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THE PREVALENCE OF *KLEBSIELLA PNEUMONIAE* CARBAPENEMASE AND METALLO-BETA-LACTAMASES/OXACILLINASE ENZYMES IN DRUG-RESISTANT BACTERIAL ISOLATES FROM STUDENTS' TOILET FACILITIES IN A NIGERIAN PHARMACY SCHOOL

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ABSTRACT

Enterobacteriaceae are human pathogens commonly responsible for pneumonia, urinary tract infections, peritonitis and septicemia. In recent years, there has been a rise in carbapenem resistance, particularly in these *Enterobacteriaceae*. This resistance is caused by the production of enzymes that inactivate carbapenems, known as Carbapenemases. Toilet infections are common among the female population and infections caused by carbapenem-resistant Enterobacteriaceae pose a huge challenge, especially as they cause an increase in the cost of treatment as well as treatment failure. This study was designed to determine the presence and incidence of *Klebsiella pneumoniae* Carbapenemase/Metallo beta-lactamase/Oxacillinase enzymes among drug-resistant isolates from the female students' hostel toilet facilities in Nnamdi Azikiwe University, School of Pharmacy, Agulu. Samples were first collected and processed, then isolated and purified. Microbial identification and characterization were carried out. The isolates were then subjected to antimicrobial susceptibility testing and subsequently to carbapenem susceptibility testing using a Rosco kit to determine the enzymes implicated in resistance. From the result, it was observed that *100%* of *Escherichia coli* strains produced oxacillinase enzyme and 6% of the strains produced metallo beta-lactamase, with no strain producing *Klebsiella pneumoniae c*arbapenemase. In *Klebsiella pneumoniae*, all strains produced oxacillinase, 38% produced metallo beta-lactamase and 5% produced *Klebsiella pneumoniae* Carbapenemase. For *Salmonella spp.*, 23% produced metallo beta-lactamase and 9% produced *Klebsiella pneumoniae* Carbapenemase and all strains of *Salmonella spp.* produced oxacillinase enzyme. In *Pseudomonas aeruginosa*, 19% of the resistant strains produced *Klebsiella pneumoniae* Carbapenemase and 4% produced metallo beta-lactamase. This study confirmed the presence of *Klebsiella pneumoniae* Carbapenemase, Metallo-beta lactamase and Oxacillinase enzymes among the drug-resistant isolates in the female student hostel toilet facilities.

Keywords: Enterobacteriaceae, Carbapenem, *Klebsiella pneumoniae*- Carbapenemase, Metallo beta-lactamase,

INTRODUCTION

Antibiotics, often known as antimicrobial agents, are drugs that, by killing or preventing the development of disease-causing bacteria, can eradicate or stop their proliferation (1). Antibiotic misuse and irrational prescription are key health factors affecting both developed and developing nations, including Nigeria. These issues may be worsened by governments' inability to provide adequate funding for the purchase of high-quality pharmaceuticals and by the absence of effective measures to prevent the cross-border sale of counterfeit and subpar antibiotics (1). The ability of bacteria to endure

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and proliferate in the presence of antimicrobial drugs that are clinically significant is known as bacterial resistance (2). The first indication of antimicrobial resistance was seen shortly after penicillin was discovered. *Escherichia coli* was the first bacterium to produce penicillinase, rendering penicillin inactive, and Abraham and Chain were the first to discover it in 1940 (3). Human health is seriously threatened by antimicrobial resistance, and impoverished countries like Nigeria have been disproportionately affected. The extensive and careless use of antimicrobial agents is the primary cause of this catastrophe (4). Due to the apparent implications, including treatment failures, longer hospital stays, and nosocomial infections, the rising prevalence of antibiotic-resistant bacteria is a source of concern for doctors and patients (5).

A wide range of infections, including *Salmonella, Enterobacter, Citrobacter, Shigella, Proteus, Serratia,* and *Escherichia coli*, are caused by members of the Enterobacteriaceae family of Gram-negative bacteria. The most frequent human organisms that cause infections such as pneumonia, UTIs, peritonitis, and septicemia belong to the Enterobacteriaceae family (6). These are commensals that cause opportunistic diseases and are commonly linked to a range of community-acquired and nosocomial infections (7). A potent beta-lactam antibiotic with a wide spectrum of activity against both Gram-positive and Gram-negative bacteria is the carbapenem. Like other beta-lactams, carbapenems work by attaching to the proteins that bind penicillin, so preventing the bacterial cell wall from being synthesized (8).

