Role of outer membrane proteins in virulence of Klebsiella ozaenae and antibiotic sensitivity

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Abstract. In the present study, the association between outer membrane proteins loss, virulence and antibiotic sensitivity was investigated in K. ozaenae. An outer membrane proteins deficient K. ozaenae was derived from local clinical K. ozaenae isolate. Virulence was assessed by comparing the clearance of viable complete K. ozaenae with that outer membrane proteins deficient K. ozaenae in the spleen, liver, appendix, and blood of injected laboratory mice. Antibiotic susceptibility was tested by disc diffusion method. It was recorded significant differences at a P value of 0.05 between the logarithm of the viable number of the complete K. ozaenae and logarithm of the viable number of outer membrane proteins deficient K. ozaenae that retrieved from the spleen, liver, appendix, and blood. It was observed that the logarithm of the viable number of complete K. ozaenae increased gradually during 18, 24, 48 and 72 hours compared with the logarithm of the viable number of outer membrane proteins deficient K. ozaenae which increases during 18-24 hours, and then decreased in all organs. Susceptibility tests showed that complete K. ozaenae isolate was resistant to most used antibiotics including amoxicillin, cephalothin, ceftazidime, cefotaxime, amikacin, tetracycline, nalidixic acid, erythromycin, trimethoprim and sensitive to only imipenem and gentamicin. Whereas outer membrane deficient K. ozaenae isolate was resistant to all antibiotics used in our study.

Keywords: Klebsiella ozaenae, Outer membrane proteins, virulence, antibiotic sensitivity

Introduction

Klebsiella ozaenae, a subtype of K. pneumonia, has been a renowned reason of chronic inflammatory disease of the upper airways. Lately there have been recorded cases confirmed its invasiveness and leading to another infections other than the upper respiratory tract including mastoiditis otitis media, liver abscess, soft tissue infections, urinary tract infections, bacteremia associated with neutropenia, meningitis and pneumonia (Farmer and Kelly, 1991; Ng et al., 2009; Kumar et al., 2013). The outer membrane is a unique structure of the gram negative bacteria cell wall. It consist of lipopolysaccharide (LPS), proteins, and phospholipids. The main structural proteins of the Enterobacteriaceae family outer membrane are porins, a heat-modifiable protein, and murein lipoproteins (Verstreate et al., 1982). The outer membrane proteins supplies diffusion conduits for low-molecular weight hydrophilic materials, like antibiotics, nutrients, and toxic salts. In addition, it composes a permeable impediment for hydrophobic molecules. Outer membrane proteins contribute stately to virulence of bacteria, inhibit the alternative pathway of complement activation, implicate in formation of biofilm on abiotic surface, participate in
adherence and invasion of host cells (Chen et al., 2010; Kim et al., 2016). Various outer membrane proteins have been celebrated to be significant in virulence of K. pneumoniae, comprising outer membrane protein A (OmpA), murein lipoprotein (LppA) and peptidoglycan-associated lipoprotein (Pal). Omp A involves in protection of K. pneumoniae from neutrophil phagocytosis and serum components killing, binds to bronchial epithelial cells, macrophages and dendritic cells, driving to elevated production of cytokine. K. pneumoniae mutant that lack Omp A was weaken in a mouse model. Moreover, Outer membrane proteins probably participate in solidity and permeability of the bacterial membrane in capsule and LPS independent fashion and innervate K. pneumoniae against certain antibiotics and anionic detergents (Hsieh et al., 2013; Paczosaa and Mecsas, 2016). In our study, we inspected the role of outer membrane protein in virulence of Klebsiella ozaenae and antibiotic sensitivity.

**Materials and Method**

1- Bacterial isolate source: A local clinical isolate of Klebsiella ozaenae isolated in our previous study was used.

