



Detection of Carbapenem-Resistant *Klebsiella Pneumoniae* Isolates from Clinical Specimens in Nnamdi Azikiwe University Teaching Hospital, Nnewi

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ABSTRACT

Background: Carbapenem-resistant *Klebsiella pneumoniae* is emerging rapidly worldwide. The accurate and prompt identification of *Klebsiella pneumoniae* carbapenemase (KPC) is a key to limiting the spread of these bacteria. Increasingly, *Klebsiella* bacteria have developed antibiotic resistance most recently to the class of antibiotics known as carbapenems.

Aim: The study was done to detect carbapenem-resistant *Klebsiella pneumoniae* isolates from clinical specimens in Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria.

Methodology: *K. pneumoniae* clinical isolates were collected and processed at Nnamdi Azikiwe University Teaching Hospital Medical Microbiology and Parasitology laboratory from August 2012 to February 2013. Fifty-two *Klebsiella pneumoniae* recovered from different specimens namely; wound swabs (14), urine (26), sputum (7), and endocervical swab/high vaginal swab (5) were analyzed. Antimicrobial susceptibility testing (AST) was determined by agar disc diffusion method using Mueller-Hinton agar according to Clinical and Laboratory Standards Institute recommendations. Modified Hodge test (MHT) was carried out on clinical isolates that showed reduced disc sizes to carbapenem and resistant to the third generation cephalosporin. Polymerase chain reaction (PCR) was carried out to detect the presence of blaKPC gene. Results: Antibiotics susceptibility results revealed that meropenem and Imipenem showed the highest sensitivity (100%) against the isolates tested. Ertapenem showed 94.2% sensitivity. *K. pneumoniae* was isolated more from female patients 30(57.7%) than from male patients 22(42.3%). The result of the single PCR also revealed the absence of blaKPC gene. The PCR and MHT results showed strong consistency when compared.

Conclusion: Care must be taken in the prescription and administration of carbapenem as it is the last resort for many bacterial infection. Infection control policies should be strictly adhered to.

Key Words: *K. Pneumonia*, Antimicrobial susceptibility testing (AST), Parasitology, Carbapenem

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative, non-motile, encapsulated, lactose fermenting, facultative anaerobic, rod shaped bacterium found in the normal flora of the mouth, skin, and intestines (Ryan and Ray, 2004).

K. pneumoniae is an important cause of human infections or diseases which are usually nosocomial or hospital-acquired. Diseases caused by *K. pneumoniae* include; urinary tract in-

fections, pneumonia, septicemia, and soft tissue infections (Podschun and Ullmann, 1998). The diseases caused by *K. pneumoniae* can result in death for patients who are immunodeficient. Capsular polysaccharide and lipopolysaccharide O side chain are two of the most important virulence factors of *K. pneumoniae* (Cortés *et al.*, 2002).

Increasingly, *Klebsiella* bacteria have developed antibiotic resistance most recently to the class of antibiotics known as carbapenems (LA County Department of Public Health,

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2010). Detection of carbapenemase-producing isolates in the microbiology laboratory is difficult (Tenover *et al.*, 2006). Acquisition of a *bla*_{KPC} gene alone does not always confer resistance to carbapenems, as defined by current breakpoints of the Clinical and Laboratory Standards Institute (CLSI). Some KPC isolates show low-level resistance to carbapenems; with elevated minimal inhibitory concentrations (MICs) (2 to 4 µg/mL) or with reduced disk zone diameters but within the susceptible range.

Emergence of carbapenem resistance in health care settings is increasingly attributed to the production of β-lactamases capable of hydrolysing carbapenems (Bilavsky *et al.*, 2010). Carbapenemases are sub-class of β-lactamase enzymes that are classified by their specific resistant mechanisms, (such as AmpC and others), this research will concentrate on *Klebsiella pneumoniae* carbapenemase (KPC). The KPC enzyme confers resistance to all β-lactam agents including penicillins, cephalosporins, monobactams, and carbapenems (Smith *et al.*, 2003; Alba *et al.*, 2005).