Carbapenems are recognized as the last line of antibiotic treatment against resistant organisms. The availability of carbapenem-hydrolyzing enzymes, known as carbapenemases, greatly hamper the use of carbapenems as a last resort, despite their stability against most beta-lactamases (9). A pathogen's *in vitro* insensitivity to any carbapenem and/or a confirmed release of the enzyme carbapenemase are indicators of carbapenem resistance (10). The majority of carbapenemases are found on plasmids, which are genetic components that are mobile and easily exchanged among Enterobacteriaceae (11). Resistance to other types of antimicrobial drugs and all conventional beta-lactams is frequently present in carbapenem-resistant patients (10). Thus, severe infections caused by Enterobacteriaceae resistant to carbapenem have a significant impact on morbidity, mortality, and medical expenses (12).

Production of carbapenemases is the most important mechanism responsible for carbapenem resistance. These enzymes cause the breakdown of the amide bond present in the beta-lactam ring thus altering their antimicrobial efficacy. The Ambler classification proposed in 1980 is used to classify the carbapenemases into four groups from A - D. These enzymes are further divided into two group, namely - serine carbapenemases which include Amber Classes A, B and D and metallo carbapenemases (zinc) which include Ambler Class B enzymes. Carbapenemases can also be classified into three key groups; the Ambler Class A beta-lactamase (KPC), Class B metalloenzymes and Class D (OXA-48 type) oxacillinase (13). The big five enzymes: KPC, NDM, OXA-48, IMP, and VIM are the most well-known members of the highly diverse group of beta-lactamase enzymes known as carbapenemase enzymes (14). The host's plasmid or chromosome contains the genes encoding for the class "A" carbapenemases. Penicillins, monobactam, imipenem, meropenem, and early cephalosporins are hydrolyzed by class "A" carbapenemases. The enzymes clavulanate and tazobactam block the Class A carbapenemases (15). Penicillinases are the first class of carbapenemases, known as Ambler class A.

KPC (*Klebsiella pneumoniae* carbapenemase) is the most prevalent enzyme in this group, although other enzymes are also present, including Guyana extended-spectrum-lactamase (GES), imipenemase (IMI), and non-metallo carbapenemase (NMC) (13). Class B carbapenemases, also known as the metallo-lactamases, need zinc at their active sites (16). Among the carbapenemases, MBLs are currently becoming the more troublesome beta-lactamases because they can hydrolyze beta-lactam antibiotics except monobactams (e.g., aztreonam) and are not inhibited by the classical serine beta-lactamase inhibitors (e.g., clavulanic acid, tazobactam, sulbactam), diazabicyclooc- tane (DBO) beta lactamases (avibactam, relebactam), or boronic acid inhibitors (vaborbactam) (17). Thus the metallo beta-lactamases can be differentiated from Classes A and D beta-lactamases by their utilization of zinc instead of a catalytic serine in the active site- mediated hydrolysis of beta-lactams (18). They are inhibited by metal chelators such as ethylenediaminetetraacetic acid (EDTA) and dipicolinic acid (DPA) but not clavulanic acid or other clinically used beta-lactamase inhibitors. They often have profiles of hydrolysis against all beta lactams except for the monobactams and can confer a high level of resistance when combined with alterations in membrane permeability and ESBL production (18). The class D beta lactamases were named oxacillinases (OXA) because they cleave oxacillin as well as penicillin, differentiating them from class “A” beta lactamases (18). The majority of multi-resistant strains found in France at this time are caused by oxacillinases. Because they can hydrolyze oxacillin more efficiently than benzylpenicillin, class D beta lactamases are also referred to as oxacillinases or OXA beta lactamases (19). Five distinct groups of Ambler class D enzymes are thought to have the ability to hydrolyze carbapenem, though this is primarily suggested by their capacity to increase carbapenem MICs in less permeable environments, like Acinetobacter, where all OXA enzymes are classified as carbapenemases (20).

