DETECTION OF NPMA GENE IN ENTEROBACTERIACEAE ISOLATED FROM CLINICAL SPECIMENS IN A TERTIARY HOSPITAL IN NIGERIA.

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ABSTRACT

BACKGROUND: Antimicrobial resistance has become of utmost concern to the global public health and the emergence of high-level aminoglycoside resistance has threatened the synergistic use of β-lactam and aminoglycoside antibiotics in the treatment of infections, especially those due to Gram-negative bacteria, thus resulting in increasing morbidity and mortality, as well as treatment cost. The aim of the study was to determine the presence of high-level aminoglycoside resistance npmA gene in Enterobacteriaceae isolates recovered from clinical specimens in a tertiary hospital.

METHODS: A total of 419 non-duplicate bacteria isolated from clinical specimens were screened for Enterobacteriaceae using the Microbact 12A Gram negative bacilli semi-automated identification system. Antimicrobial susceptibility tests were performed on the Enterobacteriaceae isolates using commercially available gentamicin antibiotic discs. Gentamicin (120µg) was used for phenotypic confirmation of high-level aminoglycoside resistance. All the resistant isolates were examined for the presence of npmA gene using conventional polymerase chain reaction.

RESULTS: Out of the 419 isolates 250 (60.1%) were confirmed Enterobacteriaceae, 30.4% (76/250) were resistant to 30µg of gentamicin, while 4% (10/250) of the Enterobacteriaceae isolated were resistant to 120µg gentamicin. However, the npmA gene was not detected in any of the high-level aminoglycoside resistant isolates.

CONCLUSION: Despite the phenotypic prevalence of high-level aminoglycoside resistance observed in the Enterobacteriaceae isolates, the *npmA* gene was not detected. The absence of the *npmA* gene therefore suggests that other factors may be responsible for the phenotypic high level aminoglycoside resistance recorded in this study.

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INTRODUCTION

Antimicrobial resistance is just one of the many challenges facing global public health and is still at its peak. It contributes to morbidity and mortality, lengthens hospital stays, raises treatment costs, and limits the antimicrobial options available [1,2]. The severity of bacterial infections has increased throughout time, largely resulting from antibiotic resistance abilities of the causative organisms.[3,4] Aminoglycosides are potent medications against life-threatening bacterial infections, and the possession of genes

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responsible for resistance to them are major contributors to the antimicrobial resistance burden being experienced today.[5,6]

The majority of Gram-negative bacteria have developed clinically meaningful degrees of resistance to various antibiotic classes, despite the fact that there is significant regional variability in the frequency of resistance.[7] Various antibiotic-resistant bacteria have previously evolved and spread from affluent healthcare institutions.[2] Polymyxins and aminoglycosides are now available as therapeutic alternatives for the treatment of severe infections caused by Carbapenem Resistant Enterobacteriaceae (CRE) after the emergence of carbapenemase synthesis in Enterobacteriaceae in recent years. Aminoglycosides became even more important in the treatment of CRE infections as a result of the growth in polymyxin resistance, particularly among isolates that expressed the KPC-2 gene.[8]

Aminoglycoside-modifying enzymes (AMEs) play a major role in the resistance of Gram-negative organisms to aminoglycosides, rendering the antibiotic inactive by altering the -OH or -NH2 groups.[9] AMEs can be classified into three classes: ATP (and/or GDP)-dependent aminoglycoside phosphotransferases (APHs), acetyl-coenzyme A (CoA)-dependent aminoglycoside acetyltransferases (AACs), and ATP-dependent aminoglycoside nucleotidyltransferases (ANTs).[10] Posttranscriptional alterations and mutations (nucleotide substitution), though they are less frequent, have been linked to aminoglycoside resistance. Transmissible 16S rRNA methyltransferases (16S RMTases), which function by decreasing the affinity of practically all aminoglycosides for 16S rRNA via ribosomal methylation, have become a novel and concerning source of aminoglycoside resistance in the last ten years.[11]

Aminoglycosides are potent antibiotics that are bactericidal and attach to the 16S rRNA aminoacyl-tRNA recognition site (A-site), which is a part of the 30S ribosomal subunit of bacteria cells. They then assault the bacterial ribosome to inhibit protein synthesis.[12] They work particularly well against Gram-negative bacteria and complement beta lactam antibiotics in the treatment of several Gram-positive diseases.[13] The *npmA* (methylating the N1-A1408 region of the site-A) offers a wide range of aminoglycoside resistance in addition to being resistant to gentamicin, tobramycin, and amikacin.[2,14] Bacteria which possess this gene will be resistant to a combination therapy of beta lactam antibiotics and any of the aminoglycosides. This study was thus, aimed at determining the presence of npmA gene among Enterobacteriaceae isolates from clinical specimens of patients accessing healthcare in a tertiary institution in Nigeria.

