Characterization of Hypervirulent Extended-Spectrum \(\beta\)-Lactamase-Producing \textit{Klebsiella pneumoniae} Among Urinary Tract Infections: The First Report from Iran

\textbf{Introduction}: This study was conducted to identify the hypermucoviscosity, iron acquisition, and capsule serotypes of \textit{K. pneumoniae} strains isolated from urinary tract infections among community-acquired patients (CA) and assess the frequency of plasmid-mediated quinolone resistance (PMQR) and extended-spectrum beta-lactamases (ESBL) genes between classic and hypervirulent strains.

\textbf{Materials and Methods}: A total of 105 \textit{K. pneumoniae} were isolated from CA-UTI. Demographic data related to the underlying diseases and clinical manifestations were further collected. Antibiotic resistance pattern and molecular characterization were compared among ESBL-positive, ESBL-negative, hypervirulent, and classic isolates.

\textbf{Results}: The results revealed that 52.4% of the isolates were confirmed as ESBL producers and 11 (10.5%) were considered as hypervirulent \textit{K. pneumoniae} (hvKp). Ciprofloxacin and nalidixic acid were the most inactive antibiotics with resistance rates of 68.6% and 64.8%, respectively. Molecular characterization revealed that 7.6% of all the isolates carried \textit{k1} and 66.6% carried \textit{K2} genes. The most frequent ESBL gene was \textit{blaSHV}, 63.8%, followed by \textit{blaTEM} 59.0%, and \textit{blaCTX-M} 58.1%. ESBL genes were significantly more in hvKp than in \textit{Kp}. Moreover, 61 (84.7%), 47 (65.2%), and 16 (22.2%) of isolates harbored \textit{qnrB}, \textit{qnrS}, and \textit{qnrA}. ESBL genes were detected in all hvKps, and \textit{blaSHV} was observed in 90.9% of hvKp (\textit{P value}= 0.048, 95%).

\textbf{Discussion}: This study reported the high frequency of antimicrobial and multidrug resistance among hvKp isolates. Coexistence of PMQR and ESBL genes in hvkp indicates the necessity to enhance the clinical knowledge and management of hvKp infections.

\textbf{Keywords}: hypervirulent \textit{Klebsiella pneumoniae}, urinary tract infections, capsular group, ESBL, \textit{qnr}

\textbf{Introduction} \textit{Klebsiella pneumoniae} is a common Gram-negative organism with worldwide distribution. It causes nosocomial and community-acquired infections, liver abscess, and urinary tract infections, mostly in patients with underlying diseases.\(^2\) This organism is among the top three pathogens of international concern documented in the World Priority List of Antibiotic-Resistant Bacteria released by the World Health Organization (WHO) in 2018.\(^3\) Over the past two decades, \textit{K. pneumoniae} has been the second most prevalent etiological agent of community-acquired (CA) urinary tract
infections (UTI).4 There are mainly two pathotypes that pose a threat to public health: hypervirulent K. pneumoniae (hvKp) and classical K. pneumoniae (cKp). The most common subtype of the K. pneumoniae strains is classic K. pneumoniae (cKp); unlike this subtype, the new “hypervirulent” K. pneumoniae (hvKp) with hypermucoviscosity has emerged as a clinically important infectious agent causing extremely invasive infections such as liver abscesses in both healthy and immunocompromised individuals.5,6 These strains can cause serious CA infections in healthy individuals, along with the reported global dissemination of the growing antibiotic resistance.7,8 Therefore, hvKp increases the public health costs, restricts the selection of antibiotics, and causes failure in medical therapy, leading to mortality and increased morbidity.9

The hvKp strains are of hypermucoviscous type due to the overproduction of the capsular polysaccharide. Hypermucoviscosity is a crucial in vitro parameter for identifying hvKp.10 Moreover, several virulence genes such as the regulator of mucoid phenotype A gene (rmpA) and aerobactin (iucA) have been identified to be associated with the hypervirulent variant of K. pneumoniae.11 Furthermore, the capsule, particularly serotypes K1 and K2, has long been known as a major virulence factor in K. pneumoniae.12

