

Original Research Article

<http://dx.doi.org/10.20546/ijcmas.2016.503.024>

Multiplex Polymerase Chain Reaction for *Klebsiella pneumoniae* Metallo- β -lactamase Causing Neonatal Sepsis in Mansoura Children University Hospital in Egypt

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ABSTRACT

The present study was conducted to detect the presence of MBLs among the isolates of *Klebsiella pneumoniae* by phenotypical methods and genotypic multiplex polymerase chain reaction (PCR) method among neonates with sepsis. The study included neonates who were admitted to the neonatal intensive care unit (NICU) and had suspected sepsis after hospital admission. A full laboratory sepsis screen for each neonate was performed and included cerebrospinal fluid analysis, blood cultures using BACT/Alert blood culture system and urine culture. Aerobic bacteria were identified using Microscan automated microbiological system. Bacterial isolates defined as *Klebsiella pneumoniae* were further subjected to microbiological laboratory studies including antibiotics susceptibility tests by disc diffusion method, determination of MBLs production by double discs and combined disc methods and determination of genotypes of MBLs-*Klebsiella pneumoniae* by multiplex PCR for VIM, IMP and NDM genes. MBLs-*Klebsiella pneumoniae* was detected among 48.1% by double discs, combined disc and genotypes respectively. The most common identified gene responsible for MBLs-*Klebsiella pneumoniae* was VIM (69.2%) followed by IMP and NDM (23.1% for each) and mixed genotypes were identified in 23.1%. We can conclude from this study that carbapenemase production is a common finding among the isolates of *Klebsiella pneumoniae* among neonates with sepsis. Both double discs and combined discs methods are accurate as screening tool for detection of resistance to carbapenem drugs. Genotypes responsible for MBLs determined VIM and IMP genes as the main prevalent genes beside NDM. Extensive studies should be carried out to determine the prevalence of MBLs- *Klebsiella pneumoniae* other geographic locations in Egypt to assess the magnitude of the problem. Antibiotics tigecycline and colistin are suitable for treatment under strict laboratory supervision and strict infection control practices and antibiotic policies should be strengthened to avoid the blowout of these microbial bugs in our hospitals.

Keywords

Klebsiella pneumoniae,
IMP,
VIM,
NDM

Article Info

Accepted:
15 February 2016
Available Online:
10, March 2016

Introduction

Neonatal sepsis is a significant health problem associated with recognizable high risks of morbidity and mortality. Several factors attribute to such grave condition among with but not limited to prematurity, low birth weight and prolonged hospital stay. The expanded use of antimicrobial therapy has led to colonization and subsequent infections by virulent bacterial pathogens (1). Various bacterial pathogens are associated with this infection either Gram positive cocci such as coagulase negative *Staphylococcus* and group B *Streptococcus* and Gram negative bacilli like *Escherichia coli* and *Klebsiella* species(2).

The frequency of isolated bacterial pathogens differs according to the geographic localities and according to impact of antibiotics prescription selecting resistant pathogens. In developing countries, like Egypt, multiple drug resistant organisms are reported to increase due to many factors. *Klebsiella pneumoniae* (*K. pneumoniae*) is associated with these infections in Egypt as previously reported (3, 4).

Klebsiella pneumoniae isolates are shown to have multiple mechanisms mediating antibiotic resistance. Isolates have been reported to express extended spectrum beta-lactamases (ESBLs), AmpC beta-lactamases, 16S rRNA methylases, aminoglycoside modifying enzymes and metallo-beta-lactamase (MBL) (5).

MBL producing bacteria hydrolyze wide group beta-lactam antibiotics including cephalosporins, penicillins, carbapenems, cephamycins, yet it remains susceptible to aztreonam. The hydrolysis capacity of MBL are not suppressed by the presence of other β - lactamase inhibitors such as clavulanate and sulbactam (6). These enzymes are

classified as class B beta-lactamases depending on the chemical structure and

amino acid sequence homology and to group 3 according to the Bush classification according to their substrate and inhibitors used for their detection (7,8). It is known that the action of MBL require the presence of zinc ions for the hydrolysis of beta-lactam antibiotics thus its action is inhibited in presence of metal-chelating substances like ethylenediaminetetra acetic acid (EDTA) (8). The genes coding for MBL are part of the chromosome and can be transferred by horizontal gene transfer from resistant species to susceptible species (6). MBLs were reported firstly to be common among *Pseudomonas aeruginosa* and *Acinetobacter spp.*, later on, it has emerged to members of *Enterobacteriaceae* (9).

