Sumerianz Journal of Biotechnology, 2022, Vol. 8, No. 2, pp. 1-13
ISSN(e): 2617-3050, ISSN(p): 2617-3123
Website: https://www.sumerianz.com
DOI: https://doi.org/10.47752/sjb.8.2.1.13

Original Article Open Access

Prevalence of Antibiotic Resistant Genes in Enterobacteriaceae from Public Healthcare Centres in Imo State Nigeria

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Article History

Received: 16 September 2024

Revised: 13 December 2024

Accepted: 24 March 2025

Published: 29 June 2025

How to Cite

Ibe E.C. Okechi R.N. Nwabunnia E.Emeka-Nwabunnia I (2025) .Prevalence of Antibiotic Resistant Genes in Enterobacteriaceae from Public Healthcare Centres in Imo State Nigeria. *Sumerianz Journal of Biotechnology*, 8.2.1.13

Abstract

The present study was targeted at investigating the prevalence of antibiotic resistant genes in Enterobacteriaceae from some public healthcare facilities in Imo State, Nigeria. Using randomized complete block sample design, four public hospitals [Imo State University Teaching Hospital, Federal Medical Centre, Owerri, Aboh-Mbaise General Hospital, and Imo State Specialist Hospital] were randomly selected for the study. Swabs were collected from different items and wards, isolation and culture purification were done using standard procedures. Kirby-Bauer disc diffusion method was employed to determine the antibiotic sensitivities of the isolates against 10 different antibiotics. The presence of some resistant genes were determined using forward and reverse primers specific for bla_{TEM}, *qnr*A, and aac(3)-1 genes. The results showed that *P. aeruginosa*, *E. coli*, *K. pneumoniae*, and *Shigella* species were the Enterobacteriaceae isolated in this study. Also, the highest mean number of Enterobacteriaceae were isolates from ISUTH (20.67±1.45), followed by ISSH (19.67 ± 0.33), while FMC, Owerri had the least (18.00±1.15). The isolates showed multiple resistances to most of the antibiotics tested. All the isolates were susceptible to Gentamicin, while area of technical uncertainty was noticed for Septrin, Cefalexin, and Streptomycin antibiotics on some isolates. Molecular characterization showed the presence of the three resistant genes investigated. The prevalence of bla_{TEM}, *qnr*A, and aac(3)-1 resistant gene-bands were 80.0 %, 70.0% and 60.0 %, respectively. These resistant genes were most abundant in *K. pneumoniae* (93.33 %) and least abundant in *Shigella* species (33.34 %). The study concluded that the three resistant genes tested were present in Enterobacteriaceae isolated from the healthcare facilities.

Keywords: Prevalence; Antibiotic resistance genes; Enterobacteriaceae; Healthcare facilities; Antibiotic sensitivities.

1. Introduction

Pathogens associated with healthcare acquired infections are usually referred to as nosocomial pathogens. Nosocomial infection is one of Africa's great health challenges, and the emergence of antimicrobial resistant

pathogens has made it more threatening [1]. Nosocomial infection or "healthcare associated infection (HCAI)" refers to infections that occur in patients receiving medical care in a hospital or healthcare facility. This infection wasn't in the patient as at the time of admission but may occur during treatment for other illness or after the patient is discharged [2]. According to the World Health Organisation, nosocomial pathogens can be responsible for occupational infections among medical staff. Infections acquired by visitors in a healthcare facility or by staffs working in a healthcare facility can also be considered as nosocomial [3]. An infection is said to have occurred when a foreign organism (capable of utilizing the host's body to sustain itself, reproduce, and colonize) enters the body of a host and cause harm. Such organisms are generally referred to as pathogens, and they inhabit the body fluids or body sites generally believed to be sterile [4]. Nosocomial pathogen may originate from the patient's own endogenous flora, or from cross contamination (contact with staff), environment, and contaminated hospital equipment (e.g., needles and catheters) [5, 6].

Nosocomial infections are usually caused by bacteria, virus, fungi, and in some cases protozoan parasites [7]. Bacteria are the most common nosocomial pathogens, as it accounts for about 80 % of reported nosocomial infection [8]. Majority of bacterial nosocomial infections are caused by pathogens belonging to the bacteria family *Enterobacteriaceae*. Prevalent members of *Enterobacteriaceae* associated with healthcare acquired infections include *Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella* spp., and *Serratia marcescens* [9]. These bacteria possess vital pathogenic features (such as antiphagocytic capsule, antigenic phase variation, capsule sequestration of growth factor, and endotoxin production) that enable colonization within the host [10]. *Enterobacteriaceae* are responsible for numerous nosocomial infections such as pneumonia (in immune-compromised patients), urinary tract and wound infections, meningitis (in neonates), gastroenteritis, and respiratory tract infection [9]. Some species of *Enterobacteriaceae* (*E. coli* and *Klebsiella* species) are of more medical significance due to their high resistance to antibiotics (e.g., Carbapenem) [11].

