Study of *OmpK35* and *OmpK36* Expression in Carbapenem Resistant ESBL Producing Clinical Isolates of *Klebsiella pneumoniae*

Amina Amal Mahmoud Nour El Din, Reem Abdel Hameed Harfoush, Hadir Ahmed Said Okasha, Dina Aly El Sayed Kholeif

Department of Medical Microbiology and Immunology, Faculty of Medicine, Alexandria University, Alexandria, Egypt

Email: hadir.okasha@alexmed.edu.eg

Received 18 July 2016; accepted 8 August 2016; published 11 August 2016

Copyright © 2016 by authors and Scientific Research Publishing Inc.
This work is licensed under the Creative Commons Attribution International License (CC BY).

http://creativecommons.org/licenses/by/4.0/

Abstract

Background: Carbapenem resistant extended spectrum β-lactamase (ESBL) producing *Klebsiella pneumoniae* (*K. pneumoniae*) is increasing worldwide. Carbapenem resistance (CR) has been attributed not only to production of carbapenemases but also to permeability barriers due to outer membrane proteins (*OmpK35* and *OmpK36*) disruption. Objective: Phenotypic detection of CR among ESBL producing *K. pneumoniae* isolates, followed by the evaluation of the role of *ompK35* and *ompK36* gene expression among carbapenem resistant *K. pneumoniae* (CR-KP) isolates. Methods: 100 ESBL producing *K. pneumoniae* isolates were included in this study. Minimum inhibitory concentration (MIC) of imipenem was performed for all isolates by broth microdilution method. For CR-KP isolates, phenotypic detection of *K. pneumoniae* carbapenemase (KPC), metallo-β-lactamase (MBL) and AmpC enzymes was performed followed by Realtime qRT-PCR to detect and quantify *ompK35* and *ompK36* gene expression. Results: 42% of our isolates were carbapenem resistant, and all of them were KPC producers either singly or in combination with MBL and/or AmpC production. Reduced expression of both *ompK35* and *ompK36* was detected in (52.38%) of CR-KP isolates, while reduced expression of *ompK36* or *ompK35* alone was found in (2.38%) and (33.33%) respectively. Twenty of 42 CR-KP isolates (47.62%), showing reduced *ompK35* and *ompK36* expression, exhibited high level resistance (HLR) (>32 µg/ml) to imipenem. There was a significant correlation between reduced expression of *ompK36* and increase MIC values (*p* < 0.05). The combined production of MBL or AmpC together with reduced expression of *ompK36* and/or *ompK35* resulted in significant increase in imipenem MIC (*p* < 0.05). Conclusion: The combined *OmpK35/OmpK36* loss resulted in HLR. However *OmpK36* seems to play a major role in those strains. Imipenem MIC was markedly increased among *K. pneumoniae* showing carbapenemase and/or AmpC production together with loss of *OmpK35* and/or *OmpK36*.

Keywords
OmpK35, OmpK36, K. pneumoniae, Carbapenem Resistant

1. Introduction

Carbapenems are widely used in the treatment of extended spectrum \(\beta\)-lactamase (ESBL) producing *Klebsiella pneumoniae* (*K. pneumoniae*). However, as carbapenems are more frequently utilized, an increasing number of carbapenem resistant *K. pneumoniae* (CR-KP) has been observed worldwide. The emergence of CR-KP is alarming, as antimicrobial treatment options are limited [1].

Carbapenem resistance (CR) in *K. pneumoniae* has been attributed mainly to enzymatic degradation by carbapenemases namely class A *Klebsiella pneumoniae* carbapenemase (KPC), class B metallo-\(\beta\)-lactamase (MBL) or class D oxacillinases (OXA) [2]. However, other mechanisms for CR in *K. pneumoniae* exist. These include the combination of outer membrane porin expression disruption and production of \(\beta\)-lactamases that have weak carbapenemase activity such as AmpC among the strains [3] [4].

Two major porins, namely OmpK35 and OmpK36 have been described in *K. pneumonia* [5]. Loss of outer membrane proteins (OMPs), including OmpK35 and OmpK36 plays a role in CR in *K. pneumoniae* strains acting as a permeability barrier. As a result, carbapenems reach low concentrations in the periplasmic space and their activity may be then compromised by enzymes with carbapenemase activity, leading to CR [6] [7]. Moreover, recent studies emphasize the importance of characterization of porin expression in KPC producing *K. pneumoniae* as this identifies isolates which are most susceptible to the combination of colistin and carbapenems for treatment, and indicating that in the future, molecular characterization of CR-KP isolates might be a practical tool for identifying effective antimicrobial combination regimens [8] [9].