The class D enzymes include the OXA-24, OXA-25, OXA-26, OXA-27, and OXA-40 from Acinetobacter baumannii isolates. OXA-23 was found in A. baumannii and Proteus mirabilis (21). These enzymes target penicillins (particularly the amino-, carboxy- and ureidopenicillins) and other narrow-spectrum cephalosporins such as cephalothin. They also weakly hydrolyze the carbapenems, and have limited activities against the broad-spectrum cephalosporins -mostly ceftazidime and many beta-lactam inhibitors such as clavulanate, sulbactam, and tazobactam (19).

Urinary tract infections (UTIs) are common in developing countries like Nigeria, where sanitary hygiene may be inadequate or lacking and they are especially common in females. Some of these infections are caused by carbapenem-resistant Enterobacteriaceae and pose a huge challenge as they cause an increase in the cost of treatment as well as treatment failure. Enterobacteriaceae like *Klebsiella pneumoniae* and *Escherichia coli* are implicated in most urinary tract infections (22); however, therapy failure is typical due to resistance in these organisms and the basis for resistance is often enzymatic (23). This study seeks to identify the pathogenic organisms present in the toilet facilities and determine the resistant strains and the enzymes (KPC/MBL/Oxacillinase) responsible for the resistance.

MATERIALS AND METHODS

Site of Study

The study was conducted at the Faculty of Pharmaceutical Sciences, Agulu, Nnamdi Azikiwe University, Anambra State, Southeast Nigeria. The samples were sourced from the

floor and seat of toilet facilities of the female hostel housing undergraduate Pharmacy students.

Sample Size

There were 56 toilets, and the sample was 112. This sample size was determined using Andrew Fischer's formula for the determination of sample size.
The confidence level used in this study was 95% and z-score of 1.96 and a sample size of 60 (number of toilets to be sampled in the course of the study) was obtained from the equation:

$$Sample\ Size = \frac{(Z-score^2)(Std\ Dev)(1-\text{Std Dev})}{CI^2}$$

Where Z = z-score which corresponds to the level of confidence
Std Dev =Standard deviation
CI = Confidence interval

Microbiological Investigations

Isolation

A total of 60 samples were collected, comprising 30 samples from the toilet seats and 30 samples from the toilet floors. The samples were processed by aseptically culturing them in triple-strength nutrient broth. Afterwards, samples were cultured onto selective agar via the streak plate technique. Selective agars used were Salmonella Shigella agar, Cetrimide agar, MacConkey agar, and Nutrient agar. Macroscopic and microscopic analysis were carried to identify and characterize the isolates. Gram character was carried out using the Gram staining techniques while biochemical characterization of the isolates was achieved by subjecting them to biochemical tests, including the catalase test, oxidase test, Indole test, and citrate utilization test, to confirm their identity.

Testing for Susceptibility of Samples to Antibiotics for the Detection of Resistance
The identified organisms were isolated, and the antibiogram of the organisms was determined. This was carried out by the use of antibiotic multidisc (Celtech Diagnostic) to identify the resistant strains. The colonies were first collected using a flamed wire loop and diluted in test tubes containing distilled water or nutrient broth in adherence to McFarland's standard. The Petri dishes containing the Mueller Hinton agar were inoculated with the organisms prepared in line with McFarland's standard. Then the given multi-antibiotic disc was placed on the surface of the agar and incubated at 37°C for 24 hours.

Storage of Resistant isolates

The resistant strains obtained from the preceding test were stored by culturing them on double strength agar in Bijou bottles and storing them in a refrigerator at a temperature of 4℃. This was done to slow down the growth of the organisms, thus ensuring their viability when employed for future microbial investigations.

Determination of the presence of enzymes among multidrug-resistant isolates

The Rosco confirmation kit, an *in-vitro* diagnostic kit used for beta-lactamase identification, was employed. It includes tablets that are used with the disc diffusion method for *in vitro* qualitative identification of the mechanisms of microbial resistance. The confirm kit is designed to identify carbapenemases in Enterobacteriaceae, namely KPC, MBL, and OXA-48.

