

Original Research Article

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## Outer Membrane Proteins as a Mechanism of Resistance in Multidrug-Resistant *Klebsiella* Clinical Isolates

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### ABSTRACT

#### Keywords

Outer membrane proteins, Multidrug-resistant (MDR) *Klebsiella* isolates, Minimal inhibitory concentrations (MICs), PAGE.

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This study was designed to investigate the role of the outer membrane proteins as a mechanism of resistance in *Klebsiella* isolates collected from Tanta university Hospital in Tanta, Egypt and to correlate between the Outer membrane proteins (OMPs) profiles and the resistance patterns of the tested isolates. The outer membrane of gram-negative bacteria plays a significant role in a variety of functions and is composed of a bilayer containing phospholipids, lipopolysaccharide and outer membrane proteins (OMPs). Two major non-specific porins, namely Omp K35 and Omp K36 have been described in *Klebsiella pneumoniae*. A total of 13 multidrug-resistant (MDR) *Klebsiella* isolates were obtained. Antibiotic susceptibility test was performed by disk diffusion method. Minimal inhibitory concentrations (MICs) were determined by broth microdilution method for the selected antibiotics. Analysis of the outer membrane profile was performed on 12% separating acrylamide gel and vertical polyacrylamide gel electrophoresis (PAGE) apparatus. It was found that The relation between the resistance patterns and OMPs profiles of the tested isolates was difficult to be determined as strains having the same resistance pattern may exhibit different OMPs profiles and vice versa

### Introduction

The outer membrane of gram-negative bacteria plays a significant role in a variety of functions and is composed of a bilayer containing phospholipids lipopolysaccharide and outer membrane proteins (OMPs). One family of OMPs the porins, are present in large amounts in the outer membrane and form water filled channels that permit the diffusion of small hydrophilic solutes across the membrane [1].

Two major non-specific porins, namely Omp K35 and Omp K36 have been described in *K.pneumoniae*. Omp K35 and Omp K36,

porins were found to be the homologues of porins OmpF and OmpC from *E.coli* respectively. OmpK36 porins allow the diffusion of a wide variety of molecules, including bacterial nutrients and antimicrobials [2, 3].

Alterations in the composition and structure of outer membrane alter antibiotic permeation leading to drug resistance. Numerous outer membrane changes have been correlated with antibiotic resistance including porin-deficiency and lipopolysaccharide (LPS) structural changes [4]. In some instances at

least, it has been argued that the outer membrane barrier alone is not sufficient to provide substantial resistance to antimicrobial agents [4]. Loss of porins in *K. pneumoniae* strains producing extended-spectrum  $\beta$ -lactamases (ES $\beta$ Ls) has been shown to cause resistance to ceftazidime, increased resistance to third-generation cephalosporins and monobactams, and decreased susceptibility to fluoroquinolones [5].

## Materials and Methods

### Isolation and identification of clinical isolates

A total of 13 MDR (multidrug-resistant) *Klebsiella* isolates were obtained from patients in different departments including Neonatology, oncology and ICU (intensive care units) admitted to Tanta University Hospital, Tanta, Egypt.

Clinical specimens including urine, blood and tracheal aspirates were examined and identified by standard microbiological procedures and biochemical reactions [6, 7].

### Reference Strains

*Escherichia coli* strain ATCC® 25922™ (American Type culture collection, USA) was given by NAMRU -3 in Cairo to the Microbiology Diagnostic and Infection Control Unit (MDICU) in the department of Medical Microbiology and Immunology, Faculty of Medicine, Mansoura University.

### Antimicrobial susceptibility testing

Susceptibilities of the isolates were tested against 23 antimicrobials of different classes using the Kirby–Bauer method as recommended on Mueller–Hinton agar with commercially available antimicrobial disks (Oxoid, UK), according to Clinical Laboratory Standards Institute [8, 9].

### Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations of 23 imipenem resistant isolates were determined by broth microdilution method against 6 antimicrobial agents. Pure forms of 11 antibiotics were used (Manufacturers: E.I.P.I.CO, CID and Pharco B International, Egypt).

