Ertapenem resistance among extended spectrum β-lactamases 
producing Klebsiella pneumoniae

Azita Leavitt¹, Inna Chmelnitsky¹, Raul Colodner², Itzhak Ofek³, Yehuda 
Carmeli¹, Shiri Navon-Venezia¹*

¹The Laboratory for Molecular Epidemiology and Antibiotic Research, Division of 
Epidemiology, Tel Aviv Sourasky Medical Center affiliated to the Sackler Faculty of 
Medicine, Tel Aviv University, Tel Aviv, Israel. ²Clinical Microbiology Laboratory, 
Ha'Emek Medical Center, Afula, Israel ³Department of Clinical Microbiology and 
Immunology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

Running title: Ertapenem resistant Klebsiella pneumoniae and inoculum effect

This work was performed in partial fulfillment of the requirements for a Ph.D. degree 
of Azita Leavitt, Sackler Faculty of Medicine, Tel Aviv University, Israel

* Correspondence: Shiri Navon-Venezia

Division of Epidemiology

Tel Aviv Sourasky Medical Center

6 Weizmann St.

Tel Aviv 64239, Israel

Phone: 972 3 692 5644

Fax: 972 3 697 4966

E-mail: shiri_nv@tasmc.health.gov.il
ABSTRACT

Ertapenem resistance in *Klebsiella pneumoniae* is rare. We report on ertapenem non-susceptible phenotype among 25 out of 663 (3.77%) extended-spectrum beta-lactamases-producing *K. pneumoniae* isolates in a multicenter Israeli study. These isolates originated from six different hospitals and were multi-clonal, belonging to 12 different genetic clones. Repeat testing using Etest and agar dilution confirmed ertapenem non-susceptibility in only 15/663 (2.3%) of the isolates. Molecular mechanisms of ertapenem resistance in seven single clone resistant isolates, was due to the presence of a ESBL genes (CTX-M-2 in four isolates, CTX-M-10 and OXA-4 in one isolate, SHV-12 in one isolate and SHV-28 in one isolate) combined with the absence of OMPK36. 7/10 isolates initially reported as ertapenem non-susceptible, and subsequently classified as susceptible, showed an inoculum effect with ertapenem but not with imipenem or meropenem. Population analysis detected the presence of an ertapenem resistant subpopulation in a frequency of $10^{-6}$. These rare resistant sub-populations carried multiple ESBL genes including TEM-30, SHV-44, CTX-M-2 and CTX-M-10, and lacked OMPK36. The clinical and diagnostic significance of the results should be further studied.
INTRODUCTION

*K. pneumoniae* has been found to be the most common species to produce ESBLs (6), and in some countries the prevalence of ESBL production approaches 50% (25). Antimicrobial co-resistance among these ESBL-producing isolates limits the number of drugs useful against these strains (30), leaving carbapenems to be the most reliable agents (19, 35). In Tel Aviv medical center, 45% of *K. pneumoniae* isolates have an ESBL-producing phenotype (24) and ertapenem is an optional therapy for nosocomial infections caused by these pathogens.

Ertapenem is a 1-β-methyl carbapenem which reached clinical use in 2001 (28). It is highly active against ESBL-producing and high level AmpC-producing Gram-negative bacteria (22) and is an important agent to treat these infections as it does not likely to lead to carbapenem resistance in *Pseudomonas* (8, 23).

Emergence of ertapenem resistance in *K. pneumoniae*, not related to the production of carbapenemases such as metallo-enzymes or KPC-enzymes, is rare and has been studied only in single cases (36). In these cases resistance was associated with the production of an ESBL and deficiency in the expression of outer membrane proteins OMPK35 and OMPK36 (17, 37). Reports on the incidence of ertapenem resistance are limited; however, one report on ESBL-producing *K. pneumoniae* isolates collected from intra-abdominal infections found that 10.9% were ertapenem resistant (26).

Accurate susceptibility testing of *K. pneumoniae* isolates to ertapenem is critical for choosing the appropriate antibiotic therapy for treating infections caused by ESBL-producing strains. It has been recommended previously that imipenem and meropenem can be surrogate markers for susceptibility to ertapenem (15), although difficulties to attain precise susceptibility testing results for imipenem and meropenem in clinical microbiology laboratories have been described previously (34), due to degradation of
the drug leading to possible false susceptibility results (13, 31, 33, 36) even when using automated systems such as MicroScan and VITEK systems (6, 7). No studies were performed regarding ertapenem susceptibility testing.