KPC represents an emerging bacterial resistance mechanism and is currently more prevalent in the north-eastern part of the U.S. (New York, New Jersey and Maryland), although it has been seen with more frequency in other parts of the country (Martin *et al.*, 2010). KPC-producing bacteria have also caused outbreaks in Israel and recently have become an emerging public health concerns in several regions worldwide, such as China, Latin America and Greece (Nordmann *et al.*, 2009). Though there have been increasingly, reported cases of carbapenem-resistant *Klebsiella pneumoniae* worldwide, there is limited data in Africa. We decided to carry out this research due to the high incidence of medical tourism by Nigerians in places where KPC has been reported. Therefore, this work was centred on detection of *Klebsiella pneumoniae* carbapenemase as it is the prevalent and most documented mechanism of resistance by carbapenem-resistant *Klebsiella pneumoniae*.

Given the limited therapeutic options available, the accurate detection of KPC-possessing *Klebsiella pneumoniae* is crucial in controlling its spread. The current guidelines for the phenotypic detection of KPC-producing organisms in US hospitals are based on reduced susceptibility to carbapenems, which has to be confirmed by the Modified Hodge Test (MHT) (CLSI, 2009).

MATERIALS AND METHODS

Isolates Collection and Identification

Out of 702 bacterial isolates examined from Nnamdi Azikiwe University Teaching Hospital Medical Microbiology and Parasitology laboratory Nnewi, 52 were isolated as presumptive *K. pneumoniae*. Isolates were collected from urine, sputum, wound swab, high vaginal swab and endocer-

vical swab samples. The collected isolates were processed using standard routine Microbiological methods. Isolation was done by sub-culturing the presumptive *K. pneumoniae* on MacConkey and EMB (Eosin Methylene Blue) agars. The plates were incubated at 37°C for 24hours. Confirmation of *K. pneumoniae* was based on morphology, gram staining and standard biochemical tests.

Antibiotics susceptibility testing

Antibiotics susceptibility testing was determined using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar plates as recommended by Clinical and Laboratory Standard Institute (CLSI, 2009).

The standard commercial disks used and their concentrations are as follows: Ciprofloxacin (5µg), Imipenem (10µg), Ceftazidime (30µg), Cefotaxime (30µg), (Abtek biological, UK), Ertapenem (10µg), Aztreonam (30µg), Piperacillin/Tazobactam (10µg), Gentamicin (10µg), and Cefepime (30µg), (Oxoid, UK), Meropenem (10µg) (Mast Diagnostics Ltd, UK).

Three to five discrete colonies of *K. pneumoniae* isolates selected from an 18-24hour agar plate were touched with a sterile wire loop and suspended in 5ml of sterile saline in a bijoux bottle. The suspension was mixed thoroughly to obtain a homogenous mixture. The turbidity of the suspension was then adjusted to match the 0.5 McFarland turbidity standard.

Each of the isolates was uniformly and aseptically inoculated into a well dried Mueller-Hinton agar, in plates by spread plate method, as follows; a sterile swab stick was dipped in the suspension, squeezed by the side of the bottle before streaking over the sensitivity plates. The appropriate antibiotic discs were placed aseptically on the agar using sterile forceps. Inoculated plates were incubated at 37°C for 18-24hours after which the diameters of the inhibition zones were measured and recorded (as susceptible, intermediate or resistant). All susceptibility tests were carried out in duplicates. *Klebsiella pneumoniae* ATCC 13883 was used as control strain.

Modified Hodge Test

This is a phenotypic test which could be used to determine if reduced susceptibility to carbapenems is mediated by a carbapenemase. It's the most easily performed confirmatory test for KPCs.

Modified Hodge test (MHT) was performed first by streaking a susceptible *Escherichia coli* isolate on a Mueller-Hinton plate, after which a carbapenem disk (Meropenem) was placed on the centre. Isolates suspected of carbapenemase production then were streaked from the disk to the outer margin of the plate. This test was also repeated in duplicates. Quality control was performed using *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC BAA 1705.

