Prevalence and genotyping of extended spectrum β-lactamase in nosocomial Klebsiella pneumoniae in neonatal care unit
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Abstract:
Background: Klebsiella pneumoniae is one of the most common nosocomial pathogens in NICUs, as it is an important part of the niche of potential sources of nosocomial NICU infections. This pathogen with its expression of extended spectrum β-lactamases and the potential spread of this pattern of resistance is forming an interesting field of research.

Objectives: Studying the expression of ESBL producing Klebsiella pneumoniae in NICUs with special emphasis on the genetic varieties of this phenomenon.

Patients and methods: The study had covered a time scale of eight months, and entailed the collection of a spectrum of samples constituting the diversity of the reservoir of Klebsiella pneumoniae strains in NICUs. This included collection of 100 samples from working environment, 44 working personnel samples, in addition to 105 different clinical samples. Of these samples, any Klebsiella pneumoniae strain was subjected to routine disk diffusion susceptibility testing, and double disk diffusion for detection of ESBL. ESBL producing Klebsiella pneumoniae isolates were further subjected to real-time multiplex PCR amplification to detect the presence of TEM and/or SHV genes using consensus gene sequences of both genes. Results were interpreted using specific melting curve peaks.

Results: Klebsiella pneumoniae was isolated from all sample categories. The isolation frequency was 11 Klebsiella pneumoniae strains of the 100 environmental samples, 7 of the 44 working personnel samples, 8 of the 45 blood culture samples, 20 of 39 lower respiratory samples, one of the 6 pyogenic samples, one of the 4 CSF samples, and two of the 11 urine samples. The overall isolation was 50 Klebsiella pneumoniae strains from 249 different samples. Of the 50 isolated Klebsiella pneumoniae strains, 33 (66%) were ESBL producers. The isolates that expressed only TEM were 6 (18.2%), and those that expressed only SHV were 13 (39.4%), and the producers of both TEM and SHV were 12 (36.4%), and the non TEM or SHV producers were 2 (6%).

Conclusion: The prevalence and transmissibility of ESBL among nosocomial Klebsiella pneumoniae are forming a great challenge in accurate diagnosis and management for clinicians and laboratory workers. The presence of TEM and SHV and occurrence of other antimicrobial resistance mechanisms together with ESBL confirm the cross resistance in nosocomial NICU Klebsiella pneumoniae and other Enterobacteriaceae as well. Multiplex PCR assay is recommended in tracing the nature and course of ESBL genotype propagation among common Gram negative bacilli in the environment and among hospital employees, especially in NICU.

Key words: ESBL, Klebsiella pneumoniae, NICU, TEM, SHV, multiplex PCR.

Abbreviations: NICU: neonatal care unit; ESBL: extended spectrum β-lactamase, TEM: Temoniera; SHV: Sulphydryl variable; CLSI: Clinical and Laboratory Standards Institute; DDDT: double disk diffusion test; PBS: Phosphate buffer saline; CFU: colony forming unit.
**Introduction:**
Multidrug resistant Gram negative bacilli belonging to the family Enterobacteriaceae have been increasingly responsible for infections among the neonates admitted to the Neonatal Intensive Care Unit (NICU) in many countries including Egypt. *Klebsiella pneumoniae* constitutes a majority of these pathogens. With the emergence of Extended Spectrum β-Lactamase (ESBL) producing *K. pneumoniae* as the predominant pathogen, the third generation cephalosporins, which have been used extensively as a life saving first-line antibiotic among septicemic neonates are rendered useless, significantly increasing the morbidity and mortality in the NICUs. Many outbreaks of *K. pneumoniae* infections in the NICU have frequently been shown to have an environmental reservoir. Imipenem, cefepime and amikacin can be suggested as the drugs of choice. Length of NICU stay was found to be an independent risk factor associated with ESBL-producing *E. coli* and/or *K. pneumoniae* acquisition status of neonates. Irrespective of the primary source, the lower digestive tract of the colonized neonates is the main reservoir of these micro-organisms, and cross contamination is presumably hand carried by the attending staff. These strains usually exhibit cross-resistance to other antibiotics, such as aminoglycosides. Therapeutic options are therefore limited.

Extended-spectrum β-lactamases (ESBLs) are a rapidly evolving group of β-lactamase enzymes produced by the Gram negative bacteria, which have the ability to hydrolyze all cephalosporins and aztreonam but are inhibited by clavulanic acid. ESBLs cannot hydrolyze carbapenemes or cephemycins. Most ESBLs are generally mutants of classical TEM and SHV genes.