In a previous nation-wide surveillance study in Israel 663 ESBL-producing *Klebsiella* spp. were screened for their susceptibility properties. 95% of these isolates were susceptible to ertapenem, and 98.8% and 95% for imipenem and meropenem, respectively (12). With high prevalence of ESBLs in our country, and the need for optional antibiotic therapies, we aimed to explore the molecular mechanisms that render resistance to ertapenem among these isolates. Moreover we aimed to explain discrepancies in ertapenem MIC testing using agar-based susceptibility-testing methods, and to examine the effect of exposure to ertapenem on these isolates.
MATERIALS AND METHODS

Study design

A total of 663 *Klebsiella* spp. with confirmed ESBL-producing phenotype were collected over a 6-month period between January and December 2004 from 10 major Israeli hospitals. ESBL detection tests were performed based on the Kirby-Bauer disk diffusion test described previously (24). The isolates were subjected to MIC testing by Etest for various antibiotics including the carbapenems imipenem meropenem and ertapenem (12). 25 of these *Klebsiella* isolates were found to be non-susceptible to ertapenem (MIC≥8) and were the subject of this study.

Pulsed-field gel electrophoresis (PFGE)

The genetic relatedness of all ertapenem non-susceptible *K. pneumoniae* isolates was analyzed by PFGE. Bacterial DNA was prepared and cleaved with 20U SpeI endonuclease (New England Biolabs, Boston, MA) as previously described, and DNA macrorestriction patterns were visually compared and interpreted according to the criteria established by Tenover et al (29).

Susceptibility testing

All 25 ESBL-producing isolates that were identified as ertapenem non-susceptible in a previous study (12) were subjected to a repeated MIC testing using Etest and agar dilution in the current study. Ertapenem MIC testing was performed according to the Clinical and Laboratory Standards Institute guidelines (11) using cation adjusted Mueller Hinton agar (Hy-Labs, Rehovot, Israel) supplemented with increasing amounts of ertapenem (Merck, Research Laboratories, Rahway, NJ). The tested inoculum used in all susceptibility testing was 0.5 MacFarland unless stated otherwise. Ertapenem MICs were determined in the presence and absence of 2 µg/ml clavulanic acid or 0.4 µg/ml.
mM EDTA to determine the contribution to resistance of class A and class B \(\beta\)-lactamases, respectively.

**Study of isolates with discrepant ertapenem susceptibility results**

Ertapenem susceptibilities discrepancies (greater than 1-fold dilution difference) between repeated agar-based testing methods were further explored by examining the effect of inoculum size on susceptibility testing of carbapenems and by performing population analysis studies.

**Inoculum effect experiments** Organisms were grown on Mueller-Hinton agar plates (Hy-Labs, Rehovot, Israel) over night. A \(10^8\) CFU/ml inoculum was prepared by suspending a sufficient number of colonies in MH broth to achieve a 0.5 McFarland suspension (corresponding to 0.1 OD at 600nm). Susceptibility testing using Etest and agar dilution methods was performed with \(10^5\) and \(10^7\) cfu containing inocula. A positive inoculum effect was defined as an eight-fold or greater increase in ertapenem MIC on testing with the higher inoculum (20).

**Population analysis** Cultures were grown over night in MH broth and were serially diluted with saline. 100 \(\mu\)l of each dilution was plated on freshly prepared ertapenem containing MH plates (0.25-64 \(\mu\)g/ml). Colonies were counted after over-night incubation at 37°C and the viable count was plotted against ertapenem concentration (38).

