Molecular determinants of β-lactamase producing *Klebsiella pneumoniae* in Mansoura University Neonatal Intensive Care Unit

**Abstract**

**Background:** The emergence of β-lactamase-producing *Klebsiella pneumoniae*, represents a significant diagnostic and therapeutic challenge to the management of infections caused by this organism. This prospective study aimed to study the frequency of β-lactamase production by *K. pneumoniae* in neonatal intensive care unit (NICU) of Mansoura University Children’s Hospital in Egypt.

**Methods:** This study was conducted over a period of 36 months from September 2010 to August 2013, where 684 samples were collected from different body sites of neonates in the NICU. Microbial isolation, identification and antimicrobial susceptibility testing were carried out. β-lactamase production by *K. pneumoniae* isolates was confirmed by phenotypic methods and PCR amplification of related genes using a six-gene panel for the amplification of the *bla*\_CMY-2, *bla*\_DHA, *bla*\_ACC, *bla*\_SHV, *bla*\_TEM and *bla*\_CTX-M genes. In vitro transformation and conjugation were carried out to detect plasmid meditated AmpC β-lactamase resistance by transmission to *E. coli*.

**Results:** *K. pneumoniae* was isolated at a rate of 12.6% and β-lactamase production was detected in 62.8% of the isolates. The most commonly detected β-lactamase gene was *bla*\_SHV (51.9%), followed by *bla*\_CMY-2 (16.7%), *bla*\_DHA (13%), *bla*\_TEM (9.2%), *bla*\_CTX-M (7.4%) and *bla*\_ACC (1.9%). Additionally, some strains carried combinations of two or three genes. The plasmid carrying *bla*\_CMY-2 was 100% successfully transformed.
Introduction

Beta-lactamase producing *K. pneumoniae* has been increasingly noticed as a problematic pathogen in different hospital settings. Moreover, the widespread and misuse of antibiotics, continues to increase resistance in Beta-lactamase drugs and to reduce the chance for cure and hamper infection control measures in hospitals worldwide (1,2).

Beta-lactamases are a group of enzymes that inhibit ß-lactam drugs by breaking their active ring structure. The most important ß-lactamases are those produced by *Enterobacteriaceae*. These enzymes are mostly responsible for therapeutic failure to this crucial group of drugs (3).

Two types of AmpC class A ß-lactamases are known to inhibit third generation cephalosporins, and these spread rapidly through chromosomal and plasmid mediated enzymes in Gram-negative bacteria. The plasmid mediated process is more critical being transferable among different species of *Enterobacteriaceae*. Extended spectrum ß-lactamases (ESBLs) resist all ß-lactams, except cephemycins and carpenbens and include a classical type that is a mutant of TEM and SHV and especially the CTX-M type (4). Moreover, AmpC class C (AmpC) ß-lactamases are clinically important, since these mediate resistance to broad range cephalosporins including cephemycins and being resistant to clavulanes (5). Chromosomal AmpC enzymes are inducible and non-transferable. The transferable plasmid mediated AmpC genes favored their dissemination among *Enterobacteriaceae*. Plasmid-encoded *blaCMY*, *blaDHA*, and *blaACC* determinants producing class C enzymes are described with increasing frequencies (6).

This work aimed to investigate the prevalence of *K. pneumoniae* as a nosocomial pathogen and its molecular resistance determinants in the NICU of Mansoura University Children’s Hospital.

Patients and Materials

Study population

This prospective study included neonates suspected of having nosocomial infection (NI) after 48 hours of admission to the NICU. A thorough history, clinical monitoring and laboratory tests were performed and documented on standard forms. Clinical sepsis evaluation was performed and divided into five categories as described by Grether

Conclusion: This study demonstrates that *K. pneumoniae* is a common multidrug resistant isolate in neonatal ICU and associated with production of ß-lactamases in a significant number of those isolates. This feature could represent a real risk for failure of therapeutic options.

Key words: *K. pneumoniae*, ESBL, Egyptian neonates.
and Nelson (7); (1) Frequent episodes of apnea and bradycardia; (2) seizure or change in the level of consciousness; (3) tachycardia, dyspnea or increase in respiratory support; (4) signs of septic shock; and (5) fever or temperature instability. Neonates diagnosed with early-onset sepsis were excluded. A case was defined as any neonate who developed \( K.\ pneumoniae \) nosocomial infection diagnosed by a positive culture. Parents of neonates have signed informed consent and the study protocol was approved by the ethics committee at Mansoura Faculty of Medicine.

