Phylogenetic Analysis of Antibiotic Resistance Genes and Virulence Genes of *Klebsiella* species *in silico*

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ABSTRACT: A total of twelve isolates were screened for virulence and antibiotic resistance genes associated with *Klebsiella pneumoniae* infections. Virulence and antibiotic resistance genes were detected by *in silico* PCR amplification. Iron uptake protein *entB* was detected in 66.67% (n=8) of the isolates while no isolate was found to harbour chelating agent *irp2*. Iron uptake system *kfu,* involved in purulent tissue infections and capsule formation, was identified in 25% (n=3) of the isolates. Regulator of mucoid phenotype A, *rmpA* was not found in any of the isolates. The *wabG* gene, responsible for urinary tract infections was found in seven *K. pneumoniae* strains. Five *uge* positive strains might play role in the pathogenicity of *K. pneumoniae* infections. About 83.33% of the isolates were positive for type 1 fimbriae *fimH1* while no type 3 fimbriae *mrkD* gene was found. Complement reaction blocked by plasmid *traT* gene was not observed in *Klebsiella* species while eight isolates harboured outer membrane lipoprotein, *ycfM* which protects *Klebsiella* species from antibiotics. Antibiotic resistance genes bla_{TEM} and bla_{SHV} were detected in 33.33% (n=4) and 66.67% (n=8) of the isolates while 25% isolates carried both bla_{TEM} and bla_{SHV} genes. Genotype 1 carried *fimH1* and *ycfM* genes while all the virulence genes studied were present in genotype 2 and 3. The bla_{SHV} gene was detected in all the genotypes while bla_{TEM} gene was found in only genotype 1 and 3. The findings of this study would be helpful to predict virulence gene associated with *Klebsiella* infections. This data also helps us to choose antibiotics for treating *Klebsiella* infections. By assessing the genotypic distribution of antibiotic resistance gene, correct antibiotic can be used to treat the infection. This could help reduce emergence of antibiotic resistance since it is known that incorrect choice of antibiotics contributes to antibiotic resistance.

Key words: *Klebsiella*, virulence gene, antibiotic resistance gene, PCR, genotype.

INTRODUCTION

Pathogenic bacteria *Klebsiella* belongs to Enterobacteriaceae family and responsible for pneumonia, bacteremia, urinary tract infections, pyogenic liver abscesses, wounds and burns infections.[1] Several studies reported that most of the nosocomial infections in Egypt were caused by *Klebsiella* species.[2,3] Shon *et al.* reported a new hypermucoviscous *K. pneumoniae* strain in Asian countries that was responsible for community-acquired primary liver abscesses, endophthalmitis or metastatic meningitis.[4] Western countries recognized sporadic cases of *K. pneumoniae* infections as reported by Compain *et al.*[5] The virulence factors, encoded by various virulence genes, determine the pathogenicity of *K. pneumoniae* infections and these virulence factors are responsible for many kinds of diseases through attacking the mammalian immune system.[6] Several studies found biofilm formation, capsule synthesis, iron uptake, hypermucoviscosity, lipopolysaccharides formation as virulence factors which are involved in the pathogenicity of *K. pneumoniae* infections.[7,8]

The clinical features of *K. pneumoniae* infections mostly depend on the mode of actions of virulence factors encoded by virulence genes. Recent studies reported that *K. pneumoniae* was responsible for an acute liver abscess in China, Kuwait and Iraq.[8,11]

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K. pneumoniae infections are particularly worrisome because of the resistance grown to most of the antibiotic drugs available, creating a serious situation reminiscent of the pre-antibiotic era. Antibiotic-resistant infections, therefore, are a substantial health and economic burden to patients and their families. Antibiotic resistant infections commonly occur due to extensive use of invasive procedures and abuse of antibiotics. Carbapenem-resistant Enterobacteriaceae (CRE) are a group of bacteria that have become resistant to nearly all available antibiotics, including carbapenems. Previously enterobacteriaceae infections were treated with carbapenems antibiotics[12] but prevalence of carbapenem-resistant *Klebsiella* species increased dramatically from 2001 to 2011.[13] Gram-negative enterobacteriaceae such as *Escherichia coli* and *K. pneumoniae* harboured an enzyme called New Delhi metallo-beta-lactamase (NDM-1) which makes them resistant to virtually all beta-lactams, including carbapenems. Yeh *et al.* demonstrated that *K. pneumoniae* infections are hard to treat due to resistant properties to third generation cephalosporins like cefotaxime, ceftriaxone and ceftazidime.[14] *K. pneumoniae* harbours plasmid encoded beta-lactamase genes (SHV, TEM and C-TXM) which conferred resistance to many types of antibiotics.[15] Antibiotic-resistant infections add considerable costs to the nation's already overburdened health care system. When first-line and then second-line antibiotic treatment options are limited or unavailable, health care professionals may be forced to use antibiotics that are more toxic to the patients and frequently more expensive. Coordinated efforts to implement new policies, renew research efforts, and pursue steps to manage the problem are greatly needed. As an alternative to *in vitro* methods for identifying bacteria, various *in silico* methods have been developed. *In silico* study in medicine is thought to have the potential to choose the effective antibiotics while reducing the need for expensive lab work and clinical trials.

