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RESEARCH ARTICLE OPEN ACCESS

Contribution of Capsule to Virulence and Antibiotic Sensitivity of *Klebsiella ozaenae* Identified by Phenotypic and Molecular Methods

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Abstract

The respiratory colonizer *K. ozaenae* can cause a broad spectrum of infections, and it always misdiagnoses by phenotypic procedures. The role of capsule has not been proved in *K. ozaenae* yet. One hundred (100) clinical specimens were taken from patients suffering different infections. After phenotypic identification by cultural, microscopic, and biochemical tests, the suspected *K. ozaenae* isolates subjected to molecular identification using 16S rRNA gene. The non-capsulated *K. ozaenae* isolate was prepared from higher muco-viscous capsulated *K. ozaenae* isolate. Mice were injected intraperitoneally by capsulated and non-capsulated *K. ozaenae*, then bacterial burden in the spleen, liver, and blood was compared and histopathological lesions were detected in liver. Susceptibility of *K. ozaenae* with and without capsule to different antibiotics concentrations was tested at 600nm wavelength. Phenotypically, 3 (3%) isolates of *K. ozaenae* were identified, while results of 16S rRNA gene were concordant in 2/3 (2%) isolates and discordant in 1/3 (1%) isolate. Significant differences were showed between the increased log number of capsulated *K. ozaenae* isolate and decreased log number of non-capsulated *K. ozaenae* isolate that recovered from mice spleen, liver, and blood. Severe pathological lesions were observed in mice liver infected by capsulated *K. ozaenae* compared with non-capsulated *K. ozaenae* isolate. No-significant differences were found between the growth of capsulated and non-capsulated *K. ozaenae* isolate treated with the same antibiotic concentration. 16S rRNA are useful molecular tool to avoid misidentification of *K. ozaenae*. Removal of capsule decreases virulence of *K. ozaenae*, but not affect its sensitivity to antibiotics.

Keywords: *Klebsiella ozaenae*, capsule, virulence, 16S rRNA, antibiotic sensitivity.

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INTRODUCTION

K. pneumoniae divided into three subspecies with different biochemical reactions, but homologous DNAs: *K. pneumoniae* subsp. *pneumoniae, K. pneumoniae* subsp. *rhinoscleromatis*, and *K. pneumoniae* subsp. *ozaena*[1,2]. *K. pneumoniae* subsp. *ozaenae* discrete from *K. pneumoniae* subsp. *pneumoniae* by a negative reaction to malonate test and voges proskauer[3]. *K. ozaenae* is known to colonize the nasopharynx and implicate as a causative agent of atrophic rhinitis or ozena[4]. *K. ozaenae* has been emerging as opportunistic pathogen associated with elderly patients, immunocompromised patients, alcoholics, diabetics and AIDS patients. Furthermore, it has been documented as causative responsible of invasive infections including pituitary abscess, cerebral abscess, splenic abscess, hepatic abscess, bacteremia, meningitis, pneumonia, cholecystitis, urinary tract infection, and soft tissue infections[5–8].

Klebsiella has a number of factors that involved in its virulence and pathogenicity, including capsule, fimbrial and non fimbrial adhesins, lipopolysaccharide and siderophores[9]. Capsular polysaccharide is the key agent of *Klebsiella spp*. Its different composition in different clinical isolates leads to differences in the serotypes of these bacteria, and it is directly responsible for the initial stages of pathogenesis. In addition, it is associated with mucosal epithelial cells, and its presence affects the process of building the adhesion proteins necessary for the adhesion and colonization of the intestinal tract of mice[10]. Unlike other intestinal tract bacteria, *K. pneumoniae* has a thick capsular polysaccharide important in settling, gluing, surviving, and multiplying within the host[11]. It has been observed that capsulated strains were virulent, whereas strains without capsule have minimal virulence or a virulent[12]. The capsule is an extracellular substance composed of hydrophilic polysaccharide arranged in repetitive units that condense in the form of a closely linked layers surrounding the bacterial cell, they immediately react with the outside environments providing protection from host immunity by impairing phagocytosis or block killing by bactericidal serum factors[13,14]. In addition, it confers a physical barrier to detrimental antimicrobial agents, restricts the interaction of the cell surface with antimicrobial peptides[15,16]. Capsular polysaccharide in both Gram negative and Gram positive bacteria has been predicted to impede the antibiotic molecules movement which confer bacteria additional intrinsic mechanism to resist antibiotics[17]. The current study was delineated to isolate and identify *K. ozaenae* from different clinical infections by phenotypic and molecular methods, and detect the role of capsule in virulence of *K. ozaenae* by comparing the bacterial burden of capsulated *K. ozaenae* isolate with non-capsulated isolate in the spleen, liver and blood of a mouse model through recovering these isolates from the mentioned tissues and determining their log number, with detection the histopathological lesions in liver of inoculated mice, in addition evaluating the role of *K. ozaenae* capsule in antibiotic sensitivity by comparing the growth of capsulated *K. ozaenae* isolate with non-capsulated at different antibiotics concentrations.

