



SJMLS - 4 (4) - 009

Antimicrobial Susceptibility of New Delhi Metallo Beta Lactamase - 1(NDM-1) producing Bacteria isolated from major hospitals in Kano-Nigeria to Colistin and TigecyclineAhmad, A.S.^{1*}, Arzai, A.H.², Yusuf, I.², Dabo, N.T.³, Takalmawa, H.U.⁴, Umar, F.A.¹ and Rabi'u, M.A.⁴

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Abstract

The continued rise in the rate of resistance of clinical pathogens to common antibiotics in our hospitals has left the prescribers with option of using colistin, tigecycline and related drugs in treating serious infections. Resistance mediated by New Delhi Metallo Beta lactamase (NDM-1) is widely underreported especially in our facilities. This study aimed at screening for carbapenemase, metallo beta lactamase (MBL) and specific NDM-1 producing clinical isolates from major tertiary hospitals in Kano-Nigeria and determining their susceptibility to colistin and tigecycline. A total of 500 antibiotic resistant bacterial isolates belonging to the family *Enterobacteriaceae* and *Pseudomonas* were screened phenotypically for carbapenemase and MBL production using Modified Hodges and EDTA Disc Synergy tests respectively. blaNDM-1 gene was detected among potential NDM-1 producers by Polymerase Chain Reaction (PCR) using NDM-1 specific primers. Susceptibility of the carbapenemase, MBL and confirmed NDM-1 producing bacteria to colistin and tigecycline was carried out using disc diffusion method. Of the total isolates screened, 162 (32.4%) and 43(8.6%) were carbapenemase and MBL producers respectively. While highest prevalence of carbapenemase was found in *S. typhi* (66.7%), followed by *S. paratyphi* (50%), *K. pneumoniae* (36.9%), *K. oxytoca* (33.3%), and *E. coli* (31%), MBL prevalence was higher in *Proteus mirabilis*, *K. pneumoniae*, and *Ps. aeruginosa*. Among the isolates positive in phenotypic confirmation test (i.e. carbapenemase and MBL), gene blaNDM-1 was detected in 3 *E. coli* and 1 *K. pneumoniae*

bringing the overall frequency of NDM-1 producers to 0.8%. While the carbapenemase producing isolates are about 96% sensitive to colistin and tigecycline, MBL and NDM-1 were all 100% sensitive using disc diffusion method. Carbapenemase, MBL and NDM-1 producing isolates occurred among the pathogens commonly isolated in the study area and are highly susceptible to Colistin and tigecycline.

Keywords: Carbapenemase, New Delhi Metallo Beta Lactamase-1, colistin, tigecycline

Introduction

The rate of consumption and abuse of antibiotics in developing countries is increasing at alarming rate and is the major cause of increasing resistance of bacteria to commonly prescribed and reserved antibiotics. Resistance of bacteria especially GNB to common antibiotics such as penicillin, tetracycline and co-trimoxazole have been documented in Nigeria. Previous studies have reported enzyme production (beta lactamase) as the main cause of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter* sp resistance to penicillins and cephalosporins. For hard to treat infections caused by antibiotic resistant pathogens, single or combinations of antibiotics such as carbapenems, flouoroquinolones, aminoglycosides are often used. However, recent reports have shown the presence and dissemination of Carbapenemase, ESBL, AmpC producing clinical bacteria isolated from both hospitalised and non-hospitalised patients in Nigerian hospitals. The organisms have also been isolated from the intestinal microbiota of

healthy human and animals across US, Asia, Africa and Europe. NDM-1-producing bacteria have been recovered from many infections such as urinary tract infections, pneumonia, septicaemia, wound and device-associated infections. New Delhi metallo beta lactamase-1 (NDM-1) is a kind of β -lactamase enzyme that cleaves the amide bond of β -lactam ring and provides resistance against major classes of β -lactam antibiotics. The enzyme has zinc ions at its active site, which helps in hydrolysing all beta lactam antibiotics excluding aztreonam.