The aim of this study was to characterize the prevalence of virulence and antibiotic resistance genes in 12 *Klebsiella* species. Pulsed-field gel electrophoresis (PFGE) analysis was used to assess the genotypic distribution of virulence and resistance genes of *Klebsiella* strains.

MATERIALS AND METHODS

Strains used in the study. Strains used in the study are summarized in Table 1.

Primers used in the study. Primers used for detection of antibiotic resistance genes are summarized in Tables 2 and 3.

Table 1. Name of the isolates.

Sl. No.	Isolate
1	NC_018106 *Klebsiella oxytoca* E718
2	NC_016612 *Klebsiella oxytoca* KCTC 1686
3	NC_011283 *Klebsiella pneumoniae* 342
4	NC_022566 *Klebsiella pneumoniae* CG43
5	NC_022082 *Klebsiella pneumoniae* JM45
6	NC_016612 *Klebsiella pneumoniae* KCTC 2242
7	NC_012731 *Klebsiella pneumoniae* NTUH-K2044
8	NC_018522 *Klebsiella pneumoniae* subsp. *pneumoniae* 1084
9	NC_016845 *Klebsiella pneumoniae* subsp. *pneumoniae* HS11286
10	NC_009648 *Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578
11	NC_021232 *Klebsiella pneumoniae* subsp. *rhinoscleromatis* strain SB3432
12	NC_013850 *Klebsiella variicola* At-22

Table 2. Primers for detection of virulence genes.

Gene	Primer sequence (5'-3')	Amplicon size (bp)
entB	ATTTCCTCAACTTCTGGGGC AGCATCGGTGGCGGTGGTCA	371[7]
irp-2	TCCCTCAATAAAGCCCACGCT TCGTCGGGCAGCGTTTCTTCT	287[16]
kfu	GAA GTG ACG CTG TTT CTG GC TTT CGT GTG GCC AGT GAC TC	797[17]
wabG	CGGACTGGCAGATCCATATC ACCATCGGCCATTTGATAGA	683[18]
rmpA	ACTGGGCTACCTCTGCTTCA CTTGCATGAGCCATCTTTCA	535[14]
uge	TCT TCA CGC CTT CCT TCA CT GAT CAT CCG GTC TCC CTG TA	534[19]
fimH1	ATGAACGCCTGGTCCTTTGC GCTGAACGCCTATCCCCTGC	688[7]
mrkD	CCACCAACTATTCCCTCGAA ATGGAACCCACATCGACATT	226[7]
ycfM	ATCAGCAGTCGGGTCAGC CTTCTCCAGCATTCAGCG	107[7]
trtT	GGTGTGGTGCGATGAGCACAG CACGGTTCAGCCATCCCTGAG	288[7]

Table 3. Primers for detection of antibiotic resistance genes.

Gene	Primer sequence (5’-3’)	Amplicon size bp
bla_{SHV}	GGCCGCGTAGGCATGATAGA CCCGGCGATTTGCTGATTTC	714[20]
bla_{TEM}	CAGCGGTAAGATCCTTGAGA ACTCCCCGTCGTGTAGATAA	643[20]
bla_{VIM}	GTTTGGTCGCATATCGCAAC AATGCGCAGCACCAGGATAG	389[21]
bla_{IMP}	GAAGGCGTTTATGTTCATAC GTACGTTTCAAGAGTGATGC	587[21]
$bla_{\text{OXA-48}}$	GCGTGGTTAAGGATGAACAC CATCAAGTTCAACCCAACCG	438[22]
$bla_{\text{NDM-1}}$	GGTGCATGCCCGGTGAAATC ATGCTGGCCTTGGGGAACG	660[23]

PCR amplification. An online software, http://insilico.ehu.eus/PCR/ was designed to perform *in silico* PCR amplification.[24,25]

PFGE digestion. Pulse-field gel electrophoresis (PFGE) digestion and construction of the dendrogram was done *in silico* using the website http://insilico.ehu.es/digest/.[24,25] The enzyme used for the digestion was *Xba*I and lambda ladder was used to compare the bands.