The scheme for preparing dilutions of antimicrobial agents and the methodology was according to CLSI guidelines [9].

### ESBL detection by Double Disc Diffusion Synergy Test (DDST)

Isolates with resistance to any of the 3 GC (Third generation cephalosporins) were subjected to the standard DDST [10]. Disks containing (30 $\mu$ g) of aztreonam, ceftazidim, cefepime, ceftriaxone and cefotaxime were placed around a disk of AMC (20  $\mu$ g of amoxicillin plus 10  $\mu$ g of clavulanate) in a distance of 30 mm center to center.

### Analysis of OMPs

The outer membrane proteins (OMPs) of all the recovered *Klebsiella* isolates were prepared and the protein contents were analyzed as described by El-Banna *et al.*, [11].

The OMPs were obtained after treatment of cell membranes recovered by ultracentrifugation using High intensity ultrasonic processor (Cole Parmer, USA) with Sodium dodecyl sulphate (SDS) (Sigma, USA). Analysis of the outer membrane profile was performed on 12% separating acrylamide gel and one dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were visualized by staining with Coomassie Brilliant Blue R-250 (CBBR-250) (Sigma, USA).

## Results and Discussion

A total of 13 MDR *Klebsiella* isolates (4 urine, 5 blood, 1 ETT and 1 wound, 2 unknown) were obtained from different department of Tanta University Hospital. Out of them only 2 isolates (15%) were *Klebsiella oxytoca* and the rest 11 isolates (85%) were *Klebsiella pneumoniae*. The antimicrobial resistance patterns and MIC of the examined isolates were illustrated in tables 1 and 2.

All of 13 *Klebsiella* isolates were (multidrug-resistance) MDR, all (100%) of the isolates were resistant to Ampicillin, Amoxicillin, Cephadrine, Cefaclor, Cefotaxime and Cefoperazon and all of them were sensitive to Imipenem. The phenotypic screening of ESBLs by double disc diffusion synergy test showed that 13 (100%) of the obtained *Klebsiella* isolates were ESBLs producers.

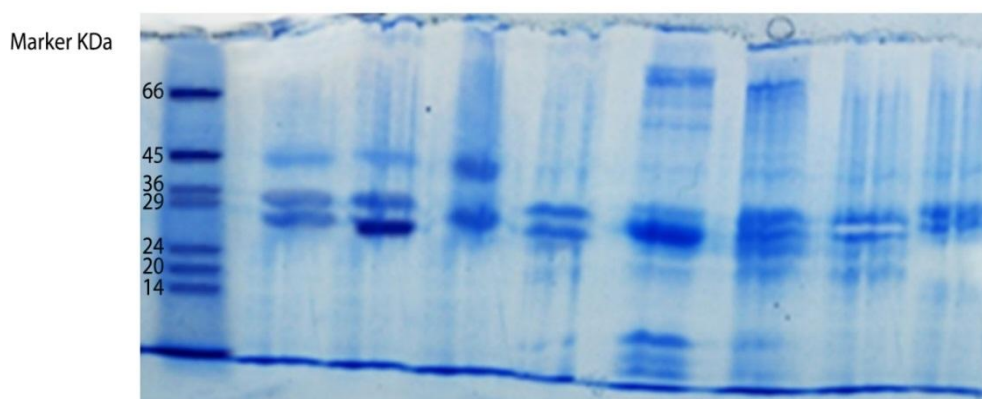
The outer membrane proteins (OMPs) of the 13 *Klebsiella* isolates were prepared. Polyacrylamide gel electrophoresis of the protein contents was performed. Representative photos are shown in figures 1

and 2. Visual examination of the electropherograms revealed that different protein bands at molecular weights of 66 to 14 KDa can be detected, overproduced or lost in the different electropherograms obtained. Proteins with molecular weights of 45 KDa and 36 KDa represent major proteins as they were detected in almost all the tested isolates.