**beta-lactamases analysis**

**Phenotypic screening** The production of ertapenem hydrolyzing enzymes by all ertapenem resistant isolates was performed by an ertapenem inactivation bioassay (40) performed on MH agar plates. A suspension of *E. coli* ATCC 25922 equivalents to a 0.5 McFarland standard was inoculated on a large Mueller-Hinton agar plate as for disk diffusion. Then, five ertapenem disks were applied evenly spaced on the plate, four on
the periphery and one in the center of the plate. Crude extract of the organism to be tested for the presence of carbapenemase was prepared by sonication and a loop was used to make a 15-mm streak of crude extract on each side of the ertapenem disk on the periphery of the plate (the center disk served as the control). Four different organism suspensions were used on each plate. KPC-3-producing clinical strain of *Klebsiella pneumoniae* (strain 490) and *E. coli* ATCC 25922 were used as positive and negative controls, respectively, in bioassays and hydrolysis assays for the detection of carbapenem-hydrolyzing activity. The plates were incubated at 37°C for 18 to 20 h. Alterations in shape of the zones of inhibition around the test organism was examined. Screening for production of metallo-β-lactamase was performed by disk approximation tests using EDTA and 2-mercaptopyruvic acid (2). The presence of β-lactamases with ertapenem-hydrolyzing activity of selected ertapenem resistant clones was further analyzed by performing ertapenem hydrolysis assays. Hydrolysis of 0.1 mM imipenem and ertapenem (Merck, Hoddesdon, United Kingdom) was monitored in crude extracts of these isolates by UV spectrophotometry at 299 nm and 294 nm, respectively in 10 mM phosphate buffer (pH 7.0) (9). Activity was standardized relative to protein concentration, which was determined by the Bio-Rad protein microassay (based on the Bradford method). Bovine serum albumin was used as the standard. Ertapenem hydrolysis assays were performed also in the presence of EDTA (0.1 mM, pH 8.0).

**Molecular analysis** Identification of *bla*ESBLs, *bla*KPC and plasmid-mediated AmpC genes in all isolates was determined by PCR on 1µl cell lysates using specific primers designed for identifying known class D and class C β-lactamases genes including *bla*TEM, *bla*SHV (29) *bla*OXA (1, 10, 16, 27), *bla*CTX-M (32), *bla*SPM (9), *bla*ACT, *bla*MIR (3), *bla*CMY, *bla*FOX and *bla*MOX (21) and *bla*KPC (7). The PCR reaction conditions were as follows: 15 min at 95°C, 35 cycles of 1 min at 94°C, 2 min at 68°C, 3 min at 72°C,
followed by an extension step of 10 min at 72°C. PCR reactions were performed with Hot-StarTaq DNA polymerase (Qiagen, Hilden, Germany) and the resulting PCR products were analyzed in a 1.5% agarose gel with ethidium bromide staining and UV light. PCR products were sequenced and analyzed with an ABI PRISM 3100 Genetic Analyzer (PE Biosystems), using the DNA Sequencing Analysis Software and 3100 Data collection Software version 1.1. The nucleotide and the deduced protein sequences were analyzed and compared using software available via the Internet at the NCBI website (http://www.ncbi.nlm.nih.gov).

**Outer membrane protein analysis**

Outer membrane proteins (OMPs) were prepared from selected ertapenem resistant and susceptible isolates grown in Mueller-Hinton broth in the absence or presence of 4 µg/ml ertapenem according to method of Wu et al (39). Cell membrane proteins were obtained by disrupting a logarithmic culture with a VCX 600 sonicator (Misonix, NY, USA). Cell debris was removed by centrifugation at 8000 rpm for 20 min at 4°C, and the supernatant was subjected to ultracentrifugation at 40,000 rpm for 1h at 4°C to collect the membranes. Membranes were then solubilized in 1.5% sodium lauryl sarcosinate for 30 min at room temperature. The suspension was centrifuged at 8000 rpm for 45 min at 4°C and the pellet containing the outer membrane proteins was resuspended in 100 µl 0.05M Phosphate buffer (pH 7.0). Protein concentrations were determined using Bradford assay and equal amount of proteins were analyzed in SDS-polyacrylamide gel electrophoresis.