TEM and SHV genes were first described in *Klebsiella pneumoniae* from Western Europe. With both of these groups of enzymes a few point mutations at selected loci within the structural gene encoding the enzyme give rise to the extended-spectrum phenotype. TEM and SHV-type ESBLs are most often found in *E. coli* and *K. pneumoniae*. However, they are also found in Proteus spp., Enterobacter spp. and other members of the Enterobacteriaceae.

The present study was carried out to determine the prevalence of TEM and SHV genes responsible for ESBL production amongst the ESBL positive *Klebsiella pneumoniae* species isolated from the patients admitted to a neonatal care unit, and from surveillance of *Klebsiella pneumoniae* from working staff by hand impression and nasal swabs, air samples, and swabs of different surfaces and equipments in the neonatal care unit.
**Material and methods:**

**Samples:**
This study included collection of 100 samples from working environment (air samples, swabs from incubators, phototherapy units, trolley, sink, resuscitation equipment, suction apparatus and hand washing soaps) and 44 working personnel samples as they constitute a potential source of infection (from hand prints, nasal swabs). Clinical samples were collected from neonates presented with symptoms and signs of nosocomial infections. These included blood cultures (45 samples), lower respiratory tract specimens by tracheal aspirates and bronchoalveolar lavage (39 samples), pyogenic lesion specimens from infected umbilical stumps and other skin and soft tissue lesions (6 samples), CSF (4 samples) and urine using infant urine collection bag (11 samples). Hand impression and nasal swabs were taken from the medical and nursing staff of the NICU. The air sampling was done using settle plates exposed to the NICU air for half an hour. The samples were transported to the microbiology laboratory, and were processed immediately. All the samples were processed and the isolates identified by standard bacteriological methods. Culture of all samples was made on appropriate media (BACTEC blood culture vials for blood culture, and blood agar and MacConkey agar for other samples). Isolation of well identifiable colonies was made, as well as Gram staining, Oxidase testing and final identification of all Gram negative bacilli as *Klebsiella pneumoniae* or other bacteria by the conventional biochemical reactions (negative oxidase, negative indole, positive urease, positive citrate, A/A with gas triple sugar iron agar reaction, K/K lysine iron agar reaction, absence of motility and negative ornithine decarboxylase test).

All *Klebsiella pneumoniae* isolates were subjected to routine disk diffusion susceptibility testing by the following antibiotics: cephalothin, gentamicin, amikacin, ampicillin, cefotaxime, imipenem, and amoxycillin/clavulanic acid according to the instructions of CLSI.9 Also, all *Klebsiella pneumoniae* isolates were subjected to double disk diffusion for detection of ESBL, using commercially available ceftazidime (30 μg), cefpodoxime (30 μg) and cefotaxime (30 μg) with concomitant use of disks containing the same antibiotics with the same concentrations supplemented with clavulanic acid (10 μg).10 The inoculum and incubation conditions were the same as for standard disk diffusion recommendations. A more than 5 mm increase in zone diameter
for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone was designated as ESBL positive.

The amplification of TEM and SHV genes was done for the following gene sequences (Consensus sequences that are compatible with 110 sequences of both TEM and SHV variants supplied from the GenBank Database:11

**SHVF:** TCAGCGAAAAACACCTTG,

**SHVR:** TCCCCGAGATAATCACC

**TEMF:** CTTCTGTTTTGTCTCAGCCA,

**TEMR:** TACGATACGGGAGGGCTTAC

Overnight *Klebsiella pneumoniae* isolates were first suspended in PBS (5x10^5 to 1x10^6 CFU/ml). Bacterial cells were centrifuged for 10 minutes at 8000xg to concentrate the bacterial cells in the pellet. Most of the supernatant was discarded, and the remaining pellet was processed in 100 μl of PBS. Bacterial DNA extraction was done by the aid of MagNA Pure Compact nucleic acid isolation kit I (Roche) and automated MagNA Pure compact instrument; using DNA Bacteria purification protocol supplied from Roche. Samples were subjected to cell wall disruption and protein digestion by lysis buffer and proteinase K. Released DNA was bound to magnetic glass particles, and a magnetic separation of the bead DNA complex was done. After that DNA was eluted at high temperature during removal of magnetic glass beads. The amplification and detection of TEM and SHV consensus sequences was done by real-time PCR technique using LightCycler instrument and LightCycler – DNA Master SYBR Green I kit supplied from Roche (a ready to use reaction mix containing Taq DNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye and 10 mM MgCl₂). Synthetic TEM and SHV control genes were developed by Pioneer company (South Korea). Reaction conditions started with denaturation step (one cycle lasting one minute at 95°C), followed by amplification step (45 cycles of one second at 95°C, then 10 seconds at 50°C, and 15 seconds at 72°C). A melting curve analysis was performed after the last amplification cycle. Cooling was made at 40°C for 30 seconds. The interpretation was made by LightCycler software version 4.05. TEM melting curve peak was at 88.59 °C, and that of SHV was at 77.53 °C. Analysis of every sample was made to detect the presence of any of the genes in them.