The KPC/MBL and OXA-48 confirm kit (98015) and the KPC+MBL detection kit (98010) were used to detect beta-lactamases in

Enterobacteriaceae. The confirm kit 98025 is used with *Pseudomonas* to identify KPC and MBL. The kits had cartridges of tablets that contained 10 micrograms of Meropenem (10 micrograms of Imipenem in the case of *Pseudomonas* spp.) alone and in combination with inhibitors of the various beta-lactamases. The inhibitors were used to differentiate isolates with resistance mechanisms from those without. Kit 98015 contains a cartridge of 30 micrograms of Temocillin tablets to detect OXA-48 or similar producing isolates. Using a pure sample of the culture, a suspension of the organisms was prepared and tested according to McFarland 0.5 standard. A sterile swab was used to spread the suspension uniformly over the surface of the Mueller Hinton agar plate, and the tablets were placed carefully with adequate spacing on the plates using a tablet dispenser. The agar plates were incubated at 35℃ for 16-20 hours, and the resulting inhibition zone diameters were measured.

RESULTS

Table 1: Sample collection from the site of study

Location	Number of samples collected	Percentage
Toilet floors	30	50%
Toilet seat	30	50%
Total	**60**	**100%**

A total of 29 isolates of *Klebsiella pneumoniae*, 20 isolates of *Escherichia coli*, 30 isolates of *Pseudomonas aeruginosa*, 44 isolates of *Salmonella spp.* and 1 isolate of *Serratia marcescens* were isolated from the samples.

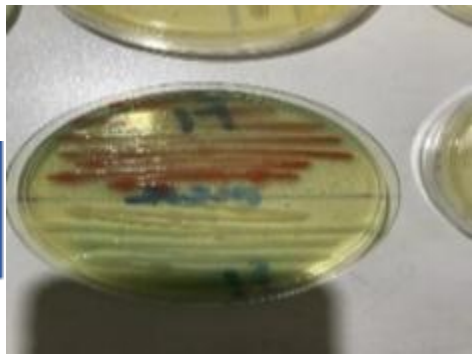
DISCUSSION

From the students' toilet facilities, 60 samples were collected. The *Klebsiella pneumoniae* colonies were pink, mucoid, opaque and had a convex shape, while the *Escherichia coli* colonies were red, dry, opaque and convex-shaped colonies in MacConkey agar. The *Pseudomonas aeruginosa* colonies were light green, mucoid, opaque, shiny and raised, *Salmonella* colonies were colourless with a black center, the *Shigella* colonies were colourless and the *Serratia marscens* colonies were red in Nutrient agar. The colonies were collected and streaked on nutrient agar to obtain purer isolates. Twenty-nine (29) isolates of *Klebsiella pneumoniae*, twenty (20) isolates of *Escherichia coli*, thirty (30) isolates of *Pseudomonas aeruginosa*, forty-four (44) isolates of *Salmonella* spp. and one isolate of *Serratia marcescens* were obtained from the samples.

Following the Gram-staining technique carried out, the Gram character of all isolates was negative (all isolates were Gram-negative organisms). Microscopically, *Klebsiella pneumoniae* appeared as dispersed, short oval rods, *Escherichia coli* appeared as long slender rods, *Salmonella* and *Shigella* appeared as slender red while *Pseudomonas aeruginosa* appeared as long slender pink rods upon microscopic investigation.

The necessary biochemical tests were conducted to confirm the identities of the isolates. Oxidase test was done to differentiate the family Pseudomonadaceae from Enterobacteriaceae. *Pseudomonas aeruginosa* is oxidase positive while *Klebsiella pneumoniae, Salmonella-Shigella* spp, *Escherichia coli*, and *Serratia marcescens are* oxidase negative. Indole test and Simmons citrate utilization test were used to differentiate *Escherichia coli* from *Klebsiella pneumoniae*

Samples Collection and Processing



Microbial Identification and Characterization

Confirmation for the identity of Isolates

Antibiotics Susceptibility Testing

Carbapenem Susceptibility Testing

Figure 1: Flowchart depicting the methodology employed in determining carbapenem-resistant isolates as well as enzymes implicated in resistance

RESULTS

Table 1: Sample collection from the site of study

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Toilet floors	30	50%
Toilet seat	30	50%
Total	**60**	**100%**

Table 2: Antibiogram of the Carbapenemase-producing isolates

Antibiotics	No of Susceptible organisms	No of Intermediate organisms	No of Resistant organisms	Total
Augmentin	12	3	109	124
Cefotaxime	18	2	104	124
Imipenem/Cilastatin	9	11	104	124
Ofloxacin	45	10	69	124
Gentamycin	47	1	76	124
Nalidixic acid	31	2	91	124
Nitrofurantoin	78	3	43	124
Cefuroxime	23	0	101	124
Ceftriaxone/sulbactam	24	19	81	124
Ampiclox	45	23	56	124
Cefexime	15	0	109	124
Levofloxacin	20	21	83	124