The greatest threat to the utilization of antibiotic medications in the treatment of bacterial nosocomial infection is the emergence and distribution of resistance in the pathogens [12]. Antibiotic resistance is the capability of bacteria to resist the action of antibiotic medication that was previously effective against the bacteria [13]. The four main mechanisms by which bacteria exhibit resistance are drug inactivation or modification, alteration of target or binding site, alteration of metabolic pathway and reduced drug accumulation [14]. Most of the prevalent nosocomial bacteria are multidrug resistance that are either acquired [e.g., extended-spectrum β-lactamase (ESBL) or naturally resistant [9]. The treatment of nosocomial infection has become more challenging because the efficacy of available therapy is reduced, and the outcomes of death cases are increasing [1]. There have been reports of higher occurrences of hospital acquired infections in developing and under-developed countries than developed countries [15]. High rate of hospital acquired infection prevalence have also been recorded in Nigeria compared to other sub-Saharan countries, with mortality rate of 0.7 to 2.5% [16]. The study also noted that nosocomial infection prevalence increases annually in the southwest region of Nigeria. According to Bereket, *et al.* [9], most of the commonly identified nosocomial bacteria are multidrug resistant. It has become evident that most of the serious nosocomial infections are caused by bacteria that are resistant to common antibiotics, making the treatment of these infections to be more challenging and expensive [6].

Studies on hospital acquired infections from health facilities in parts of Nigeria have been reported [17-19]. However, there are limited data on the antibiotic resistivity patterns of hospital acquired Enterobacteriaceae species and the prevalence of their resistance genes in healthcare facilities in the South-eastern Nigeria. This study therefore investigated the prevalence of antibiotic resistance genes from Enterobacteriaceae from selected public healthcare centres in Imo State, Nigeria.

2. Methodology

2.1. Sampling Technique

The randomized complete block sampling design was adopted for the study. Four public health care centres [Federal Medical Centre Owerri (FMC), Imo State University Teaching Hospital (ISUTH), Aboh-Mbaise General Hospital (AMGH), and Imo State Specialist Hospital (ISSH), Umuguma] within the State were selected at random, for the study.

2.2. Sample Collection

Swab samples were collected from various units in the wards and staff apparels. The places/items from where the samples were collected include floor, bed cover, bed pan, and hospital staff apparel (protective gown and hand gloves) within maternity ward, surgical theatres, and intensive care unit of each hospital.

Swabbing of the surfaces of each item was done using appropriately-labelled sterile swab sticks soaked in saline water. Several portions on each item were swabbed, with more than one swab stick being used on each object. Three hundred and sixty (360) swabs were made with these swab sticks, which were immediately replaced back in the tubes and thereafter, sent to the laboratory for analyses [20].

2.3. Isolation of Bacterial Pathogens

The isolation and characterizations of the bacterial pathogens were as described earlier by the authors [21].

2.4. Antibiotic Sensitivity Test

Antimicrobial susceptibility testing was carried out on all the isolates using the Kirby-Bauer disc diffusion method as recommended by the Clinical and Laboratory Standards Institute [22]. Sterile petri-dishes of Mueller Hinton agar were prepared according to the manufacturer's specifications. A 3.8 g portion of Mueller Hinton agar

was dissolved in 100 mL sterile distilled water. The mixture was heated with intermittent agitation for complete dissolution. After which it was autoclaved at 121 ^{o}C, 15 psi, for 15 minutes, allowed to cool and dispensed into sterile petri-dishes. Colonies of overnight cultures were suspended in 5 ml sterile water and vortexed (VMX3-28) and the suspension was then brought to 0.5 McFarland standards (equivalent to $1.0x10^{8}$ cfu/ml). This was done by matching the turbidity of the bacterial suspension with that of 0.5 McFarland standard solution (prepared from $BaCl_2$ and Conc. H_2SO_4 solutions). Thereafter, a sterile cotton wool swab was inserted into each test tube containing the standardized bacterial suspension, rotated with firm pressure on the side wall of the test tube to remove excess fluid and thereafter used to inoculate the entire surface of the Mueller Hinton agar plates, in duplicates.

Antibiotic disks containing 10 antibiotics namely, Tarivid 10 µg, Pefloxacin 10 µg, Ciproflox 10 µg, Augmentin 30 µg, Gentamicin 30 µg, Streptomycin 30 µg, Cefalexin 10 µg, Nalidixic acid 30 µg, Septrin 30 µg, Ampicillin 30 µg was placed on each inoculated plate. All plates were incubated at 37 ^{o}C for twenty-four hours and the diameter of zones of inhibition measured to the nearest millimetre, using a transparent metre rule (CLSI, 2003). The bacterial isolates were designated as Sensitive (S), Resistant (R), or Intermediate (I) based on CLSI standards.