2- Preparation of outer membrane proteins deficient K. ozaenae isolate: Removal of outer membrane proteins from the K. ozaenae was induced based on method described by (Carlone et al., 1986) as follows: 2-4 colonies from overnight pure culture of K. ozaenae were transferred in a tube containing 10 ml of brain heart infusion broth. After incubation in shaker with 100 cycles / min for 18-24 hours, mixed bacterial suspension was centrifuged at 10,000 rpm / min for 10 minutes, the precipitate was suspended in 1,5 ml of the 10 N of HEPES solution and then the suspension centrifuged at 18000 rpm for 2 minutes at 4C0, the supernatant was collected and sub cultured to ensure the viability of outer membrane proteins deficient K. ozaenae isolate.

3- Virulence test: 10⁸ CFU \ ml of complete and outer membrane deficient K. ozaenae isolates suspensions were prepared. Distinct two groups, each one was composing of 12 male mice, were injected intraperitoneally, the first group injected with complete isolate suspension, and the second group was injected with outer membrane deficient K. ozaenae isolate suspension. At each period of 18,24,48, and '72 hours after inoculation three mice from distinct groups were immolated and the blood was drawn directly from the heart. Also parts from the spleen, liver, and appendix were collected to calculate numbers of complete and outer membrane deficient K. ozaenae isolates by colony count method (Alwazny et al., 2008; Chen et al., 2010).

4- Antibiotic susceptibility pattern: Susceptibilities of complete and outer membrane deficient K. ozaenae isolates were examined against nine antibiotics of different classes using disc diffusion method on Muller-Hinton agar medium according to Clinical Laboratory Standards Institute (Bauer et al., 1996; CLSI, 2010).

5- Statistical analysis: Results of the current study were statistically examined by using independent t test. Significant result determined when a P value was of 0.05.

**Results**

1. This study was carried out to detect the role of outer membrane proteins in virulence of K. ozaenae by comparing the logarithm of the viable number of the complete K. ozaenae with the logarithm of the viable number of outer membrane proteins deficient K. ozaenae that obtained from different organs of injected laboratory mice. It was observed that the logarithm of the viable number of whole bacteria increased during 18, 24,48 and 72 hours compared with the logarithm of the viable number of outer membrane proteins deficient K. ozaenae which increases during 18-24 hours, and then decreased in all organs: the spleen, liver, appendix, and blood( Figures 1,2,3,4). In addition, it was recorded significant differences at a P value of 0.05 between the logarithm of the viable number of the complete K. ozaenae and logarithm of the viable number of outer membrane proteins deficient K. ozaenae that retrieved from different organs.
2. The role of outer membrane proteins in antibiotic sensitivity of *K. ozaenae* was detected by comparing susceptibilities of complete and outer membrane deficient *K. ozaenae* isolates against nine antibiotics of different classes using disc diffusion method. It was found that complete *K. ozaenae* isolate was resistant to most used antibiotics including amoxicillin, cephalothin, ceftazidime, cefotaxime, amikacin, tetracycline, nalidixic acid, erythromycin, trimethoprim and sensitive to only imipenem and gentamicin. Whereas outer membrane deficient *K. ozaenae* isolate was resistant to all antibiotics used in our study (Table-1).

![Figure 1](image1.jpg)

**Figure (1)** Numbers of complete and outer membrane deficient *K. ozaenae* cultured from mice spleen.

![Figure 2](image2.jpg)

**Figure (2)** Numbers of complete and outer membrane deficient *K. ozaenae* cultured from mice liver.
Figure (3) Numbers of complete and outer membrane deficient *K. ozaenae* cultured from mice appendix.

Figure (4) Numbers of complete and outer membrane deficient *K. ozaenae* cultured from mice blood.

Table (1): Antibiotic susceptibility pattern for *K. ozaenae* with and without outer membrane

| Antibiotics   | Complete *K. ozaenae* | Outer membrane detached *K. ozaenae* |
|---------------|------------------------|--------------------------------------|
| Amoxicillin   | R                      | R                                    |
| Impenem       | S                      | R                                    |
| Cephalothin   | R                      | R                                    |
| Ceftazidime   | R                      | R                                    |
| Cefotaxime    | R                      | R                                    |
| Gentamicin    | S                      | R                                    |
| Amikacin      | R                      | R                                    |
| Tetracycline  | R                      | R                                    |
| Naldixic acid | R                      | R                                    |
| Erythromycin  | R                      | R                                    |
| Trimethoprim  | R                      | R                                    |
Discussion

