Molecular characterization of bla_{KPC}, bla_{NDM1}, and *acrA* Genes in *Klebsiella Pneumoniae*

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ABSTRACT

Klebsiella pneumonia (*K. pneumonia*) harboring bla_{KPC}, bla_{NDM1}, and *acrA* genes are highly resistant and inactivate all β-lactam antibiotics including carbapenems. This study aimed to molecularly characterization of these enzymes in *K. pneumonia* isolates. A total of sixty-seven clinical isolates of multiple drug-resistant (MDR) *K. pneumonia* were collected from different sites of infection, characterized and subjected to antimicrobial susceptibility testing, double-disc diffusion Synergy test (DDST) and Modified-Hodge test (MHT). bla_{KPC}, *acrA* , bla_{NDM1} genes amplified by PCR. *K. pneumonia* isolates carrying these resistance genes were fully identified by 16S rRNA sequencing and the relatedness of isolates was analyzed through their phylogenetic tree. The most active antibiotics against *K. pneumonia* isolates were colistin (100%). Extended-spectrum β-lactamases (ESßLs) and Carbapenem- resistance were detected in 40% and 34.3% of tested isolates respectively. PCR amplifications revealed that 6 isolates harboured bla_{KPC} and 2 isolates harboured bla_{NDM1} genes. The gene of *acrA* gene was detected in 4 isolates. The identification of *K. pneumoniae* isolates was confirmed by 16S rRNA sequencing. We conclude that, the early management of *K. pneumonia* infections needs efficient antibiotic therapy. Colistin is the drug of choice in treatment of these types of infection**.**

Keywords: *Klebsiella pneumonia, Carbapenem, Colistin, bla_{KPC} gene   , bla_{NDM1} gene.*

1. Introduction

The extensive use of antimicrobial agent's results in the appearance of Multiple-drug resistance (MDR) strains of *Klebsiella* in hospitals (Wu et al., 2012 & Babakhani et al., 2015). Gram-negative bacteria have different mechanisms of resistance to antimicrobial agents, including; the production of β- lactamases, restricted outer membrane permeability, active antibiotic efflux or combination of these resistance strategies. The production of β- lactamases is reported to be a major resistance mechanism among *Klebsiella* isolates (Pages et

al., 2009 & Kuo et al., 2007). In the last decade, a notable increase in ESßLs -producing *Klebsiella pneumoniae* isolates was reported from various parts in the world (Messai etal., 2008 & Guillermo et al., 2013). The abuse of carbapenems against ESßLs -producing Gram-negative micro-organisms leads to the emergence of carbapenem-resistant strains (Wang et al., 2011). The production of carbapenemases especially *K. pneumoniae* carbapenemase (KPC) is the most important mechanism of enzymatic resistance in *K. pneumonia* isolates (Lee et al., 2009). Infections with Metallo ß lactamases (MβLs) *K. pneumoniae* strains need to be treated with drugs such as colistin or tigecycline (Yong et al., 2009). New Delhi metallo-beta-lactamase (NDM-1) was imported from India and spread to the United Kingdom in 2010; after that, NDM-1 cases has been identified worldwide (Rolain et al., 2010; Marra, 2011& Kumarasamy et al., 2010). Bacteria harboring blaNDM-1gene are resistant to carbapenems and also to aminoglycosides. In addition, there is a genetic element accompanied blaNDM-1gene, which encodes efflux pump. So, all of the previous reasons make blaNDM-1gene more important in causing infections by MDR , and in some cases Extensive drug resistance (XDR) microorganisms (Magiorakos et al., 2012)**.**

2. Material and methods

2.1. Isolation and identification of clinical isolate

Sixty-seven of MDR *Klebsiella* isolates were collected and isolated from different sites of infection including; blood, sputum, urine and wound discharge, in over a period from August 2012 to August 2015. Clinical specimens were examined and identified by standard microbiological procedures and biochemical reactions (Cheesbrough, 1993& Collee& Miles, 1996). Confirmation of identification was performed by 16s rRNA sequencing of purified PCR product and Phylogenetic tree were achieved.

2.2. Antibiotic susceptibility testing

The antimicrobial susceptibility testing for the isolated *Klebsiella* species was performed using the Kirby–Bauer disc diffusion method on Mueller–Hinton agar according to Clinical and Laboratory Standards Institute guidelines (CLSI). (Bauer etal., 1996 & CLSI., 2012).