NDM-1 has been reported almost worldwide with varying prevalence, but grossly under reported in Africa and Nigeria in particular. Infections caused by NDM-1 bacteria are difficult to treat and have been reported to increase morbidity, mortality and length of stay in hospitals. The chemotherapeutic options for treating NDM-1-producing Enterobacteriaceae infection are limited.

Colistin and tigecycline are both agents for which many strains still have MICs below the clinical breakpoint. Their activities are still promising, which may make them an alternative therapeutic option against carbapenem resistant Enterobacteriaceae (CRE), NDM-1 and other multidrug resistant pathogens.

New Delhi metallo- β -lactamase-1 gene (*bla_{NDM-1}*) codes for NDM-1. An association with other resistance mechanisms makes majority of Enterobacteriaceae with *bla_{NDM-1}* gene extensively resistant to antibiotics and susceptible only to colistin and less consistently, tigecycline. Dissemination of the plasmid borne *bla_{NDM-1}* through horizontal gene transfer is a potential threat to the society and NDM-1 producers collected worldwide are all multidrug resistant but many remain susceptible to fosfomycin, colistin and tigecycline. The current increase in number of detections of multi drug resistant bacteria from different samples (both clinical and environmental samples) in Nigeria is worrisome, and need urgent surveillance intervention to plan for control actions. Similarly, reports of pockets of resistance of MDR to colistin and tigecycline in some parts of the globe, has necessitated the search for

susceptibility of NDM-1 isolated from clinical samples from Nigeria to colistin and tigecycline. Therefore, this research is aimed at determining the antimicrobial susceptibility of NDM-1 producing clinical bacterial isolates to colistin and tigecycline in Kano-Nigeria.

Materials and method

Collection of Clinical Isolates

A total of 500 consecutive, non-repetitive clinical bacterial isolates were collected from Microbiology Departments of Aminu Kano Teaching Hospital, Muhammad Abdullahi Wase Specialist Hospital and Murtala Muhammad Specialists Hospital, Kano, Nigeria after obtaining ethical clearance from the respective ethical committees. The isolates collected from blood, urine and catheter tip samples over a period of 12 months; include *E. coli* (187), *Klebsiella pneumoniae* (130), *K. oxytoca* (3), *Proteus mirabilis* (87), *P. vulgaris* (29), *Pseudomonas aeruginosa* (56), *Salmonella typhi* (6), and *S. paratyphi* (2).

Selection of the various isolates were based on their susceptibility profile, and therefore only isolates that are resistant to at least 3 classes of antibiotics such as ciprofloxacin (5 μ g), cefpodoxime (30 μ g), ceftazidime (30 μ g), ceftriaxone (30 μ g), ceftiofloxacin (30 μ g), amoxicillin (20 μ g), ofloxacin (30 μ g), levofloxacin (30 μ g), and augmentin (30 μ g) (Oxoid, UK) were selected. The identities of the selected isolates were confirmed using biochemical tests.

Preliminary and confirmatory screening of isolates for Carbapenemase production

The isolates recovered from the hospitals were screened for carbapenemase production according to the Clinical and Laboratory Standard Institute (CLSI) guidelines. Disc diffusion screening test was employed using Imipenem (IPM) [10 μ g] and meropenem (MEM)[10 μ g] discs. The IPM and MEM were placed on the surface of Mueller Hinton Agar (MHA) plates using sterile forceps about 25 mm apart, and the plates were incubated for 24 hours at 37 °C after which zones of inhibitions were read. Any isolates that shows zone of inhibition of \leq 19 mm for MEM and IPM were further

subjected to Modified Hodges Test to confirm carbapenemase production. Disc diffusion technique described by CLSI was adopted. In brief, a McFarland suspension of *E. coli* ATCC 25922 was streaked with sterile cotton swab on surface of MHA plates. This was allowed to stand for 10 minutes at room temperature. Two discs; one with MEM and other with IPM, were placed onto the plate 25 mm apart lying diagonally opposite to each other. Aseptically, a 10 μ l loop was used to pick three to five colonies each test bacteria grown overnight on Mueller Hinton Broth was inoculated onto MHA plate in a straight line from the edge of one disc to another. The plates were incubated at 37 °C for 24 hours.