SDS-PAGE was performed according to the method of Bradford et al. (4), using a Mini protein cell Electrophoresis System apparatus with prepared 10% SDS-polyacrylamide gel (BioRad). Samples were boiled for 5 min prior to loading and then separated at a constant voltage of 150 volt in a running buffer of 1 x Tris/Glycine/SDS (BioRad) and
visualized by Coomassie Blue staining (Gibco-BRL). After electrophoresis, protein bands of interest were excised from the gel, washed in a 200 mM NH4HCO3/50% acetonitrile solution and dried in a speedVac. Protein were rehydrated in a 20 µg/ml trypsin solution (Promega, Madison, WI) and incubated for 16 h at 37°C. Peptides were extracted from gel slices by diffusion in water, and were identified by liquid chromatography mass spectrometry (LC-MS/MS) using an UltimateTM nano HPLC system (LC Packings, Amsterdam, The Netherlands) and a Qstar Pulsar mass spectrometer (Applied Biosystems, Foster City, CA). The MS data were analyzed using the Mascot protein identification software (Matrix Science, London, U.K). For outer membrane protein analysis of ertapenem susceptible strains possessing an inoculum effect, prior to protein analysis bacteria were grown in MH broth in presence of 4 µg/ml ertapenem to obtain the resistant population only.
RESULTS

Occurrence of ertapenem resistance among *Klebsiella pneumoniae* isolates and their clonal relatedness

25 of 663 ESBL-producing *Klebsiella* species (3.77%), collected from 6 hospitals in Israel, were found to be resistant (18 isolates, MIC≥16 µg/ml) or intermediate (7 isolates, MIC>4-8 µg/ml) to ertapenem by Etest (Table 1, 2). All isolates but one (isolate 20, MIC>32 µg/ml) were susceptible to imipenem (MIC50 1 µg/ml). As for meropenem, four isolates were intermediate (MIC 6-8 µg/ml) and one isolate (isolate 20) was resistant (MIC >32 µg/ml) (MIC50 3 µg/ml) (Table 1). Ertapenem resistant isolates originated from blood (12), wound (6), bone marrow (1), catheters (2) and peritoneal fluid (1).

All 25 ertapenem non-susceptible isolates were subjected to genotyping that revealed twelve different genetic clones; clone A was the most prevalent PFGE clone present in eight out of the 25 isolates (32%) and detected in 4 of the 6 studied hospitals. In hospital-1 the dominant clone was A, but in hospital 2 and 5 the dominant clones were clones K and D respectively, clones that were absent in other hospitals (Table 1).

Ertapenem susceptibility testing

The 25 ertapenem non-susceptible isolates included in this study were collected from different hospital clinical labs throughout Israel. When these ESBL-producing isolates arrived in our lab MIC testing of carbapenems was repeated and revealed discrepancies (Table 1).

Of the 25 isolates with initial ertapenem MIC >4 µg/ml ten isolates (40%) were defined as susceptible to ertapenem (MIC of 0.125 -4 µg/ml) upon repetitive testing by both Etest and agar dilution; 2 were intermediate; and 13 were resistant (Table 2). Repetitive MIC testing for imipenem and meropenem did not vary or varied in ± one dilution.
Moreover, significant discrepancies in ertapenem susceptibility results were observed from different Etest measurements as a result of appearance of resistant colonies within the zone of inhibition. The discrepancies observed in MIC testing did not correlate with a specific genetic clone or clones. Overall, we found reasonable agreement between Etest and agar dilution testing (Table 2), however in, 3 isolates we noted discrepancy in MIC between Etesting and agar dilution susceptibility (>2 dilutions difference).

The 10 isolates initially classified as non-susceptible and latter found susceptible were subjected for a further detailed analysis. The other 13 isolates which showed ertapenem resistant profile constantly (2% of the ESBL-producing Klebsiella) were categorized as ertapenem resistant isolates, of which 7 representative single-clone-isolates were further studied for mechanisms of ertapenem resistant.

**Inoculum effect studies**

Ten isolates reported as ertapenem resistant initially, and susceptible on repetitive detection (Table 1, marked in bold), were subjected to inoculum effect experiments. Seven of 10 isolates tested (70%) showed an inoculum effect (more than 8-fold increase in ertapenem MIC); MIC50 increased from 0.5 to 8 by Etest and from 2 to 32 µg/ml by agar dilution (Table 3). All 10 isolates showing a positive inoculum effect for ertapenem were tested for their susceptibility to imipenem and meropenem at high inoculum size; MIC for imipenem increased with inoculum size from MIC50 of 0.19 µg/ml to MIC50 of 0.5 µg/ml, but an inoculum effect was not observed. As for meropenem, MIC50 increased from 0.064 µg/ml to 1.5 µg/ml at high inoculum, and four of seven isolates showed an inoculum effect; none of them though changed their classification from susceptible to intermediate or resistant.
Population analysis studies