2.5. Molecular Analysis

DNA extraction: The bacterial genomic DNA extraction from the bacterial cells was carried out using DNA purification kit (QIAGEN) according to the manufacturer's instructions [23]. Sample tubes of 1.5-ml were labelled for each bacterial colony, while 1.25 ml of the bacterial culture was transferred into the labelled tube and centrifuged at 20,000 rpm for 5 minutes, and the liquid portion decanted. Thereafter, 180 µl of enzymatic lysis buffer was added to the tube and vortex for 10-20 s, it was then incubated at 37 ^{o}C for 30 minutes. Twenty-five (25 µl) microliters of proteinase K and 200 µl of buffer AL was added to the tubes and then vortex briefly. It was incubated at 56 ^{o}C for 30 minutes, and 200 µl of 100% ethanol was added, then the mixture was also briefly vortexed. Using a micropipette, the entire content (≈600 µl) was transferred to a labelled spin column; centrifuged at 10,000 rpm for 1 minute then the column was removed from the collection tube and placed in a new collection tube. Five hundred (500 µl) microliters of buffer AW1 was added to the column and centrifuged at 10,000 rpm for 1 minute. The column was removed from the collection tube and placed in a new collection tube. Five hundred (500 µl) microliters of buffer AW2 was added to the column and centrifuged at 20,000 rpm for 3 minutes. The tube was carefully removed from the centrifuge, the column was transferred to a 1.5 ml tube and 200 µl of buffer AE was added to the column. Then it was allowed to stand at room temperature for 1 minute. Centrifuged at 10,000 rpm for 1 minute. The column was discarded, and the DNA appropriately stored [23].

PCR amplification: Three Primers (Table 1), corresponding to the polymorphic region of *bla*$_{TEM,}$ *gnr*A, and aac(3)-1 gene were used to amplify the extracted DNA Briefly, 1 µl of the forward and reverse primers was added to 2.5 µl Taq polymerase buffer 10x containing a final concentration of 1 mM $MgCl_2$, 0.2 mM dNTPs and 0.2 µl Taq polymerase (5 U/µl) in a final reaction volume of 25 µl. PCR conditions were initial denaturation at 95 °C for 5 min, 34 cycles at 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. Final extension at 72 °C for 10 min was done. The results were visualized on 1.5 % agarose gel stained with ethidium bromide and photographed using gel documentation system [24].

Table-1. The primers used for polymerase chain reaction (PCR)

Gene		Sequence (5' 3')	Size (bp)
bla$_{TEM}$	F	ATGAGTATTCAACATTTCCG	20
	R	GTCACAGTTACCAATGCTTA	
*qnr*A	F	AGAGGATTTCTCACGCCAGG	20
	R	TGCCAGGCACAGATCTTGAC	
aac(3)-1	F	TTCATCGCGCTTGCTGCYTTYGA	23
	R	GCCACTGCGGGATCGTCRCCRTA	

2.6. Statistical Analysis

The data acquired from the research were analyzed and represented in Charts and Tables. The mean and standard error values were determined using formulas below.

$$\text{mean} = \frac{\sum fx}{\sum f} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

$$\text{Standard error} = \frac{\sigma}{\sqrt{n}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

3. Results

3.1. Enterobacteriaceae Species from Materials Utilized in Healthcare Centres

Enterobacteriaceae species were isolated from materials utilized in the four healthcare centres. The mean highest number of Enterobacteriaceae species was observed in ISUTH (20.67 ± 1.45), followed by those from ISSH (19.67 ± 0.33). Samples collected from FMC, Owerri showed the least number of Enterobacteriaceae species (18.00 ± 1.15) (Figure 1). The mean number of Enterobacteriaceae species observed in sample materials from AMGH (18.67 ± 1.20) was slightly lower than those observed in ISSH. From the error bars in Figure 1, it can be observed that there is more disparity in the observed mean number of Enterobacteriaceae species in samples from ISSH, compared to the other healthcare centres.