The aim of this study was to phenotypically detect CR among ESBL producing *K. pneumoniae*, followed by the evaluation of the role of *ompK35* and *ompK36* gene expression among CR-KP isolates.

2. Materials and Methods

2.1. Bacterial Isolation

A total of 100 *K. pneumoniae* isolates were collected from clinical specimens delivered to the Microbiology lab of Alexandria main university hospital (AMUH). The isolates were identified using standard biochemical reactions [10] and verified using API 20 E system (bioMerieux, Marcy l’Etoile, France). Phenotypic detection of ESBLs was performed as guided by CLSI (2014) [11]. The organism was considered ESBL producer when the inhibitory zone of ceftazidime was ≤22 mm, ceftriaxone was ≤25 mm, aztreonam ≤27 mm and when there was a difference of ≥5 mm between the zones of ceftazidime and ceftazidime-clavulanic acid discs [11].

2.2. Detection of CR

Minimum inhibitory concentration (MIC) of imipenem was performed for all isolates by broth microdilution method, and the results were interpreted according to the CLSI breakpoints (sensitive ≤1 μg/mL, intermediate =2 μg/mL and resistant ≥4 μg/mL) [11] [12].

2.3. Phenotypic Detection of Carbapenemases

Modified Hodge test (MHT) was performed according to CLSI guidelines [11]. Class A and B carbapenemases were further identified using carbapenemase inhibition tests (the combined disk method). For class A, KPC enzyme production was suspected when there is ≥5 mm increase of inhibition zone diameter around imipenem/phenylboronic acid disk (10 μg/400 μg) than imipenem disk (10 μg) [13]. For class B, MBL detection is based on the synergy between MBL inhibitor (EDTA) and imipenem. This was achieved when there was a ≥7 mm increase of inhibition zone diameter around imipenem/EDTA (10 μg/750 μg) than imipenem disk (10 μg) [14].

2.4. Phenotypic Detection of AmpC Enzyme

Done according to Rand et al. [15] by Modified Hodge test using cefoxitin. Briefly, a cefoxitin-susceptible 0.5
McFarland adjusted *E. coli* indicator strain (ATCC 25922) was inoculated on Mueller Hinton agar plate and the cefoxitin disk was placed in the center of the plate. The test organism (*K. pneumoniae*) was streaked from the edge of the cefoxitin disk to nearly the edge of the plate. If the test organism expresses AmpC, it hydrolyzes the cefoxitin and shows growth of the indicator *E. coli* along the intersection of the streak and the zone of inhibition from the cefoxitin disk.

2.5. Analysis of *ompK35* and *ompK36* Gene Expression

Total RNA was obtained from 1 ml (about 10^8 cells/ml) of mid-logarithmic growth-phase bacterial cultures with ISOLATE II RNA Mini kit (Bioline Ltd, UK) [16]. Reverse transcription and Real-time PCR were performed with the SensiFAST™ SYBR No-ROX One-Step Kit (Bioline Ltd, UK) using StepOne™ Real Time PCR (Applied Biosystems, Foster, CA, USA). The qPCR reactions were performed in a 20 μL final volume containing the selected primers for *ompK35*, *ompK36* and *rpoB* (housekeeping gene) (Table 1). The reaction conditions consisted of initial denaturation at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C, 11 s at 54°C and 22 s at 72°C [8]. The expression levels were expressed in cycle threshold (*Ct*) units. Expression of each gene was normalized to that of the housekeeping gene *rpoB*. The relative expression of *ompK35* and *ompK36* genes was compared to that of a control isolate that is known to express both porins (*K. pneumoniae* ATCC 13883). To guarantee the specificity of the amplification products, a melting curve analysis was performed. No amplification of unspecific products was observed.

2.6. Ethics

This study was approved by the Ethical Review Committee, Faculty of Medicine, Alexandria University, Egypt.

2.7. Statistical Analysis

Data were fed to the computer using IBM (NY, USA) SPSS software package version 20.0. Qualitative data were described using number and percent. Quantitative data were described using mean and standard deviation. For normally distributed data, comparison between two independent populations was done using Student’s t-test. Correlations between two quantitative variables were assessed using Pearson coefficient. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

3. Results

In the present study, the highest isolation of ESBL producing *K. pneumoniae* isolates was from urine specimens (33%), followed by sputum and bronchoalveolar lavage (BAL) specimens (31%), then pus specimens (28%). The lowest isolation percentage was from blood and aspirated fluids (7% and 1%) respectively.