MATERIALS AND METHODS

Specimens collection and bacterial identification

A total of 100 different clinical specimens were collected from patients who were admitted to General Al-Qasim hospital in Babylon province in Iraq. The specimens (blood, urine, sputum, stool, wounds swabs, burn swabs, ear swabs, and throat swabs) were plated on MacConkey agar and incubated at 37°C for 24 hours. Suspected pink and mucoid colonies were transferred and purified to individual colonies and phenotypically identified by cultural, microscopic, and biochemical tests according to the methods adopted by[18]. Suspected *K. ozaenae* isolates were further subjected to molecular identification using specific primer based on 16S rRNA gene for confirmed isolation.

Molecular identification of *K. ozaenae*

Bacterial RTP® extraction Kit supplemented by (InVitek- Germany) was used to extract the genomic *K. ozaenae* DNA. Oligonucleotide primers was designed using the clone manager software to amplify 16S ribosomal RNA gene from the isolated *Klebsiella pneumoniae* subsp. *ozaenae* based on the published corresponding sequence of 16S r RNA gene of *Klebsiella pneumoniae* subsp. *ozaenae* strain ATCC 11296 (NCBI Reference Sequence: NR_119276. 1). The forward primer sequence was: 5' AGGCCTAACACATGCAAGTC 3' and the

reverse primer sequence was: 3'GTAGCTTAAC CTTCGGGAGG5'. PCR reactions were implemented in a total volume of 50µl mixture using Thermo Scientific™ Phusion High-Fidelity DNA Polymerase (Fisher scientific) containing 25µL of 2X Phusion Master Mix, 0.5µM for each primer, 100 ng/ 50µL of genomic *K. ozaenae* DNA, and water exempt from nuclease. The parameters of PCR reaction were as follow: 98°C for each of the first and second denaturation steps for 30 and 10 seconds, annealing at 54°C (30 seconds); the extension step was done for 1 minute at 72°C, and the last extension step for 5 minutes at 72°C. Negative control (without DNA) PCRs were utilized to avoid false positive results. The amplified PCR products were electrophoresed using 1% agarose gels and spotted with ethidium bromide and imaged using a Bio-Rad ChemiDoc MP.

Detection the presence of capsule

The presence of capsule in *K. ozaenae* isolates was detected using negative staining method[19]. The higher muco-viscous phenotype of *K. ozaenae* was detected using string test[18].

Preparation of non-capsulated *K. ozaenae* isolate

Non-capsulated *K. ozaenae* isolate was prepared from higher muco-viscous *K. ozaenae* isolate by elimination of capsular polysaccharide through rapid agitation method[20] as following: 2-4 pure higher muco-viscous *K. ozaenae* colonies were inoculated into 10 ml of phosphate buffer saline, gently shaken up by hand then shaken up by vortex for 5 min, washed twice with phosphate buffer saline then centrifuged for 2 minutes at 18000 rpm and suspended with phosphate buffer saline. Thereafter washed twice by phosphate buffer saline, shaken up by vortex, and then centrifuged for 3 minutes at 18000 rpm and suspended with phosphate buffer saline. The suspension then checked for sterility and stored at 4°C.

Experimental animals

Swiss mice, Albino Balb/c, Mus musculus, white, aged 8-12 weeks, ranged between 20-24 grams. The mice were obtained from the Embryonic Research Institute in Baghdad province and placed in cages inside the animal house, taking into account the cleanliness of drinking water, feed and sterilization from time to time. All procedures were completed in accordance with guide for the care and use of laboratory animals[21].