On one hand, fluoroquinolones such as ciprofloxacin and levofloxacin as well as β-lactam antibiotics are commonly recommended for complicated UTI treatment.13 On the other hand, there has been a global increase in extended-spectrum β-lactamases (ESBL)-producing Enterobacteriaceae and fluoroquinolone resistance.14 Plasmid-mediated quinolone resistance (PMQR) determinants have been further reported. PMQR includes Qnr (quinolone resistance) proteins (qnrA, qnrB, qnrC, qnrD, and qnrS) which protect the topoisomerase IV and DNA gyrase from quinolone inhibition, which acetylates aminoglycoside and ciprofloxacin and reduces their activity.15 PMQR genes are mostly on the same plasmid as ESBL genes.16 Resistance plasmids harboring genes encoding ESBLs can be transferred by conjugation, which helps disseminate PMQR determinants in different Enterobacteriaceae species.17

Multidrug-resistant (MDR) K. pneumoniae isolates are defined by non-susceptibility to at least one agent in three or more antibiotic categories, which severely limits antimicrobial treatment options.18 Hence, the coexistence of MDR-ESBLs and PMQR genes is a main concern, particularly in the community status.19,20 ESBL producers have been associated with resistance to non-β-lactam antimicrobials, including fluoroquinolones, aminoglycosides, and sulfonamides, which are often used to treat and prevent diseases. Additionally, plasmid-mediated quinolone resistance (PMQR) genes are associated with ESBL production, and the spread of co-expressing PMQRs and ESBL isolates contributes to the growing concerns regarding MDR isolates.21 Also, hvKp strains are of major importance as they are hypervirulent and highly transmissible.18

To our knowledge, there is a paucity of information concerning the frequency of hvKp in CA-UTI and their antimicrobial susceptibility. Therefore, the objective of this study was to identify the hypermucoviscosity, iron acquisition, and capsule serotypes of K. pneumoniae strains isolated from CA-UTI; the frequency of PMQR (qnrA, qnrB and qnrS) and ESBL genes (blaSHV, blaTEM, blaCTX-M) in ESBL and non-ESBL producer isolates was further assessed. In addition, cKp and hvKp strains were compared in terms of antimicrobial susceptibility and molecular characterizations.

Materials and Methods
A cross-sectional study was conducted at Milad Advanced Medical laboratory, Isfahan, Iran, between April and December 2019. All patient data accessed complied with the relevant data protection and privacy regulations. This study protocol was approved by the Ethics Committee of Isfahan University of Medical Sciences (IR.MUI.MED.REC.1398.388), but no human participants were included. Urine samples were collected from outpatients who were suspected of UTI and had not consumed any antibiotics for the past two weeks. Samples were collected from the clean-catch midstream urine of adults and children aged less than two years using a sterile urine bag or a suprapubic catheter for sample collection. Demographic data and information related to underlying diseases and clinical manifestations were further collected.4 Urine samples were transferred to the microbiology laboratory at Isfahan University of medical science, then cultivated on blood agar and EMB media (Merck, Germany) for 24 hours. As described previously, a minimum colony count of 10⁵ CFU/mL was considered as positive urine culture for midstream urine.22 Cases of mixed infection were excluded where they might reflect contamination with normal flora of the periurethral, vaginal, and perianal regions.23 All isolates were primarily screened by their pigment production, colony morphology, and Gram-staining techniques. Isolates were then identified by conventional biochemical tests and standard culture methods. In this regard, citrate and urea positive, oxidase, indole,
methyl red, and motility negative strains were identified as *K. pneumoniae*.