MATERIALS AND METHODS

Study Area

This study was carried out in Nnamdi Azikiwe University Teaching Hospital, Nnewi, a tertiary health care facility in Anambra state, South-eastern part of Nigeria. This tertiary healthcare facility serves as a major referral center for clients from Anambra state and other southeastern parts of Nigeria. Ethical Approval Ethical approval was obtained from the Nnamdi Azikiwe University Teaching Hospital Research and Ethics Committee (NAUTH-REC) to allow for the collection of clinical samples.

Sample size determination

The sample size for this study was calculated using; $N = (z^2pd)/d^2$

Where N = minimum sample size required

Z = reference of normal distribution for the desired confidence interval at 1.96% for 95% confidence interval

P = standard of deviation

Q = 1-P

D = desired level of precision

Sample Collection

A total of 419 non-duplicate isolates collected from the Medical Microbiology Laboratory of Nnamdi Azikiwe University Teaching Hospital (NAUTH) were used in this study. These isolates which had been recovered from various clinical samples over a period of one month including; urine (200), stool (38), wound swabs (38), high vaginal swabs (25), semen (40), blood (35), ear swabs (25) and sputum (18).

Identification of Isolates

Primary re-identification of the isolates was performed by sub-culturing each isolate onto a freshly prepared MacConkey agar (MCA) and was incubated at $35^0C \pm 2^0C$ for 18 to 24 hours. Following incubation after 24hrs, isolates which showed lactose-fermenting colonies were further subjected to oxidase test and finally confirmed using Microbact 12A semi-automated identification kit (Oxoid Ltd., England). Microbact 12A is a commercial test kit composed of 12 standardized biochemical tests substrates in 12 micro-wells respectively used to identify Enterobacteriaceae.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed on all isolates confirmed as Enterobacteriaceae using the Modified Kirby-Bauer disc diffusion method on Mueller-Hinton agar according to the CLSI guidelines.[15] Commercially available antimicrobial discs (Oxoid Ltd Basingstoke, UK) were used to determine the drug susceptibilities of the isolates. The antibiotic discs used for testing include Gentamicin (CN: 30µg and 120 µg). A lawn of each bacterial inoculum equivalent to $1.5 X 10^8$ CFU/ml, was made on the surface of a Mueller-Hinton agar (Oxoid, UK) plate using a sterile swab stick and allowed to dry for 3-5 minutes. Antibiotics were then placed on the lawn, and the plates incubated aerobically at $35\text{-}37^0C$ for 16-18 hours. The zones of growth inhibition around each antibiotic disc were measured and reported based on the guidelines of the CLSI.[15]

Phenotypic Determination of Aminoglycoside Resistance

Following the antimicrobial susceptibility tests done using 120µg gentamicin discs, the test isolates which demonstrated a diameter of the zones of inhibition less or equal 6mm were consider phenotypically to be high level gentamicin resistant.[15] These were then subjected to molecular assay for the aminoglycoside resistance gene (*npmA*).

Molecular Detection of *npmA*

Gene Five milliliters of an overnight broth culture of the bacterial isolate grown in Luria Bertani (LB) medium (Oxoid) were spun at 14000rpm for 3minutes. The cells were re-suspended in 500µl of normal saline and heated at 95^oC for 30 minutes. The heated bacterial suspension was fast cooled on ice for 10 minutes and spun for 3minutes at 14000rpm. The supernatant was transferred to a 1.5ml microcentrifuge tube and stored at -20^oC for other downstream reactions. The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer and a conventional PCR reaction was conducted with an initial denaturation at 95^oC for 5 minutes, followed by 34 cycles of denaturation at 95^oC for 30 seconds. Annealing at 52^oC for 40 seconds and

extension at 72ºC for 1 minutes 20 seconds. A final extension at 72ºC for 7 minutes was then conceded.

Table 1: The primers and sequences used for DNA amplification.

Primer	Sequence	Base pair	Ref.
***npmA*-F**	CGGGATCCAAGCACTTTCATACTGACG	981	17
***npmA*-R**	CGGAATTCCAATTTTGTTCTTATTAGC		

RESULTS

Out of the 419 bacteria isolated, 250(59.7%) were identified as belonging to the Enterobacteriaceae family. The isolates included species in the following frequencies of occurrence; *Escherichia coli* (90), *Klebsiella pneumoniae* (44), *Klebsiella oxytoca* (25). The lowest occurring organisms were *Citrobacter sakazakii, Enterobacter aerogenes, Enterobacter clocaea* and *Proteus alkalifaciens*, all represented with one isolate each.