In this study, CR was detected in 42% of the 100 ESBL producing *K. pneumoniae* isolates where 12 isolates (12%) exhibited low level resistance (LLR) (≥4 - 32 µg/ml) and 30 isolates (30%) exhibited high level resistance (HLR) (>32 - 512 µg/ml).

Out of the 42 CR-KP strains, 20 (47.62%) were isolated from ICUs, 12 (28.57%) from surgical wards and 10 isolates (23.81%) from internal medicine wards. The highest isolation was observed among sputum and BAL specimens, where 16/42 isolates (38.1%) were identified, followed by urine and pus specimens (12/42, 28.57%) each, and blood culture specimens (2/42, 4.76%).

### Table 1. Nucleotide sequence of primers used for real time quantitative PCR analysis of mRNA expression of *ompK35* and *ompK36* genes [8].

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ompK35</em></td>
<td>Forward</td>
<td>GCAATATTCTGGCAGTGGTGATC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACCATTTTTCATAGAAGTCCAGT</td>
</tr>
<tr>
<td><em>ompK36</em></td>
<td>Forward</td>
<td>TTAAAGTACTGTCCCTCCTCTGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCAGAGAAGTAGTGCCAGACCGTCA</td>
</tr>
<tr>
<td><em>rpoB</em></td>
<td>Forward</td>
<td>AAGGCAGATCCAGCTTGTCAGC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGACGTTGATCTGTCACCACCATCA</td>
</tr>
</tbody>
</table>
By the phenotypic detection methods, all 42 isolates were KPC producers whether singly in 9/42 isolates or in combination with other enzymes: [KPC/AmpC] and [KPC/AmpC/MBL] in 13/42 isolates for each, and [KPC/MBL] in 7/42 isolates.

The analysis of expression levels of \(ompK35\) and \(ompK36\) genes by real time qRT-PCR showed that among the 42 isolates, only 5 isolates (11.91%) showed normal expression of both porins (OmpK35 and OmpK36), one isolate (2.38%) showed reduced expression of \(ompK36\) only, 14 (33.33%) showed reduced expression of \(ompK35\) only while reduced expression of both \(ompK35\) and \(ompK36\) occurred in a significant number of isolates (22/42, 52.38%) \((p = 0.011)\).

When comparing the MIC results with the expression levels of porins, among the 30 HLR isolates, 20 isolates exhibited reduced expression of both \(ompK35\) and \(ompK36\) and 9 isolates exhibited reduced expression of \(ompK35\) only and this was statistically significant \((p < 0.05)\).

Meanwhile, among the 12 LLR isolates, 5 isolates showed normal expression of both porins, 5 isolates showed reduced expression of \(ompK35\) alone while 2 isolates only showed reduced expression of both \(ompK35\) and \(ompK36\).

To study the contribution of reduced expression of these porins to the increase of imipenem MIC values, the correlation between \(ompK35/ompK36\) genes expression and MIC values was analyzed. Our data demonstrated a significant correlation between reduced expression of \(ompK36\) and increased MIC values, while this correlation could not be established between reduced expression of \(ompK35\) and high MIC values (Figure 1 and Figure 2).

Our data demonstrated that the combined effect of MBL production together with reduced expression of \(ompK35\) and \(ompK36\) resulted in significant increase in imipenem MIC. Furthermore, the combined effect of AmpC production together with reduced expression of \(ompK35\) resulted in significant increase in imipenem MIC (Table 2).

4. Discussion

CR is a major threat to the antimicrobial treatment of infections with multidrug resistant organisms. One report cites that these resistant strains can contribute to death in up to 50% of patients who become infected. The optimal treatment of these infections is not well established and the clinical outcome data remain sparse [17].

![Figure 1](image-url)
The present study demonstrated that (42%) of ESBL producing *K. pneumoniae* isolates were CR-KP. Similar and variable results were detected by different studies [18]-[20]. These discrepant results might be attributed to the different antibiotic susceptibility pattern of *K. pneumoniae* isolated all over the countries and the diverse antibiotic policies that may aid in selection of certain antibiotic resistant pathogen than another. The high prevalence of CR-KP in the current study may be attributed to the excessive empirical use of carbapenems in our hospital for infections with multidrug resistant pathogens.

In this study, the highest percentage of CR-KP were isolated from ICUs, similar to a study by Carrér *et al.* [21] and Arnold *et al.* [22]. This can be attributed to the nature of patients admitted to this section; many of our patients were immunocompromised, subjected to invasive procedures and devices, complicated or end-stage cases referred from other hospital wards, where they had prolonged and numerous antibiotic therapies, with long periods of hospital stay.