**String Test**
Isolates were considered positive for the hypermucoviscosity phenotype after subculture on blood agar at 37°C overnight if an inoculation loop touching the surface of the colony generated a viscous string of 5 mm or more when pulled away from the plate.24

**Antimicrobial Susceptibility Testing**
The antibiotic susceptibility profile was determined on Muller–Hinton agar (Merck, Germany) plates using the disk diffusion method (the modified Kirby–Bauer assay) as described by the Clinical and Laboratory Standards Institute (CLSI 2019).25 Quality control strains were included to monitor the test performance. *K. pneumoniae* clinical isolates were tested for their susceptibility to the following antimicrobial agents: amoxicillin-clavulanic acid (20/10 μg), cefotaxime (30 μg), ceftazidime (30 μg), cefixime (5 μg), cefepime (30 μg), imipenem (10 μg), meropenem (10 μg), gentamicin (10 μg), tobramycin (10 μg), amikacin (30 μg), trimethoprim–sulfamethoxazole (30 μg), nitrofurantoin (300 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), levofloxacin (5 μg), ofloxacin (5 μg), and piperacillin-tazobactam (100/10 μg).25,26 All disks were obtained from ROSCO Company, Denmark.

**Screening of ESBL Producers**
The preliminary screening test to detect ESBL activity was performed by the disk diffusion method according to the CLSI guidelines (2019). The inhibition zone size of ≤22 mm for ceftazidime (30 μg) and ≤27 mm for cefotaxime (30 μg) indicated ESBL production.27 The phenotypic confirmatory test for ESBL was carried on by combination disk method using cefotaxime (30 μg) and ceftazidime (30 μg) alone and in combination with clavulanic acid (10 μg).28,29 ESBL activities were identified by ≥5 mm increase in zone diameter around the disk with the antibiotic combined with clavulanic acid.30,31 In brief, Muller Hinton agar plates were swabbed with 0.5 mL of overnight bacterial culture; after 10 minutes the antibiotic disk was placed on the plates and incubated for 24. Afterwards, the inhibition zone was measured and reported in diameter.32 *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as the quality control strains.25,33

**Molecular Detection of Capsular Types and Virulence Genes**
Genomic DNA was extracted from all *K. pneumoniae* isolates as described previously.34 Polymerase chain reaction (PCR) for virulence-associated genes (such as *rmpA* and *iucA*) was carried out among the string test positive isolates. Moreover, using *K1* and *K2* specifying primers, all isolates were tested for the presence of *K. pneumoniae* capsular types.5

**Molecular Detection of ESBL and PMQR Genes**
ESBL genes (*blaSHV, blaTEM, blaCTX-M*) were detected in all isolated uropathogens.35 In addition, isolates that were phenotypically resistant to at least one fluoroquinolone were screened for *qnrA, qnrB*, and *qnrS* genes. PCR primers were previously described.10 Amplification was conducted as follows: initial denaturation at 95°C for five minutes, followed by 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for five minutes. The PCR products were analyzed by electrophoresis with 1% agarose gel in 1X Tris–Acetate–EDTA buffer. The gels were stained with stain load dye (CinnaGen Co, Iran) and the PCR products was visualized under UV light.36,37

**Statistical Analysis**
The relationship among antibiotic resistance, ESBL production, and PMQR determinants was evaluated by the Chi-square test or Fisher’s exact test. P-values≤0.05 were considered as statistically significant. The data were analyzed using the Statistical Package for Windows v.19.0 (SPSS Inc., Chicago, IL, USA).

**Results**
**Clinical Characteristics**
Over the eight-month study period, 105 *K. pneumoniae* were isolated from the urine samples of CA patients in Isfahan. The mean age of patients was 46.50 years (1–81 years), and they comprised 32 (30.5%) males and 73 (69.5%) females. According to the demographic data of the patients, most had underlying diseases, including diabetes mellitus (33.5%), kidney transplant (13.3%), chronic renal insufficiency (20.9%), and immunosuppression (11.4%).