The following commercial antimicrobial discs (Oxoid Ltd., Basingstoke, UK) were used Ampicillin (10 μg); amoxacillin–clavulanic acid (20/ 10 μg); aminoglycosides: amikacin (30 μg) and gentamicin (10 μg); carbapenems: imipenem (10 μg); cephalosporins: cefradine (30 μg), cefotaxime (30 μg); and ceftazdime (30 μg); fluoroquinolone: ciprofloxacin (5 μg); and norfloxacin (10 μg); tetracycline (30 μg); Amphenicols: chloramphenicol (30 μg); Nitrofuran: nitrofurantoin (300 μg); Cyclic polypeptide: Colistin Sulphate (10 μg); Macrolide: Erythromycin (15 μg); and Folate-pathway inhibitors: trimethoprim/sulphamethoxazole (1.25/ 23.75 μg). *E. coli* ATCC 25922 strains was used for quality control. Isolates that showed resistance to at least three classes of antibiotics were considered as MDR strains.

2.3 Minimum inhibitory concentrations (MIC) determination

The MICs of imipenem resistant isolates were evaluated by a standard broth microdilution method according to CLSI guideline (CLSI, 2009) toward six antimicrobial agents including; imipenem, ciprofloxacin, cephradine, chloramphenicol, cefotaxime and amikacin (Pure powders Manfacturers: E.I.P.I.CO, CID and Pharco B International, Egypt). Reaszurin dye was used as an indicator of bacterial growth (Elshikh etal., 2016).
ESßLs detection by Double Disc Diffusion Synergy Test (DDST).

All isolates resistance to cefotaxime (30 μg); and or ceftazdime (30 μg); were subjected to the standard DDST (Spanu et al., 2002). Discs containing (30μg) of aztreonam, ceftazidim, cefepime, ceftriaxone and cefotaxime were placed around a disc of AMC (20 µg of amoxicillin plus 10 µg of clavulanate) in 30 mm center to center.

2.4 Screening of carbapenemases producing isolates

Carbapenemase phenotype was detected by a meropenem or imipenem (MIC ≥2 µg/mL) or isolates resistant to imipenem and or meropenem were considered as screening positive. The positive isolates were further confirmed by using modified Hodge test (CLSI, 2009).

2.5 Modified-Hodge test (MHT)

This test is done according to Shivaprasad et al., (2014). First, a 0.5 McFarland dilution of the *E. coli* ATCC 25922 in 5 mL of saline or broth was prepared. Then, a 1:10 dilution was prepared and streaked on a Mueller Hinton agar plate as for the routine disc diffusion procedure. A 10µg imipenem disc was placed in the center of the test area. 3-5 colonies of each tested isolate grown overnight were inoculated in a straight line out from the edge of the disc to the edge of the plate. The plate was incubated overnight at 35 ± 2 ˚C in ambient air for 16-24 hours.

2.6 Imipenem - EDTA combined disc diffusion test (CDDT) for MβLs

Test was carried out as described in *Yong* et al.*,* (2002). Two imipenem (10 µg) discs were placed on the surface of an agar plate and 10 µl of 0.5 M EDTA solutions was added to one of them to obtain a desired concentration of 750 µg. Plates were incubated for 16 to 18 hours at 35˚C. If inhibition zone increased with the imipenem-EDTA disk ≥7 mm than the imipenem disc alone it was considered MβLs positive.

2.7 Genotype detection
2.7.1 Plasmid and genomic DNA extraction

Plasmid extraction was carried out using alkaline lysis method according to the manufacturer's instructions on the plasmid isolation kit (Thermo Scientific, Waltham, MA, USA). Extracted plasmids were separated by electrophoresis, stained with ethidium bromide and visualized under UV illumination. Total DNA extraction was carried out using the Spin column genomic DNA mini-preps kit (Qiagen, Germany).
2.7.2 Molecular analysis of resistance genes

PCR assays were performed by convention methods to detect carbapenemases (blaKPC, and blaNDM-1genes); metallo-beta-lactamase and acrA gene for acrAB efflux system, the presence of `target genes was examined in single PCR using specific oligonucleotides listed in (Table 1) (Dandachi et al., 2016& Pakzad etal., 2013).

Table 1. The primers sequence used for detection of *bla*KPC, *bla*NDM-1 and *bla acr*A Genes.