Molecular Analysis

DNA Amplification

Polymerase Chain Reaction (using the PCR machine; PTC-200 BY MJ Research) was carried out to detect the presence of bla_{KPC} gene. The primers used are as follows; bla_{KPC}-F1: TCGCTAAACTCGAACAGG and bla_{KPC}-R1: TTACTGCCCGTTGACGCCCAATCC. The PCR reaction system contained 0.1-1.0µM for each primer. Cycling parameters were; initial denaturation at 93°C for 3 minutes, followed by 35 cycles of 1 minute at 93°C, 30 seconds at 56°C, and 1 minute 30 seconds at 72°C. The PCR amplification was ended by a final extension at 72°C for 10 minutes.

RESULTS

Of the 702 bacterial isolates analyzed for the presence of *K. pneumoniae*, 52 were identified as *K. pneumoniae*. Sources of the isolated *K. pneumoniae* were; wound swab 14(26.9%), urine 26(50.0%), sputum 7(13.5%), and ECS/HVS 5(9.6%), as shown in Table 4.1. The prevalence of *K. pneumoniae* clinical isolates was found to be 7.4%.

Table 4.1 shows the percentage distribution of *K. pneumoniae* in clinical samples from NAUTH.

Table 4.2 outlined the percentage distribution of *K. pneumoniae* amongst male and female patients.

Fig. 4.1 shows the age distribution of *K. pneumoniae* amongst male and female patients in NAUTH. Among the male patients, age interval of 40-49 was the modal class, while the mean was 39.95. The modal class for the female patients was 50-59 while the mean was 45.83.

The Antimicrobial susceptibility pattern of the 52 *Klebsiella pneumoniae* clinical isolates was evaluated. Table 4.3 shows the results of the test. 10(19.2%) clinical isolates of *K. pneumoniae* that showed reduced susceptibility and resistance to third generation cephalosporin were subjected to polymerase chain reaction (PCR) to detect if bla_{KPC} gene was present. Plate 1: shows the result. Bla_{KPC} gene was not detected from the *K. pneumoniae* isolates tested.

Table 4.1: Percentage distribution of *K. pneumoniae* in clinical samples from NAUTH, Nnewi.

| Specimen type | Number of specimens | Number of isolates (%) |
|---------------|---------------------|------------------------|
| Wound swab | 187 | 14 (26.9) |
| Urine | 306 | 26 (50.0) |
| Sputum | 111 | 7 (13.5) |
| ECS/HVS | 98 | 5 (9.6) |
| Total | 702 | 52 |

ECS= Endocervical swab; HVS= High vaginal swab

Table 4.2: Percentage distribution of *Klebsiella pneumoniae* amongst male and female patients of NAUTH, Nnewi.

| Patients | <i>K. pneumoniae</i> (%) |
|----------|--------------------------|
| Male | 22 (42.3) |
| Female | 30 (57.7) |
| Total | 52 |

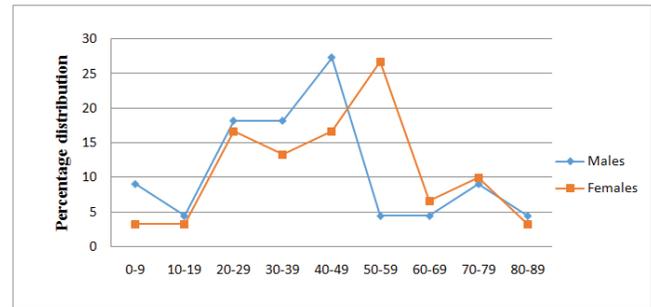


Figure 4.1: Age distribution of *K. pneumoniae* amongst male and female patients in NAUTH

Table 4.3: Antimicrobial susceptibility profile of *K. pneumoniae* isolates from NAUTH.