Bacterial virulence assay

Capsulated and non-capsulated *K. ozaenae* isolates were grown and then suspended at 10^8CFU \ ml of phosphate buffer saline. Separate two groups of mice (12 mice in each one) were intraperitoneally injected, the first one with capsulated *K. ozaenae* suspension, while the second group with non-capsulated *K. ozaenae* suspension. Three mice from each group were sacrificed at18-24-48-72h interval post-injection, and cardiac blood, liver and spleen tissues were sampled. Viable numbers of capsulated and non-capsulated *K. ozaenae* isolates were enumerated in the samples by colony count method. At 72h after inoculation, the liver samples of inoculated mice were processed to detect histopathological changes[22–24].

Antibiotic growth test

Different antibiotics (ampicillin, amoxicillin, doxycycline, and cephalexin, amikacin, naldixic acid**)** with final concentrations of 12.5, 25, 50, 75, 125, 250, 500 µg/ml were prepared with final concentration 10^8CFU\ml of capsulated and non-capsulated *K. ozaenae* isolates. After incubation at 37°C for 18h, bacterial growth turbidity was determined by measuring the absorbance at optical density of 600nm wavelength[25].

Statistical analysis

The mean of replicate samples was specified and levels of statistical significant differences between experimental groups were calculated by independent t test using SPSS version 20. The results with a P value of ≤ 0.05 were treated to be significant.

RESULTS

Isolation percentage of *K. ozaenae*

The results of cultural, microscopic, and biochemical tests revealed that out of total of 100 different clinical specimens, 3 (3%) isolates were positive for *K. ozaenae* (table 1). Confirmative molecular identification of *K. ozaenae* using 16S rRNA gene as a molecular marker showed that the blood isolate and the stool isolate were positive for 16Sr RNA gene with (2%) of isolation, where the urine isolate was negative for 16Sr RNA gene (Fig. 1).

Detection of *K. ozaenae* capsule

Negative staining method showed that all three isolates of *K. ozaenae* had capsule. The *K.*

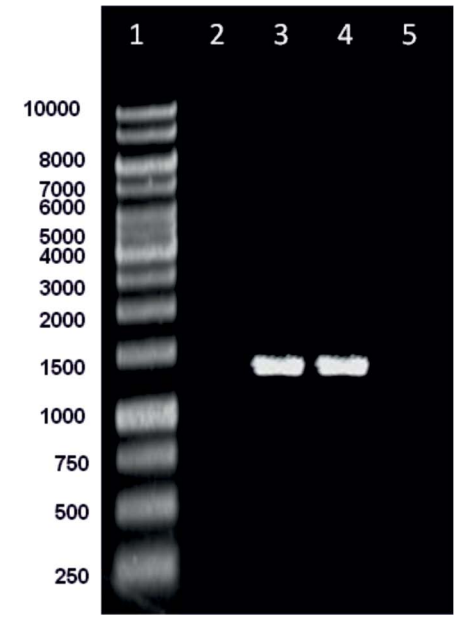

Fig. 1. PCR analysis of the 16Sr RNA gene of *Klebsiella pneumoniae subsp. ozaenae* isolated in the current study. Lanes are: (1) Molecular marker, (2) negative urine isolate for 16Sr RNA gene (3,4): positive blood and stool *K. ozaenae* isolates for 16Sr RNA gene (5) negative control. Product expected size is 1413 bp.

ozaenae isolate that isolated from blood specimen characterized by production of colonies with string-like growth more than 5 mm. This higher muco-viscous *K. ozaenae* isolate was used in preparation the non-capsulated *K. ozaenae* isolate as described in materials.

Effect of capsule in virulence of *K. ozaenae*

This study conducted to evaluate the role of capsule in virulence of *K. ozaenae*. Bacterial burden in spleen, liver and blood of mice intraperitoneally inoculated with 10^8 CFU\ ml of capsulated and non-capsulated *K. ozaenae* isolates was detected at18-24-48-72h intervals post-inoculation by determination the log of viable number of bacteria recovered from spleen, liver and blood. The results indicated significant differences between the increased log number of the capsulated *K. ozaenae* isolate and decreased log number of non-capsulated *K. ozaenae* isolate that recovered from mice organs as explained in (Fig. 2, 3, 4). The log number of capsulated *K. ozaenae* isolate recovered from mice spleen, liver and blood raises with time inoculation from 18 to 72 hours, whereas the log number of non-capsulated *K. ozaenae* isolate recovered from mice spleen, Liver and blood shows a little increase at 24 h then declines obviously at 48h and 72h post inoculation. Liver sections were prepared from mice at 72 hour after inoculation with capsulated