Out of the 105 positive urine cultures, 55 (52.4%) were confirmed as ESBL producer based on the combination disk method. MDR phenotype was detected in 59 out of
105 (56.1%) isolates. Of these 105 isolates, 94 (89.5%) were considered as cKp. Therefore, 11 isolates (10.5%) were string test positive and had two virulence genes (iucA and rmpA), hence considered as hvKp. As shown in Table 1, among these hvKp isolates, nine were females (81.8%), five (45.4%) were identified as MDR, and seven (63.6%) produced ESBL phenotype. Patients suffering from hvKp isolates had risk factors such as diabetes (7/11 (63.6%)), kidney transplant (3/11 (27.2%)), and immunosuppression (2/11 (18.1%)). Clinical and microbiological characteristics of the hvKp isolates are shown in Table 1.

**Antimicrobial Resistance**

The results revealed that ciprofloxacin and nalidixic acid were the most inactive antibiotics with resistance rates of 68.6% and 64.8%, respectively. However, the most effective antibiotics were meropenem, imipenem, amikacin, tobramycin, and piperacillin-tazobactam with resistance rates of 6.7%, 7.6% and 9.5%, respectively. There was no significant association among antimicrobial resistance, age, and gender (P>0.05). According to statistical analysis, the resistance rates of cefixime, cefotaxime, ciprofloxacin, ofloxacin, levofloxacin, and nalidixic acid were significantly higher in ESBL-positive than in ESBL-negative strains (P ≤0.05). The resistance rates to most tested antimicrobial agents were lower in hvKp than in cKp isolates. Nonetheless, cefixime, trimethoprim–sulfamethoxazole, nitrofurantoin, and carbapenems had higher resistance rates. Table 2 depicts the complete results of antibiotic resistance patterns and compares the ESBL-negative and ESBL-positive isolates.

**Molecular Analysis**

**Capsular Types**

In this study, 8/105 (7.6%) isolates carried k1 capsule gene, all belonging to hvKp (P <0.0001). Furthermore, 70/105 (66.6%) carried K2 capsule gene, with only three (27.3%) associated with hvKp strain (P=0.005, Table 3). Twenty-seven isolates did not have k1 and K2 genes.

**Detection of ESBL Genes**

Although ESBLs were phenotypically detected in 55 (52.4%) of the isolates, electrophoresis analysis revealed that all isolates had at least one ESBL gene. Table 3 represents the frequency of these genes among ESBL-positive and negative isolates. The most frequent ESBL gene in the tested isolates was blaSHV 67 (63.8%), followed by blaTEM 62 (59.0%) and blaCTX-M 61 (58.1%). ESBL genes were more observed in hvKp than in cKp. According to the table, blaSHV was found in 90.9% of hvKp group and 60.6% of cKp group (P= 0.048).

**Detection of PMQR Genes**

In the current study, ESBL-producing isolates had a high level of resistance to all tested quinolones compared with non-ESBL-producing ones. In addition, 72 isolates that were resistant to at least one quinolone antibiotics were tested for qnr genes. The most common qnr gene was qnrB 61 (84.7%), followed by qnrS 47 (65.2%) and qnrA 16 (22.2%). Table 4 describes the distribution of qnr genes among ESBL-positive, ESBL-negative, hvKp, and cKp strains.

Results revealed that qnr subtypes could be present alone or in association with blaCTX-M, blaTEM, and blashv genes. The simultaneous presence of two or three qnr genes was also detected. qnrBS combinations were most frequently associated with blaCTX-M (54.11%), and 59.7% (43/72) of the isolates had both qnrB and blashv genes. Statistical analysis showed that unlike ESBL genes, qnr genes were mostly present in cKp isolates (P-value<0.05 (Table 4)).

