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Virulent and multidrug-resistant *Klebsiella pneumoniae* from clinical samples in Balochistan

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Abstract

Klebsiella pneumoniae is an important pathogen causing hospital-acquired infections in human beings. Samples from suspected patients of *K pneumoniae* associated with respiratory and urinary tract infections were collected at Bolan Medical Complex, Quetta, Balochistan. Clinical samples (n = 107) of urine and sputum were collected and processed for *K pneumoniae* isolation using selective culture media. Initially, 30 of 107 isolates resembling *Klebsiella* spp. were processed for biochemical profiling and molecular detection using gyrase A (*gyrA*) gene for conformation. The *K pneumoniae* isolates were analysed for the presence of drug resistance and virulence genes in their genomes. The 21 of 107 (19.6%) isolates were finally confirmed as *K pneumoniae* pathogens. An antibiogram study conducted against 17 different antibiotics showed that a majority of the isolates are multidrug resistant. All the isolates (100%) were resistant to amoxicillin, cefixime, amoxicillin-clavulanic acid, cefotaxime, and ceftriaxone followed by tetracycline (95.2%), ciprofloxacin and gentamicin (76.2%), sulphamethoxazol (66.7%), nalidixic acid (61.9%), norfloxacine (42.9%), piperacillin-tazobactam (23.8%), cefoperazone-sulbactam (19%), and cefotaxime-clavulanic acid (33.3%), whereas all the isolates showed sensitivity to amikacin, chloramphenicol, and imipenem. The presence of tetracycline, sulphamethoxazol-resistant genes, and extended-spectrum beta-lactamase was reconfirmed using different specific genes. The presence of virulence genes *fimH1* and *EntB* responsible for adherence and enterobactin production was confirmed in the isolates. The high virulence and drug resistance potential of these *Klebsiella* isolates are of high public health concern. Multidrug resistance and virulence potential in *K. pneumoniae* are converting these nosocomial pathogens into superbugs and making its management harder.

Sareeen Fatima and Faiza Liaqat contributed equally to the scientific work.

KEYWORDS

antibiotics, antimicrobial, nosocomial infections, opportunistic pathogens, pathogenicity

1 | INTRODUCTION

The genus *Klebsiella* falls under Enterobacteriaceae family, a Gram-negative bacteria with rod shape, lysine decarboxylase but not ornithine decarboxylase producers, and Voges-Proskauer positive.[1] *Klebsiella* species are ubiquitous in nature and can be frequently found in different environments such as water, soil,[2] and dirt. This bacteria can colonise in nasopharynx and gastrointestinal tract,[3] which makes them able to act as an opportunistic pathogen in human being.[4] This bacteria has also been reported in insects[5] and mammals.[6] Gastrointestinal colonisation is likely a common and significant reservoir among body sites in terms of risk of transmission and infection.[7]

Klebsiella pneumoniae is a clinically important member of the genus *Klebsiella*, reportedly being responsible for around 86% of human infections due to *Klebsiella*,[8] which makes it the most significant pathogen of this genus. However, species of *Klebsiella oxytoca* are reported as the second most widespread species, responsible for about 26% of infections.[1,9] *Klebsiella* spp. are considered as an important member of hospital-acquired pathogens, which are not only responsible for numerous infections of respiratory and urinary tract, but can also cause the infection of soft tissues, wounds, sepsis, and septicaemia.[9,10] The presence of virulence factor and drug resistance enhances the colonisation of *Klebsiella*, particularly in the case of nosocomial infections. The most common pathogenicity factors in this species are iron acquisition capability, possession of fimbriae, lipopolysaccharide, and so on.[11] Types 1 and 3 fimbriae present in *Klebsiella* enhanced the urinary tract infections.[12] Virulence genes enterobactin synthase component B (*entB*), aerobactin siderophore receptor (*iutA*), putative salicylate synthetase (*ybtS*), iron regulatory protein 1 and 2 (*irp-1*, *irp-2*), and ferric yersiniabactin uptake receptor (*fyuA*) responsible for siderophores production, which are present in this species, can make them able to acquire iron from human host.[13] The lipopolysaccharide and capsule increase its pathogenicity and help them avoiding phagocytosis, resulting sepsis and septic shock.[14]