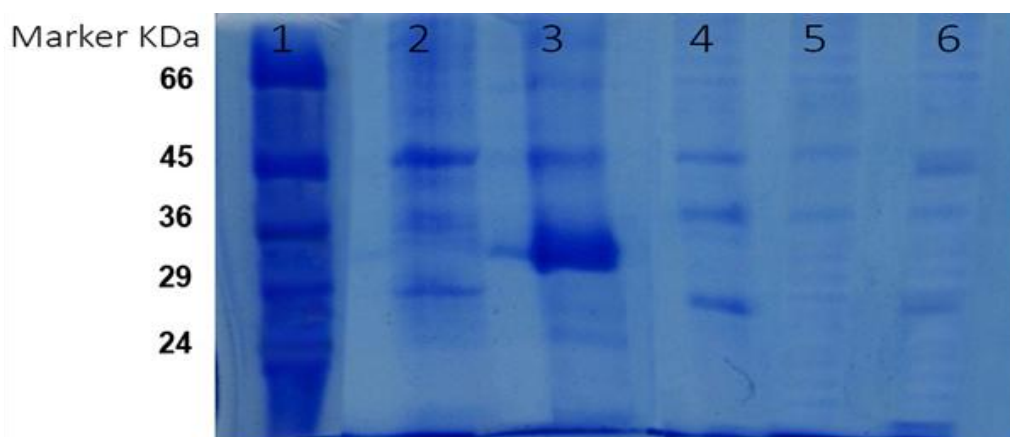
The relation between the resistance patterns and OMPs profiles of the tested isolates was difficult to be determined as strains having the same resistance pattern may exhibit different OMPs profiles and vice versa. The presence, absence or overproduction of each of the major proteins differs among the isolate as shown in table 3.

All of our isolates were MDR isolates and all of them produce ESBLs. This is in accordance with what was reported by Lopes *et al.*, [12] that *Klebsiella pneumoniae* isolates were resistant to many antibiotics mainly third generation cephalosporins and penicillins due to acquisition of plasmids that harbor genes codes for the production of extended-spectrum  $\beta$ -lactamases.

**Fig.1** SDS-Polyacrylamide gel of the outer membrane proteins of selected ESBLs producing *Klebsiella* isolates, lane 1: protein molecular weight marker; lane 2: K22; lane 3: K11; lane 4: K16; lane 5: K5; lane 6: K3; lane 7: K23; lane 8: K52 lane 9: K55



**Fig.2** SDS-Polyacrylamide gel of the outer membrane proteins of selected ESβLs producing *Klebsiella* isolates, lane 1: protein molecular weight marker; lane2:K67; lane3:K34; lane 4:K401; lane 5: K202; lane 6: K36



**Table.1** The site and Resistance pattern patterns of the examined *Klebsiella* isolates

| Isolate code | Site              | Resistance pattern   |
|--------------|-------------------|--|
|              | Department        |  |
| K22          | Urine-Oncology    | AM,AMOX, AMC,PRL, CE,CEC, CTX,CFP, CAZ, FEP,NA,OFX, CIP,LEV, TE, C, SXT,S,E, GN,TOB, AK. |
| K11          | Blood-Neonatology | AM,AMOX,PRL, CE,CEC, CTX, CAZ,CFP,FEP,NA,OFX CIP,LEV, TE, E,TOB, GN                      |
| K16          | Urine-Oncology    | AM,AMX,PRL, CE,CEC, CTX, CAZ, CFP,NA,OFX, CIP,LEV, C, E, SXT,S, TE,TOB, GN.              |
| K5           | ND                | AM,AMOX,PRL, CE,CEC, CTX, CAZ,CFP,FEP,NA,OFX, CIP,LEV, C, E, SXT,S, TE,TOB, GN.          |
| K3           | Urine-Oncology    | AM, AMX,PRL, CE,CEC, CTX, CAZ,CFP,C,E,SXT,S,TE,TOB,NA,OFX,CIP,LEV.                       |
| K23          | ETT-ICU           | AM,AMOX,PRL, CE,CEC, CTX,CAZ, CFP,NA,OFX,CIP,LEV, C, TE, TOB, GN.                        |
| K52          | Blood-Neonatology | AM,AMOX,PRL, CE,CEC, CTX, CAZ,CFP,NA,OFX, CIP,LEV, E,SXT, TE, GN,S.                      |
| K55          | Urine-Oncology    | AM, AMOX,AMC,PRL, CE,CEC,CFP, CTX, CAZ, FEP,NA,OFX,CIP,LEV, TE, C, SXT,S, E, GN.         |
| KAE          | Blood-Neonatology | AM,AMOX,AMC,PRL, CE,CEC, CTX, CAZ,CFP,FEP,NA,OFX, CIP,C, E, SXT,TE, GN,TOB, AK           |
| K34          | Wound infection   | AM,AMOX,PRL, CE,CEC,CTX,CFP,NA,OFX, CIP,LEV, C, E, SXT, S,GN.                            |
| K401         | Blood-Neonatology | AM,AMOX,PRL, CE,CEC, CTX,CFP,TE, C, E,S,TOB, GN  |
| K202         | Blood-Neonatology | AM, AMOX,AMC,PRL, CE,CEC, CTX, CAZ,CFP, FEP,C, TE, E,GN,TOB.                             |
| K36          | ND                | AM,AMOX, PRL, CE,CEC, CTX,CFP, NA,OFX, CIP,LEV,C, TE, SXT, E,GN,TOB.                     |