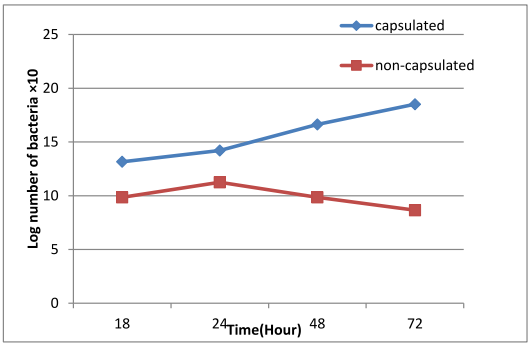

Fig. 2. Viable count of capsulated and non-capsulated *K. ozaenae* recovered from mice spleen. Each dot acts log10 CFU per gm of spleen (mean) for 3 mice.

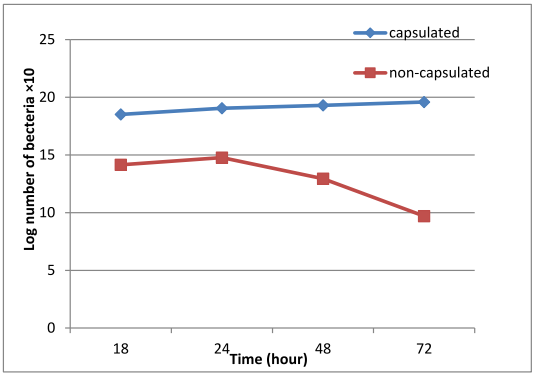

Fig. 3. Viable count of capsulated and non-capsulated *K. ozaenae* recovered from mice liver. Each dot acts log10 CFU per gm of liver (mean) for 3 mice.

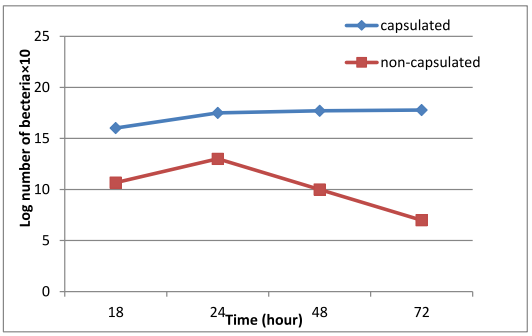

Fig. 4. Viable count of capsulated and non-capsulated *K. ozaenae* recovered from mice blood. Each dot acts log10 CFU per of blood (mean) for 3 mice.

and non-capsulated *K. ozaenae* isolates. The histopathological examination of liver sections revealed pathological lesions resulting from infection with capsulated *K. ozaenae* including Kupfer cell hyperplasia, foci of inflammation and migration of polymorphonuclear cell with congestion. The liver sections of mice inoculated with non-capsulated *K. ozaenae* isolate shows minimal lesions represented by mild degeneration (Fig. 5).

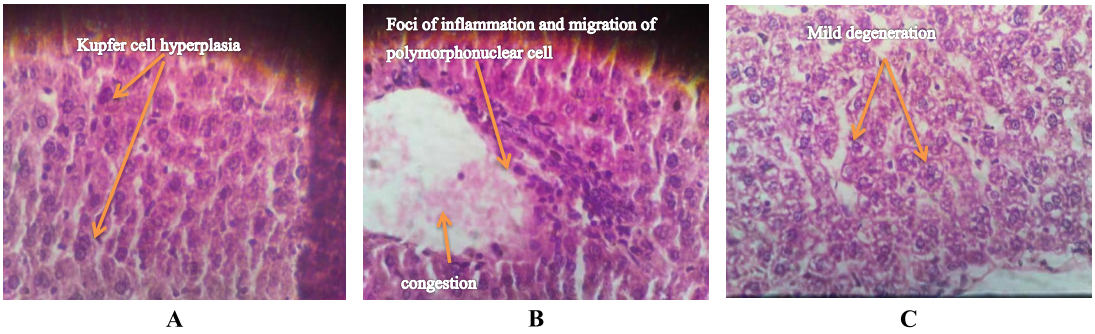

Fig. 5. Cross-liver sections from mice injected intraperitonally with (A , B) capsulated *K. ozaenae* isolate shows: A: Kupfer cell hyperplasia, B: acute inflammatory cell infiltration (foci of inflammation and migration of polymorphonuclear cell with congestion. C: with non-capsulated *K. ozaenae* isolate show :mild degeneration (H&E. 200X).