Drug resistance to multiple antibiotics in pathogens is the increasing cause of morbidity and mortality.[15] *K pneumoniae* has been reported with resistance to fluoroquinolones, aminoglycosides, and beta-lactam antibiotics. The production of beta-lactamase enzyme by *Klebsiella* species is making them resistant to a wide group of antibiotics.[16] In a majority of *K pneumoniae* isolates, the β-lactamase is encoded by chromosomal-encoded SHV-1 gene.[6] Pathogens with increased pathogenicity factors and multidrug resistance act as a superbug and are a growing public health threat to the developing and developed countries. A study was designed to record the prevalence of *K pneumoniae* in urinary tract infection (UTI) and respiratory tract infections (RTI), and to determine their multidrug resistance and virulence potential of Balochistan patients in Quetta City.

Key messages

- *Klebsiella pneumoniae* is an important nosocomial pathogen, responsible for different systemic infection in human being
- MDR *K pneumoniae* with virulence genes were isolated form the urinary tract infection and respiratory tract infection patients in this study
- the isolates (100%) in this study showed resistant to amoxicillin, cefixime, amoxicillin-clavulanic acid, cefotaxime, and ceftriaxone

2 | MATERIALS AND METHODS

2.1 | Sampling

A total of 107 samples, of which 72 were urine and 35 sputum, were collected from different patients suffering UTI and RTI in Bolan Medical Complex, Quetta. All the samples were collected aseptically in sterile sampling bottles, following safety protocols and procedures, with the patient inform consent and according to the will and satisfaction of patients. The samples' inclusion criteria were strictly limited to UTI and RTI patients. Samples were carried to the laboratory in the University of Balochistan and processed within 1 to 2 hours of collection, not later than 6 hours.

2.2 | Isolation of the target pathogen

The collected samples were aseptically inoculated to pre-prepared sterile MacConkey agar (Oxoid, UK) media and incubated at 37°C for 24 hours. Mucoid, circular, and

lactose-fermenting colonies were subculture to Eosin Methylene Blue agar (EMB) (Oxoid, UK) for conformation and differentiation with *Escherichia coli*.

2.3 | Biochemical characterisation

Presumptively selected isolates were Gram stained and biochemically characterised using biochemical tests, such as catalase, oxidase, urease, sulphur, indole, motility, methyl red, Vogues-Proskauer, and citrate utilisation.[17]

2.4 | Molecular conformation of the isolates

The preliminary conformed *Klebsiella* isolates were confirmed with the help of genotyping using *Klebsiella*-specific gene *gyrA* (F-CGCGTACTATACGCCAT GAACGTA and R-ACCGTTGATCACTTCGGTCAGG)[18] following standard protocol for DNA extraction.[19] Polymerase chain reaction (PCR) (25 μL) was prepared by mixing 12.5 μL master mix with 1 μL of each forward and reverse primers, 7.5 μL nuclease-free PCR water and DNA sample (3 μL). The mixture was processed at 94°C denaturing for 4 minutes. 94°C for 30 seconds, 55°C for 40 seconds, 72°C for 60 seconds, and 30 cycle. Final extension was performed at 72°C for 10 minutes and at a final temperature of 4°C.