| Antimicrobial agent | Susceptible (%) | Intermediate (%) | Resistant (%) |
|-------------------------|-----------------|------------------|---------------|
| Meropenem | 52 (100) | 0 (0) | 0 (0) |
| Cefepime | 51 (98.1) | 0 (0) | 1 (1.9) |
| Piperacillin/Tazobactam | 46 (88.5) | 2 (3.8) | 4 (7.7) |
| Aztreonam | 39 (75.0) | 3 (5.8) | 10 (19.2) |
| Gentamycin | 43 (82.7) | 1 (1.9) | 8 (15.4) |
| Ertapenem | 50 (94.2) | 1 (1.9) | 1 (1.9) |
| Ceftazidime | 49 (94.2) | 0 (0) | 3 (5.8) |
| Cefotaxime | 50 (96.2) | 0 (0) | 2 (3.8) |
| Imipenem | 52 (100) | 0 (0) | 0 (0) |
| Ciprofloxacin | 45 (86.2) | 3 (5.8) | 4 (7.7) |

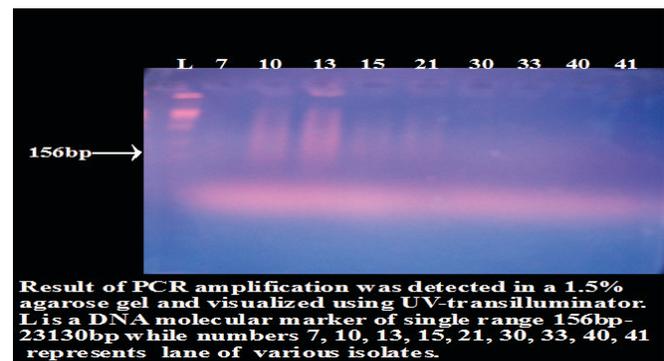


Plate 1: Agarose gel electrophoresis result for the detection of bla_{KPC} gene.

DISCUSSION

With the increased dependence on carbapenem antibiotics, KPCs are a major public health concern. Bacterial isolates producing KPCs are able to hydrolyze a broad spectrum of β -lactams including the penicillins, cephalosporins, carbapenems and monobactams. They have the potentials to spread rapidly in hospital environments to cause nosocomial infections with high mortality rates (Hirsch and Tam, 2010). Carbapenems are a class of β -lactam antibiotics with a broad spectrum of antibacterial activity. They are one of the antibiotics of last resort for many bacterial infections, such as *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (Smith, 2010).

In this study, a total of 52 clinical isolates of *Klebsiella pneumoniae* were isolated from 702 clinical bacterial isolates. Sources of *K. pneumoniae* clinical isolates were as follows; wound swab, urine, sputum, and ECS/HVS. *K. pneumoniae* was predominantly isolated from urine samples 26(50%) followed by wound swab 14(26.9%), then sputum and ECS/HVS 7(13.5%) and 5(9.6%) respectively. This result conforms to the research done by Jombo *et al.*, (2011) who reported urine specimen to have the highest number of *Klebsiella* isolates followed by wound swab or pus, sputum and then ECS/HVS. Iroha *et al.*, (2011), reported that out of 390 samples collected, *K. pneumoniae* was isolated from 150 samples (urine 72, HVS 12, wound swab 16, and sputum 50). In their research, urine specimen constituted the largest number of *K. pneumoniae* isolates. Sarathbabu *et al.*, (2012), also isolated *K. pneumoniae* predominantly from urine samples in hospitalised patients in India. *K. pneumoniae* is an opportunistic bacterium which affects the elderly and the immunocompromised. Therefore, the isolation of *K. pneumoniae* more frequently from urine specimen could be due to the presence of indwelling devices such as catheter in patients associated with the diseases. Presence of these devices in the body leads to the adherence of Gram-negative organisms such as *Klebsiella* on the surfaces of these devices which eventually promotes the establishment and development of the disease. Unlike the mucous membrane which serves as the first line of defence against bacteria and other pathogens, these invasive devices do not have such protective properties rather, it predisposes one to infections.