**Results:**

Working place environmental samples were 100 samples (45 of them gave significant growth for one or more bacterial isolate, while 55 were negative). *Klebsiella pneumoniae* was isolated in 11 environmental cultures. Working staff cultures from hand prints, nasal swabs were 44 (of them 30 showed significant growth for one or more bacterial isolate,
while 14 were negative). *Klebsiella pneumoniae* constituted 7 of the positive growth for working staff cultures.

Regarding samples taken from neonates presented with infections in different sites, 45 blood cultures were made (19 of them were positive, and *Klebsiella pneumoniae* was isolated from 8 of these cultures). Lower respiratory aspirate cultures were 39, and of them 29 cultures were positive including 20 *Klebsiella pneumoniae* isolates. Also *Klebsiella pneumoniae* was isolated from pyogenic infections (1 isolate), CSF cultures (1 isolate) and urine (2 isolates). The overall *Klebsiella pneumoniae* isolation was 50 isolates from 249 different environmental and patient cultures (23.9 % isolation percentage from all samples) (Table 1).

Using double disk diffusion technique for detection of ESBL phenotype in *Klebsiella pneumoniae* isolates, it was revealed that ESBL was present in 33 (66%) of the isolated *Klebsiella pneumoniae*. After that, Multiplex PCR was made to detect the presence of TEM and/or SHV gene sequences among Klebsiella pneumoniae isolates. The overall data revealed that TEM gene was present in 6 (18.2%), SHV in 13 (39.4%) and both TEM and SHV genes in 12 (36.4%) of ESBL producing isolates (Table 2).

The 50 *Klebsiella pneumoniae* isolates were tested for antimicrobial susceptibility by routine Kirby-Bauer test. The results are presented in table (3) regarding susceptibilities of antimicrobial panel with ESBL and non-ESBL producing isolates.

### Table (1): Outcome of culture of different samples:

<table>
<thead>
<tr>
<th>Sample (no)</th>
<th>Results</th>
<th>Number of <em>Klebsiella pneumoniae</em> isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Environmental samples (100)</td>
<td>45 (18 mixed)</td>
<td>11</td>
</tr>
<tr>
<td>(12 air, 22 incubators, 18 phototherapy units, 1 trolley, 7 sink, 15 resuscitation equipment, 15 suction apparatus, 10 soaps)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Working personnel (44)</td>
<td>30 (17 mixed)</td>
<td>7</td>
</tr>
<tr>
<td>(hand prints, nasal swabs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood culture (45)</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>Lower Resp. Infection (39)</td>
<td>29</td>
<td>20</td>
</tr>
<tr>
<td>Pyogenic lesions (6)</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>CSF (4)</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>
Table (2) Phenotypic and molecular results of ESBL testing in *Klebsiella pneumoniae* isolates:

<table>
<thead>
<tr>
<th>ESBL status</th>
<th>Number (%) of <em>Klebsiella pneumoniae</em> isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESBL producers</td>
<td>33 (66%)</td>
</tr>
<tr>
<td>Non ESBL producers</td>
<td>17 (34%)</td>
</tr>
<tr>
<td>Only TEM producers</td>
<td>6 (12%) of all isolates</td>
</tr>
<tr>
<td></td>
<td>18.2% of ESBL isolates</td>
</tr>
<tr>
<td>Only SHV producers</td>
<td>13 (26%) of all isolates</td>
</tr>
<tr>
<td></td>
<td>39.4% of ESBL isolates</td>
</tr>
<tr>
<td>Both TEM &amp; SHV producers</td>
<td>12 (24%) of all isolates</td>
</tr>
<tr>
<td></td>
<td>36.4% of ESBL isolates</td>
</tr>
<tr>
<td>Non TEM or SHV producers</td>
<td>2 (4%) of all isolates</td>
</tr>
<tr>
<td></td>
<td>6% of ESBL isolates</td>
</tr>
</tbody>
</table>

Table (3): Resistance by Kirby-Baur disk diffusion (KBDD) according to CLSI for *Klebsiella pneumoniae* isolates:

<table>
<thead>
<tr>
<th>Drug</th>
<th>Resistance of <em>Klebsiella pneumoniae</em> isolates</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-ESBL-producing (17 isolates)</td>
<td>ESBL producing (33 isolates)</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>16 (94%)</td>
<td>32 (97%)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>9 (53%)</td>
<td>29 (88%)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>8 (47%)</td>
<td>30 (91%)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>10 (59%)</td>
<td>33 (100%)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0 (0%)</td>
<td>2 (6%)</td>
</tr>
<tr>
<td>Amoxycillin/clavulanic A.</td>
<td>3 (18%)</td>
<td>12 (36%)</td>
</tr>
<tr>
<td>Cotrimoxazole</td>
<td>11 (65%)</td>
<td>27 (82%)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>4 (12.1%)</td>
<td>29 (87.9%)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>3 (9.1%)</td>
<td>30 (90.9%)</td>
</tr>
</tbody>
</table>
Cefpodoxime | 2 (6.1%) | 31 (93.9%)

**Discussion:**
*Klebsiella pneumoniae* has rapidly become the most common ESBL producing organism, making it difficult to eradicate this organism from the high risk wards such as intensive care units. This problem is highly encountered in neonatal intensive care units, where bacteria are exposed to great antibiotic pressure. Furthermore, many of these units residents are particularly vulnerable to infection because they are immunocompromised or have an easy avenue of access for bacteria. In this study different environmental, working personnel and clinical samples were collected aiming at studying the frequency and molecular characteristics of *Klebsiella pneumoniae* isolates.

The clinical samples culture results showed that *Klebsiella pneumoniae* is a highly respectable causative pathogen especially in neonatal bacteremia (8 of 19 positive blood cultures) and lower respiratory infections (20 of 29 isolated respiratory pathogens), in addition to CSF, urine and pyogenic infections.
Klebsiella pneumoniae was also isolated from the environment (11 isolates from 100 environmental samples) and from the working personnel samples (7 isolates from 44 working personnel samples). This denotes that Klebsiella pneumoniae is an important part of the niche of potential sources of nosocomial NICU infections.

ESBL detection was made using double disk diffusion test (DDDT) as a proven routine method for detection of ESBL, and it was found that 33 of 50 (66%) Klebsiella pneumoniae isolates were expressing this phenotype.

Similar results were obtained by many other authors all over the world. In India, in a study conducted by Krishna et al (2007), it was stated that after culturing different environmental samples from various sites in NICU monthly for a period of six months, where ESBL producing K. pneumoniae were isolated from all the sites except room air at least on one occasion. ESBL producing K. pneumoniae was always isolated from one of the incubators, medicine trolley and sink; while at least one of the health care workers carried it in the hands four out of six times tested. The overall percentage of ESBL production among studied K. pneumoniae in their study was 71.93%. ESBL producing K. pneumoniae with similar antibiogram were also isolated from the clinical samples obtained from the neonates. They concluded that widespread use of third generation cephalosporins as a preemptive antibiotic for suspected cases of septicaemia may have contributed to emergence of ESBL producing K. pneumoniae in addition to other risk factors.

In another study by Lal et al., (2007), a total of 204 multidrug-resistant isolates of Klebsiella pneumoniae taken from clinical samples; blood (n=108), urine (n=15), pus (n=2) and sputum (n=79) were obtained from a tertiary care facility to be screened for ESBL resistance by phenotypic and molecular studies. Eighty six per cent (175 of 204) of the isolates were found to be resistant to at least one of the 3rd generation cephalosporins, of which 97.1 per cent (170) of Klebsiella sp. isolates were confirmed to be positive for ESBL by double disk diffusion and E-test.

Similar results regarding the very high prevalence of ESBL producing in ICUs generally and specifically in NICUs, were documented all through the past decade all over the world: in Spain, Germany, Kuwait, and Australia. In Africa, ESBLs have been reported in Tunisia, Morocco, Senegal, Nigeria, South Africa, and Kenya. In Egypt, it was reported previously by El Kholy et al., (2003) that ESBL production and 3rd generation cephalosporins resistance among Klebsiella isolates was 40% in clinical samples taken from five hospitals in the greater Cairo region. In a study conducted from 2002 to 2008 in different localitons in...
Europ, there was a relatively stable rate of detection of ESBL-positive *Klebsiella pneumoniae* isolates (16.4% in 2002 to 17.9% in 2008).24