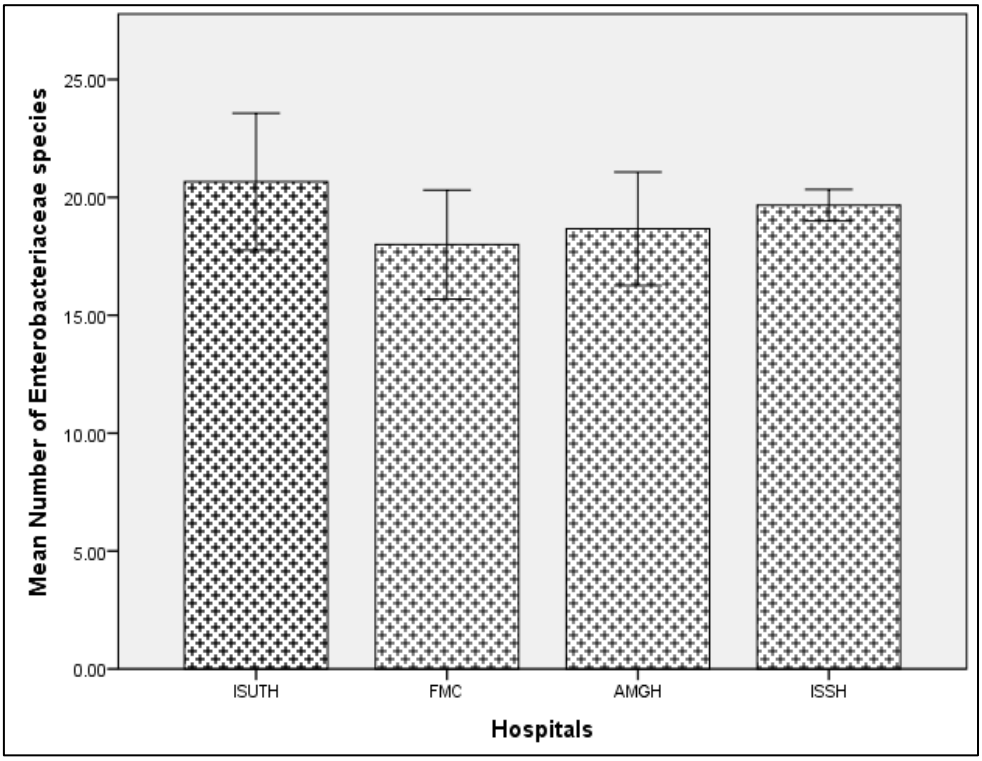
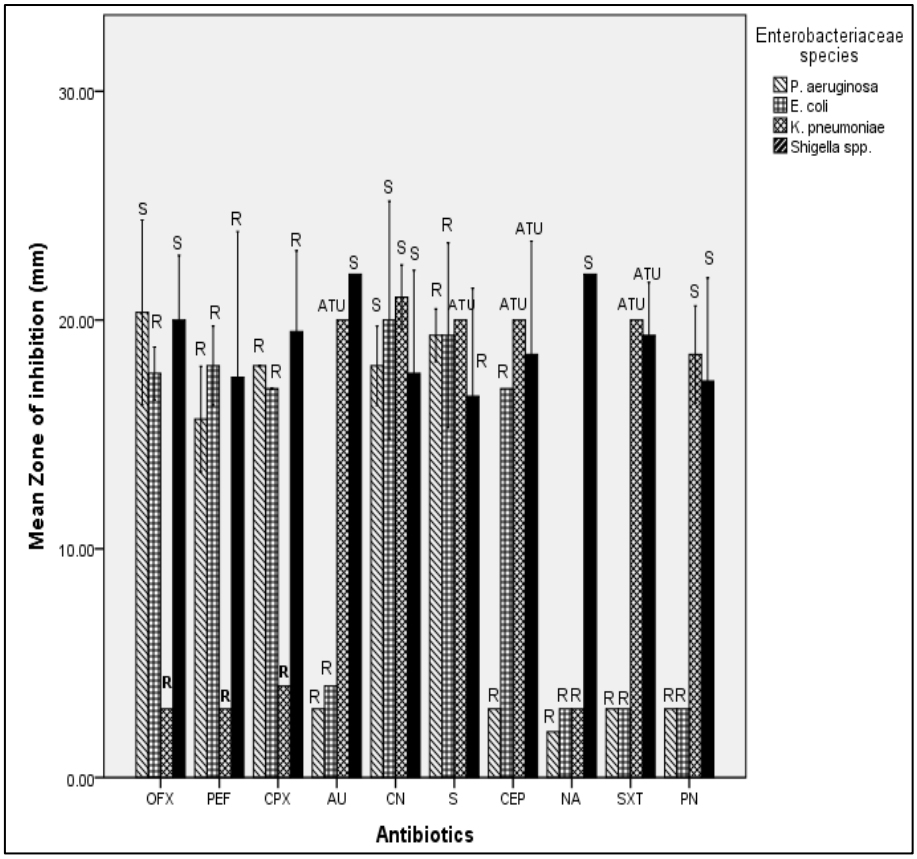


Figure-1. Mean number of Enterobacteriaceae species isolated from the four healthcare centres in Imo State

3.2. The Antibiotic Resistance Potential of the Bacterial Isolates

The antibiotic resistivity of the bacterial isolates to the multiple antibiotics is represented in the Figure 2. The isolates were resistant to most of the antibiotics investigated *Pseudomonas aeruginosa*, *E. coli*, *K. pneumoniae*, and *Shigella* species were resistant to Pefloxacin (PEF) and Ciproflox (CPX). *Escherichia coli* and *K. pneumoniae* were resistant to Tarivid (OFX), while *P. aeruginosa* and *Shigella* species were susceptible to Tarivid. *Pseudomonas aeruginosa* and *E. coli* were resistant to Augmentin (AU). *Shigella* species was susceptible to Augmentin, while *K. pneumoniae* showed area of technical uncertainty towards Augmentin antibiotics. All the bacterial isolates were however susceptible to Gentamicin. *Pseudomonas aeruginosa*, *E. coli* and *Shigella* species were resistant to Septrin (S); while an area of technical uncertainty was observed for Septrin against *K. pneumoniae*. *Pseudomonas aeruginosa* and *E. coli* were resistant to Cefalexin (CEF); there was area of technical uncertainty for the zone of inhibition observed for Cefalexin against *K. pneumoniae* and *Shigella* species. *Shigella* species was the only isolate susceptible to Nalidixic acid (Figure 2). *Pseudomonas aeruginosa* and *E. coli* were resistant to Streptomycin (SXT) and Ampicillin (PN). An area of technical uncertainty was observed for *K. pneumonia* and *Shigella spp* against Streptomycin. *K*lebsiella *pneumonia* and *Shigella* species were both susceptible to Ampicillin antibiotics.