2.5 | Antibiogram studies

The antibiogram studies of the conformed *K pneumoniae* were conducted by using the Kirby-Bauer technique of disc diffusion with Mueller-Hinton agar (MHA) (Oxide, UK).[20] The target bacteria were spread over the surface of sterile MHA plates using sterile cotton swab. Antibiotics discs amoxicillin (AML 10 μg), cefixime (CFM 5 μg), sulphamethoxazol (SXT 25 μg), tetracycline (TE 30 μg), nalidixic acid (NA10 μg), ciprofloxacin (CIP 5 μg), norfloxacin (NOR 10 μg), imipenem (IMP 10 μg), amikacin (Ak 20 μg), gentamycin (CN 10 μg), chloramphenicol (C30 μg), cefotaxime (CTX 30 μg), amoxicillin-clavulanic acid (AMC 30 μg), piperacillin-tazobactam (TZP 110 μg), cefoperazone-sulbactam (SCF 105 μg), ceftriaxone (CRO 30 μg), and cefotaxime-clavulanic acid (CTX + CLA 30/10 μg) were placed over the surface of the bacterial lawn and incubated at 37°C for 16 to 24 hours. The zone of inhibitions of each antibiotic was recorded in millimetre (mm) and it corresponds to the CLSI standard values of respective antibiotics.[21]

2.6 | Extended-spectrum beta-lactamase production

The extended-spectrum beta-lactamase (ESBL) production test was performed by double discs ceftriaxone (CRO 30 μg) and cefotaxime-clavulanic acid 30/10 μg (BD).[22]

2.7 | Molecular conformation of drug resistance genes in the isolates

The phenotypically evaluated resistance in *Klebsiella* isolates was confirmed against specific genes corresponding resistance to selected antibiotics through PCR. Tetracycline resistance was confirmed by gene *tetB* using primers F-CCTTATCAT GCCAGTCT TGC, R-ACTGCCGT TTTTTCGC C,[23] while *Sul1* gene using primer F-CGGCGTGGGCTACCT GAACG and R-GCCGATC GCGTGAAGTTCCG responsible for sulphonamide-resistant[24] and *SHV* gene for ESBL production using primer F-ATTTGTCGCTTCTTTACTCGC and R-TTTATGGCG TTACCTTTGACC.[25]

2.8 | Molecular detection of virulence factors

The virulence factors associated with *K pneumoniae* were determined by targeting specific gene *fimH* encoding for type 1 fimbriae, the main virulence factor of the bacteria, using primer F′-ATGAACGCCTGGTCCTTTGC, R-GCT GAACGCCTATCCCCTGC,[26] and *EntB* gene responsible for enterobactin production using primer F-ATTTCC TCAACTTCTGGGGC and R-AGCATCGGTGGCGGT GGTCA.[27]

3 | RESULTS

Thirty isolates were preliminary identified as *K pneumoniae* out of the total (107) samples based on initial screening. Of which 21 of 107 isolates were biochemically and molecularly confirmed as *K pneumoniae* with the help of Gyrase A (*gyrA*) gene (Figure 1), making 19.6% prevalence of the pathogens in the patients. All the isolates were collected from the urine (72) and sputum (35) samples of the patient suspected with *K pneumoniae* associated RTI and UTI. The *K pneumoniae* isolates of 20.8% (15/72) were from urine and of 17.1% (6/35) from sputum.

All the gyrase A gene-positive *K pneumonia* isolates (21) were processed for antibiogram studies against