Figure 1.

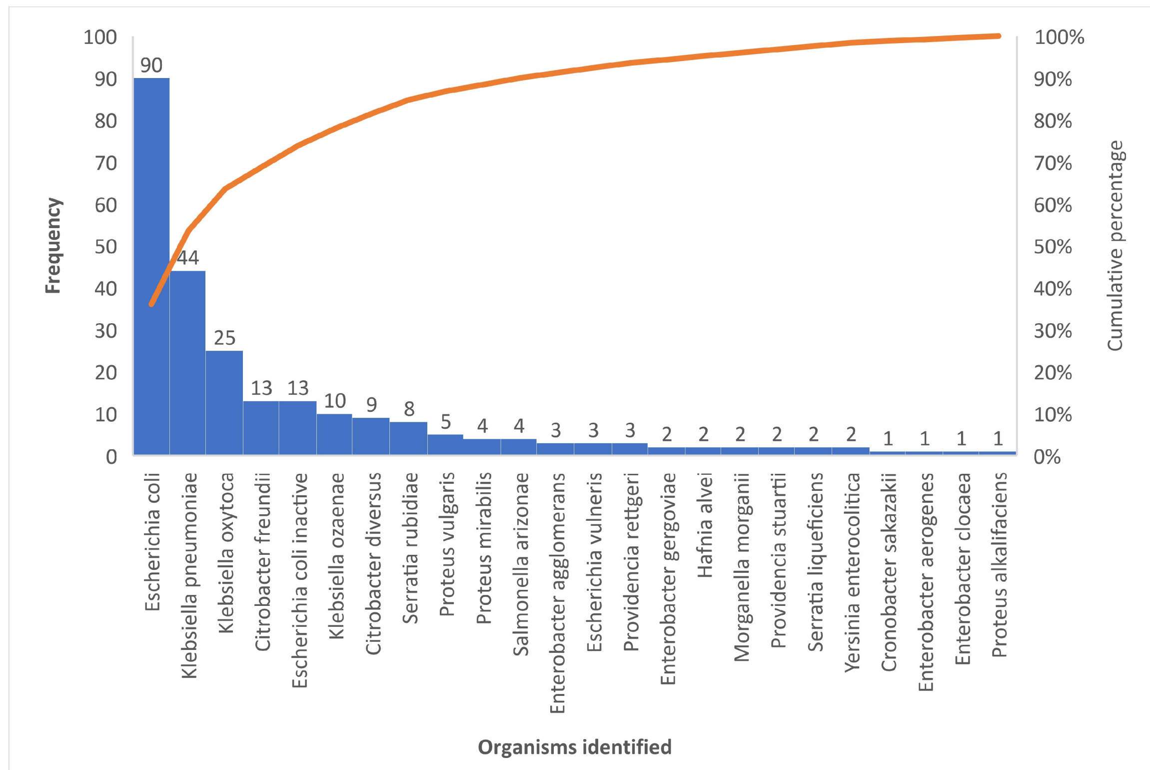


Fig. 1: A Pareto plot showing the distribution of the different microorganisms identified with a cumulative percentage.

Out of the 250 Enterobacteriaceae, 76(30.4%) of the isolates were resistant to the 30µg gentamicin disc (low level aminoglycoside resistance) which included *Escherichia coli* (n=30), *Klebsiella pneumoniae* (n=19), *Klebsiella oxytoca* (n=7), *Citrobacter freundii* (n=4), *Klebsiella ozaenae* (n=4), *Serratia rubidiae* (n=3), *Enterobacter agglomerans* (n=2), *Citobacter diversus*