Note:AM:Ampicillin,AMX:Amoxicillin, AMC: Amoxicillin-Clavulante, PRL: Piperacillin, CE: Cephradine, CEC: Cefaclor, CTX: Cefotaxime,CAZ: Ceftazidime, CFP: Cefoperazon FEP:Cefepime, IMP: Imipenem, AK: Amikacin, GN:Gentamycin, S:Streptomycin, TOB:Tobramycin, NA:Nalidixic acid, CIP:Ciprofloxacin OFX:Ofloxacin, LEV: levofloxacin, SXT:Cotrimoxazol, TE:Tetracyclin, E: Erythromycin, C: Chloramphenicol.

NK: Not Known

ETT-ICU: Endotracheal Tube – Intensive Care Unit.

**Table.2** The MIC values of the examined *Klebsiella* isolates

| Code        | Levo | Moxi | Gemi | Gati | CIP   | GN    | AK    | CFP   | CAZ  | CTX   | FEP   |
|-------------|------|------|------|------|-------|-------|-------|-------|------|-------|-------|
| <b>K22</b>  | 32   | 64   | 256  | 64   | 512   | >1024 | >1024 | >1024 | 256  | >1024 | 256   |
| <b>K11</b>  | 32   | 128  | 128  | 128  | 32    | 64    | -     | 512   | 256  | 512   | >1024 |
| <b>K16</b>  | 64   | 128  | 128  | 128  | 128   | 128   | -     | 128   | 64   | 256   | -     |
| <b>K5</b>   | 16   | 64   | 32   | 32   | 128   | >1024 | -     | >1024 | 128  | 1024  | >1024 |
| <b>K3</b>   | 16   | 64   | 32   | 32   | 256   | -     | -     | >1024 | 256  | >1024 | -     |
| <b>K23</b>  | 32   | 128  | 16   | 8    | 64    | 512   | -     | >1024 | 256  | >1024 | -     |
| <b>K52</b>  | 16   | 128  | 64   | 32   | 128   | 256   | -     | 512   | 64   | 256   | -     |
| <b>K55</b>  | 128  | 128  | 128  | 128  | >1024 | >1024 | -     | 1024  | 256  | >1024 | >1024 |
| <b>KAE</b>  | -    | 16   | 8    | 64   | 128   | >1024 | 128   | >1024 | 1024 | >1024 | 1024  |
| <b>K401</b> | 32   | 128  | 128  | 128  | -     | >1024 | -     | 1024  | -    | 128   | -     |
| <b>K202</b> | -    | 256  | 8    | -    | 16    | >1024 | -     | >1024 | 1024 | >1024 | 128   |
| <b>K36</b>  | 16   | 256  | 64   | 16   | 64    | 256   | -     | 1024  | -    | 128   | -     |

Note: Levo: Levofloxacin, Moxi: moxifloxacin, Gemi: gemifloxacin, Gati: gatifloxacin, CIP: Ciprofloxacin, GN: Gentamycin, AK: Amikacin, CFP: Cefoperazon, CAZ: Ceftazidime, CTX: Cefotaxime, FEP: Cefepime.