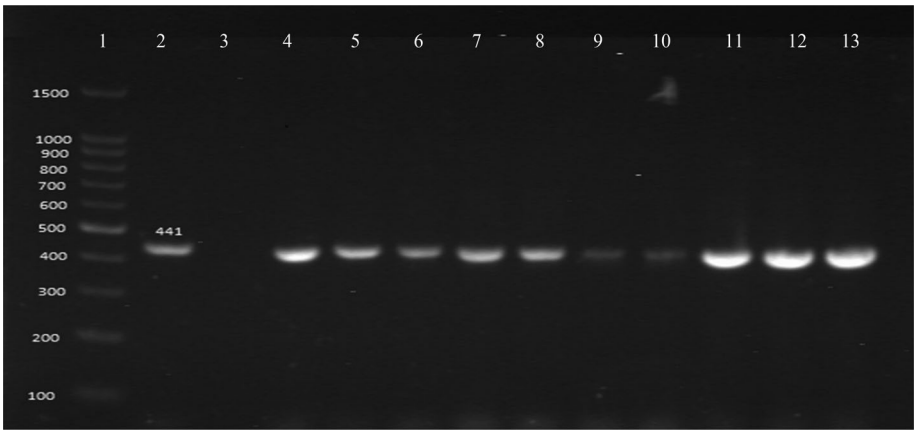

FIGURE 1 Polymerase chain reaction assay result for *Klebsiella* identification; line 1 = DNA size ladder 100 bp (GeneDireX); line 2 = reference strain for *gyrA*; line 3 = negative control; line 4–13 = positive samples

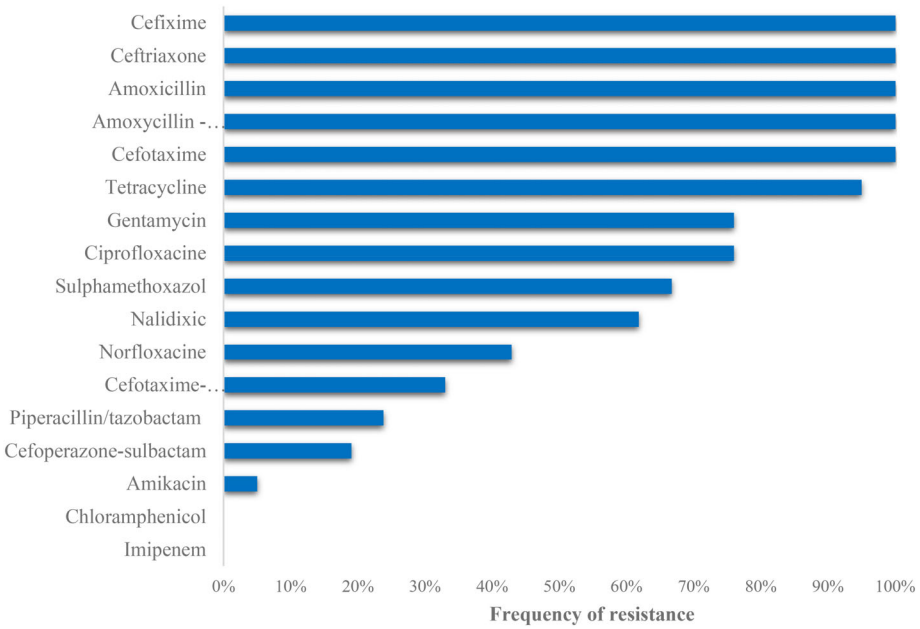

FIGURE 2 Drug resistance pattern of *K pneumoniae* clinical isolates against antibiotics used

17 common antibiotics. The antibiogram studies revealed that all the isolates were multidrug resistant to the tested antibiotics (Figure 2). The antibiogram of the isolates showed 100% (21/21) resistance to CFM, AML, CTX, CRO, and AMC, whereas TET 95.2% (20/21), CIP and CN 76.2% (16/21), and SXT 66.7% (14/21). Resistance against NA were found to be 61.9% (13/21), while 42.9% (9/21), 33.33% (7/21), and 23.8% (5/21) against NOR, CTX+C, and TZP, respectively.

The lowest percentage of resistance was found against SCF 19% (4/21), AK 4.8% (1/21), C, and IMP 0% (0/21). It was found that out of 21 isolates only 7 (33.3%) were positive for ESBL production. All of the isolates showed resistant to third-generation cephalosporin.

In this study, imipenem and chloramphenicol were found to be highly active (100%) antibiotics against the *K pneumoniae* isolates, whereas only one (4.8%) isolate showed resistance to the drug amikacin. It was noted that out of 21 isolates, two isolates showed multidrug resistance to 14 and 13 antibiotics used in the study, while six isolates showed resistance to 11 antibiotics and four to 10 antibiotics used. Two isolates showed resistance to 12 and five isolates to 9 antibiotics, respectively. One isolate was found resistant to 8 antibiotics and the other to 6 antibiotics used. This study confirms the extensive multidrug resistance potential of the *K pneumoniae* clinical isolates.