Primer name	Primer sequence (5' to 3')	Product size(bp)
blaKPC	F: 5´-CGTTGACGCCCAATCC-3´ R: 5´- ACCGCTGGCAGCTGG-3´	390 bp
blaNDM-1	F: 5´-GGTTTGGCGATCTGGTTTTC-3´ R: 5´-CGGAATGGCTCATCACGATC-3´	621 bp
bla acrA	F: 5´-ATGAACAAAAACAGAGG-3´ R: 5´-TTTCAACGGCAGTTTTCG-3´	495 bp

The amplifications of target bla KPC were carried out using 1 µl of plasmid DNA template in 25 µl of final reaction volume containing 12.5 µl of PCR Master Mix 1× (Promega, USA), and 1.0 µmol l−1 of each primer (macrogen, Korea). Target blaKPC genes was amplified using the initial denaturation at 95 ˚C for 2 min, cyclic denaturation at 95 ˚C for 20 s, annealing at 55 ˚C for 30 s, elongation at 72 ˚C for 40 s for 35 cycles and final extension at 72 ˚C for 3 min in a thermocycler (Thermo scientific, USA), The correct size amplified product was detected by agarose gel electrophoresis (1.0-1.5% m/v concentration, 1X TAE buffer at 100 V for 1 hour). Positive and negative controls and a 100 bp DNA ladder (Promega, USA) were included in each batch of reactions (Venkatachalam et al., 2012).

Molecular detection of NDM-1 and acrA genes were amplified in phenotypically carbapenem resistant *Klebsiella* isolates; they prepared by adding 10 µl of Master Mix (buffer, Mg CL2, dNTP's and Taq polymerase), 1 µl of each primer, and finally 3 µl of the free Dnase/Rnase water. Each PCR reaction was performed by using 15 µl of the prepared Master Mix and 5 µl of extracted DNA in a final volume of 20 µl. PCR conditions for NDM-1 include initial denaturation at 94 ˚C for 10 min, cyclic denaturation at 94 ˚C for 30 s., annealing at 58 ˚C for 40 s., elongation at 72 ˚C for 50 s. for 36 cycles and final extension at 72 ˚C for 5 min then cooled at 4 ℃ for ∞ in a thermocycler (Thermo scientific, USA), PCR conditions for acrA genes include initial denaturation at 94 ˚C for 5 min, cyclic denaturation at 94 ˚C for 1 min., annealing at 52 ˚C for 1 min., elongation at 72 ˚C for 1 min.

for 30 cycles and final extension at 72 ˚C for 5 min then cooled at 4 ºC for ∞ in a thermocycler (Thermo scientific, USA) PCR products were detected in 1.5% agarose gel.

2.7.3 Identification using 16S rRNA gene sequence analysis

A PCR gene fragment of 16S rRNA was amplified from the purified genomic DNA of carbapenem resistant *K. pneumonia* isolates using the universal primer set, 8F 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492 5′-GGTTACCTTGTTACGACTT-3′, with thermal cycling as described earlier (Radwan etal., 2010). The BLASTn results of 16S rRNA sequences were retrieved and aligned with the sequences of bacterial isolates using Clustal*W* embedded in MEGA 6 (Molecular Evolutionary Genetics Analysis) software (Tamura etal.,2013). Phylogenetic analysis was conducted based upon 16S rRNA gene data using maximum likelihood (ML) analyses. Alignment gaps were treated as missing data. ML analysis was conducted using a heuristic search with tree bisection-reconnection branch swapping and 100 random addition sequence replicates.

*2.7.4 Purification of PCR product and Nucleotide Sequencing*

A volume of 50 µl of PCR product was purified by the aid of PureLink™ PCR Purification Kit (Thermo Fisher, USA) according to the manufacturer's instructions to be sequenced. DNA sequencing was performed at Central Lab, Faculty of Medicine, and Menoufia University, Egypt.

Results

Antimicrobial susceptibility pattern of 67 *Klebsiella* isolates showed that 23 (34.3%) were imipenem resistant isolates, MHT identified in 15 isolates out of 23 (65.2%) isolates as carbapenemase producers by developing cloverleaf shaped inhibition zone. MBLs activity was detected in 19 out of 23 (82.6%) isolates by CDDT. A total of 59 (88%) isolates out of 67 *Klebsiella* isolates were resistant to cefotaxime and or ceftazidime and were tested by DDST.