To examine the possibility of existence of ertapenem resistant sub-population, two *Klebsiella* isolates that showed an inoculum effect on ertapenem (isolates 5 and 7), were subjected to population analysis. The distribution of the population of the two isolates in term of their resistance to ertapenem was compared with two isolates that did not possess an inoculum effect, with an ertapenem resistant isolate (MIC of 64 µg/ml) and with an ertapenem susceptible isolate (MIC <1 µg/ml), (Figure 1). Population analysis revealed the existence of an ertapenem resistant subpopulation at a frequency of 1.5x10^-6, demonstrating pre-existence of rare ertapenem resistant subpopulations among ertapenem susceptible isolates that demonstrated resistant MICs at first.

Molecular mechanisms of ertapenem resistance

Analysis of β-lactamases

Seven single clone ertapenem resistant isolates and the two isolates that possessed an inoculum effect with ertapenem were chosen for molecular characterization of the resistance mechanisms involved in ertapenem resistance. All isolates possessed ESBL genes. Seven isolates possessed CTX-M-type (isolates 3, 5, 7, 15, 18 and 160 - CTX-M-2; isolates 5, 7 and 11 - CTX-M-10), four possessed SHV-type (isolates 5 and 7 - SHV-44, isolate 17- SHV-12 and isolate 20- SHV-28); Isolates 5 and 7 possessed TEM-30, and isolate 11 possessed in addition OXA-4. All isolates produced additional β-lactamases such as SHV-1 (5 isolates), TEM-1 (1 isolate), and OXA-2 (4 isolates). Other β-lactamases belonging to Ambler class-A including *bla*<sub>KPC</sub>, class B, or plasmid mediated AmpC or OXA-type carbapenemases were not detected. Crude enzyme preparations from all isolates did not exhibit ertapenem hydrolyzing activity in bioassays, and did not hydrolyze ertapenem and imipenem according to
spectrophotometric assays compare to 46.5 mU/mg (where U = µmol imipenem/min) for control isolate carrying KPC.

**Outer membrane analysis**

OMP analysis was performed on seven stable ertapenem resistant strains, on two susceptible isolates possessing an inoculum effect, and on a *K. pneumoniae* ATCC strain 13883 (Figure 2A). SDS-PAGE revealed the presence of two main OMPs with apparent molecular weights of 35 and 36 kDa in ertapenem susceptible strains, and demonstrated the absence of OMP36 in six of the seven stable ertapenem resistant isolates. Analysis of OMP36 by mass spectrophotometer identified this OMP as OMPK36. OMP35 (with apparent molecular weight of 35kDa) was identified as OMPA. The OMPs profile obtained from two ertapenem susceptible isolates that showed an inoculum effect was similar to ertapenem susceptible isolates (Figure 2A). OMP analysis performed on these two strains after growth in the presence of ertapenem, revealed lack of OMPK36, similar to the OMP pattern of resistant strains, confirming the existence of an ertapenem resistant sub-population in the presence of the antibiotic (Figure 2B).
DISCUSSION

This study describes an occurrence of 3.7% of ertapenem resistance among large collection of ESBL-producing *K. pneumoniae* hospital isolates in Israel. Most of these isolates were susceptible to imipenem and or meropenem. Ertapenem resistance existed in multiple genetic clones of *K. pneumoniae* from various origins and was not due to clonal spread.

Molecular characterization of these ertapenem non-susceptible isolates revealed lack of carbapenemases, and indicated the presence of ESBL genes together with changes in permeability as a result of outer membrane proteins changes. This mechanism was reported earlier in clinical strains of *K. pneumoniae* such as SHV-2 and CTX-M enzymes (14, 17, 37). Moreover it has been shown that other ESBL genes (SHV and CTX-M) as well as β-lactamases such as OXA-2 confer ertapenem resistance when introduced on a plasmid into a cured *K. pneumoniae* strain (18). Our ertapenem resistant strains carried various ESBL genes such as CTX-M-2 (not reported previously), CTX-M-10, SHV-12 and SHV-28 (not reported previously), together with loss of OMPK36. One isolate, isolate 18 (MIC 12 μg/ml), possessed CTX-M-2 but did have OMPK36 (Figure 2A), possibly suggesting other mechanism involved in ertapenem resistance and thus should be further investigated.