The outer membrane proteins of gram negative bacteria interferes with the host and plays an important role in adhesion of bacteria with host cells. They also play a role in resistance to host immunity and protection of bacteria from the phagocytosis and serum complement (Kay et al., 1981). In current study, the significant differences at a P value of 0.05 between the logarithm of the viable number of the complete \textit{K. ozaenae} and logarithm of the viable number of outer membrane proteins deficient \textit{K. ozaenae} that retrieved from the spleen, liver, appendix, and blood of injected laboratory mice indicated that outer membrane proteins are one of virulence factors engaged in dissemination and proliferation of \textit{K. ozaenae} in host cell, and have a critical role in protection from early host defense mechanism. However, other virulence factors, such as capsule or lipopolysaccharide may play a role in dissemination and proliferation of outer membrane proteins deficient \textit{K. ozaenae} in host cell, and modulate inflammation (Cortes et al., 2002; Paczosaa and Mecsas, 2016). Deletion of the genes necessary for the outer membrane protein OmpK36 formation in \textit{K. pneumoniae} resulted in enhanced serum resistance, increased neutrophils uptake, and less virulence (Chen et al., 2010).

The \textit{K. ozaenae} isolate was multi-resistant to most antibiotics used in our study, but was sensitive only to imipenem and gentamicin. This finding came in line to another report (Huang et al., 2012) which isolated one extremely multi resistant strain of \textit{K. ozaenae} from a patient in the intensive care unit of a Chinese tertiary hospital. This \textit{K. ozaenae} isolate was resistant to ampicillin, aztreonam, amikacin, ampicillin/sulbactam, trimethoprim–sulfamethoxazole, ceftriaxone, cefepime, cefotetan, ceftazolin, ceftazidime, tobramycin, gentamicin; levofloxacin; ciprofloxacin; piperacillin/tazobactam and ertapenem; while sensitive only to imipenem. Furthermore, Gassama-Sow et al (2010) isolate 2 strains of \textit{K. ozaenae} from adults with diarrhea, these strains were resistant to amoxicillin, amoxicillin-clavulanic acid, ticarcillin, trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol, oxacillin, and ciprofloxacin. But our result is not compatible with Sugawara et al (2016) who isolate \textit{K. ozaenae} resistant to cloxacillin, oxacillin, benzylpenicillin, and ampicillin, but was sensitive to cephalosporins of third generation fourth generation. Subspecies of \textit{K. pneumoniae} partake similar susceptibility to different antibiotics (Stock and Wiedemannm, 2001). The appearance of resistance isolates may due to systems of innate efflux pump, the capacity of isolates to acquire resistance factors, and the widespread and inappropriate employment of antibiotics which embolden resistant clones selection (Du et al., 2014).

The outer membrane deficient isolate exhibit resistance to all antibiotics due to loss of porins, which act to insert the antibiotic into the bacterial cell. This result was compatible with Sugawara et al (2016) who found that elimination of ompK35 gene led to a two fold raises in the MICs of benzylpenicillin, ampicillin, imipenem and three to four fold increases in the MICs for cephalosporins. In addition, it compatible with the finding of (Chen et al., 2010) who found loss of OmpK36 resulted in increased antimicrobial resistance. The resistance to gentamicin may result from loss of some outer membrane proteins, which reduces the permeability of the antibiotic and its entry into the cell. Gram-negative bacteria have diverse porins in their outer membrane that share in modification of cellular permeability, and the loss of porins is one of the important process involved in increasing antibiotic resistance and is frequently noticed in antibiotic resistant strains (Martinez, 2008; Xuan et al., 2009). Two main porins OmpK35 and OmpK36 in \textit{K. pneumoniae} are often lost in multi resistant strains, particularly in combination with Extended Spectrum - Lactamase producers (Yigit et al., 2003; Kim, et al., 2007).

Conclusion

Loss of outer membrane proteins in \textit{K. ozaenae} resulted in reduced virulence and increased antimicrobial resistance.
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