The SXT, TET, and ESBL resistances were genotypically (*Sul1*, *tetB*, and *SHV*) reconfirmed using specific primers (Figure 3A-C). The *tetB* gene was confirmed in all the isolates showing 100% resistance of the isolates against tetracycline, whereas *Sul1* gene corresponding to sulphamethoxazol resistance was present in 14/21 (66.7%) isolates and SHV in 7/21 showing that 33.3% were positive for ESBL production. Pathogenicity of pathogens is dependent on the presence of virulence factors,

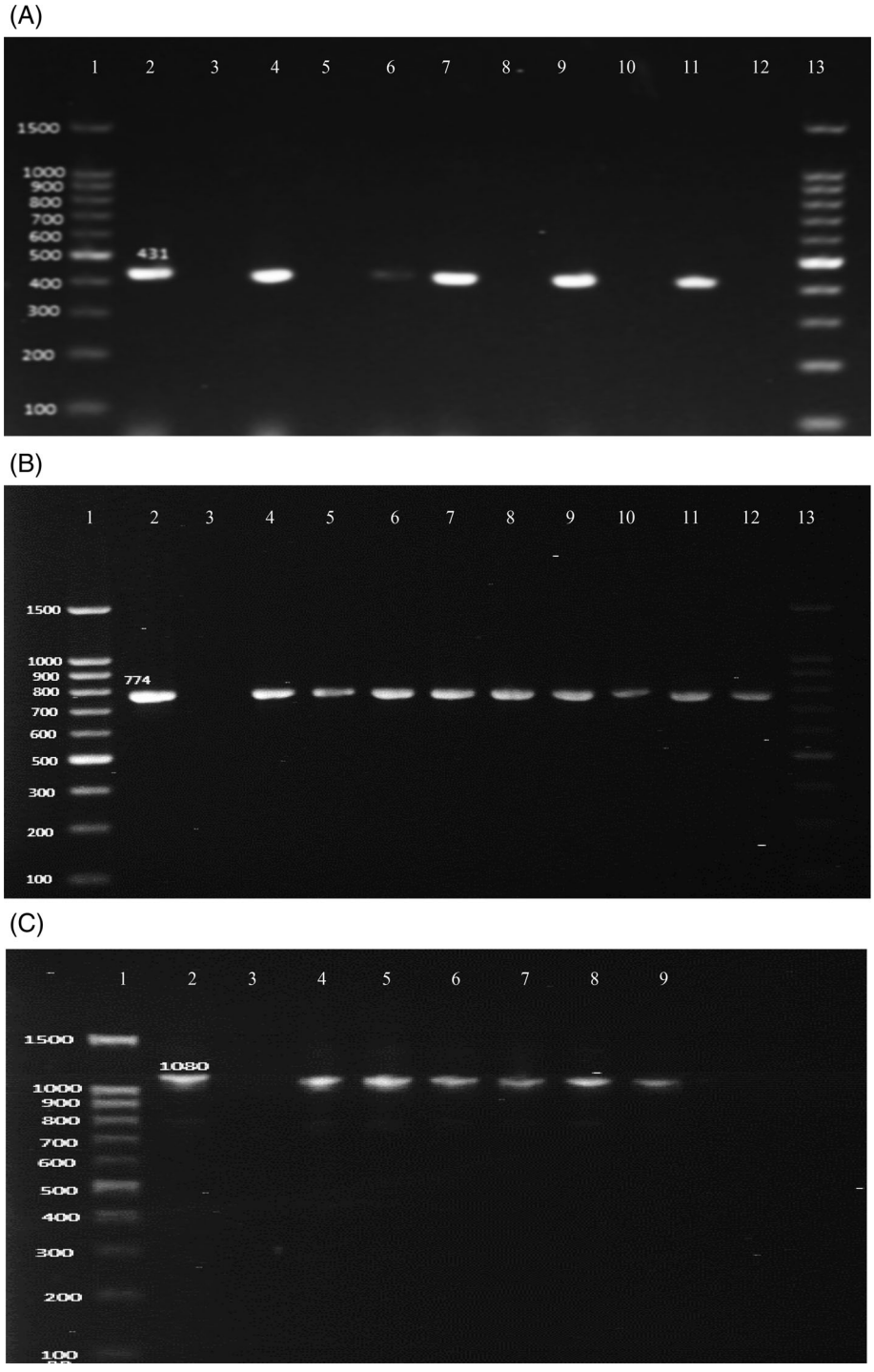

FIGURE 3 A, PCR results for Sul1 (431 bp) drug resistance gene; line 1 and 13 = DNA size ladder 100 bp (GeneDireX); line 2 = reference strain for *Sul1* gene positive; lines 3, 5, 8, 10, and 12 = negative control; lines 4, 6, 7, 9, and 11 = positive samples. B, PCR results for *tetB* (774 bp) drug resistance gene; lines 1 and 13 = DNA size ladder 100 bp (GeneDireX); line 2 = reference strain for *tetB* gene positive; line 3 = negative control; lines 4–12 = positive samples. C, PCR results for SHV (1080 bp) ESBL resistance gene; line 1: DNA size ladder 100 bp (GeneDireX), line 2: reference strain for SHV gene positive; line 3: negative control; lines 4–9 = positive samples

which are directly proportional to the higher pathogenicity, resulting in more complicated infection.

In this study, we evaluated the presence of common and selected virulence factors associated with *K pneumoniae* clinical isolates. The virulence factors are important to show the clinical and nosocomial importance of the isolates. The 21 confirmed *K pneumoniae* isolates were analysed for the presence of *fimH* gene responsible for improved adherence properties and *entB* gene of enterobactin production. All the isolates found bearing the targeted genes, with 100% presence of type 1 fimbriae and enterobactin production (Figure 4A,B), showed the high pathogenicity potential of these isolates (Table 1).

4 | DISCUSSION

K pneumoniae is an important member of ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *K pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas*