**Sample collection**

Samples from different body sites; blood, urine, cerebrospinal fluid, endotracheal aspirate of mechanically ventilated neonates and wounds of surgically operated neonates were collected. Processing of the samples was done according to standard protocols used in the Microbiology Diagnostic and Infection Control Unit (MDICU)/Department of Medical Microbiology and Immunology, Mansoura faculty of medicine. \( K.\ pneumoniae \) isolates were identified by colonial morphology, Gram-stained smears, culturing the isolates on MacConkey’s agar (Oxoid, UK) at different temperatures 4°C, 10°C and 42°C to differentiate \( K.\ pneumoniae \) from other Klebsiella spp. (8), and using biochemical reactions (oxidase, catalase, gelatin liquefaction test, DNAase production and IMVC). Further identification to the species level was carried out using API 20E. Isolates were stored with 50% sterile glycerol at -80°C.

**Antimicrobial susceptibility testing**

Susceptibility tests were performed using the Kirby–Bauer disc diffusion method and Muller-Hinton agar (Oxoid, UK) according to the Clinical Laboratory Standards Institute guidelines (CLSI) (9). The following antibiotics were used; gentamicin (10µg), ceftazidime (30 µg), cefpime (30 µg), aztreonam (30 µg), imipenem (10µg) and ampicillin/sulbactam (20 µg) (Oxoid, UK).

**Screening for extended spectrum \( \beta \)-Lactamases production**

All \( K.\ pneumoniae \) isolates that showed resistance to any of the \( \beta \)-lactam drugs were tested for the presence of ESBL. Detection of ESBL was performed using double disk synergy (DDS) test on Mueller-Hinton agar. It was defined as an increase in the zone of inhibition of ≥ 5 mm with cefotaxime discs when they were tested in combination with discs containing clavulanic acid (10). The class C \( \beta \)-lactamase resistance of AmpC phenotype was detected by the AmpC disc test and is defined as a flattening or indentation of the ceftoxin inhibition zone in the vicinity of a sterile disc of 6 mm moistened with 20µl of sterile saline and inoculated with multiple colonies of the tested organism after overnight incubation at 37°C (11). A negative test showed undistorted zone. \( E.\ coli \) ATCC 25922 was used as a control strain.

**Plasmid content and transmissibility**

Plasmid DNA was extracted from the \( \beta \)-lactamase producing \( K.\ Pneumoniae \) isolates using the alkaline lysis method described by Birnhoim and Doly (1979) (12). \( E.\ coli \) LE392 strain was made competent using calcium chloride. Plasmid DNA (0.1-1mg in water) was mixed with (100ml of fresh or 200ml of competent cells stored at -80°C) and placed on ice for 30 minutes. The cells were then heat shocked by incubation at 42°C for 90-120 seconds and then back to ice for another 2 minutes (13). Conjugation was carried out by broth mating (at a ratio of 1:4) to test the transmissibility of \( \text{bla}_{CMY-2} \) plasmid from the \( K.\ pneumoniae \) isolates harboring this plasmid as donors to a recipient \( E.\ coli \) LE392 strain which was made competent using calcium chloride \( E.\ coli \) ATCC 25922 strain (14). The transformants and transconjugants were selected on Luria-Bertani agar containing ceftazidime (10 µg/ml) (Oxoid, UK).
Characterization of the transformants and transconjugants

Detection of beta-lactamase production in transformants and transconjugants, was confirmed phenotypically by the ability to grow on Luria-Bertani agar containing ceftazidime (10 µg/ml) and genotypically by isolation of the \( \text{bla}_{\text{CMY}-2} \) plasmid, comparing its size with that of the donor strain. PCR amplification of \( \text{bla}_{\text{CMY}-2} \) gene was accomplished using primers listed in Table 1.

PCR for \( \beta \)-lactamase genes

For those isolates that met the criteria for either the ESBL resistance or the AmpC phenotype, PCR was carried out using a six-gene panel for the amplification of the \( \text{bla}_{\text{CMY}}, \text{bla}_{\text{DHA}}, \text{bla}_{\text{ACC}}, \text{bla}_{\text{SHV}}, \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{CTX-M}} \) genes. The following thermal cycling program was used: 95°C for 5` followed by 35 x [95°C for 1`; 55°C for 1`; 72°C for1` then 72°C for 10`]. The amplified DNA fragments were visualized after electrophoresis of 10µl of the PCR samples using a 1% agarose gel (15, 16).