RESULTS AND DISCUSSION

Genetic diversity of the isolates was determined using pulsed field gel electrophoresis (PFGE) by *Xba*I restriction digestion. This enzyme recognized the T'CTAG_A sequence and isolates clustered into four genotypic groups according to their banding patterns (Figure 1). Genotype 3 was found to be the most prevalent (50%) followed by genotype 1 (25%), 2 (16.67%) and 4 (8.03%) as shown in Figure 2.

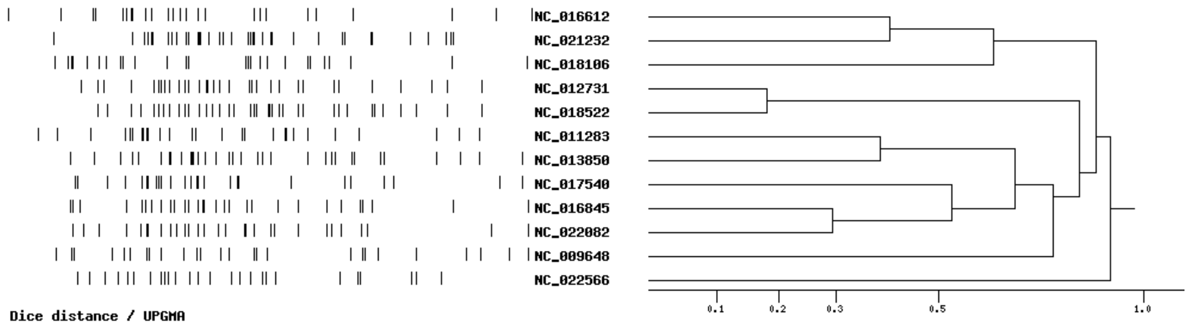

Figure 1. Phylogenetic diversity of *Klebsiella* species identified by PFGE.

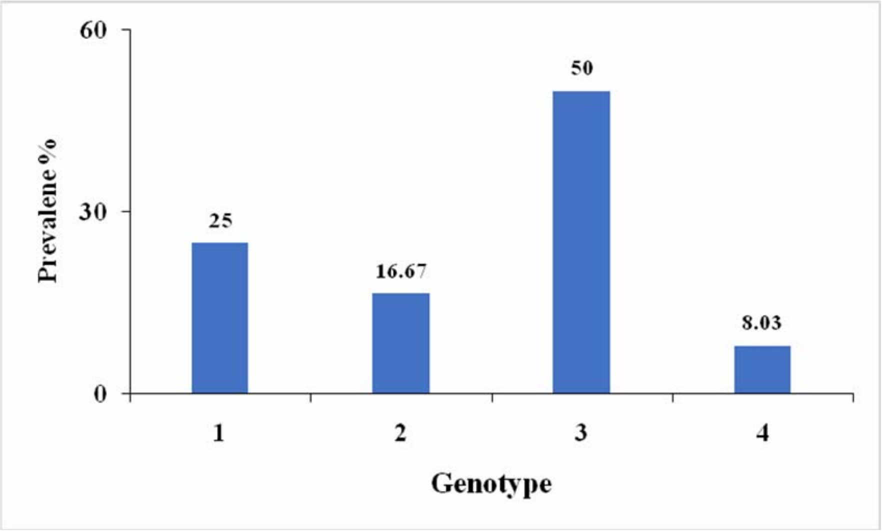

Figure 2. Prevalence of genotypes.