MBLs production by clinical isolates from *Klebsiella pneumoniae* represents therapeutic challenges. Infections with MBLs *Klebsiella pneumoniae* strains need to be treated with drugs such as tigecycline or colistin, which clinicians are becoming increasingly dependent on for treatment of such infections (10)

Thus laboratory detection of MBLs *Klebsiella pneumoniae* among clinical isolates has a major influence on therapeutic trends in clinical situations. There are many phenotypic methods proposed by CLSI for detection of MBLs (11,12). Genotypic methods are available for detection of genes coding MBL. The common reported MBLs genes are the VIM and IMP types, with recently recognized NDM-1, gene started to spread (13).

Studies about the incidence of MBLs *Klebsiella pneumoniae* among clinical isolates from Egypt especially in neonates are scarce (14).

The present study was conducted to detect the presence of MBLs among the isolates of *Klebsiella pneumoniae* by phenotypical methods and genotypic multiplex polymerase chain reaction (PCR) method among neonates with sepsis.

Materials and Methods

The study was carried out in Mansoura University Children Hospital between March 2014 till November 2015. The study included 100 neonates who were admitted to the neonatal intensive care unit (NICU) and had suspected sepsis after hospital admission. Sepsis was defined according to Egyptian Neonatal Network (EGNN) by finding at least 3 criteria (15) i-the presence of risk factors for development of sepsis like prematurity, (ii) the presence of two or more clinical signs of sepsis (poor reflexes, lethargy, respiratory distress, bradycardia, apnea, convulsions, abdominal distension, and bleeding),(iii)abnormal hemogram and positive CRP and positive culture.

A full laboratory sepsis screen for each neonate was performed and included cerebrospinal fluid analysis blood cultures using BACT/Alert blood culture system and urine culture. Aerobic bacteria were identified using Microscan automated microbiological system (Beckman coulter). Hematologic parameters, including a complete blood picture and CRP were measured at the time of sepsis evaluation. Parenteral antibiotics were started immediately after the samples for the infection screen had been obtained.

Bacterial isolates defined as *Klebsiella pneumoniae* were further subjected to microbiological laboratory studies including antibiotic susceptibility tests by disc diffusion method, determination of MBLs production by double discs and combined

discs methods and determination of genotypes of MBLs-*Klebsiella pneumoniae* by multiplex PCR.

Antibiotic Susceptibility by Disc Diffusion Method

Antimicrobial susceptibility testing for isolated *Klebsiella pneumoniae* was performed by the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Oxoid) according to the recommendations of the CLSI (2010) (11). The antibiotics used were: ampicillin (10 µg), cephalexin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefepime(30 µg), aztreonam (30 µg), piperacillin/tazobactam (100/10 µg), imipenem (10 µg), meropenem (10 µg), amikacin (30 µg), tigecycline (15 µg) and colistin (10 µg).

Klebsiella pneumoniae isolate with a reduced susceptibility to meropenem or imipenem (inhibition zone diameter of ≤ 21 mm) was screened for the production of carbapenemase according to the standard guidelines (11) by both combined discs and by double discs methods.

Simply, *Klebsiella pneumoniae* suspension equal to a 0.5 McFarland standard suspension of was prepared to be used as a culture inoculum for combined discs tests and double discs test.

Detection of Metallo-beta-lactamase Production by Combined Discs Test

Freshly prepared Muller Hinton agar was inoculated with 0.5 McFarland suspension of *Klebsiella pneumoniae* and two imipenem discs were used with concentration 10 µg, one containing 10 µl of 0.1 M (292 µg) anhydrous EDTA (Sigma Chemicals, St. Louis, MO) and other with imipenem only. Plates were incubated at 37°C for 24 hours.

After incubation, the diameter of inhibition zones was measured. An increase in zone diameter of >4mm around the imipenem-EDTA disc compared to that of the plain imipenem disc alone was considered positive for MBL production (Franklin et al. (16).

Double Discs for MBLs-*Klebsiella pneumoniae*

Disks containing 10µl of the EDTA solution with concentration of 0.5M were prepared using sterile EDTA solution added to sterile blank 6mm disk prepared from Whatman filter. Freshly prepared Muller Hinton agar was inoculated with 0.5 McFarland suspension of *Klebsiella pneumoniae* and 10 µg meropenem disk was placed at the center of the plate and the 10µl of the EDTA disk was placed at a distance of 10mm center to center, and the plate was incubated at 37°C overnight. The zone around the meropenem disk extended on the side nearest the EDTA >7mm indicated organism was a MBL producer.