Table 3: Enzymatic basis of resistance for the resistant isolates

Isolates	*Klebsiella pneumoniae* Carbapenemase (%)	Metallo-beta lactamase (%)	Oxacillinase (%)	Total (%)
Escherichia coli	0 (0)	1 (6)	17 (100)	17 (100)
Klebsiella pneumonia	1 (5)	8 (38)	21 (100)	21 (100)
Salmonella spp	3 (9)	8 (23)	35 (100)	35 (100)
Pseudomonas aeruginosa	5 (19)	1 (4)	-	27 (100)

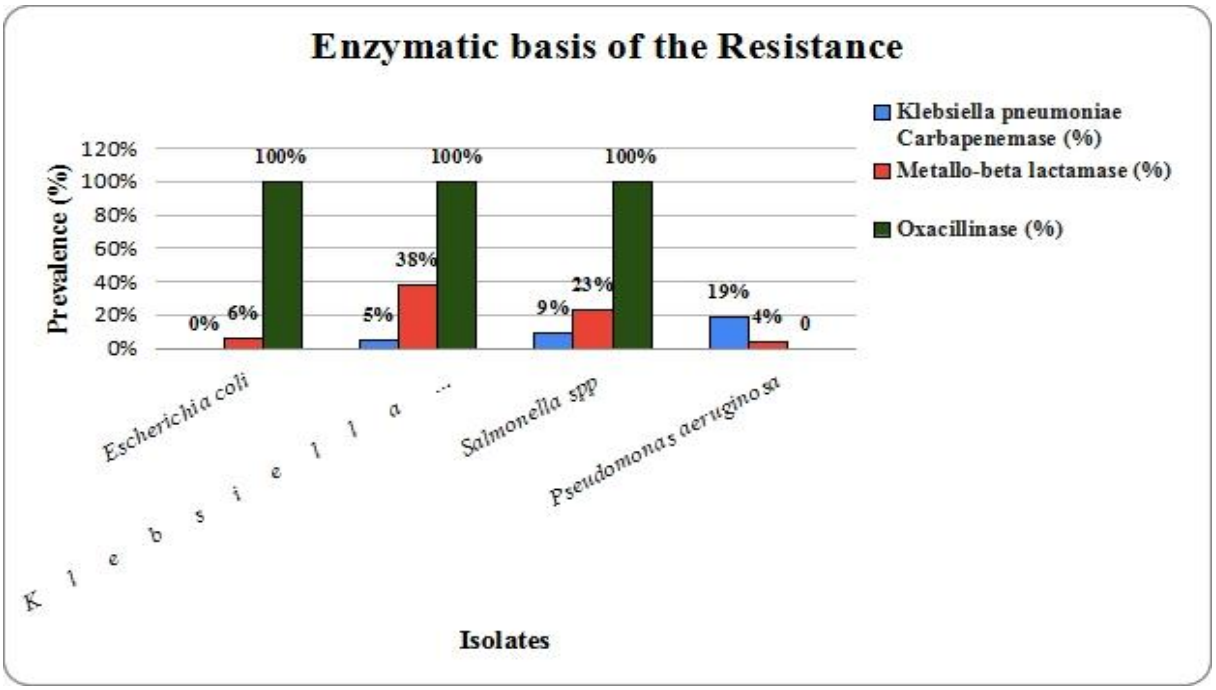

Figure 2: Prevalence of *Klebsiella pneumoniae* Carbapenemase, Metallo-beta Lactamase and Oxacillinase in resistant organisms