Phenotypic Screening for Metallo beta lactactamase using EDTA Disc Synergy Test

EDTA disc synergy test was performed as described by the CLSI guidelines using Disc diffusion techniques with two MEM discs; one with MEM (10 μ g) and other containing 10 μ l of 0.1M anhydrous EDTA (292 μ g). Any bacteria that produced a diameter of >4 mm around the disc with MEM-EDTA and not around the disc with MEM alone was considered positive for MBLs. *Escherichia coli* ATCC 25922 was used as negative control.

Amplification of blaNDM-1 gene using Polymerase Chain Reaction (PCR)

All isolates positive in phenotypic confirmation test carried out were subjected to PCR technique to detect blaNDM-1 gene encoding NDM. Genomic DNA extraction of the test bacteria and that of *Klebsiella pneumoniae* NCTC 13443 (positive control strain) was carried out by standard kit protocol (ThermoFisher Scientific). Amplification of the blaNDM-1 genes was carried out in a Thermal Cycler using the primers NDM-Fm (5'-ggtttggecgatctggttttc-3') and NDM-Rm (5' cggaatggctcatcagatc-3') for 35 cycles. PCR mixture was carried out in 50 μ l micro test tubes, which comprised of 1.5 μ l of NDM-1 primers, 0.2ml of dNTPS, 1.5mM MgCl₂, 14mM tris-HCl Buffer (PH 8.2), 1.0 μ l Taq polymerase, 2 μ l of the template and the final volume was balanced with sterile molecular grade distilled water. The cycling conditions used for the amplification are: initial denaturation 94°C for 5

minutes, denaturation 94°C for 30 seconds, annealing 43°C for 30 seconds, extension 72°C for 1 minute, and final extension 72°C for 10 minutes (Olowe and Grobbel, 2010). The amplicons were run on 1.5% agarose gel in TAE buffer at 120 volts for 1 hour. The DNA bands were visualized using UV light box (Gel documentation Unit). The purified amplified PCR products were sequenced. The blaNDM-1 gene sequences were pasted into basic local alignment search tool (BLAST) in NCBI website and compared with available nucleotide sequence database.

Antimicrobial susceptibility Test

The susceptibility of carbapenemase, MBLs, and confirmed NDM-1 producers to colistin and tigecycline was determined by agar Diffusion method. Isolates were streaked on the surface of MHA by swabbing. The plates were allowed to stand for 10 minutes at room temperature. Two discs, one of colistin (10 μ g) and other tigecycline (15 μ g) (Oxoid, UK), were placed onto the agar plate. A strain producing a diameter of > 14mm and > 21mm for colistin and tigecycline respectively was considered susceptible. *E. coli* ATCC 25922 was used as the control strain

Results

The overall susceptibility of the selected isolates from all the hospitals to the commonly prescribed antibiotics showed high level of resistance to β -lactam antibiotics and non β -lactam antibiotics such as augmentin, cefpodoxime, ceftriaxone, cefoxitin, ofloxacin, levofloxacin, chloramphenicol, co-trimoxazole and tetracycline. Majority of the isolates showed reduced susceptibility to amoxicillin, augmentin, cefpodoxime, ceftriaxone and ofloxacin with the exception of *P. aeruginosa* which is totally resistant to all antibiotics but showed enhanced sensitivity to cefoxitin (Table 1).