**Table.3** The outer membrane protein patterns in the examined *Klebsiella* isolates

| Isolate code | OMPs changes |    |    |    |    |    |    | Pattern code |
|--------------|--------------|----|----|----|----|----|----|--------------|
|              | 66           | 45 | 36 | 29 | 24 | 20 | 14 |              |
| <b>K22</b>   | -            | +  | +  | +  | -  | -  | -  | <b>I</b>     |
| <b>K11</b>   | -            | +  | +  | ++ | -  | -  | -  | <b>II</b>    |
| <b>K16</b>   | -            | ++ | -  | ++ | -  | -  | -  | <b>III</b>   |
| <b>K5</b>    | -            | -  | +  | +  | -  | -  | -  | <b>IV</b>    |
| <b>K3</b>    | +            | -  | +  | ++ | -  | -  | +  | <b>V</b>     |
| <b>K23</b>   | +            | -  | +  | +  | -  | -  | +  | <b>VI</b>    |
| <b>K52</b>   | -            | -  | +  | +  | -  | -  | -  | <b>IV</b>    |
| <b>K55</b>   | -            | -  | +  | +  | -  | -  | -  | <b>IV</b>    |
| <b>K67</b>   | -            | +  | +  | +  | -  | -  | -  | <b>I</b>     |
| <b>K34</b>   | +            | +  | -  | ++ | -  | -  | -  | <b>VII</b>   |
| <b>K401</b>  | -            | +  | +  | +  | -  | -  | -  | <b>I</b>     |
| <b>K202</b>  | -            | -  | -  | -  | -  | -  | -  | <b>VIII</b>  |
| <b>K36</b>   | -            | -  | -  | -  | -  | -  | -  | <b>VI</b>    |

-absent, + present, ++overproduced

Gram negative bacterial outer membranes are poorly permeable to both hydrophobic and hydrophilic molecules. So, most antimicrobial agents other than  $\beta$ -lactam must cross the membrane in order to reach their intracellular drug targets and so require the presence of porin to bypass the asymmetric bilayer of phospholipid and lipopolysaccharide

membrane [13]. Consequently, loss of porins ompK 35 and ompK 36 led to an increase in carbapenem, ciprofloxacin, and chloramphenicol resistance [14].

Porins are outer membrane proteins (OMPs) that allow the nonspecific diffusion of small molecules into the bacterial cell. Most of the



studies about OMPs have been carried out with *Escherichia coli*, in which two major porins (OmpC and OmpF) have been characterized. Loss of either of them has been related to antibiotic resistance [15]. Decreased permeability can produce significant levels of resistance that may be increased when it is combined with enzymatic inactivation [15]. In *K. pneumoniae*, two main porins have been characterized: OmpK35 (the homolog of OmpF) and OmpK36 (the homolog of OmpC) [3, 16].

Recently, loss of the OmpK36 porin has been associated with both ceftazidime resistance and increases in cephalosporin and quinolone MICs [5].

In our study the OMPs of the ESBLs-producing *Klebsiella* isolates were studied. The electrophoretic profile of the selected isolates showed the presence of different protein bands in the electropherograms of the tested isolates. These bands have molecular weights from 66 to 14 KDa. Proteins with molecular weights of 36 and 29 KDa represent major proteins as they were detected in almost all the tested isolates.

In our work we cannot correlate between the OMPs profiles and the resistance patterns of the tested isolates as isolates which had more protein bands not differ greatly in resistance pattern from other isolates which had less or no protein bands.

This finding is similar to other reports [5,17] which reported that the specific contribution of alterations in porin expression and the resistance patterns were difficult to determine, because other mechanisms of resistance are commonly present in porin-deficient strains (such as production of  $\beta$ -lactamases or aminoglycoside-modifying enzymes, modified topoisomerases or energy-dependent efflux systems).

## Conflict of Interest

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

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