Molecular Detection of MBLs-Genes by Multiplex PCR

Klebsiella pneumoniae isolates identified by screening methods to be MBLs producers were subjected to genotypes studies.

Bacterial DNA Extraction

Isolation of Bacterial DNA was obtained from colonies grown overnight on blood agar using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Extracted DNA was kept frozen at -70°C until amplification.

Multiplex PCR

Amplification procedures were performed using specific primers for three genes

namely VIM, IMP and NDM according to primers sequences summarized in table 1. Total DNA from isolates was subjected to multiplex PCR using a PCR kit (Qiagen) according to the manufacturer's instructions. Briefly, 200pg of DNA was added to 20 microns of amplification mixture and the amplification was performed by ABI 9700 thermocycler (Applied Biosystems, Carlsbad, CA, USA) (19). The amplified products were separated in 1.5% agarose gel ethidium bromide. The gel was run at 70 V for 1 hour. The gel images were taken under ultraviolet light and 100 bp ladder molecular weight markers was used to measure the molecular weights of amplified products.

Statistical Analysis

All the statistical analyses were performed using SPSS16. Summary of measures was reported as mean ± standard deviation (SD) for quantitative variables and percentages for categorical variables. The differences in distribution were evaluated using the chi-square test for categorical variables. Value ≤ 0.05 was considered statistically significant.

Results and Discussion

Demographic data and culture results for the studied neonates are summarized in table 2. The studied neonates with suspected sepsis were mainly preterm 60% with male 45% and females 55%. Previous antibiotics therapy was prescribed for 20% of those patients. Survival rate was 85%. Blood culture was positive in 55% of cases.

The isolated bacterial pathogens were *Staphylococcus* species 28 (50.9%) and *Klebsiella pneumoniae* 27(49.1%), figure 1.

MBLs-*Klebsiella pneumoniae* was detected among 13 (48.1%) by double discs, combined discs and genotypes respectively, table 3.

The most common identified gene responsible for MBLs-*Klebsiella pneumoniae* was VIM (69.2%) followed by IMP and NDM (23.1% for each) and mixed genotypes were identified in 23.1%, table 4.

In comparison for susceptibility for antibiotics between MBLs and non MBLs *Klebsiella pneumoniae* there was significantly reduced susceptibility among MBLs-*Klebsiella pneumoniae* compared to non MBLs- *Klebsiella pneumoniae* to ampicillin, cefotaxime, cefipime, ceftazidime, ceftriaxone, meropenem, imipenem, piperacillin/ tazobactam (P=0.003, P=0.07, P=0.02, P=0.01, P=0.01, P=0.001, P=0.001, P=0.01), while susceptibility to amikacin, tigecycline, aztreonam, and colistin had no statistically significant difference, table 5.

Sepsis in neonates is one of common health problem associated with morbidity and mortality worldwide. The problem is claimed to be responsible for around of 30-50% of total neonate's deaths in developing countries (20, 21). In the present study the mortality rate among the studied neonates was 15% the majority were preterm. Prematurity is a major implicated risk factor for developing neonatal sepsis as it leads to many interventional invasive procedures like parenteral nutrition, mechanical ventilation and prolonged hospital stay (22, 23).

In the present cohort of neonates, the isolated bacterial pathogens were *Staphylococcus* species and *Klebsiella pneumoniae*. Similar data were reported previously denoting that Gram positive organisms account for about 70% of all late onset sepsis and the most common Gram-negative organism causing neonatal sepsis was *Klebsiella pneumoniae* (24-26). The main source of bacterial pathogens causing neonatal sepsis in NICU is the colonization

of the infant's body sites like skin from the environment leading to late onset sepsis (27).

Among 100 neonates studied for sepsis, 55% were confirmed to have bloodstream infection by using blood culture. This rate is comparable to rates reported in other developing countries where the rates ranged from 37% up to 55.6 % (29-31). In contrast, very low rates (2.27 %) was reported by *li., et al., 2013* (32), which can be explained by the strict adherence to infection control guidelines in health care settings to prevent health care associated infections in NICU in these countries.