Twenty seven isolates (40.3%) showed positive production of ESβLs by DDST. The higher percentage of resistance in the tested isolates was observed against ampicillin (100%) and cephradine (97 %) followed by erythromycin (94%). The isolates showed low percentage of resistance towards colistin (0%), nitrofurantoin and imipenem (%34.3) respectively. Twenty-three *Klebsiella* isolates showed various levels of resistance with different MICs profiles. The MICs for imipenem and ciprofloxacin were 4 to ≥ 128 µg/ mL.

Prevalence of carbapenems resistance-associated genes among 23 clinical *Klebsiella* isolates showed that 6 isolates having blaKPC and 2 isolates were positive for blaNDM-1gene (Fig. 1). While in ciprofloxacin-resistant strains, four isolates were harbored acrA gene and consequently contain acrAB efflux system (Fig. 2).
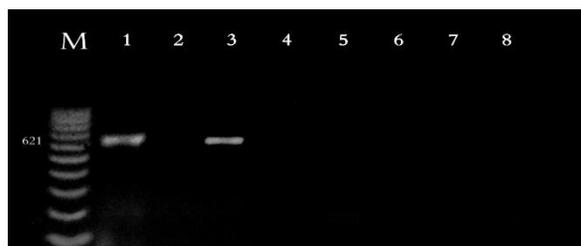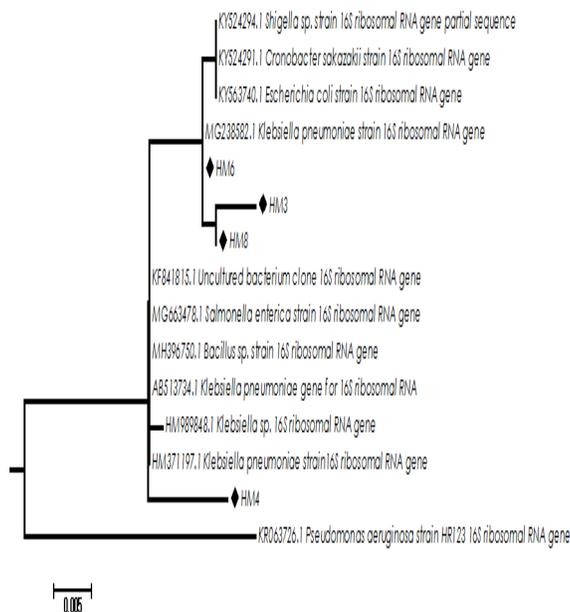

Figure 1. Molecular detection of blaNDM-1 on agarose gel Lane M is a 100-base-pair DNA molecular weight ladder, samples in lanes 1 and 3 are positive for the blaNDM-1 gene.
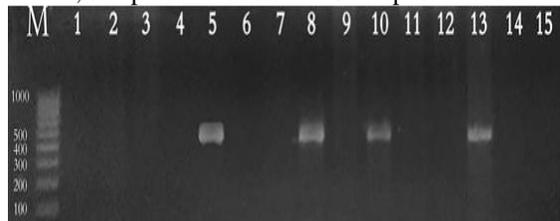
Figure 2. PCR amplification of acrA gene in ciprofloxacin-resistant *Klebsiella* isolates. Lane M: 100 bp DNA size marker; Lane1: negative control. Lane 5, 8, 10, 13 PCR product of *acr*A gene (495 bp).

Phylogenetic tree analysis for 16S rRNA was constructed by NCBI tool which proves the isolates that harbor blaKPC and blaNDM-1 isolates were identified as *K. pneumoniae*, as, HM6 HM3 and HM8 were closely affiliated to *K. pneumoniae (MG238582.1)*. The sequences of HM4 were fully related to *K. pneumoniae (KR063726.1)* as in (Fig. 3).

KY524294.1 Shigella sp. strain 16S ribosomal RNA gene partial sequence
KY524291.1 Cronobacter sakazakii strain 16S ribosomal RNA gene
KY563740.1 Escherichia coli strain 16S ribosomal RNA gene
MG238582.1 Klebsiella pneumoniae strain 16S ribosomal RNA gene
HM6
HM3
HM8
KF841815.1 Uncultured bacterium clone 16S ribosomal RNA gene
MG663478.1 Salmonella enterica strain 16S ribosomal RNA gene
MH396750.1 Bacillus sp. strain 16S ribosomal RNA gene
AB513734.1 Klebsiella pneumoniae gene for 16S ribosomal RNA
HM989848.1 Klebsiella sp. 16S ribosomal RNA gene
HM371197.1 Klebsiella pneumoniae strain 16S ribosomal RNA gene
HM4
KR063726.1 Pseudomonas aeruginosa strain HR123 16S ribosomal RNA gene

0.005

Figure 3. Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (Tamura etal., 2013). The tree with the highest log likelihood (-982.2599) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 18 nucleotide sequences. All positions with less than 100% site coverage were eliminated. That is, less than 0% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 592 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Jukes &Cantor, 1969).