Antimicrobial susceptibility testing revealed that all *K. pneumoniae* isolates were susceptible to meropenem and Imipenem (100% sensitivity each). Fifty (94.2%) *K. pneumoniae* isolates showed susceptibility to ertapenem. High antimicrobial drug susceptibility pattern was also observed among other antimicrobial drugs used; Cefepime 51(98.1%), Piperacillin/tazobactam 46(88.5%), Aztreonam 39(75%), Gentamycin 43(82.75), Ceftazidime 49(94.2%), Cefotaxime 50(96.2%), and Ciprofloxacin 45(86.2%). This result is similar to that reported by Iroha *et al.*, (2011), who in their

research reported resistance to the commonly used antimicrobial agents such as chloramphenicol and ampicillin while carbapenems, aminoglycosides, cephalosporins, fluoroquinolones and monobactam showed greater sensitivity. This result is an indication that the antimicrobial drugs used in this study have not been grossly misused in this environment.

Varying degree of resistance (1-3 of the antimicrobial drugs used) was observed among the *K. pneumoniae* clinical isolates. Aztreonam showed highest resistance (19.2%) while lowest antimicrobial drug resistance was observed in cefepime (1.9%) and ertapenem (1.9%). It is worthy of note here that ertapenem non-susceptibility is not specific for carbapenemase production especially in areas where carbapenemase production is uncommon (Woodford *et al.*, 2007). Among the carbapenems, *K. pneumoniae* showed no resistance to meropenem and imipenem. However, (1.9%) resistance and moderate susceptibility (1.9%) was observed in ertapenem. Meropenem and ertapenem is a more sensitive indicator than imipenem for the detection of KPC (CLSI) using disc diffusion.

Results from this research showed that *K. pneumoniae* infection was observed more in female patients (57.7%) than in their male counterpart (42.3%). The age distribution amongst the male patients showed that the age interval of 40-49 occurred more frequently (27.3%) and therefore, is the modal class while the mean was calculated to be 39.95. Amongst the female patients, the modal class was observed to be 50-59 while the calculated mean was 45.83. Age distribution pattern of subjects infected with *K. pneumoniae* ranged from 7months to 87years. There was no statistical significant difference between the distribution of *K. pneumoniae* isolates amongst male and female patients.

The outcome of the single PCR amplification showed that *Klebsiella pneumoniae* carbapenemase was not detected among *Klebsiella pneumoniae* isolates in NAUTH. Comparing the Modified Hodge Test (MHT) result and that of PCR, one can observe that both results showed 100% sensitivity for the detection of KPC activity. This finding is consistent with those of Lijun *et al.*, (2012). Here Lijun and associates investigated the presence of *K. pneumoniae* carbapenemase genes from 159 clinical Gram-negative isolates resistant to several classes of β -lactam antibiotics. The sensitivity and specificity of MHT by this result have shown to exceed 90%; however, several reports have noted the occurrence of false positive results when the MHT was used to detect carbapenemase in ESBL-producing isolates (Carvalhoes *et al.*, 2010; Wang *et al.*, 2011). The absence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) in NAUTH Medical Microbiology and Parasitology Laboratory as of the time of this research could be as a result of the exorbitant prices of carbapenems which makes them not to be readily availability, and therefore not easily misused.

CONCLUSION

Based on the outcome of this study, *Klebsiella pneumoniae* demonstrated a high resistance to Aztreonam and Gentamycin compared to other antimicrobial drugs used. Therefore, care must be taken in the prescription and administration of these antimicrobial agents. Strict infection control policies (such as hand washing hygiene, use of gowns, contact precautions etc) should be put in place to control the growing incidence of antibiotics resistance. Antimicrobial drugs should be bought only from certified local manufacturers and importers. This will help to reduce the high level of counterfeit drugs in the country. Proper education of the public through jingles and adverts should also be considered to discourage self-medication. With the outcome of the antimicrobial susceptibility testing (AST), one can conclude that if these antimicrobial agents are used judiciously, it will go a long way in providing the desired treatment efficiencies and at the same time minimising resistance.

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