Results

Out of the 684 collected samples, 86 (12.6%) yielded positive culture results for \( K. \) pneumoniae isolates. These were recovered from 35 blood samples (40.7%), 20 wound swabs (23.3%), 16 urine samples (18.6%) and 15 endotracheal aspirates (ETA) (17.44%). All isolates were sensitive to imipenem while none of them showed sensitivity to ampicillin. \( \beta \)-lactamases production was

### Table 1. Specific primers used for molecular amplification of the \( \text{bla} \) genes

<table>
<thead>
<tr>
<th>( \text{bla} ) gene</th>
<th>Oligonucleotide sequence</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| \( \text{bla}_{\text{CMY2}} \) | F TGG CCA GAA CTG ACA GGC AAA  
R TTT CTC CTG AAC GTG GCT GGC | 461  | (15) |
| \( \text{bla}_{\text{DHA}} \) | F AAC TTT CAC AGG TGT GCT GGG T  
R CCG TAC GCA TAC TGG CTT TGC | 405  | (15) |
| \( \text{bla}_{\text{ACC}} \) | F AAC AGC CTC AGC AGC CGG TTA T  
R TTC GCC GCA ATC ATC CCT AGC | 346  | (15) |
| \( \text{bla}_{\text{TEM}} \) | F ATA AAA TTC TTG AAG ACG AAA  
R GAC AGT TAC CAA TGC TTA ATC A | 867  | (16) |
| \( \text{bla}_{\text{SHV}} \) | F GGG TTA TTC TTA TTT GTC GC  
R TTA GCG TTG CCA GTC CTC | 930  | (16) |
| \( \text{bla}_{\text{CTX-M}} \) | F CGA TGT GCA GTA CCA GTA A  
R TTA GTG ACC AGA ATC AGC GG | 585  | (17) |
phenotypically detected in 62/86 (72%) isolates as shown in Table 2.

The distribution of b-lactamase producing *K. pneumoniae* according to different body sites is shown in Table 2. The results revealed a higher detection among blood sample isolates; 31 (50%), followed by wound swab samples 15 (24.2%). Ten isolates (16.1%) were from urine samples and 6 samples (9.7%) were isolated from ETA samples. ESBL production was found in 43/86 of the total isolates (50%). The frequencies of ESBL genes varied among isolates with 28, 5 and 4 isolates being positive for *bla*SHV, *bla*TEM and *bla*CTX-M genes respectively. Two of these isolates contained dual resistance determinants for *bla*SHV and *bla*CTX-M. Class C (AmpC) b-lactamases were detected in 19 (22.1%) isolates. Molecular genetic basis for AmpC production determinants were as follows: 9, 7 and 1 isolates producing *bla*CMY-2, *bla*DHA and *bla*ACC, respectively.

Table 2. Distribution of 62 phenotypically detected beta-lactamase producing *K. pneumoniae* from different clinical samples.

<table>
<thead>
<tr>
<th>Sample site</th>
<th><em>K. pneumoniae</em> isolates</th>
<th>No.</th>
<th>%</th>
<th>No. (%) lactamases producing <em>K. pneumoniae</em> isolates</th>
<th>ESBL</th>
<th>AmpC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>31</td>
<td>50</td>
<td>26</td>
<td>26 (69.4)*</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Wound</td>
<td>15</td>
<td>24.2</td>
<td>09</td>
<td>09 (26.2)</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>10</td>
<td>16.1</td>
<td>05</td>
<td>05 (26.2)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>ETA</td>
<td>06</td>
<td>9.7</td>
<td>03</td>
<td>03 (26.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>100</td>
<td>43</td>
<td>43 (69.4)*</td>
<td>19</td>
<td>30.6</td>
</tr>
</tbody>
</table>

*Represent 50% of the total *K. pneumoniae* isolates

Table 3. Distribution of gene resistance determinants in the 54 beta-lactamase producing *K. pneumoniae*

<table>
<thead>
<tr>
<th>Molecular resistance determinants of Beta lactamase</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>bla</em>SHV</td>
<td>28 (51.9%)</td>
</tr>
<tr>
<td>2. <em>bla</em>TEM</td>
<td>5 (9.2%)</td>
</tr>
<tr>
<td>3. <em>bla</em>CTX-M</td>
<td>4 (7.4%)</td>
</tr>
<tr>
<td>4. <em>bla</em>CMY-2</td>
<td>9 (16.7%)</td>
</tr>
<tr>
<td>5. <em>bla</em>DHA</td>
<td>7 (13%)</td>
</tr>
<tr>
<td>6. <em>bla</em>ACC</td>
<td>1 (1.9%)</td>
</tr>
<tr>
<td>7. <em>bla</em>SHV + <em>bla</em>CTX-M</td>
<td>2*</td>
</tr>
<tr>
<td>8. <em>bla</em>TEM + <em>bla</em>CMY-2</td>
<td>2*</td>
</tr>
<tr>
<td>9. <em>bla</em>TEM + <em>bla</em>DHA</td>
<td>1*</td>
</tr>
<tr>
<td>10. <em>bla</em>SHV + <em>bla</em>ACC</td>
<td>1*</td>
</tr>
<tr>
<td>11. <em>bla</em>CMY-2 + <em>bla</em>SHV + <em>bla</em>CTX-M</td>
<td>2*</td>
</tr>
</tbody>
</table>

*Combined genes were already calculated among the individual genes in cells 1-6.

