Detection Of Kpc And Oxa-48 Gene In Clinical Isolates Of Carbapenem Resistant *Klebsiella Pneumoniae*

Bhanu Shrestha (bhanu_totti7@yahoo.com)
Kookmin University
Anil Kumar Sah
Annapurna Research Center
Laxmi Sen Thakuri
Annapurna Research Center
Roshani Nhuchhen Pradhan
St, Xavier's College
Dharmendra Kumar Nath
St. Xavier,s College
Sameekshya Mainali
NIST
Sagar Aryal
St. Xavier's College

Research note

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Abstract

Objectives: The emergence of carbapenem resistant Klebsiella pneumoniae has been problematic in health sectors. It has been increasing widely in both hospital and community settings. The resistance acquired due to presence of Klebsiella pneumoniae carbapenemase (KPC) and Oxacillinase-48 carbapenemase (OXA-48) is of great concern. This cross-sectional study was designed to detect the carbapenem resistant gene namely blaKPC and blaOXA-48 in K. pneumoniae obtained from different clinical specimens.

Results: Among the clinical isolates tested, culture positivity was 51.31% with K. pneumoniae as predominant isolates with 28.20%. AST revealed 38 (69.09%) carbapenemase producing K. pneumoniae while MHT confirmed 31 (56.36%) isolates as carbapenemase producer phenotypically. Similarly, highest percentage of carbapenemase production was seen in sputum (45.16%), in male (67.74%), in age group 51-60 (25.80%) and in OPD (45.16%). PCR screening was done for 31 carbapenemase producing isolates to detect blaKPC and blaOXA-48 genes. Two (6.45%) isolates were positive for blaKPC gene and 9 (29.03%) for blaOXA-48 genes. Significant numbers of blaKPC and blaOXA-48 genes were detected in carbapenem resistant Klebsiella pneumoniae from clinical isolates.Keywords: Klebsiella pneumoniae, blaKPC, blaOXA-48, Carbapenem

Introduction

Antibiotic resistance is major problem in the health sector which is increasing day by day and leading global health crisis. Antibiotic resistance leads to economic burden, increased mortality rate, long stay on hospital and higher medical costs (WHO 2018). The rapid emergence of resistant bacteria is frequently evolving leading to danger of efficacy of antibiotics (Ventola 2015). Different corresponding genes responsible for resistance mechanism are consistently being identified accordingly with identification of various resistance mechanisms (Blair et al 2014). More strains of pathogen have become antibiotic resistant, and chemotherapeutic agents are being ineffective against those antibiotic resistant bacteria. Resistant bacteria are emerging globally as a threat in treating common infections caused by them in community and hospital settings (Chaudhary and Aggarwal 2004).

Carbapenems are potent β- lactam antibiotics that are used to treat serious infection in hospital settings. Carbapenems includes imipenem, meropenem, ertapenem, cephalosporins, and the broad-spectrum of penicillin. Carbapenemase producers are mostly identified among Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Acinetobacter baumannii (Bialvaei et al 2015). Among the current threat related to carbapenemase spread, KPC and OXA–48 are relevant because misuse, overuse and abuse of carbapenems can increase in the KPC and OXA–48—producing strain (Bina et al 2015). The study of carbapenem resistant Gram negative bacteria have been major concern because it is associated with the multi-drug resistant bacteria for which limited antibiotics are only available (Poirel et al 2007). Thus because of this reason early detection of carbapanemase producing isolates have become important task

In Nepal, various infections are major public health problem. Antibiotics may be prescribed by physician or other healthcare worker inappropriately and many patient self-treat with antibiotics, including prior to hospital admission, which can contribute to increased resistance rate. The irrational uses of the antibiotics have driven the evolution of antibiotic resistance, leading to increased morbidity and mortality along with increased health care cost and longer period of hospitalization (Fair et al 2014). There are several studies on phenotypic characterization however genotypic studies are limited in case of Nepal. So there is rapid need to study the occurrence of major types of genes causing wide spread of carbapenemase that explain the epidemiological features of carbapenemase producing microorganism. This study would further help in detecting the bla_{KPC} and bla_{OXA-48} genes in clinical settings of Nepal and add additional information to existing information.

Materials And Methods

The study was carried out from May 2018 to October 2018 at Annapurna Neurological Institute and Allied Science Hospital (ANIASH) and Annapurna Research Center, Maitighar, Kathmandu, Nepal. A total of 380 samples were processed out.

Sample type and collection methods:

Different samples including urine, blood, sputum, catheter tip, csf, tracheal aspirates, CVP tips were collected from an outpatient department (OPD), different wards and intensive care unit (ICU) of ANIASH. Samples were collected in the sterile container. All the samples were analyzed in microbiology laboratory of the hospital and PCR along with gel electrophoresis were performed in the ANIAS Research laboratory, Kathmandu, Nepal.

Culture and identification:

All the samples were cultured on the MacConkey agar and Blood agar, in addition to these urine sample were also cultured on the CUTI agar. Gram staining was performed to separate the Gram negative bacteria from the Gram positive bacteria. All these methods were applied as well to identify *K. pneumoniae* along with the biochemical test. All isolates confirmed to *be K. pneumoniae,* were used for further analysis.