The high rate of ESBL production in this study is attributed to the abuse of the 3rd generation cephalosporins; imposing a selective stress, leading to survival of the only ESBL expressing strains with the eventual spread of ESBL genes to other bacteria within the same species or other species. The routine disk diffusion based on Kirby-Bauer - with the use of only one of 3rd generation cephalosporin representatives or "ESBL detectors" - is less sensitive than DDDT, as concluded from the results of this study. The percentage of detection of ESBL was only 87.9, 90.9, and 93.9% with the use of cefotaxime, ceftazidime and cefpodoxime respectively. This is explained on the basis of variable expression of ESBL with different cephalosporins, and variability of growth conditions and difficulty in standardizing antimicrobial susceptibility testing.11

In this study, it was noticed that the percentage of resistance against most of the members of routine antimicrobial susceptibility testing panel was increased in ESBL producing strains compared to non-ESBL producing strains. This may be explained by the fact of cross-resistance of other antimicrobial resistance mechanisms - such as aminoglycoside resistance, other classes of β-lactamases and even carbapenemases - with ESBL as a part of plasmid mediated transfer not only of one gene, but of other bound resistance genes as well.25

For the sake of epidemiological studying and better understanding of the molecular nature of ESBL producing *Klebsiella pneumoniae*. This resistance is mainly associated with the production of enzymes called Temoniera (TEM) and sulphhydryl variable (SHV) that are generally plasmid-mediated.26 In a long term study conducted by Bagattini et al., (2006) covering 8 years of studying and tracing the plasmids carrying blaSHV and blaTEM, it was found that the selection of ESBL-producing clones and the transfer of these genes between different clones were responsible for the spread of *K. pneumoniae* in the neonatal intensive care unit.27

So, a simple multiplex PCR analysis was used to amplify consensus sequences that are shared in most studied SHV and TEM genes worldwide. To study the existence of either or both genes in the studied isolates, it was evident that a large number (36.4%) of ESBL producing *Klebsiella pneumoniae* were expressing both TEM and SHV genotypes. TEM alone was isolated in 18.2% and SHV alone was isolated in 39.4%. Neither of TEM or SHV was present in 6% of isolates. From these data we can conclude that the most common genotype expressing ESBL in the isolated *Klebsiella pneumoniae* in the presented study was SHV alone, followed by the presence of both SHV and TEM genes, followed by TEM
alone. The absence of the two studied genes was recorded only in a minority of samples, denoting the specific high impact of these genes (either single or combined existence) in this pattern of resistance. Also, the study of the presence of TEM and/or SHV in any epidemiological survey may give an opportunity to classify isolates into one of 4 potential genotypes (only TEM, only SHV, both of them, or neither of them), aiding in the tracing and containment of the incriminated strain in any nosocomial outbreak.

Similar researches were made all over the world; stressing on the molecular background of ESBL producing Klebsiella pneumoniae. Perilli et al., (2002) studied the distribution and prevalence of ESBLs belonging to the TEM and SHV families in 448 ESBL-producing clinical isolates of Enterobacteriaceae collected from 10 different Italian hospitals by direct sequencing of PCR-amplified genes. They found that Klebsiella pneumoniae was the most common producer of SHV-type ESBLs. The results of the two later studies were in agreement with the results of the presented study that the most frequent genotype is the SHV one.

The genotype screening pattern is highly influenced by specific local factors. Another spectrum of genotypes was obtained by Lal et al., (2007) in their study in an Indian tertiary care hospital. They found that the majority of the ESBL positive clinical isolates of Klebsiella pneumoniae carried both TEM and SHV genes followed by TEM alone.

The above data are forming a trial to make a basis of epidemiological and genetic understanding of ESBL production. The presence of both TEM and SHV and occurrence of other antimicrobial resistance mechanisms together with ESBL in the studied strains confirm the eventual cross resistance and free transfer of related genetic material in nosocomial NICU Klebsiella pneumoniae. This study design is also applicable on other Enterobacteriaceae that can express ESBL. Multiplex PCR assay is recommended in tracing the nature and course of ESBL genotype propagation among common Gram negative bacilli that may catch these genes in the environment and among hospital employees, especially in NICU.

It is highly recommended to apply the following advices in the NICU to minimize nosocomial neonatal infections:

- Handling neonates should be minimized. Measures should be taken to minimize the risk of transmission of pathogens from mother to infant. NICU staff should perform hand washing before and after handling infants, as well as on entering or leaving NICU. Also, equipment and supplies should not be shared between infants.
- Aseptic techniques for invasive procedures and their standard precautions must be strictly applied to minimize organisms spread through blood, body fluids and other secretions.
- Ultimate care and disinfection must be provided during dealing with reusable devices, in order to prevent transmission of potential multi-drug resistant pathogens.
- Regular surveillance and follow-up incidence of ESBL producing *Klebsiella pneumoniae*.

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