comparable to those

![GeneRuler 100 bp DNA Ladder (M) (a) SHV-universal (938 bp amplification product), (b) CTX-M-gesamt (968 bp amplification product) in *Klebsiella pneumoniae* strains (Lane 1-6). GeneRuler 100 bp DNA Ladder (M)](image)
obtained from the study by Araque-Calde son et al. (2008) with MIC values of the same range. Thus, this close similarity would suggest that environmental B. cepacia complex may be a potential source of infection of immune-compromised or hospitalized patients (Nazik et al., 2018), at least in Nigeria. Burkholderia spp. from pharmaceutical wastewaters in this study was intrinsically resistant to ciprofloxacin which agrees with previous investigations (Walsh & Duffy, 2013), where it was also reported that clinically relevant Burkholderia spp. was intrinsically resistant to ciprofloxacin.

There are little or no data on spread of multidrug resistance S. maltophilia in the community (colonization) and environment in Nigeria. The detection of this potentially pathogenic microorganism with clinically relevant resistance in the wastewater sampled in our study is an indication of potential risk for the microbiological pollution of water resources. In addition, resistance to trimethoprim/sulfamethoxazole, the drug of choice for treatment, is also burdensome (Ismael et al., 2017; Juhász, Pongrácz, Iván, & Kristóf, 2015). This is the first confirmation of S. maltophilia isolate with multidrug resistance from a pharmaceutical wastewater in Nigeria.

Chryseobacterium indologenes is a healthcare-associated pathogen (H CAP) and has been widely reported to cause life-threatening infections in patients, especially ones on chronic immunosuppressant drugs (Mehta & Pathak, 2018). Intrinsic resistance to several antibiotic and multidrug resistances to major antibiotics like we observed in our study has also been reported by Mehta and Pathak (2018) in their case report. They emphasized concerns on how this affected the effective empirical treatment of this bacterial infection, more so with the scant availability of data from literature which has been challenging. Detection of C. indologenes in wastewaters from pharmaceutical industries is not adequately documented; this seems to be the first report of multidrug-resistant C. indologenes isolated from our study in Nigeria.

Among the antimicrobials evaluated, the carbapenems (imipenem and meropenem) are the most powerful antibiotics used as therapy for severe infections. However, the resistance mechanism of these bacteria to the carbapenems is not the production of the carbapenamase enzyme but could be due to intrinsic characteristics, mutation, porin loss, or efflux pump resulting to their ability to become resistant to the carbapenems. The resistance determinants in these bacteria from the pharmaceutical wastewaters correspond to that of their closely related clinically relevant species. Alouache et al. (2013) highlighted the fact that there is a similarity in the antibiotic-resistance mechanisms in the clinical setting and the environment. The environment has a role to play, being the source of dissemination of resistance genes. This suggests that the evolution of intrinsic resistance in environmental and clinical bacteria developed along the same line.

This study has shown that MRGN bacteria with resistant determinants are widespread in pharmaceutical wastewaters from the environment in Nigeria. Most previous studies in Nigeria of the antibiotic-resistance profile of pathogenic bacteria have been directed toward clinical isolates. The detection of these potentially pathogenic microorganisms with clinically relevant antibiotic resistance in the pharmaceutical wastewaters is an indication of potential risk for the microbiological pollution of water resources. The environment has a role to play, being the source of dissemination of resistance genes, and so continuous surveillance of the environmental reservoirs of MDR bacteria is necessary to prevent their further spread. However, further comparative analyses on the genetic relatedness of the MRGN strains found in the pharmaceutical wastewaters of this study with their pathogenic counterparts are necessary, and there is need to identify possible transmission pathways to evaluate the risk for public health in Nigeria.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTION

AIO has made major contribution to the research by acquisition and analysis of data, and writing the manuscript; EOU involved in the conception of research and writing the manuscript; and SUN was key to the conception and supervision of research.

DATA AVAILABILITY STATEMENT

The raw data underlying the main results of this study will be archived.

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