The alarming notice in the present study regarding isolated *Klebsiella pneumoniae* was the high percentage (48.1%) of MBLs strains. Resistance to carbapenem has been reported in previous reports from different regions (33-35). In recent study the MBLs *Klebsiella* strains isolated from Italian ICU were found among 59.2% of the isolates (36). In Europe, carbapenem-resistant *K. pneumoniae* is reported to be high and even increasing in some countries (37). Carbapenem-resistant *K. pneumoniae* isolates are frequently found to be carbapenemase-producing, these results indicate the potential needs for active screening of patients at high-risk of acquiring these strains with adequate implementation of infection control guidelines and the stepwise use of antimicrobials (37, 38, 39)

Resistant to carbapenems among *K.pneumoniae* are worrisome because these antibiotics are last line antibiotics for treatment of such bacterial pathogens. Moreover, the presence of carbapenems resistance due to the presence of carbapenemases are usually associated with extensively drug- or pandrug-resistant

leaving few or no effective treatment options (38-41). In Egypt, we could not find data except from limited studies on few isolates confirming the emergence of MBLs- *Klebsiella pneumoniae* (14).

Prompt start of efficient antibiotic therapy is mandatory for early management of neonatal sepsis and must depend upon proper studies of the distribution of pathogens in each hospital and their susceptibility patterns according to laboratory findings. In a setting where MBLs producers are reported, empirical

treatment of sepsis should ideally include drugs that will be effective against these pathogens. All the isolated *K. pneumoniae* with MBLs production in the present study were found to be resistant to both imipenem and meropenem. These isolates also had a high level of resistance to ampicillin, the third generation cephalosporins as well as to the beta-lactam/beta-lactamase inhibitor combination used in this study compared to non MBLs- *Klebsiella pneumoniae*. This is well known finding reported previously (42).

Table.1 Primers Sequences, Used for Genes Amplifications

Gene	Sequence	bp	Ref.
VIM	F-5/ GATGGTGTGGTTCGCATA-/3 R-5/ CGAATGCGCAGCACCAG-/3	390	(17)
IMP	F-5/ GGAATAGAGTGGCTTAAAYTCTC-/3 R-5/ CCAAACYACTASGTTATCT-/3	232	(18)
NDM	F-5/ CACCTCATGTTTGAATTCGCC-/3 R- R-5/ CTCTGTCACATCGAAATCGC-/3	984	(19)

Table.2 Demographic Data and Culture Results of the Studied Neonates

Parameter	No(100)%
Sex	
Male	45 (45%)
Females	55 (55%)
Full term	40 (40%)
Preterm	60 (60%)
Survival	
No	15 (15%)
Yes	85 (85%)
Previous antibiotics therapy	
Yes	20 (20%)
No	80 (80%)
Culture	
No growth	45 (45%)
Positive culture	55(55%)

Table.3 Metallobetalactamase Determination by Phenotypic and Genotypic Methods

	No. %
MBLs- <i>Klebsiella pneumoniae</i> by combined disc	13(48.1%)
MBLs – <i>Klebsiella pneumoniae</i> by genotypes	13(48.1%)

Table.4 Genetic Distributions among MBLs-*Klebsiella pneumoniae*

Genotypes	No. %
VIM	9(69.2%)
IMP	3 (23.1%)
NDM	3 (23.1%)
Mixed	3 (23.1%)

Table.5 Comparison between Antibiotics Susceptibility between MBLs- and Non MBLs –*Klebsiella pneumoniae* Species

Antibiotics	MBLs- <i>Klebsiella pneumoniae</i> (n=13)	Non MBLs- <i>Klebsiella pneumoniae</i> (n=14)	P value
amikcin	5 (38.5%)	10 (71.4%)	0.4
ampicillin	1 (7.6%)	11(78.6%)	0.003
azteronam	8 (61.5%)	14(100%)	0.4
cephalexin	3(23.1%)	11(78.6%)	0.02
cefipime	2 (15.4%)	10(71.4%)	0.02
tigecycline	13 (100%)	14(100%)	0.9
colistin	12 (92.3%)	14 (100%)	0.6
cefotaxime	3(23.1%)	12(85.7%)	0.07
ceftazidime	2(15.4%)	14(100%)	0.01
ceftriaxone	3(23.1%)	12(85.7%)	0.01
meropenem	0(0%)	14(100%)	0.001
imipenem	0(0%)	14(100%)	0.001
piperacillin/tazobactam	2(15.4%)	14(100%)	0.01