Two isolates contained both *bla*CMY-2 and *bla*TEM, one isolate had both *bla*TEM and *bla*DHA resistance genes, one more isolate possessed *bla*SHV and *bla*ACC. Two isolates possessed triple resistance genes (*bla*CMY-2, *bla*SHV, and *bla*CTX-M) (Table 3). The (9) *K. pneumoniae* isolates carrying isolated *bla*CMY-2 plasmid were used as donors for both transformation and conjugation experiments. Transmissibility of antibiotic resistance plasmid *bla*CMY-2 was 100% successful by transformation to the competent *E. coli* LE392. Conjugation experiment was (77.8%) successful, 7 of the 9 *K. pneumoniae* isolates which were selected as donors.
Discussion

*Klebsiella pneumoniae* has a feature of being one of the most common species harboring beta lactamases. Extensive use of antibiotics has contributed greatly to the emergence of multidrug resistant *Klebsiella* strains that cause hospital infections (18).

This study indicated that *K. pneumoniae* was responsible for 12.6% of nosocomial infection and (50%) of those isolates were ESBL producers. Higher ESBL production of *K. pneumoniae* clinical isolates (67%) was also reported by Abdel-Hady et al. (19) in Egyptian neonates hospitalized in ICU. However, a much lower incidence (2.97%) was reported by Romero et al. (20) in Spanish patients and healthy person. Therefore, wide range of incidence of ESBL production among organisms including *K. pneumoniae* can be attributed to the source of isolates, different antibiotic policies, empirical treatment and the age of patients enrolled in different studies. A study in an Egyptian University Hospital in 2010 reported the ESBL production rate of 76.5% in *K. pneumoniae* (21). The high rate of ESBL producers among clinical isolates in the developing world is troublesome due to lack of financial support for effective infection control and limited sources of effective antimicrobials. These factors have a clear impact on the morbidity and mortality related to infections caused by ESBL producers.

This study demonstrated that *bla*SHV was the most common resistance determinant accounting for 51.9% of the *K. pneumoniae* isolates, followed by *bla*CMY, *bla*DHA, *bla*TEM and *bla*CTX-M at rates of 16.7%, 13 %, 9.2% and 7.4% respectively. These findings are lower than the results reported by Guiqing Wang and co-workers in USA (22) who detected *bla*SHV and *bla*TEM genes at rates of 84.3% and 50.4%, respectively. In contrast, the study of Parveen et al. (2011) in India, reported *bla*CTX-M as the most common ESBL-type among *K. pneumoniae* isolates from blood, and 92% of their strains were ESBLs-producers, including high rates prevalence of *bla*TEM (82%) and *bla*SHV 46% (23).

The present study showed that the occurrence rate of class C beta lactamases (Amp C) is (30.6%) among our *Klebsiella* isolates, and this figure is much higher than reports from other countries like Korea (3.1%) (24) and the United States (1.2%) (25). Additionally, our result was also less than those reported recently from Cairo (34.8%) and Korea (39.3%), respectively (26, 27). While this study demonstrated that *bla*CMY-2 is the most prevalent gene (51.9%), followed by *bla*DHA (13.2%). The study of Al-Agamy (28) in Egypt, reported less prevalence rate of *bla*CMY-2 (5%) and slightly more rate of *bla*DHA (23.5%). Furthermore, a second Egyptian study by the same author demonstrated *bla*CMY, as a sole gene among AmpC beta lactamase genes in the *Klebsiella* isolates (29), whereas a Turkish study has found that all their *K. pneumoniae* isolates carried MOX group genes but *bla*DHA and *bla*ACC genes were not detected (30). All these results confirm the fact that these ESBL types are widely different within the same and each country.

In conclusion, this study demonstrates that the majority of *Klebsiella* isolates are multidrug resistant and producers of beta lactamases. The potential of these isolates in horizontal spread of resistance genes represent a real risk for failure of therapy by third generation cephalosporins.
References


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