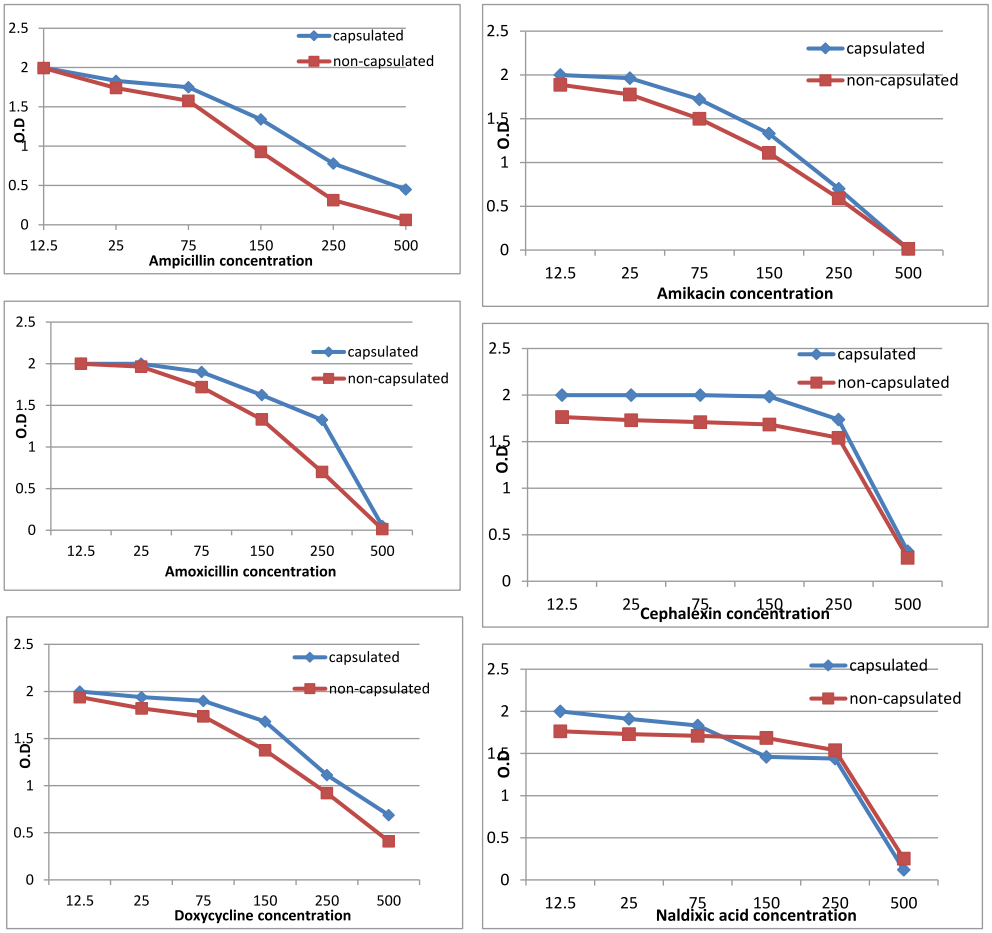

Fig. 6. Changes in growth of capsulated and non-capsulated *K. ozaenae* isolates at different antibiotics concentrations.

Table 1. Specimens profile and isolation percentage of *K. ozaenae* identified by phenotypic and molecular methods

Types of specimens	Numberof specimens	Number of *K. ozaenae* Isolates identified by phenotypic method (%)	Number of *K. ozaenae* isolates identified by molecular method (%)
Blood	10	1 (1%)	1 (1%)
Urine	25	1 (4%)	0
Sputum	10	0	0
Stool	25	1 (1%)	1 (1%)
Wounds swabs	5	0	0
Burn swabs	5	0	0
Ear swabs	10	0	0
Throat swab	10	0	0
Total	100	3 (3%)	2 (2%)

Effect of capsule in antibiotic sensitivity

To evaluate the role of capsule in susceptibility of *K. ozaenae* bacteria to antibiotics, the study compared between the growth of capsulated and non-capsulated *K. ozaenae* isolates at different concentrations for ampicillin, amoxicillin, doxycycline, cephalexin, amikacin, and naldixic acid by measuring the absorbance of growth turbidity at 600nm wavelength. In general, it was noticed that non-capsulated *K. ozaenae* isolate showed non-significant lower growth compared to the capsulated *K. ozaenae* at the same antibiotic concentration as explained in (Fig. 6). It is noted that the rate of growth gradually decreases with increased antibiotic concentration for *K. ozaenae* isolates with and without capsule.