Antibiotic susceptibility testing and Modified Hodge Test:

Kirby Bauer disc diffusion method was employed to check the sensitivity and resistivity of isolates against antibiotic. Results were interpreted according to Clinical and Laboratory Standard Institutes guidelines. Carbapenem class of antibiotics (Ertapenem, Meropenem, Imipenem) were used to check the Carbapenemase resistance.

Modified Hodge Test (MHT) is a simple phenotypic test for detection of presence of carbapenemase enzyme in bacteria. *E. Coli* ATCC 25922 was along with resistant isolates and 10 µg meropenem in the center of the test area. *K. pneumoniae* was streaked from the edge of the disk to the edge of the plate during the process. After ambient incubation the plate for a clover leaf-type indentation at the intersection of the *K. pneumoniae* and the *E. coli* 25922, within the zone of inhibition of the carbapenem susceptibility disc were examined.

Plasmid DNA extractions:

Carbapenemase resistant *Klebsiella pneumoniae* was preserved on Tryptic soya broth for DNA extraction process. DNA was extracted by Alkaline hydrolysis methodin which *K. pneumoniae* strains was cultured in LB (Luria Bertani) broth at 37°C overnight.. Finally the plasmid preparation was stored at 4°C or –20°C.

Amplification of the carbapenem resistance gene (bla_{OXA-48} and bla_{KPC}) by PCR:

For the process positive control, negative control, blank and sample were prepared. For the sample: 8 µl PCR water, 13 µl Master mix, 0.5 µl Forward primer, 0.5 µl Reverse primer and 3 µl of Plasmid DNA were mixed. For bla_{OXA-48}: Initial denaturation: 94°C for 10 minute; Denaturation: 94°C for 40 second; Annealing: 62°C for 40 second; Extension: 72°C for 1 minute; Final extension: 72°C for 7 minute conditions were used. For bla_{KPC}: Initial denaturation: 95°C for 5 minute; Denaturation: 95°C for 45 second; Annealing: 52°C for 45 second; Extension: 72°C for 1 minute; Final extension: 72°C for 8 minute conditions were used. OXA- 48 F was 5'GCTTGATCGCCCTCGATT3' and OXA–48 R was 5'GATTTGCTCCGTGGCCGAAA3' with the amplicon of 281bp. Similarly, KPC F was 5'ACGACGGCATAGTCATTTGC3' and KPC R was 5'CATTCAAGGGCTTTCTTGCTGC' with amplicon of 538bp.

Detection of PCR products by gel electrophoresis:

The amplified products were characterized by performing gel electrophoresis with 1.5 % agarose gel made in w/v tris-acetate-EDTA. 0.3 µl of Ethidium bromide was used in the gel as tracking dye. The gel was allowed to solidify on the plastic cast with comb. After proper solidification 1 µl of 100bp DNA ladder was loaded, 3 µl positive controls and 3 µl negative controls was loaded on the well. 3 µl of PCR amplicons were respectively loaded on the wells.

Results

During the study period, 380 non-repetitive samples were processed and 195 (51.31%) samples showed growth while 185 (48.68%) showed negative culture results. Out of 195 organisms isolated from various

specimens, *Klebsiella pneumoniae* were predominant isolates with number of 55 (28.20%). Among the 55 *K. pneumoniae* isolated, the highest percentage of isolates was obtained from sputum (40%), in the OPD (50.9%), among male patient 31 (56.4%) and from the age group 51–60. Among the 10 different antibiotics used against *K. pneumoniae* isolates, Colistin was found to be 100% sensitive to all the isolates. Ertapenem showed highest number of resistant *K. pneumoniae* isolates with the number of 40 (72.73%), followed by the 38 (69.09%) of Meropenem, and so on. All the meropenem resistant isolates were screened as carbapenem resistant. Those meropenem resistant *K. pneumoniae* were phenotypically confirmed to produce carbapenemase by Modified Hodge Test (MHT). Out of 31 carbapenemase positive *Klebsiella pneumoniae* isolates, highest percentage was observed in sputum (45.16%), in male (67.74%), in age group 51–60 (25.80%) and in OPD (45.16%).Molecular screening was done by PCR using gene specific primers for both the genes. PCR amplification of bla_{KPC} and bla_{OXA-48} genes showed among the 31 carbapenemase positive isolates, 2 (6.45%) and 9 (29.03%) isolates were found to be positive for bla_{KPC} and bla_{OXA-48} genes respectively.