Carbapenemase and Metallo beta-lactamase production

Out of the 500 clinical bacterial isolates screened for carbapenemase production, 162 (32.4%) and 43(8.6%) were found to produce carbapenemase and MBLs respectively. The highest prevalence of carbapenemase was found in *S. typhi* (4/6,

66.7%), followed by *S. typhi* (1/2, 50%), *K. pneumoniae* (48/130, 36.9%), *K. oxytoca* (1/3, 33.3%), *E. coli* (59/187, 31%), and *Proteus spp* had the least carbapenemase prevalence (Table 2). MBL prevalence however, was higher in *Proteus mirabilis* and *K. pneumoniae*, followed by *Ps. aeruginosa* and *E. coli*. No MBL was detected in *Salmonella spp* and *K. oxytoca* and was detected in one *P. vulgaris*.

Among the isolates positive in phenotypic confirmation test (carbapenemase and MBL), gene bla NDM-1 was detected in 3 *E. coli* and 1 *K. pneumoniae* (Figure 1), bringing the overall frequency of NDM-1 producers in this study to be 0.8%. The susceptibility of carbapenemase producing isolates to colistin and tigecycline were found to be 96.3% while that of MBLs and NDM-1 were all 100% (Table 3).

Table 1: Sensitivity pattern of bacteria isolated from blood, urine and catheter tips of patients attending major hospitals in Kano, Nigeria

| Name of organisms | N | No of isolates susceptible to antibiotics (% susceptibility) | | | | | | | | | | |
|----------------------|------------|--|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| | | CIP | CPD | CAZ | CXM | FOX | COT | CXC | AMC | OFL | LEV | AUG |
| <i>E. coli</i> | 187 | 4 | 0 | 0 | 0 | 1 | 0 | 1 | 2 | 3 | 2 | 2 |
| <i>K. pneumoniae</i> | 130 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 1 | 3 | 1 |
| <i>K. oxytoca</i> | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| <i>P. mirabilis</i> | 87 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>P. vulgaris</i> | 29 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>P. aeruginosa</i> | 56 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>S. paratyphi</i> | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>S. typhi</i> | 6 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 500 | 6 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 4 | 5 | 3 |

Note=CIP=ciprofloxacin, CPD=cefpodoxime, CAZ=ceftazidime, CXM=ceftriaxone, FOX=cefoxitin, COT= co-trimoxazole, CXC=cloxacillin, AMC=amoxycillin, OFL=ofloxacin, LEV=levofloxacin, AUG=augmentin, N=Number

Table 2: Prevalence of Carbapenemase, MBLs and blaNDM-1 gene producers among clinical bacterial Isolates

| Bacterial species | Isolates screened | Carbapenemases (%) | MBLs (%) | Bla _{NDM-1} gene (%) |
|----------------------|-------------------|--------------------|----------------|-------------------------------|
| <i>E. coli</i> | 187 | 59(31.6) | 16(8.0) | 3(1.6) |
| <i>K. pneumoniae</i> | 130 | 48(36.9) | 13(10.0) | 1(0.5) |
| <i>K. oxytoca</i> | 3 | 1(33.3) | 0(0.00) | 0(0.0) |
| <i>P. mirabilis</i> | 87 | 20(23.0) | 8(9.2) | 0(0.0) |
| <i>P. vulgaris</i> | 29 | 8(27.6) | 1(3.5) | 0(0.0) |
| <i>P. aeruginosa</i> | 56 | 21(37.5) | 5(8.9) | 0(0.0) |
| <i>S. paratyphi</i> | 2 | 1(50.0) | 0(0.00) | 0(0.0) |
| <i>S. typhi</i> | 6 | 4(66.7) | 0(0.00) | 0(0.0) |
| Total | 500 | 162(32.4) | 43(8.6) | 4(0.8) |