A recent study documented that fungi and bacteria secreted iron chelating agents siderophores.[6] Siderophore compounds enterobactin biosynthesis (*entB*) and yersiniabactin biosynthesis (*irp-2*) which are iron uptake proteins produced by *Klebsiella*. These chelating agents demonstrated higher affinity to extracellular ferric ions.[6] May and Okabe suggested that biofilm formation is induced by the expression of enterobactin.[26] Another study also found that ironenterobactin genes are activated when bacterial infections occurred.[27] Aljanaby *et al.* documented that all isolates were found to be positive for enterobactin, *entB* gene but yersiniabactin, *irp-2* was found in only 12 isolates.[6] Eight isolates (66.67%) were found to harbour enterobactin gene, *entB* and gave 371 bp gene product in the present study among them seven were *K. pneumoniae*. Several studies also found that *entB* gene was present in almost all *K. pneumoniae*.[7,28,29] Another important iron uptake system, *kfu* is involved in purulent tissue infections and capsule formation was reported by Aher *et al*.[30] Aljanaby *et al.* found that about 65.62% isolates had the *kfu* gene.[6] Three *K. pneumoniae* isolates were found to harbour iron uptake system, *kfu* with 797 bp gene product. No yersiniabactin biosynthesis (*irp-2*) gene was found. Genotypic distribution found that genotype 1 contained no *entB* or *kfu* gene (Figure 3). Genotype 2 contained both *entB* and *kfu* genes (100%) while genotype 4 harboured only *entB* genes (100%). About 83.33% isolates present in genotype 3 expressed *entB* genes while around 16.67% isolates in genotype 3 carried *kfu* genes.

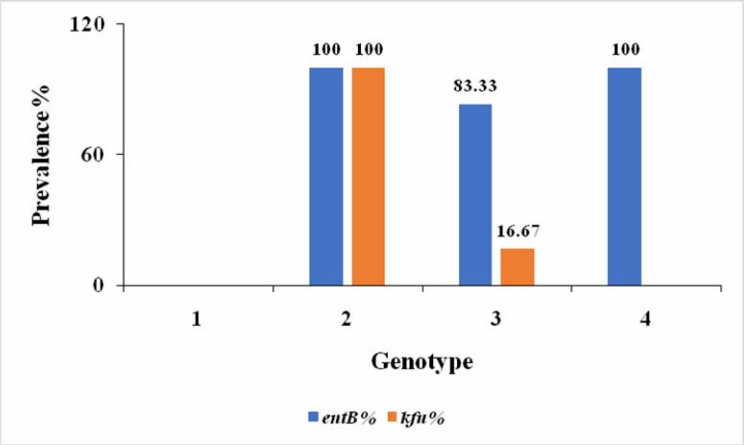

Figure 3. Genotypic distribution of *entB* and *kfu* genes.

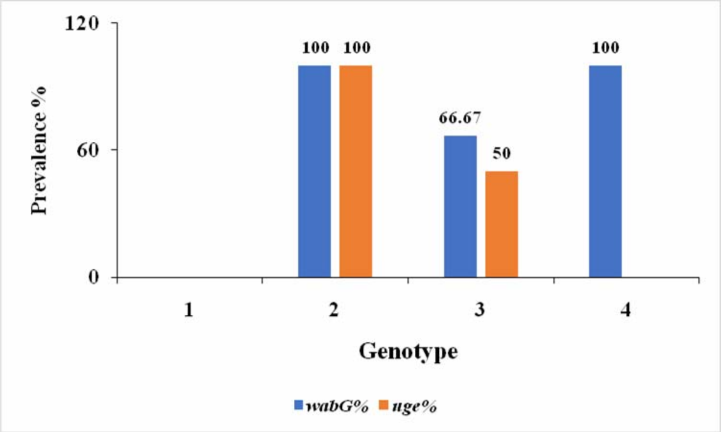

Figure 4. Genotypic distribution of *wabG* and *uge* gene

A previous study documented that virulent strains of *K. pneumoniae* produced mucopolysaccharide mass and extracellular polysaccharides.[31] Another study found that *rmpA* gene caused invasive syndrome and also found that 90% of the isolates had *rmpA* gene in people with community acquired pneumonia in South Africa and Taiwan.[32] Laboratory animal injected with mucoid strains of *rmpA* had higher mortality rate than animals injected with non-mucoid strains in the same laboratory.[32] Rivero *et al.* reported that strongly mucoviscous phenotype plasmid controlled the *rmpA* gene which is responsible for the synthesis of the regulator of the capsular polysaccharide.[33] In the study conducted by Aljanaby *et al.* 20 isolates (62.5%) had the *rmpA* gene out of the 32 strains studied.[6] The present study found no *rmpA* gene. An earlier study reported that *wabG* gene is associated with invasive and serious infections but their mechanism in disease development is still unclear.[34] Mutant *wabG* gene produced non-capsulated and less virulent *K. pneumoniae* strains in murine pneumonia model. Mutant *wabG* gene produced defective core polysaccharides and was unable to induce urinary tract infection that proved an important role in the pathogenicity of *K. pneumoniae* infections.[35] Aljanaby *et al.* documented that about 87.5% of the isolates harboured *wabG* gene.[6] Seven *K. pneumoniae* strains produced 683 bp gene product for *wabG* gene in the present study. So, these isolates might be involved in urinary tract infections. Previous findings reported that laboratory animals harbouring *K. pneumoniae* strains without *uge* were less virulent than strains with *uge* gene.[19] Present study found five (41.67%) *uge* positive *K. pneumoniae* strains. Genotype 1 contained no virulence genes. All the isolates present in genotype 2 carried *wabG* and *uge* genes but genotype 4 contained only *wabG* genes (Figure 4). Prevalence of *wabG* and *uge* genes was varied in genotype 3. The prevalence of *wabG* and *uge* genes in genotype 3 was 66.67% and 50%, respectively.