**Discussion**

To our knowledge, this is the first report on the prevalence of hvKp isolates among UTI samples in Iran. Over the recent years, hvKp has emerged as a global health issue and a potential “superbug” for clinical settings, causing a wide variety of infections among community patients. In the current study, using a positive string test, 10.5% of K. pneumoniae were identified as hypervirulent, which was confirmed by the presence of the rmpA and iucA genes. Lin et al reported lower amount of hvkp in UTI samples (27.8%). In the present study, hvkp strain caused UTI mostly among old female patients. Diabetes mellitus was the most prevalent underlying disease 7/11 (63.6%). A previous study implied that patients with diabetes ran a higher risk for symptomatic UTI. Other contributing underlying diseases were chronic renal insufficiency, kidney transplant, and immunosuppression. These results are similar to the previous findings on UTI in China.

Consistent with previous reports, the rate of resistance to common antibiotics in hvKp strains was lower than that in the cKp group with the exception of cefixime, trimethoprim–sulfamethoxazole, nitrofurantoin, and carbapenems. It is widely accepted that carbapenemase-producing hvKp (CR-hvKp) strains cause various life-threatening infections, especially in critical patients.
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<td>CFX, GEN, NAL, NIT, CTX</td>
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</table>

**Table 1 Clinical and Microbiological Characteristics of hvKp Isolates**

**Abbreviations:** AMC, amoxicillin-clavulanic acid; CFX, cefixime; CTX, cefotaxime; CAZ, cefazidime; FEP, cepime; OFLA, ofloxacim; LEVO, levofloxacim; CIP, ciprofloxacin; IMI, imipenem; MERO, meropenem; GEN, gentamycin; NAL, nalidixic acid; PTZ, piperacillin-tazobactam; SXT, trimethoprim-sulfamethoxazole; NIT, nitrofurantoin.
Table 2 Percentage of Antimicrobial Resistance of K. pneumoniae

<table>
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<tr>
<th>Total n=105</th>
<th>ESBL Positive n=55</th>
<th>ESBL Negative n=50</th>
<th>P value</th>
<th>cpk n=94</th>
<th>hvkp n=11</th>
<th>P value</th>
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<td>R (%)</td>
<td>R (%)</td>
<td>R (%)</td>
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<td>Cephalosporins</td>
<td>Cefixime</td>
<td>55(52.4)</td>
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<td>Cefotaxime</td>
<td>64(61.0)</td>
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<td>22(21.0)</td>
<td>16(29.1)</td>
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<td>Imipenem</td>
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<td>Meropenem</td>
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<td>Fluoroquinolone</td>
<td>Ciprofloxacin</td>
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<td>65(69.1)</td>
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<td>11(22.0)</td>
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Table 3 Frequency of Different ESBL Genes and Capsular Serotype. Among ESBL Positive, ESBL Negative, hvkp and cpk

<table>
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<tr>
<th>Genes</th>
<th>Total=105</th>
<th>ESBL Positive</th>
<th>ESBL Negative</th>
<th>P value</th>
<th>Within hvkp (%)</th>
<th>Within cpk (%)</th>
<th>P value</th>
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<td>67(63.8)</td>
<td>42(62.7)</td>
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<td>33(53.2)</td>
<td>29(46.8)</td>
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<td>7(63.6)</td>
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<td>blsCTX-M</td>
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<td>38(62.3)</td>
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<td>4(50.0)</td>
<td>4(50.0)</td>
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<td>6(54.5)</td>
<td>2(23.1)</td>
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<td>K2</td>
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<td>35 (50.0)</td>
<td>0.253</td>
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</tbody>
</table>

Empirical use of beta-lactam drugs for CA-UTI ensues increased rates of resistance and outbreaks. In the present study, 52.4% of isolates were ESBL producers, which is higher than that obtained in Taiwan (20.7%) and South Korea (30.0%). In Turkey (2019), on the other hand, 47% of Klebsiella spp. were ESBL producers. These differences might be attributed to the excessive use of antibiotics and differences in risk factors. Unfortunately, phenotypic ESBLs were more in hvkp strains (7 (63.6%)) than in cKp (48 (51.1%)). In a study carried out in China in 2010, only 17% of hvkp were ESBL producers. In the current study, all hvKps carried ESBL genes, implying the compatibility of plasmids containing these genes with hvKp strains.