Carbapenem resistance in *K. pneumoniae* has been attributed also to enzymatic degradation. In the current study, all 42 isolates were KPC producers. Our result was concordant with previous studies that revealed high
percentage of KPC production among their isolates [23]-[25]. This high percentage can be explained by the great potential for spread of this enzyme due to its location on plasmids in CR-KP strains [18].

The combined production of KPC/MBL/AmpC was observed among our isolates. Co-production of [KPC and MBL] was detected among 7/42 isolates (16.67%) and [KPC, MBL and AmpC] among 13/42 isolates (30.95%).

Several Egyptian studies described the combined carbapenemase production [18] [26]. Hospital infections caused by K. pneumoniae co-producing KPCs and MBLs have also been described in other regions of the world [27].

OmpK35 and OmpK36 are the major outer membrane porins of K. pneumoniae. In this study, the expression of both porins genes has been estimated to evaluate their role in CR. The results showed a reduced expression of both ompK35 and ompK36 in a significant number of isolates (52.38%) while the rest of the isolates had either reduced expression of ompK36 only (2.38%) or ompK35 only (33.33%) or normal expression of both porins in (11.91%).

These results were similar to previous studies by Hashemi et al. [28], Ma et al. [29], Ruiz [30] and Findlay et al. [31] who also reported defects in OmpK35 and/or OmpK36 production in CR-KP isolates.

As regards the carbapenem resistance level in correlation to porins expression, our findings revealed that loss of both porins contributed to HLR and clearly agree with previous studies by Davies et al. [32], Kitchel et al. [33] and Clancy et al. [9].

To elucidate the contribution of porins in CR, studies by Tsai et al. [34] and Doumith et al. [4] noted that a double deletion of ompK35 and ompK36 genes (ΔompK35/36) led to a marked increase in the MIC of meropenem and ertapenem respectively and the increased MIC could be restored to the original levels following complementation by plasmids carrying ompK35 and ompK36 genes.

Furthermore, we demonstrated a significant correlation between reduced expression of ompK36 and increased MIC values of imipenem while we failed to do so for of ompK35. These observations coincide with the findings previously published by Palasubramaniam et al. [35], Landman et al. [36] and Netikul et al. [37]. Several genetic studies also highlighted the role of OmpK36 in CR; according to Tsai et al. [34], the deletion of ompK36 gene resulted in greater drug resistance compared to the deletion of ompK35 thus, the emergence of clinical antibiotic resistance due to OmpK36 loss was predicted. Meanwhile, Clancy et al. [9] noted that a certain mutation in ompK36 gene was an independent predictor for HLR to meropenem.

In the present study, CR was attributed to the combined effect of enzymes production together with loss of OmpK35 and/or OmpK36. From previous reports, we know that KPC production combined with porins loss can result in higher carbapenem MIC [33] [36].

Our data also demonstrated that the combined production of MBL together with reduced expression of ompK35 and ompK36 resulted in significant increase in imipenem MIC. These findings were compatible with those obtained by Sho et al. [38] and Chen et al. [39]. Moreover, the production of AmpC together with reduced expression of ompK35 resulted in significant increase in imipenem MIC in accordance with previous studies by Palasubramaniam et al. [35] and Shin et al. [7].

The current study and those cited emphasize the importance of characterization of porin expression in CR-KP, as referred by Hong et al. [8] and Clancy et al. [9] who documented that screening for porin expression identifies CR-KP which are most susceptible to colistin/carbapenems combination in attempt for treatment.

5. Conclusion

The high percentage of CR calls for more rigid application of infection control measures and establishing strict antibiotic policies mainly in ICUs. Our data revealed an interplay between enzymes production and reduced expression of ompK35 and/or ompK36 that affected the CR level and hence the options of treatment available including combination therapies. It is mandatory to correctly identify patients for whom combination antimicrobial therapies are most likely to be effective based on the molecular biology of specific isolates, types of infection to be treated, underlying diseases and other host factors in order to both optimize the outcomes and minimize the potential for toxicity.

References

http://dx.doi.org/10.1073/pnas.1501049112

http://dx.doi.org/10.1128/JCM.00827-10


Submit or recommend next manuscript to SCIRP and we will provide best service for you:

Accepting pre-submission inquiries through Email, Facebook, LinkedIn, Twitter, etc.
A wide selection of journals (inclusive of 9 subjects, more than 200 journals)
Providing 24-hour high-quality service
User-friendly online submission system
Fair and swift peer-review system
Efficient typesetting and proofreading procedure
Display of the result of downloads and visits, as well as the number of cited articles
Maximum dissemination of your research work

Submit your manuscript at: http://papersubmission.scirp.org/