DISCUSSION

The respiratory colonizer *K. ozaenae* can cause an extensive infections and even mortality in immunocompromised hosts[6]. The results of phenotypic identification including cultural, microscopic, and biochemical tests revealed that the percentage of *K. ozaenae* among the examined clinical specimens was (3%). This percentage was agreement with similar study[26] used phenotypic identification and recorded (2.8%) isolation percentage of *K. ozaenae*. A misdiagnosis of *K. ozaenae* by biochemical tests has been formerly described[27], so the phenotypic identification of *K. ozaenae* was not accurate for all isolates because one isolate in our study was a negative for 16Sr RNA gene. The differences in obtained results by phenotypic and molecular diagnosis might be due to poor sensibility of the former. Molecular identification can promote the diagnostic precision because several molecular targets can offer great sequence symmetry.

K. pneumoniae is characterized by possession of capsular polysaccharide that overlay the bacteria. *K. ozaenae* isolate that isolated from blood specimen, which produced colonies with higher viscus mucus due to the production of large amounts of capsular polysaccharide expressed as higher virulent with higher muco-viscous phenotype. Furthermore this phenotype is characteristic of invasive infections[28,29].

The significant differences between the increased log number of capsulated *K. ozaenae* isolate and the decreased log number of non-capsulated *K. ozaenae* isolate recovered from liver, spleen, and blood of mice indicate that cellular and humeral bactericidal mechanisms may engage in the clearance of non-capsulated *K. ozaenae* isolate from the host, and the capsule participate in resistance of capsulated *K. ozaenae* to these mechanisms. The capsular polysaccharide is the most important virulence determinant of *Klebsiella* spp. The mutant *Klebsiella* strain that lacking or has reduced capsular polysaccharide characterized by reduction in its virulence to the mice which result from increasing the phagocytosis by polymorph nuclear leukocytes or killing of bacteria by serum factors or both[13,30]. The possible mechanisms that explain the role of the capsular polysaccharide in the protection of bacteria from phagocytosis include hydrophilicity of cell surface, the presence of the capsule interferes with the process of opsonization, and capsular polysaccharide mask the lipopolysaccharide

preventing deposition of complement[31]. It was observed that hydrophobic strains are more readily phagocytosed by polymorph nuclear leukocytes than hydrophilic ones[32]. The tissue infected with *K. ozaenae* manifested large numbers of polymorphnuclear leukocytes, and the capsule interference with these cells prevents them from phagocytosis, thus the numbers of bacteria increased, concerted with sever pathological lesions as shown with presence of inflammation foci in liver sections. The capsular polysaccharide form a soft mucus layer surrounds the solid cell wall and the frequent washing process contributes to its removal. On the other hand, this process cannot remove the lipopolysaccharide that anchor to the outer membrane, and this LPS promote opsonization of *K. ozaenae* when the capsule is detached[33].

Although the non-capsulated *K. ozaenae* isolate showed lower growth compared to the capsulated *K. ozaenae* when treated with the same antibiotic concentration, differences in the turbidity of the growth of *K. ozaenae* isolates with and without capsule were statically non-significant across all antibiotics concentrations. This indicates that the removal of capsule does not affect the sensitivity of *K. ozaenae* to antibiotics. The intrinsic mechanism that enable the Gram negative bacteria to resist antibiotics is hugely consequent from a cell envelope forming of lipopolysaccharides with little permeability to hydrophobic molecules, multidrug efflux pump system, and slow porins with weakly permeability[34–36]. Susceptibility of *K. pneumoniae* to different classes of antibiotics was not affected in the mutant capsule deficient isolates. Production of penicillinase, Amp C type enzymes, hydrolyzing β-lactamase, or shelter topoisomerase multination may expedite appearance of *Klebsiella pneumoniae* resistance to antibiotic[37].

CONCLUSION

Molecular identification offer reliable and fast method to overcome misidentification subspecies of *Klebsiella pneumoniae*. Removal of capsular polysaccharide signi cantly reduces the virulence of *K. ozaenae*, while no compulsory proof that capsule has a role in antibiotic sensitivity; thus the capsule plays an important role in the virulence of *K. ozaenae*, but has no effect in the sensitivity of *K. ozaenae* to antibiotics.

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CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

AUTHORS CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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