Additionally, MDR-hvKp was identified in 5 out of 11 (45.4%) hvKp isolates. This shows the resistance to at least one agent in three or more antimicrobial categories, which is higher than a study conducted in China (2019), where 20% MDR-hvKp was detected. Taken together, these data reveal that MDR-hvKp isolates are emerging and antimicrobial resistance is growing among these strains, which is similar to previous studies in China. Therefore, new clinical and medical interventions concerning these strains are needed.

Serotypes K1 and K2 have been generally considered as predominant virulent strains of K. pneumoniae, causing high resistance to phagocytosis. In this research, 54.5% of hvKp possessed k1 gene (p value<0.001) whereas 27.3% had K2 gene (p value= 0.005). This is in accordance with a study carried out in the US in 2018 (55% k1 vs 20% k2) and another performed in China in 2016 (33.3% k1 vs 17.2 k2).
The resistance rates of the antibiotics to ESBL-positive strains between CA-UTIs were significantly higher than those of ESBL-negative strains (P < 0.05) (Table 2). Based on these results, ESBL-producing *Klebsiella* spp. are not merely a nosocomial problem, rather they are a growing issue in CA-UTIs.

The PCR revealed the prevalence of ESBL genes in the current study. Nevertheless, we found that *bla*<sub>SHV</sub> was the most prevalent ESBL gene, followed by *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub>. In a similar investigation done in Iraq (2019), 64.7% *bla*<sub>TEM</sub>, 35.2% *bla*<sub>SHV</sub> and 41.1% *bla*<sub>CTX-M</sub> genes existed in the isolates of *K. pneumoniae*. 49

ESBL producing strains are mostly resistant to common antibiotics such as fluoroquinolones. 50 Moreover, plasmid-mediated quinolone resistance might facilitate the spread and increased frequency of quinolone-resistant strains. A high proportion of isolates (68%) in this study were resistant to fluoroquinolones. According to the findings, resistance to the tested fluoroquinolones in ESBL-producing isolates was significantly higher than in non-ESBL-producing isolates, which is similar to another study performed in the Asia-Pacific region. 51 In the current research, 46 (83.6%) of ESBL-positive *K. pneumoniae* isolates were resistant to ciprofloxacin, and their prevalence was higher than that of Meshkat (37.5%) in 2017. 52

So far, *qnr* genes have been widely detected in different parts of the world. 53 The frequencies found in the current research were higher than those reported in a Moroccan community in 2014, where the prevalence of *qnr* gene between 50% *Klebsiella* and 18.7% *E. Coli* was 23% *qnrB*, 10% *qnr A*, and 3% *qnrS*. 54 PMQR are plasmid-mediated and can be easily transmitted across the Enterobacteriaceae family members, thereby facilitating the epidemic spread of quinolone resistance through horizontal gene transfer. 55

Coexistence of PMQR and ESBL genes was identified in four out of 11 hvKp isolates. Although ESBL genes were mostly observed in hvKp than in cKp, *qnr* genes mostly existed in cKp strains (P < 0.05). These results imply that hvKp strains are capable of acquiring various types of antibiotic resistance mechanisms via the transmission of mobile genetic elements. In addition to the disconcerting antimicrobial resistance pattern of hvKp strains, these isolates underscore the need for specific surveillance programs.

**Conclusions**

In this study, the frequency of hvKp was lower than similar studies in other regions; however, the higher rates of resistance to antimicrobial agents, particularly carbapenems, and the high rates of MDR emergence indicate that these strains have emerged as major issues in healthcare centers. Moreover, the coexistence of PMQR and ESBL genes severely affects the clinical outcomes due to the complications in therapy, hence the necessity to enhance the clinical knowledge and management of hvKp infections. It is also important to take appropriate supportive measures regarding empirical antibiotic therapy so as to control the spread of resistant strains.

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**Disclosure**

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