P value < 0.01 is significant

Figure.1 Isolated Bacteria from Positive Blood Culture

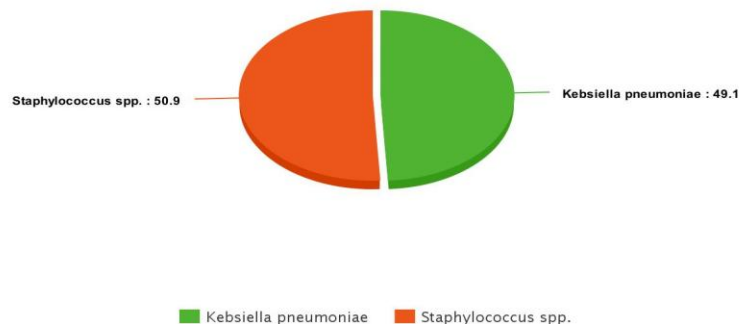
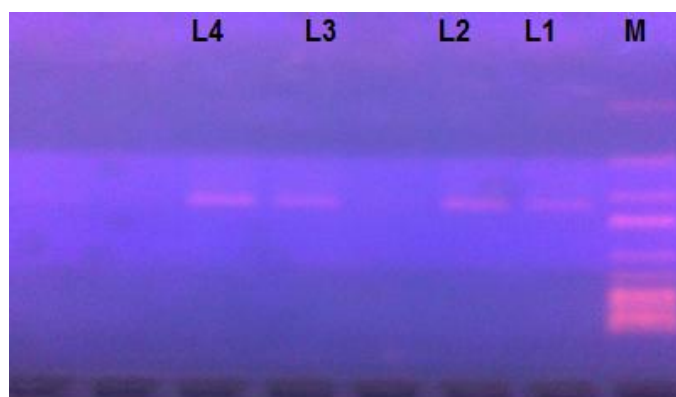


Figure.2 Marker and Positive *Klebsiella pneumoniae* for IMP Genes



M: Molecular marker
L1,L2,L3,L4 positive species for IMP gene

All of the isolated MBLs *Klebsiella pneumoniae* strains were susceptible to tigecycline and 92.3% of isolates were susceptible to colistin. These findings are similar with other reports (43-45).

Laboratory screening methods for carbapenemase production include phenotypic methods like double disc test and combined disc assay beside molecular methods such as PCR amplification, and DNA sequencing (43). In the present study, both the combined disc test and double disc methods detected MBL production positive

results within all resistant strains *Klebsiella pneumoniae* which were resistant to carbapenem antibiotics by disc diffusion test (16, 42). Franklin *et al.* (16) also reported that both methods are accurate screening tools. These results suggest that both tests can be used as a convenient routine method for detection of MBL producing *K. pneumoniae* isolates

Besides disc methods for screening of MBLs production, molecular detection of carbapenemase genes is an interesting rapid alternative, yet, its high cost and

requirement of good experience in molecular laboratory techniques limit its uses. However, its use remains essential for epidemiological studies.

The most common identified gene responsible for MBLs-*Klebsiella pneumoniae* was VIM (69.2%) followed by IMP and NDM (23.1% for each) and mixed genotypes were identified in 23.1%. Many of the carbapenem-resistant Enterobacteriaceae outbreaks identified have been related to the production of carbapenemases, the metallo-beta-lactamases genes VIM and IMP (44).

Similar results were obtained previously from Egypt 2012 on limited number of *Klebsiella pneumoniae* by Abdulall *et al.*, 2014(14) and Abdelaziz *et al.* (2013)(45), demonstrating that IMP and VIM genes were common among MBLs-*Klebsiella pneumoniae* producing strains. However, our report is the first to report the presence of NDM in Egypt.

The present study conclude from this study that carbanamenase production is a common finding among the isolates of *Klebsiella pneumoniae* among neonates with sepsis. Both double discs and combined discs methods are accurate as screening tool for detection of resistance to carbapenam drugs. Genotypes responsible for MBLs determined VIM and IMP genes as the main prevalent genes beside NDM. Extensive studies should be carried out to determine the prevalence of MBL-*Klebsiella pneumoniae* in other geographic locations in Egypt to assess the magnitude of the problem. Antibiotics tigecycline and colistin are suitable for treatment under strict laboratory supervision and strict infection control practices and antibiotic policies should be strengthened to avoid the blowout of these microbial bums in our hospitals.

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How to cite this article:

Maggie Mesbah, R., Maysaa El Sayed Zaki and Mona Abdel Latif Alsayed. 2016. Multiplex Polymerase Chain Reaction for *Klebsiella pneumoniae* Metallo- β -lactamase Causing Neonatal Sepsis in Mansoura Children University Hospital in Egypt. *Int.J.Curr.Microbiol.App.Sci*. 5(3): 194-205. doi: <http://dx.doi.org/10.20546/ijcmas.2016.503.024>