Adhesive organelles *fimH1* involved in urinary tract infections was documented by Struve *et al.*[36] Type 1 fimbriae, *fimH1* was detected in 83.33% of the isolates and produced 688 bp gene product. *K. pneumoniae* binds to endothelial cells and epithelial cells of the respiratory and urinary tracts with the help of type 3 fimbrial adhesion, *mrkD* gene. Langstraat *et al.* found that this gene binds to collagen molecules of the mammalian cells.[37] Sahly *et al.* stated that type 1 and type 3 fimbriae were found in clinical *K. pneumoniae* isolates.[38] Recent study found that all isolates from wound and blood samples contained type 1, *fimH1* and type 3 fimbriae, *mrkD* gene.[39] They also identified that isolates from sputum samples harboured type 1 fimbriae, *fimH1* while isolates from urine samples contained mainly type 3 fimbriae, *mrkD* gene. Present study found no *mrkD* gene. Biofilm formation capacity of *Klebsiella* helps to colonize in the urinary tract and is involved in chronic urinary tract infections.[6] Bellifa *et al.* found that biofilm protects bacteria from drug exposure and bacteria harbouring this biofilm forming gene are more resistant to antimicrobials.[40] El Fertas-Aissani *et al.* stated that complement reaction is blocked by plasmidic *traT* gene and this gene also play a role in bacterial conjugation.[7] Recent study found high level of *traT* gene (78.5%) in *K. pneumoniae* isolates.[39] However, no *traT* gene was detected in the present study. Aljanaby *et al.* found that all the isolates they studied harbouredouter membrane protein, *ycfM*.[6] *Klebsiella* is protected from drug exposure with the help of outer membrane lipoprotein, *ycfM*.[40] Eight isolates were detected to harbor *ycfM* gene with 160 bp gene product. Type 1 fimbriae, *fimH1* and outer membrane lipoprotein, *ycfM* genes were present in all genotypes (Figure 5). Their distribution within the genotype was almost similar. All the isolates present in genotype 2 and 4 carried *fimH1* and *ycfM* genes. About 33.33% isolates in genotype 1 expressed *fimH1* and *ycfM* genes. The *fimH1* gene was encountered at higher prevalence (100%) in genotype 3 while about 66.66% isolates in genotype 3 carried *ycfM* genes.

Resistance to beta-lactam antibiotics is acquired due to the presence of beta-lactamase gene. *K. pneumoniae* have acquired amoxicillin, ampicillin and ticarcillin resistance genes naturally.[6] Several studies found that *K. pneumoniae* harbouring AmpC beta-lactamases, metallo enzymes, SHV, extended-spectrum β-lactamases, and TEM beta-lactamases conferred resistance to meropenem, imipenem, 3rdgeneration cephalosporins and others.[22,41,42] Efflux pump is also important for virulence of *Klebsiella.*[43]