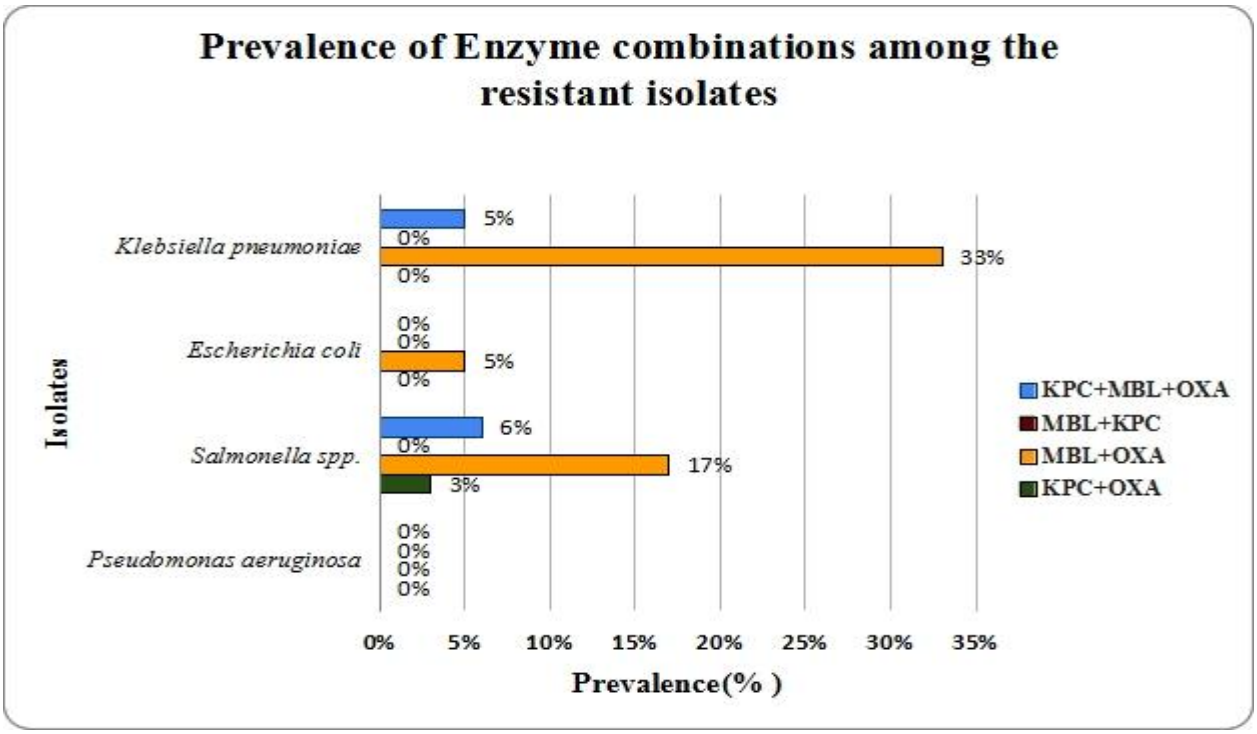

Figure 3. Prevalence of enzyme combinations among resistant isolates

as the former is indole-positive, while the latter is indole-negative. *Escherichia coli*, in addition, is negative for the citrate test and *Klebsiella pneumoniae* is positive, and with catalase test, all three organisms showed a positive result. An antimicrobial susceptibility test was carried out in triplicate for the *Klebsiella pneumoniae, Escherichia coli,* and *Salmonella* spp. Isolates, using the Gram-negative multi-antibiotic disc, and the result was interpreted using the EUCAST 2022 standard breakpoints. From the test, 17 isolates of *Escherichia coli,* 21 isolates of *Klebsiella pneumoniae*, 27 isolates of *Pseudomonas aeruginos*a, and 35 isolates of *Salmonella* spp. at the end of the study were recorded as multi-drug resistant. The resistant strains were tested to determine the mechanism by which they are resistant to the isolates using an agar tablet/disc diffusion method. Meropenem (10 µg) alone and in combination with inhibitors of different beta-lactamases was used for the Enterobacteriaceae. The inhibitors used in combination with 10 µg meropenem were dipicolinic acid (MRPDP), which is a metallo beta lactamase inhibitor, phenylboronic acid (MRPBO), a *Klebsiella pneumoniae* Carbapenemase and AmpC inhibitor, and finally cloxacillin (MRPCX). Temocillin was used to indicate the presence of OXA-48 or similar enzymes. A triple synergy combination tablet of Meropenem, Phenylboronic acid, and Dipicolinic acid was also used for some isolates as it allowed the identification of the enzymes *Klebsiella pneumoniae* Carbapenemase and metallo beta-lactamase. For *Pseudomonas aeruginosa*, Imipenem in combination with inhibitors of different beta-lactamases was used. The inhibitors used in combination with 10 µg Imipenem were dipicolinic acid (IM+DP) which is a metallo beta-lactamase inhibitor, phenylboronic acid (IM+PBO), a *Klebsiella pneumoniae* Carbapenemase and AmpC inhibitor, and finally cloxacillin (IP+CX4), which is an AmpC inhibitor and EDTA a metallo beta-lactamase inhibitor. From the results, it was observed that all *Escherichia coli* strains produced oxacillinase enzyme, and 6% of the population produced metallo beta-lactamase in combination with oxacillinase enzyme. For *Klebsiella pneumoniae,* all strains produced oxacillinase enzyme. However, it was observed that 33% of the population produced metallo beta-lactamase in combination with oxacillinase, and 5% produced metallo beta-lactamase and *Klebsiella pneumoniae* Carbapenemase in combination with oxacillinase. For Salmonella spp., 17% of the population produced metallo beta-lactamase in combination with oxacillinase, 3% produced *Klebsiella pneumoniae* Carbapenemase in combination with oxacillinase and 6% produced metallo beta-lactamase and *Klebsiella pneumoniae* Carbapenemase in combination with oxacillinase. All strains of Salmonella spp. produced oxacillinase enzyme. In *Pseudomonas aeruginosa,* 19% of the resistant strains produced *Klebsiella pneumoniae* Carbapenemase, and 4% of the resistant strains produced metallo