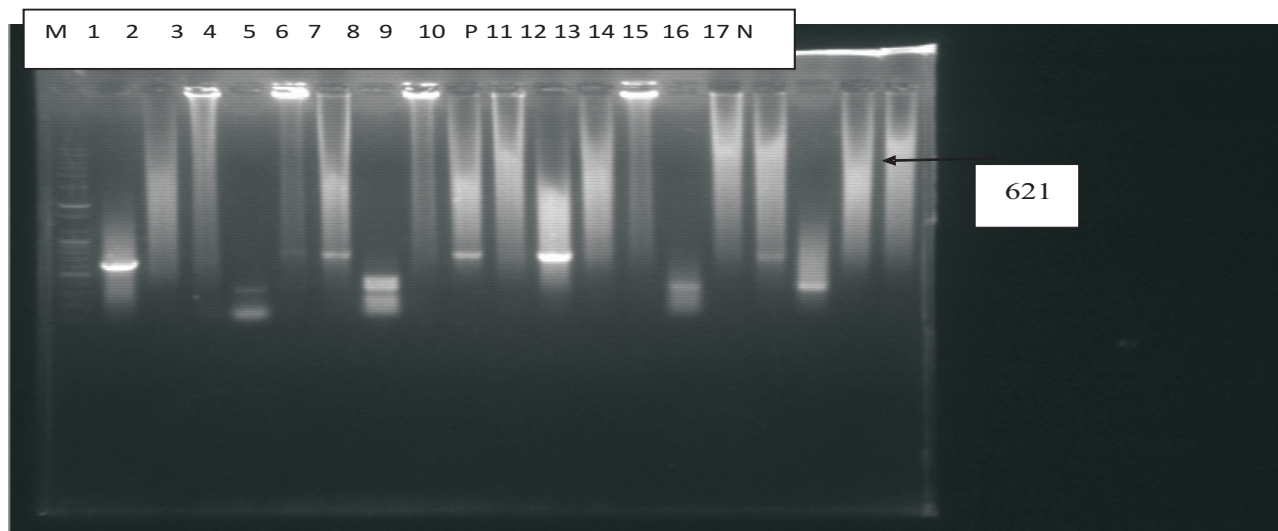


Figure 1: Gel electrophoresis of bla_{NDM-1} gene

Key

Lane M is molecular maker graduated on hundred base pair (100bp)

Lane P positive control, Lane N negative control

Lane 5, 6, 9, and 15 are positive bands (*Klebsiella pneumoniae* and three *E. coli* respectively)

Lane 1, 2, 3, 4, 6, 7, 8, 10, 11, 12, 13, 14, 16 and 17 are negative bands

Table3: Susceptibility of Carbapenemase, MBLs Producing and NDM-1 confirmed isolates to Colistin and Tigecycline

| Bacterial Isolates | N^o of Isolates Screened | N^o Susceptible to Colistin (%) | N^o Susceptible to Tigecycline (%) |
|------------------------------------|---|--|---|
| 1. Carbapenemase producers | | | |
| <i>E. coli</i> | 59 | 56(95.0) | 56(95.0) |
| <i>K. pneumoniae</i> | 48 | 46(96.0) | 46(96.0) |
| <i>K. oxytoca</i> | 1 | 1(100) | 1(100) |
| <i>P. mirabilis</i> | 20 | 20(100) | 20(100) |
| <i>P. vulgaris</i> | 8 | 8(100) | 8(100) |
| <i>P. aeruginosa</i> | 21 | 21(100) | 8(100) |
| <i>S. paratyphi</i> | 1 | 1(100) | 1(100) |
| <i>S. typhi</i> | 4 | 4(100) | 4(100) |
| Total | 163 | 157(96.3) | 157(96.3) |
| 2. MBLs producers | | | |
| <i>E. coli</i> | 16 | 16(100) | 16(100) |
| <i>K. pneumoniae</i> | 13 | 13(100) | 13(100) |
| <i>P. mirabilis</i> | 8 | 8(100) | 8(100) |
| <i>P. vulgaris</i> | 1 | 1(100) | 1(100) |
| <i>P. aeruginosa</i> | 5 | 5(100) | 5(100) |
| Total | 43 | 43(100) | 43(100) |
| 3. NDM-1 confirmed isolates | | | |
| <i>E. coli</i> | 3 | 3(100) | 3(100) |
| <i>K. pneumoniae</i> | 1 | 1(100) | 1(100) |
| Total | 4 | 4(100) | 4(100) |