R – Resistance, S – susceptible, ATU – area of technical uncertainty

Figure-2. Antibiotic resistivity of the bacterial isolates to multiple antibiotics discs

3.3. Molecular Characterization of Genes Responsible for Multi-Drug Resistivity

Three antibiotic resistant genes (*bla*$_{TEM}$, *qnr*A, and aac(3)-1) were investigated from the DNA extracted from the bacteria (Table 2). The *bla*$_{TEM}$ gene-band was the most prevalent antibiotic-resistant gene-band identified in the study (80%). The *bla*$_{TEM}$ gene-bands were observed at 1000 base pairs (bp) of the DNA ladder (Appendix ii (a,b)). The antibiotic resistant gene-band that codes for *qnr*A was observed to be the second most prevalent gene identified in the study (70%); while the aac(3)-1 antibiotic-resistant gene-band was the least prevalent gene (60%) identified in the study. The *qnr*A gene-bands were observed at 300 bp of the DNA ladder; while the aac(3)-1 gene-bands were observed between 600 and 500 bp of the DNA ladder (Appendix ii (e,f)).

Among the four bacteria identified, the three antibiotic resistant genes were more widespread in *K. pneumoniae* (93.33%), followed by *E. coli* (80%). *Pseudomonas aeruginosa* recorded 73.34 %, while *Shigella* species had the least (33.34 %) (Figure 3).

Table-2. Multi-drug resistant band detection from the isolates DNA

Bacteria	Resistivity genes			Total (%)
	bla$_{TEM}$	*qnr*A	aac(3)-1	
P. aeruginosa				
Pa01	-	+	-	6.67
Pa02	+	+	+	20.00
Pa03	+	-	-	6.67
Pa04	+	+	+	20.00
Pa05	+	+	+	20.00
E. coli				

Ec01	+	+	+	20.00
Ec02	+	+	+	20.00
Ec03	-	-	-	0.00
Ec04	+	+	+	20.00
Ec05	+	+	+	20.00
K. pneumoniae				
Kp01	+	+	+	20.00
Kp02	+	+	+	20.00
Kp03	+	+	+	20.00
Kp04	+	+	+	20.00
Kp05	+	+	-	13.33
Shigella species.				
SS01	+	+	+	20.00
SS02	-	-	-	0.00
SS03	-	-	-	0.00
SS04	+	-	-	6.67
SS05	+	-	-	6.67
Total	16	14	12	
Percentage (%)	80.0	70.0	60.0	

+ = present, - = absent

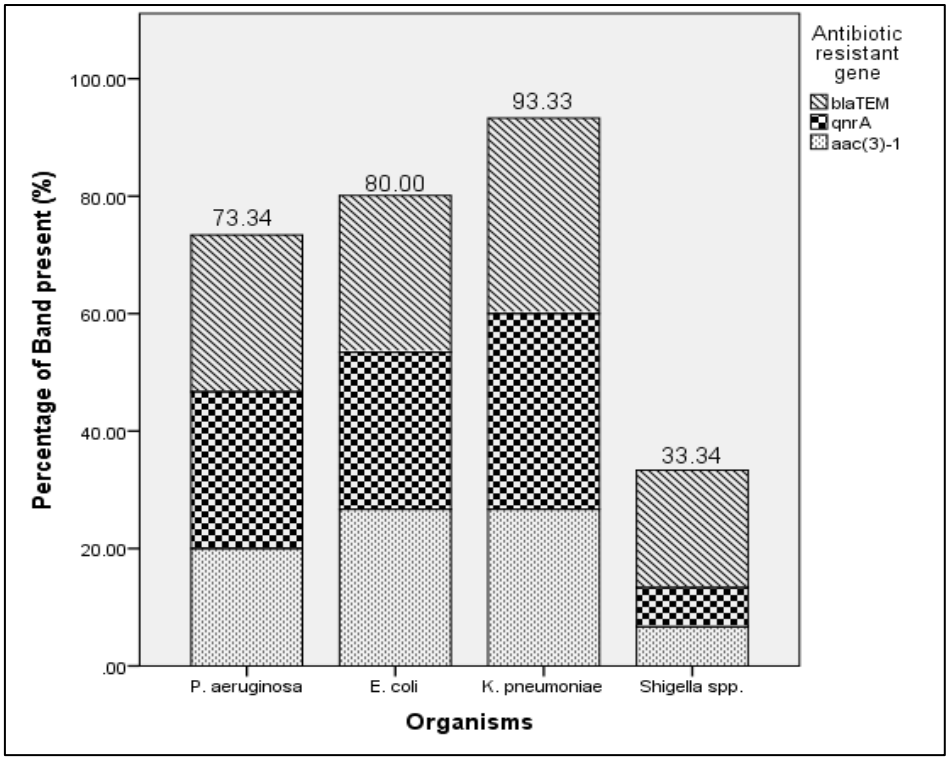


Figure-3. Percentage of gene-bands for *bla*$_{TEM}$, *qnr*A, and aac(3)-1 genes in the DNA of the isolates