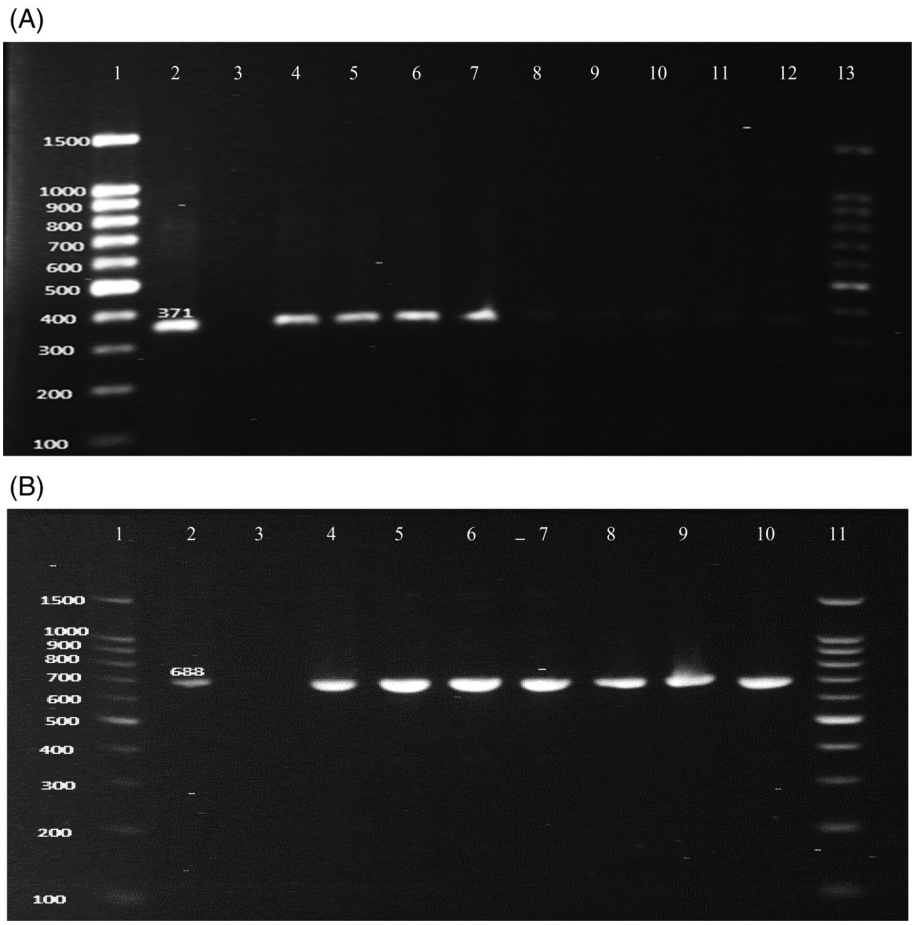

FIGURE 4 A, Polymerase chain reaction assay result for virulence gene *EntB* (371 bp); lines 1 and 13 = DNA size ladder 100 bp (GeneDireX); Line 2 = reference strain for EntB gene positive; line 3 = negative control; lines 4–12 = positive samples. B, PCR results for *fimH-1* (688 bp) virulence gene; lines 1 and 12 = DNA size ladder 100 bp (GeneDireX), line 2 = reference strain for *fimH-1* gene positive; line 3 = negative control; lines 4–11 = positive samples

TABLE 1 Percentage and number of genes detected among *K pneumoniae* clinical isolates

Gene	Urine isolates	Sputum isolates	Total
fimH1	15/15 (100%)	6/6 (100%)	21/21(100%)
EntB	15/15 (100%)	6/6 (100%)	21/21 (100%)
tetB	15/15 (100%)	6/6 (100%)	21/21 (100%)
Sul1	9/15 (60%)	5/6 (83.3%)	14/21 (66.67%)
SHV	3/15 (20%)	4/6 (66.67%)	7/21 (33.33%)
gyrA	15/15 (100%)	6/6 (100%)	21/21 (100%)

Abbreviations: *EntB*, Enterobactin synthase component B; *fimH1*, type 1 fimbrial adhesin; *gyrA*, gyrase A; *SHV*, sulphydryl variable β-lactamas; *Sul1*, sulphamethoxazol; *tetB*, tetracycline.

aeruginosa, and *Enterobacter* spp.) and potential threat as a nosocomial infection.[28] In this study, we found that 19.6% samples from suspected UTI and RTI infections are positive for *K pneumoniae* pathogen, which can lead the patients to complicated infections in case of drug resistance. In a similar study, Younis[29] reported 73.3% *K pneumoniae* prevalence in clinically diseased chicken, which is higher than our study, whereas Martin et al[30] and Zhang et al[31] reported 23% and 73.9%, respectively, prevalence of *K pneumoniae* in clinical samples. Fatima et al[32] and Chakraborty et al[33] reported 17% and 24% prevalence of *K pneumoniae* in Pakistan and Bangladesh, respectively, from clinical samples. The antibiogram studies revealed that a number of isolates are multidrug resistance to commonly used antibiotics. Increasing drug resistance in pathogenic bacteria is a great human health concern. Our studies showed that 33.3% *K pneumoniae* is ESBL positive. Taneja et al[34] reported 70.7% ESBL prevalence in *K pneumoniae* isolates from Dehli. Hayat et al[35] detected ESBL gene in 56.5% of *K pneumoniae* isolates from clinical samples in Pakistan, while Chakraborty et al[33] reported 45% ESBL prevalence in *K pneumoniae* in