Discussion

This study revealed that resistance pattern of common GNB pathogens in major hospitals in Kano, the most populous state of Nigeria is high, especially toward commonly prescribed antibiotics such as amoxicillin, cloxacillin, amoxicillin-clavulanic acid, cefpodoxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cotrimexazole and cefoxitin (30 µg). However, the number (prevalence) of isolates showing decreased susceptibility or resistance to antibiotics such as imipenem, meropenem, levofloxacin, cefpodoxime and ceftriaxone is quite higher when compared to amount reported in 2014 in the study area in 2 major hospitals . The increase in the trend of antibiotic resistance pattern observed in this study is very alarming and calls for immediate and urgent intervention, to tackle the continued circulation of counterfeit and inappropriate consumption of antibiotics in the study area.

Carbapenemase and MBLs producers were found in 32.4%, and 8.6% of the total bacterial isolates tested respectively. This and other studies have proved that majority of carbapenemase and MBL producing bacteria are multidrug resistant because they could harbor genes responsible for resistance to carbapenem (*bla*NDM-1) and other antibiotics on same plasmid . The prevalence of specific carbapenemase “NDM-1” producing *Enterobacteriaceae* was very low (0.8%), higher among *E. coli* than *K. pneumoniae*. This finding supports previous reports which reported the existence of such multi-drug resistance organisms in Ugandan and Iranian hospitals. Few more studies done with similar objectives also confirm the existence of such organisms . However, the prevalence of NDM-1 producers in this study is lower than the findings in Uganda and Iran, where 2.6 and 12.2 % prevalence were reported respectively . Similarly, the higher detection among *E. coli* coincides with the previous work in India, Pakistan, and UK, where highest prevalence of NDM-1 was reported among *E. coli* and *K. pneumoniae* .

Harboring other genes other than the specific NDM-1 genes, production of other enzymes other than carbapenemase, MBL or

modifications in outer membrane proteins or penicillin-binding proteins could be reason for observed carbapenem resistance .

Susceptibility of carbapenemase, MBL and specific NDM-1 producers to reserved antibiotics like tigecycline and colistin was impressive, as sensitivity toward both antibiotics was 100%. In recent studies done in Asia and Europe settings, it was observed that there were now pockets of resistance for either colistin or tigecycline or both .

This study however indicates that colistin and tigecycline could be used for empirical treatment of infections caused by NDM-1 producing GNB in the study area. However, they should be used with caution in hospitals, and emergence of either efflux or enzyme mediated resistance should be closely monitored in order to prolong the lifespan of these useful but toxic antibiotics.

Conclusion

It is concluded that carbapenemase, MBLs and NDM-1 producing isolates have occurred among the pathogens commonly isolated with a sharp decrease in carbapenem susceptibility. Therefore, Colistin and tigecycline could be drugs of choice for the treatment of clinical bacterial infections caused by NDM-1 producing isolates detected in Kano, Nigeria.

Conflict of interest

Authors declared that no conflict of interest exist

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Citation: Ahmad, A.S., Arzai, A.H., Yusuf, I., Dabo, N.T., Takalmawa, H.U., Umar, F.A. and Rabi'u, M.A. Antimicrobial Susceptibility of New Delhi Metallo Beta Lactamase - 1(NDM-1) producing Bacteria isolated from major hospitals in Kano-Nigeria to Colistin and Tigecycline. *Sokoto Journal of Medical Laboratory Science*;4(4): 91 - 98.

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