Significant discrepancies in ertapenem susceptibility testing using agar-based methods were observed in this study. These discrepancies varied with inoculum size and were found to be related to the existence of ertapenem resistant sub-population that carried various ESBL genes such as CTX-M-2, -10, TEM-30 or SHV-44, and their permeability was affected with ertapenem selective pressure. This is the first study that investigates discrepancies regarding ertapenem susceptibility testing and the first to
demonstrate the effect of ertapenem on the in vitro selection of resistant sub-
populations.

This study defines two appearances of ertapenem resistance in ESBL-producing *K. pneumoniae* isolates: 1. strains who have resistant phenotype under various testing conditions and independently of the inoculum size (2% of the ESBL-producing isolates tested). These strains were ESBL-producers and lacked OMPK36. 2. Strains that were susceptible under standard inoculum, but show an inoculum effect (1.5% of the ESBL-producing isolates tested); these strains were found to have a rare pre-existing resistant sub-population due to combination of common and rare ESBL and OMPK36.

Since ertapenem is increasingly used to treat ESBL infections, these findings should be further explored. While it is clear that the strains that are ertapenem-resistant under various test conditions should be reported as resistant and not be treated with ertapenem, it is not obvious how to report and treat the strains that are susceptible under standard testing conditions, but have rare ertapenem resistant sub-populations (1:1,000,000). Although rare, correct detection of these strains is important. We tried to define phenotypic markers that may assist in differentiating between the inoculum effect isolates and the ertapenem "true resistant" isolates; the later had meropenem MIC 2 \( \mu \text{g/ml} \) or greater, while non of the isolates that possessed an inoculum effect had meropenem MIC 1 \( \mu \text{g/ml} \) or above. Imipenem MIC was not a good indicator for distinguishing between them as all these isolates actually had low imipenem MICs in most cases.

Understanding the clinical significance of these strains is important; ESBL-producers are often multidrug resistant, and if ertapenem can be used to treat these infections erroneous reporting of these isolates as resistant may limit significantly the treatment options, on the other hand, if these strains are more likely to fail ertapenem
treatment, misdetection of this phenotype may have grave results. Further studies including in-vivo experimental modeling using these strains at varying inoculum size with ertapenem therapy may shed light on the clinical implications of these strains.

Our study confirms that among ESBL-producing *K. pneumoniae*, a small (2%) but likely important population of ertapenem resistant strains exists due to ESBL production combined with OMPK36 loss. Another small population (1.5%) has rare pre-existing subpopulation that may lead to false resistant test when high inoculum is used. Diagnostic implications of these findings needs to be resolved, and clinical correlates are warranted.
ACKNOWLEDGEMENTS

This work was supported by a grant from the Public Committee for the Designation of Estate Funds Ministry of Justice State of Israel.
REFERENCES


Table 1 Twenty-five ESBL-producing *K. pneumoniae* isolates included in the study

<table>
<thead>
<tr>
<th><em>K. pneumoniae</em> isolate</th>
<th>Hospital</th>
<th>PFGE clone</th>
<th>Carbapenem MIC testing (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Initial Etest&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>A</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>A''</td>
<td>&gt;32</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>G</td>
<td>32</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>H</td>
<td>16</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>A'</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>B</td>
<td>16</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>J</td>
<td>32</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>G</td>
<td>&gt;32</td>
</tr>
<tr>
<td>11</td>
<td>2</td>
<td>K</td>
<td>16</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>K''</td>
<td>&gt;32</td>
</tr>
<tr>
<td>13</td>
<td>2</td>
<td>K'</td>
<td>32</td>
</tr>
<tr>
<td>14</td>
<td>3</td>
<td>M</td>
<td>6</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>N</td>
<td>12</td>
</tr>
<tr>
<td>16</td>
<td>3</td>
<td>A</td>
<td>16</td>
</tr>
<tr>
<td>17</td>
<td>4</td>
<td>F</td>
<td>&gt;32</td>
</tr>
<tr>
<td>18</td>
<td>4</td>
<td>E</td>
<td>6</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>D</td>
<td>&gt;32</td>
</tr>
<tr>
<td>21</td>
<td>5</td>
<td>A</td>
<td>&gt;32</td>
</tr>
<tr>
<td>22</td>
<td>5</td>
<td>A</td>
<td>&gt;32</td>
</tr>
<tr>
<td>23</td>
<td>5</td>
<td>D</td>
<td>8</td>
</tr>
<tr>
<td>24</td>
<td>5</td>
<td>D</td>
<td>32</td>
</tr>
<tr>
<td>26</td>
<td>5</td>
<td>A</td>
<td>6</td>
</tr>
<tr>
<td>27</td>
<td>5</td>
<td>D</td>
<td>8</td>
</tr>
<tr>
<td>94</td>
<td>6</td>
<td>A</td>
<td>12</td>
</tr>
<tr>
<td>160</td>
<td>6</td>
<td>I</td>
<td>32</td>
</tr>
</tbody>
</table>
a Performed in the initial isolating hospital clinical lab.