Discussion

Carbapenemase producing pathogens causing infections that are difficult to treat (Ben-David etal., 2012). In this study, the tested isolates showed elevated levels of resistance to different antimicrobial agents. We found that 34.3 % of our isolates showed resistance to imipenem, This results in contrast to other studies reported that imipenem was the most effective β-lactam and all isolates were completely sensitive to it, and this was may be resulted from difference in geographical area or abuse of these drug in our country (ElKholy etal., 2003).

In our study, carbapenem-resistant isolates showed significant resistance to amikacin (82.6%), gentamicin, and ciprofloxacin (both 100%) compared with carbapenem sensitive isolates were 34%, 52.3%, and 32% respectively. These results were coincided with Nordmann et al., (2012) who observed that the carbapenemase producing isolates are usually associated with many other non-β-lactam resistance determinants, which give rise to MDR.

We also observed 100% of MβLs producers were "MDR". These results represent a potential threat to hospitalized patients by limiting the therapeutic options. In this study, among the 67 MDR *Klebsiella* isolates, 59 (88%) isolates were shown resistance to third generation's cephalosporins, of these isolates, 27 (40.3%) showed ESβLs production. Our results were somewhat higher than Shukla et al (2004), who reported that 32% of *K. pneumoniae* from 120 samples of tertiary care hospital were ESβLs producers.

Infections caused by carbapenem-resistant strains have few treatment options (Hirsch& Tam, 2010& Livermore et al., 2011) and are associated with mortality rates up to 50% (Patel etal., 2008). In our study,

carbapenem- resistance was detected in 23 (34.3%) *Klebsiella* isolates, this result agreed with other results proved that a percent was varying from 20 to 40 % in New York and Greece while higher results were shown in other studies reaching 83% in USA (Bratu et al., 2005, Giakkoupi etal., 2009& Marquez et al., 2013).

In our study, PCR amplifications revealed that 6 isolates harbor blaKPC, all of them were MDR isolates, limiting the therapeutic options to Polymixin. These results are similar to what was reported by Queenan and Bush, (2007), as they found that Carbapenemase producing pathogens causing infections are difficult to treat and have high mortality rates.

In our study, all 23 Imipenem resistant *K. pneumonia* isolates were subjected to PCR, 2 (9%) isolates were positive for blaNDM-1gene. The analysis of antibiotic resistance profiles of NDM-1 producer *K. pneumonia* strains in this study revealed that all NDM-1 producing *K. pneumoniae* isolates were MDR strains, with resistance to almost all tested antibiotics and this is in agreement with the results of other studies (Fazeli etal., 2015& Brink etal.,2012). In vitro, different studies reveal that colistin has consistent activity against MDR or pan resistant MβLs -producing isolates (Maltezou et al., 2009). In our study, all our resistant isolates were sensitive to colistin.

AcrAB efflux system is the most principal efflux pump contributing to the intrinsic resistance in *K. pneumoniae* against multiple antimicrobial agents including ciprofloxacin and other fluoroquinolones (Pakzad et al., 2013& Zavascki et al., 2010). In addition, the presence of the multidrug efflux pump system (acrAB) was significantly correlated with the MDR pattern (Wasfi et al., 2016).

Our study, demonstrated that 100% of ciprofloxacin-resistant isolates were MDR (multidrug resistant) isolates and 64% of them were resistant to tetracycline, imipenem, ceftazidime, and gentamicin. The PCR assay showed that only 4 isolates out of 23 tested isolates that harbored the acrAB gene. In contrast, Pakzad *et al.*, (2013) who reported that all ciprofloxacin-resistant isolates harbored acrAB gene.

Conclusion

K. pneumoniae was the most significant bacteria associated with human nosocomial infections. Efficient antibiotic therapy is necessary for early management of *K. pneumonia* infection. National and regional guidelines and policies must not only made but also monitored and implemented against antibiotic resistance. Colistin is the drug of choice in treatment multidrug resistant isolates.

Conflicts of interest: The authors confirm that this article content has no conflicts of interest.

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