Discussion

Infections caused by Enterobacteriaceae are common and in most cases, can be easily treated with antibiotics. Nevertheless, with the increasing rate of antibiotic resistance in these enteric bacteria, death due to the absence or high cost of potent chemotherapy has become common [25]. This study was therefore conducted to investigate the prevalence of some genes coding for antibiotics resistance among Enterobacteriaceae isolated from some public healthcare facilities in Imo State, Nigeria. The two foremost hospitals (ISUTH and ISSH) with the highest mean numbers of Enterobacteriaceae species were referral /tertiary hospitals, while the health facility with the second to the last number of the enteric bacteria was a secondary health facility (General hospital). This study indicates differences in the rate of occurrences/spread of the Enterobacteriaceae species among the studied health facilities in Imo State. This could be attributed to factors such as patient population/hospital staff ratios, available facilities, and awareness of control measures if any, among others. However, such disparities in the prevalence of Enterobacteriaceae species from various healthcare facilities, as observed in this study lend credence to the report by Azeez-Akande [26]. In the same study, higher prevalence rate was observed in University Teaching Hospitals, Ibadan, compared to the other health institutions studied. Similarly, disparities in the prevalence rate of hospital acquired infection based on the income level of the locations were also reported by WHO [27].

To ascertain the presence of multiple drug resistance (MDR) genes in the isolates, the Enterobacteriaceae species isolated were subjected to antibiotic sensitivity test using multiple antibiotic discs. Variations in antibiotics sensitivity among the isolates as observed in this study shows possible wide spread of multi-drug resistance genes in these pathogens. This result lends support to the report by Bolaji, *et al.* [12], that there is increase and widespread of antibiotic resistivity among nosocomial pathogens. According to Bereket, *et al.* [9], most of the prevalent nosocomial bacteria are multi-drug resistant. From the result (Figure 2), it could be seen that the Enterobacteriaceae species identified in this study exhibited multidrug resistance. *Escherichia coli*, *P. aeruginosa* and *K. pneumonia* showed high resistivity than *Shigella* species. In line with this finding, Tolera, *et al.* [28], reported high level of resistivity for *E. coli*, *P. aeruginosa* and *Klebsiella* species towards multiple antibiotics. Furthermore, Baka, *et al.* [7], reported *K. pneumoniae* susceptibility to majority of penicillin antibiotics. The increase in antibiotic resistivity as observed in this study could be attributed to the indiscriminatory use of antibiotics, poor infection control policies, overcrowding of hospitals and inefficient healthcare system [4, 29].

Employing molecular assay, the present research investigated the *bla*$_{TEM}$, *qnr*A, and aac(3)-1 genes. It was noticed that these genes were present in all the Enterobacteriaceae species isolated in this study. Pishtiwan and Khadija [30] in their study on the pprevalence of blaTEM, blaSHV, and blaCTX-M genes among ESBL-producing Enterobacteriaceae species in Iraqi hospital, also reported the presence of ESBL genes in all ESBL-producing isolates tested. The most globally common type of ESBL seems to be CTX-M type ESBLs with their higher incidence in most locations compared to SHV and TEM ESBLs [33]. Similarly**,** in Burkina Faso, the most prevalent ESBL resistance genes were *bla*CTX‑M (40.1%), *bla*TEM (26.2%) and *bla*SHV (5.9%) in Enterobacteriaceae [31]. In Nigeria however, the most frequent gene types among isolates from patients with surgical site infections were *bla*SHV, *bla*CTX‑M and *bla*OXA, as reported by Olowo, *et al.* [32]. Furthermore, Chukwu, *et al.* [17] reported high prevalence of *bla*$_{TEM}$ gene among clinical isolates from healthcare facilities in Lagos, Nigeria**.** Similarly, other researchers in their various studies have also reported high prevalence of *bla*$_{TEM}$ gene [29, 33, 34]. This seems to be the case in this study, where *bla*$_{TEM}$ gene was the most prevalent among the Enterobacteriaceae isolated, followed by

*qnr*A, while the aac(3)-1 gene was the least. This dominant presence of the bla_{TEM} gene reflects the high resistance of the bacteria isolated to Cefalexin (CEP).

In their study on the prevalence of *blaTEM* gene in clinical isolates of *Klebsiella pneumoniae* from hospitals in North of Iran, Sharafkhah, *et al.* [34] reported a rate of 55% among ESBL- producing *K. pneumoniae* In the same study, tetracycline, tobramycin, and ampicillin were the most active antibiotics against *K. pneumoniae* isolates, showing 85%, 81% and 73% sensitivity, respectively. In this study however, 93.335% of *K. pneumoniae* was haboured the resistant gene *blaTEM*. The bacterium was also resistant to most (80%) of the antibiotics tested except for gentamicin and ampicillin. Similarly, *Escherichia coli* was resistant to all but gentamycin antibiotics. The high resistivity of these bacteria could be attributed to the high prevalence of the three resistant genes studied among the bacterial strains isolated in this study. Such high prevalence of multi drug resistance enteric bacteria in hospitals in Western Nigeria has previously been reported by Kayode, *et al.* [18]. The presence of DNA bands for these resistant genes in these bacteria isolates, serves as a confirmatory to presence of multidrug resistant gene band in the isolates. It also indicates that the primers were relevant, and the genes were present in the chromosomal material.