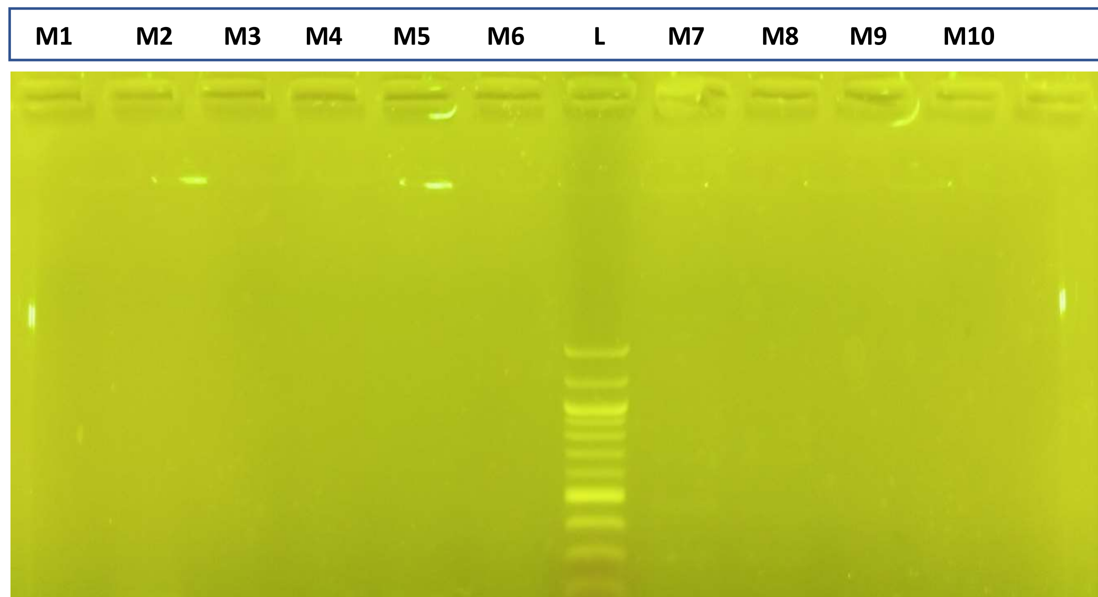
(n=1), *Hafnia alvei* (n=1), *Proteus vulgaris* (n=1), *Providencia rettgeri* (n=1), *Providencia stuartii* (n=1), *Salmonella arizonae* (n=1) and *Serratia liqueficiens* (n=1), while 10(4.0%) showed resistance to 120µg gentamicin (high level aminoglycoside resistance) and included *Klebsiella pneumoniae* (n=4), *Escherichia coli* (n=2), *Klebsiella oxytoca* (n=1), *Citobacter diversus* (n=1), *Citrobacter freundii* (n=1) and *Proteus vulgaris* (n=1)

Table 2: Showing the antibiotic susceptibility profiles of the Enterobacteriaceae isolates tested.

Class of antibiotic used	Generic name of antibiotic used and disc content	AST profile (%)		
		S	I	R
Aminoglycoside	Gentamicin 30µg	164(65.6)	10(4.0)	76(30.4)
	Gentamicin 120µg	240(96.0)	0(0)	10(4.0)

S; Susceptible, I; Intermediate, R; Resistant

All 10 isolates which were phenotypically positive for high level aminoglycoside resistance were negative for the npmA gene expression. This implies they did not habour the *npmA* gene, and the resistance must have been attributed to other mechanisms of aminoglycoside resistance.



Agarose gel electrophoresis of bacteria isolates for npmA gene. Lane1 to 10 represents negative npmA gene on the agarose. Lane L represents 100bp molecular ladder with a 1500bp molecular weight of DNA.

DISCUSSION

From this study, *Escherichia coli* and *Klebsiella species* were the most frequently isolated Gram-negative organisms from clinical specimens mostly from urogenital tract. The study recorded a 30% resistance to low level gentamicin which was lower than 66.7%[17] and 53.8%[18] previously reported in Nnamdi Azikiwe University Teaching Hospital, Nnewi, and also lower than the 50.0% and 71.7% resistance rate reported in Saudi Arabia and Iran.[19,20] This low level resistance could be attributed to the sources of the isolates or good

antibiotics stewardship practiced by the clinicians. Molecular investigation and characterization for *npmA* gene among the 10 phenotypic positive isolates revealed that none of the isolates produced *npmA* gene, which indicates that there has not been dissemination of this gene in NAUTH or the fact that the high level phenotypic resistance may be due to other non-tested genes responsible for such resistance.[16]

CONCLUSION

This study revealed that Enterobacteriaceae poses a great resistance threat to aminoglycoside antibiotics used against it in clinical settings. Although some of our isolates showed resistance to aminoglycosides tested in this study, nevertheless, the *npmA* gene which have been shown to confer this aminoglycoside resistance attribute was not detected in any of the Enterobacteriaceae isolated. This suggests that the resistant isolates may have acquired another mechanism of resistance or have undergone mutation, which made it impossible for our target primer to detect. In addition, we encourage that routine monitoring of npmA and other acquired 16S RMTases be adopted in our laboratories especially tertiary health-care facilities.

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