b Repeated ertapenem susceptibility testing performed in our lab for all the ertapenem non-susceptible isolates as described in Materials and Methods. MICs presented are the average of three independent measurements.

MIC values in bold designate isolates that changed their ertapenem susceptibility from resistance to susceptible.
Table 2 Summary of ertapenem susceptibility testing

<table>
<thead>
<tr>
<th></th>
<th>Initial Etest</th>
<th>Second Etest</th>
<th>Agar dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>I</td>
<td>R</td>
</tr>
<tr>
<td>No. of isolates</td>
<td>0</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>Range (µg/ml)</td>
<td>-</td>
<td>6</td>
<td>&gt;32</td>
</tr>
<tr>
<td>MIC50 (µg/ml)</td>
<td>-</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td>% of Total(^{a})</td>
<td>0</td>
<td>0.4</td>
<td>3.3</td>
</tr>
</tbody>
</table>

\(^{a}\) Calculated out of 663 ESBL-producing *K. pneumoniae* isolates tested
Table 3 The effect of inoculum size on ertapenem susceptibility testing of 10 ertapenem susceptible strains

<table>
<thead>
<tr>
<th>K. pneumoniae isolate no.</th>
<th>Ertapenem MIC (µg/ml) at the tested inoculum (cfu)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Etest</td>
</tr>
<tr>
<td></td>
<td>$10^5$</td>
</tr>
<tr>
<td>2</td>
<td>0.19</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>0.25</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>0.25</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>14</td>
<td>0.25</td>
</tr>
<tr>
<td>24</td>
<td>0.25</td>
</tr>
<tr>
<td>27</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Note: The values represent the MIC (µg/ml) at the tested inoculum (cfu) for each isolate.
Figure legends

Figure 1

Population analysis of ertapenem sensitive *Klebsiella pneumoniae* isolates with an inoculum effect. The distribution of ertapenem resistance among the population of two *Klebsiella pneumoniae* isolates (strains 5 and 7) with an inoculum effect was studied. Population analysis was performed and compared to an ertapenem resistant *K. pneumoniae* and to an ertapenem susceptible isolate (strain 8) with a low MIC. Population analysis showed pre-existence of rare ertapenem resistant subpopulation in ESBL-producing *Klebsiella pneumoniae* that may lead to discrepancies in ertapenem susceptibility testing.

Figure 2

Outer membrane protein profiles of ertapenem resistant *K. pneumoniae* isolates and ertapenem susceptible isolates (Panel A). OMPK36 was present in ertapenem sensitive isolates (ATCC and in isolates 5 and 7, the two sensitive isolates with a positive inoculum effect). OMPs profile of isolate 7 (possessing an inoculum effect) with and without ertapenem in growth medium demonstrated the lack of OMPK36 in the presence of the antibiotic, similar to the OMP pattern of resistant strains supporting the pre-existence of a resistant subpopulation that remains viable in the presence of ertapenem.
FIGURE 1

Ertapenem mg/l

log CFU/ml

strain 5 MIC 0.25 + inoculum effect
strain 7 MIC 0.25 + inoculum effect
strain 490 MIC 64 no inoculum effect
strain 8 MIC<1 no inoculum effect
FIGURE 2

A

OMPA
OMPK36

Ertapenem-S

Ertapenem-R

B

7+ERT
7

Ertapenem-S with an inoculum effect