Conclusion

Increase in nosocomial infections has been associated with the increase in antimicrobial resistance by the implicated organisms. This is mostly due to indiscriminate use of antibiotics, poor hospital hygiene and waste management system, hospital overcrowding, and insufficient professional hospital staff. The Imo State University Teaching Hospital showed the highest prevalence of Enterobacteriaceae species: *P. aeruginosa*, *E. coli*, *K. pneumoniae*, and *Shigella* species isolated. The study also indicated that these isolates were resistant to multiple antibiotics, with K*lesiella pneumoniae* showed more resistivity compared to the other *Enterobacteriaceae* species identified in the study. There were presence of resistance-genes: bla_{TEM}, *qnr*A, and aac(3)-1, in the chromosomes of the isolated Enterobacteriaceae. The bla_{TEM} gene was the most common resistant gene identified in the study. Antibiotics stewardship should be practiced in healthcare settings. The practice of proper diagnosis and rational prescription of antibiotics should be imbibed in the health sector to reduce the selective pressure for resistant bacteria.

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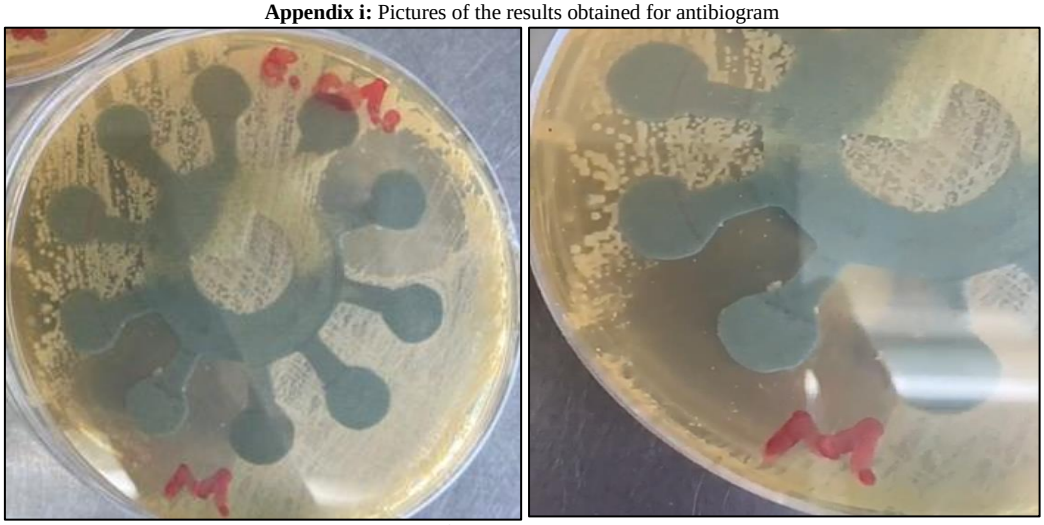
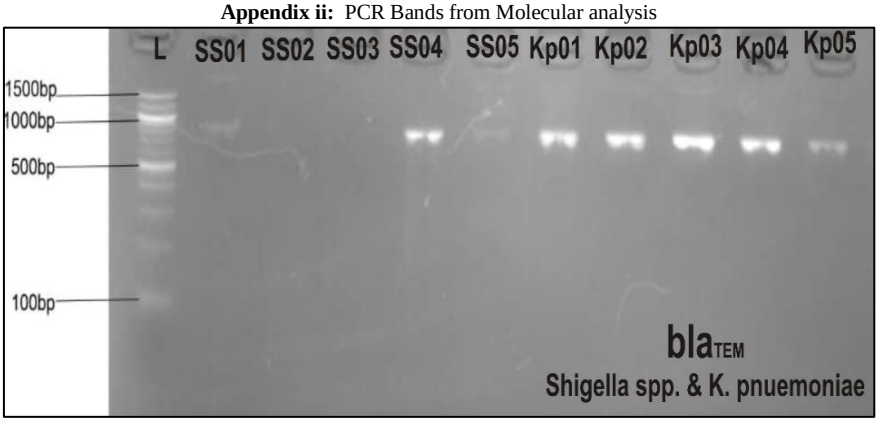
Appendix i: Pictures of the results obtained for antibiogram