beta-lactamase. It was observed from the result that 9% of all resistant organisms produced KPC and 18% produced MBL. In the Enterobacteriaceae, where presence of oxacillinase was tested, 100% of oxacillinase was produced. This was in contrast to the work by Kim *et al*. (2020) which showed that KPC had the greatest prevalence of 88.9%, while MBL (NDM-1) and (NDM-5) had a prevalence of 7.5% and 1.7% respectively and oxacillinase enzymes (OXA-181 and OXA 232) had a prevalence of 1.1% and 1.1% respectively.

It was also observed from the results that the predominant enzyme responsible for resistance was the oxacillinase enzyme, as all resistant strains produced this enzyme. It was also observed that in several organisms, more than one enzyme was responsible for resistance. After oxacillinase, metallo beta-lactamase was

the next most produced enzyme, while *Klebsiella pneumoniae* Carbapenemase was the least for Enterobacteriaceae. This was similar to the result of the work done by Yijun *et al*. (2016), in which metallo beta-lactamase was more prevalent than *Klebsiella pneumoniae* Carbapenemase (24). In *Pseudomonas aeruginosa, Klebsiella pneumoniae* Carbapenemase-producing strains were more than the metallo beta-lactamase-producing strains. These enzymes may not be the only ones responsible for resistance in *Pseudomonas aeruginosa,* as reports show that oxacillinase genes (bla oxa-sogene) are expressed constitutively in *Pseudomonas aeruginosa. Pseudomonas aeruginosa* also carries chromosomal genes for 2 beta lactamases: Class C Ceplalosporinase; AmpC and Class D Oxacillinase.

CONCLUSION

The microbial contaminants in the students' hostel toilet facilities in Nnamdi Azikiwe University, Faculty of Pharmaceutical Sciences, were isolated and identified as *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa,* and *Salmonella* spp. The majority of the isolates were resistant to at least three classes of antibiotics they were tested against; hence, they were classified as multi-drug resistant (MDR). In addition, the study also evaluated the susceptibility of the resistant isolates to carbapenems and confirmed the presence of carbapenemases, including Klebsiella pneumoniae Carbapenemase, metallo-beta-lactamase, and Oxacillinase. The outcome of this study is a cause for concern as it shows a breach in our last line of defense against microbial infection. It also emphasises how crucial good personal hygiene is and how urgently we must take action to stop the spread of infections. This can be accomplished through promoting frequent cleaning of restroom facilities, enhancing sanitation, and informing people about the risks associated with drug abuse which is a major risk factor for drug resistance

Continued Surveillance is needed since it appears that bacterial evolution has finally caught up to—and regrettably exceeded—human ingenuity.

Ethics approval and consent to participate

This was not applicable as the study does not involve experiments on animals or human subjects.

Competing interest

The authors hereby declare that they have no competing interest.

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