Figure 1: Comparative study of Carbapenem resistant *K. pneumoniae* and total *K. pneumoniae* isolates in different specimens, hospital wards, in different gender, in different age group

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Photographs 1: Gel electrophoresis of PCR amplicons of (A) bla_{OXA-48} gene (B) bla_{KPC} gene

Discussion

Production of carbapenemase by *Klebsiella pneumoniae* leads to the resistant against carbapenem. These have led to the great problem in the global health sectors. The variation among the distribution of *K. pneumoniae* in the study in compare to others is due to different geographical locations and differing in the number of specimen used. In contrast to our study, *Klebsiella pneumoniae* isolate showed higher number of isolates in urine in the study of Fortunata *et al* (2015). Similarly, the prevalence of isolates in case of sputum was slightly in accordance with the study by Ravichitra *et al* (2014) and Maniklandar *et al* (2013). Chaudhary *et al (*2014) which showed the incidence of 77.1% in male and 22.90% in female had opposite result to ours. Contradictory result was seen in case of carbapenemase producing isolates distribution among the male and female population in our study with the result of Henkhoneng *et al (*2014). Highest *K. pneumoniae* were seen in age group 51–60 which was also contradictory to study by Rai *et al* (2015) which showed highest in age group 21–30. Similarly, carbapenemase production was also high in age group 51–60.

Further the highest resistance was shown by ertapenem with 72.73% followed by meropenem, gentamicin and imipenem with 69.04%, 67.25% and 65.49% respectively. These resistance patterns indicate presence

of analogously significant percentage of carbapenem resistant in clinical isolates. As very limited study have been done in Nepal for detection of carbapenemase producer exact clarification on increasing or decreasing trend of carbapenemase producer in Nepal cannot be evaluated.

KPC have caused multiple epidemics and even more troubling are capable of propagation to other species. Carbapenem resistance are a serious problem since carbapenems were highly resistant to most other β-lactamase prior to the advent of KPC and were often used as drugs of last resort for serious Gram negative infections like *K. pneumoniae.* Among the *K. pneumoniae* isolated, 56.36% were found to be carbapenemase producer and this phenotypic confirmation was analyzed using Modified Hodge Test (MHT). Higher prevalence of KPC producing isolates of *Klebsiella* was reported by Gupta *et al* (2011) by MHT. Phenotypically sensitive isolates with meropenem were not taken for molecular analysis but there might be chance of expression of these genes in phenotypically sensitive isolates also whereas it might not be expressed in phenotypically resistant isolates also in some cases.

In the study, all the 31 phenotypically confirmed carbapenemase producing *K. pneumoniae* isolates were subjected to molecular characterization for KPC and OXA–48. The organisms were tested for the presence of bla_{KPC} and bla_{oxa-48} genes. In comparison to our finding, the study by Bina *et al* (2015) showed 80.5% isolates were positive by MHT, but all of them (100%) were negative for amplification of the bla_{KPC} gene in the PCR method. Molecular approach detects the carbapenemase gene with high sensitivity. Conversely, there is no specific phenotypic method in laboratory that detects the KPC and OXA–48 genes. In this respect, the presence of bla_{KPC} and bla_{oxa-48} genes must be confirmed by molecular method. Although MHT was thought to confirm the presence of KPC however it is now considered to be involvement of some KPCs that may or may not indicate a resistant phenotype. Thus, it is highly significant to note the false positive result during phenotypic confirmation. Further negative results from molecular analysis don't mean those isolates don't have the genes of our choice, it might have presence of those genes but unable of expression.

According to the analyses, antibiotic resistance is growing and medical societies are fast running out of treatment options. This is creating large problem in the pharmacopoeia. Early detection of these types of resistance genes such as KPC and OXA–48 would be useful tool for the identification of infection thereby helping in controlling and prevention of their spread. Thus, it is vital for every laboratories to become alert about such infection which may be threat leading to epidemic condition if not treated and controlled at the time. Therefore, phenotypic and genotypic identification technique should be performed for the specific diagnosis and research purpose.

Conclusions

Comparatively significant numbers of bla_{KPC} and bla_{OXA-48} genes were found in carbapenem resistant isolates. Therefore, early detection of these types of genes possessing *Klebsiella pneumoniae* isolates will be useful concept for choosing the most appropriate antibiotics and also for controlling transmission of such microorganism with resistance genes. Further results of this study would be very useful in

generating appropriate carbapenem resistant control policy and establishing the rapid molecular diagnostic method in Nepal.

Limitations

Due to the limitation of time and resources this study was subjected to carry out in single hospital with higher flow of patient.

Abbreviations

ICU: Intensive care unit, OPD: Outpatient department, CUTI: Chromogenic urinary tract infection agar.

Declarations

Competing interest:

We declare no any conflict of interest from our side.

Ethics approval and consent to participate:

Ethical approval was obtained from Nepal Health Research Council (NHRC), Nepal with the Reg no. 315/2018 and Ref no. 3049. Before sample collection written informed consent was taken from patient or their guardians.

Consent for publication:

Not applicable

Availability of data and materials:

The datasets generated and/or analyzed during the current study are not publicly available because it is personal research working carried out for the completion of dissertation work for partial fulfillment of Master degree in Science (Medical Microbiology) but are available from the corresponding author on reasonable request.

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Contribution of authors:

This study have been supported by various individuals but authors have the special role on the completion of the study, *BS* was responsible for composing the manuscript and designing the overall procedure of the study along with conduction of laboratory based activities, *AKS* acted as overall supervisor of the laboratory based activities, *LXT* and *SKT* helped in the molecular screening, *RNP, DKN and SM* were responsible for sample collection, transportation and processing of the sample and *SA* who supervised the overall study was also responsible for the designing the framework of the study and supervision.

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