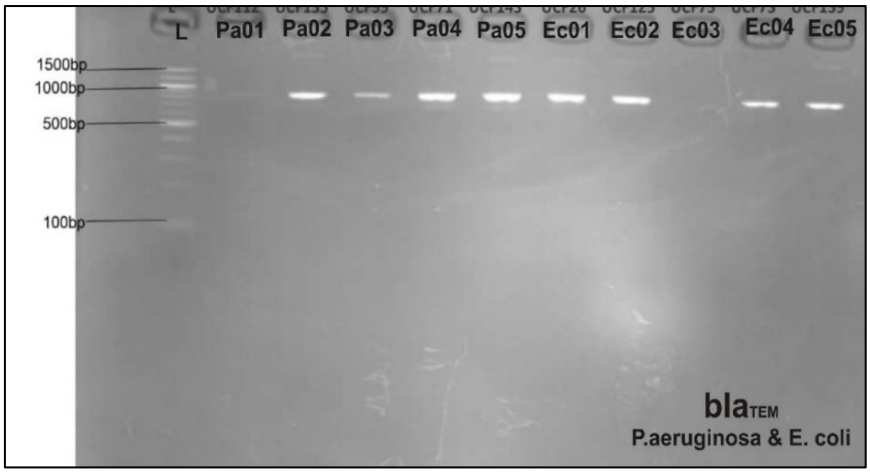


Antibiogram of Isolate on Mueller Hinton Agar

Appendix ii: PCR Bands from Molecular analysis

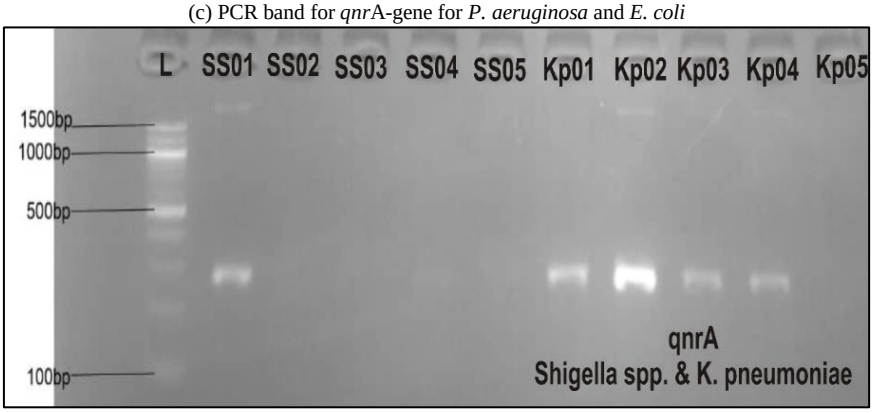


(a) PCR band for *Bla*$_{TEM}$-gene for *Shigella* species and *K. pneumoniae*

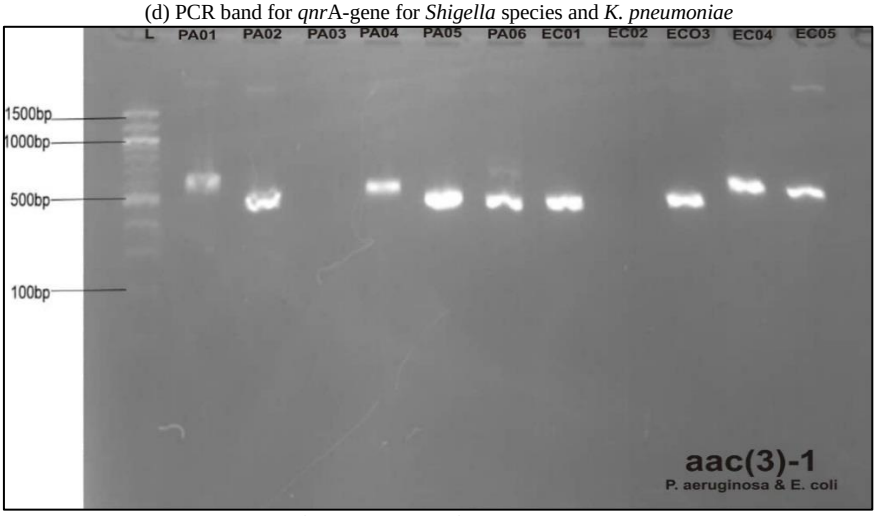


(b) PCR band for *Bla*$_{TEM}$-gene for *P. aeruginosa* and *E. coli*

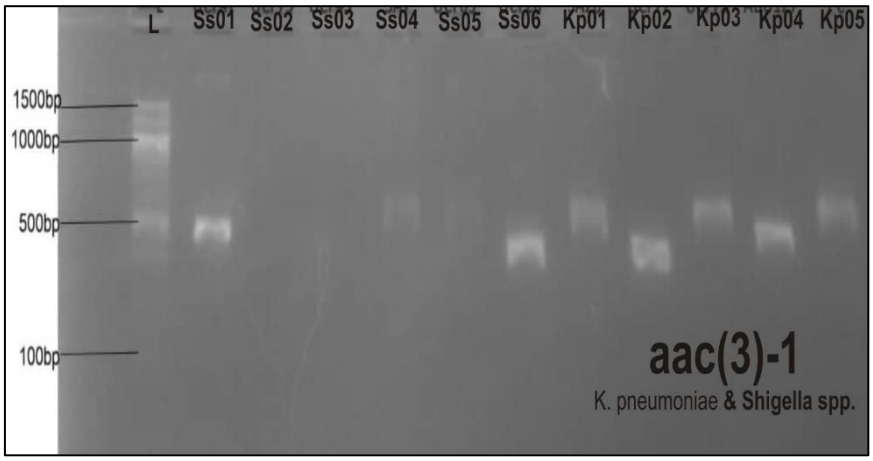
(c) PCR band for *qnr*A-gene for *P. aeruginosa* and *E. coli*



(d) PCR band for *qnr*A-gene for *Shigella* species and *K. pneumoniae*



(e) PCR band for aac(3)-1-gene for *P. aeruginosa* and *E. coli*



(f) PCR band for aac(3)-1-gene for *Shigella spp*. and *K. pneumoniae*

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