a similar study in Bangladesh. In our study, we found a higher resistance of the clinical isolates towards antibiotics AMC, CFM, AML, CTX, and CRO. Significant resistance has been reported against cephalosporin by Nijssen et al.[36] Khamesipour and Tajbakhsh[37] claimed 87.8, 43.3, and 32.2% resistance to AMC, TET, and AK while 34.4% and 26.7% of CFM and CN, respectively. Compared to his study, resistance to these four antibiotics is higher in our study. Chakraborty et al[33] reported 56% multidrug resistance *K pneumoniae* in clinical isolates from Malaysia, where they showed 100% resistance to ampicillin; 90% to amoxicillin; 45% to ceftriaxone; 40% to ciprofloxacin; 45% to co-trimoxazole; and 25%, 50%, and 35% to gentamicin, nalidixic acid, and tetracycline respectively.

In our study, we found that all the isolates were multidrug resistance to minimum 6 and maximum 14 antibiotics out of 17 used in the study. Lina et al[38] reported resistance in ceftazidime (36%), CN (27%), TET (27%), and CIP (45%) to *K pneumoniae*. It was found in a similar study that 61.2% of *K pneumoniae* are drug resistant, wherein 20.4% of the isolates showed 100% resistance to all cephalosporins (CFM, CRO, ceftazidime, and ceftizoxime), and 98% to carbenicillin, 55% to piperacillin, 32% to CTX, and 31% to ceftazidime with zero resistant to IMP.[39] These results are in agreement with our result where 100% resistance was found against cephalosporins and 0% against imipenem. In our study, the resistance rate against tetracycline, aminoglycosides, and fluoroquinolones was higher in comparison with ESBL-producing isolates, similar results have been reported by Hashemi et al.[40] Similar to our study, Taitt et al[41] reported 100% resistance to tetracycline conformed with *tetB* and 60% *Sul1* resistance genes in their study in Kenya. Gholipour et al[42] detected *SHV* genes responsible for ESBL production on 7.5% *K pneumoniae* isolates, which is less than that of our study (33.3%). The higher pattern of drug resistance in *K pneumoniae* isolates in our study can be linked to over the counter sale and extensive uses of antibiotics in animal farming.[20] Banning over the counter sale and abuse of antibiotics for animal farming is the possible solution to control the drug resistance in Pakistan.

The virulence factor such as enterobactin production helps the pathogens in biofilm formation and infection development.[43] Studies revealed that enterobactin biosynthesis is iron-uptake proteins produced by *Klebsiella*[44] because of the presence of *entB* gene in its genome.[45] We found the presence of *entB* gene in 100% *K pneumoniae* isolates, which are in compliance with Aljanaby and Alhasani[44] study. El Fertas-Aissani et al[27] also reported 100% detection of *entB* gene in *K pneumoniae* isolates in their study. Possessing fimbriae can increase the pathogenicity of pathogens.[46] *fimH1* gene responsible for type 1 fimbriae can enhance the biofilm formation capability of *Klebsiella* to colonise in urinary and respiratory tract resulting in complicated infection.[44] In our study, we find 100% presence of *fimH1* gene, and these results are supported by Xiao et al[47] study where they reported the high frequency of *fimH1* gene identification in *K pneumoniae*. It was also reported that the presence of fimbriae can enhance the drug resistance of the pathogen.[48]

5 | CONCLUSIONS

It was concluded in this study that the increased and unnecessary uses of antibiotics produce superbugs within the common nosocomial pathogens such as *K. pneumoniae*, posing a serious threat to global public health. The emergence of multidrug resistance in common virulent species of pathogens will have a great impact over the health system and economy of developing countries. The *K. pneumoniae* isolates were found harbouring virulent drug-resistant genes and were showing resistance to several common antibiotics, which are of great concern. Alternative management procedures and novel drug hunting along with wise uses of antibiotics are the possible solutions to fight the multidrug resistant pathogens.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data will be available on request.

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