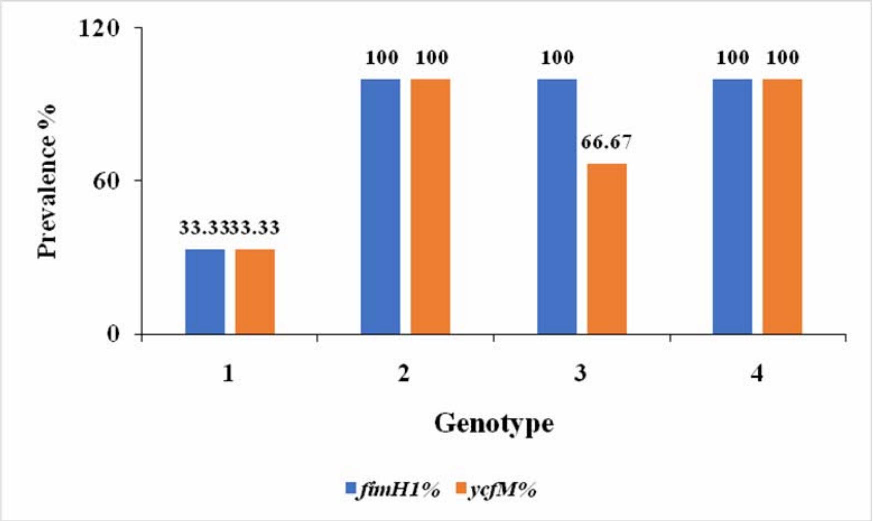

Figure 5. Genotypic distribution of *fimH1* and *ycfM* genes.

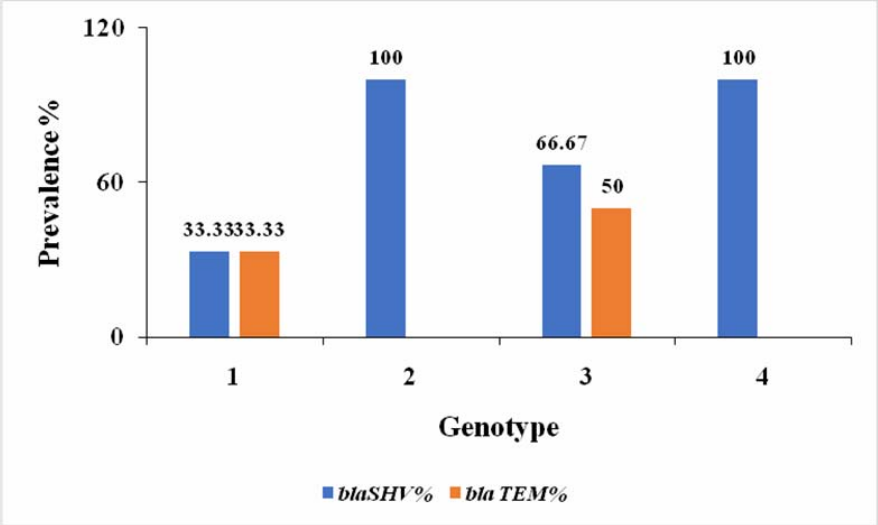

Figure 6. Genotypic distribution of bla_{SHV} and bla_{TEM} genes.

Another researcher identified the correlation of ESBL genes with virulence factors.[31] Present study detected four (33.33%) bla_{TEM} and eight (66.67%) bla_{SHV} genes. The bla_{TEM} and bla_{SHV} gene produced 643 bp and 714 bp gene product, respectively. Twenty-five percent isolates carried both bla_{TEM} and bla_{SHV} genes. Poirel *et al.* reported that Turkey first observed OXA-48 gene in *K. pneumoniae*.[22] *K. pneumonia* harbouring this OXA-48 gene was then spread to European countries and Mediterranean countries.[44] New Delhi first reported clinically significant carbapenemase producer NDM-1 (New Delhi metallo-β-lactamase)[45] which was spread to other countries.[46] Morocco[47], Oman[46], Singapore[48] and the United States[49] identified co-existing NDM-1 and OXA group carbapenemases gene in *Klebsiella* isolates. Recent study found that 58% isolates had OXA-48 genes and 2% harboured NDM-1 gene while NDM-1 and OXA-48 genes were present in 8% of the isolates.[50] Non-glucose-fermenting bacteria like *Pseudomonas aeruginosa* was found to harbor $\textit{bla}_{\text{VIM}}$ and $\textit{bla}_{\text{IMP}}$ gene[51] but a recent study detected no $\textit{bla}_{\text{VIM}}$ and $\textit{bla}_{\text{IMP}}$ in *K. pneumoniae*.[52] China, Japan and Australia had identified IMP-producing members of the Enterobacteriaceae while isolates from Italy and Greece were found to harbour VIM-producing enterobacteriaceae.[44] Antibiotic resistance gene was present in all genotypes (Figure 6). All the isolates present in genotype 2 and 4 carried $\textit{bla}_{\text{SHV}}$ genes (100%). Genotype 1 and 3 carried both $\textit{bla}_{\text{SHV}}$ and $\textit{bla}_{\text{TEM}}$ genes. About 33.33% isolates present in genotype 3 expressed both $\textit{bla}_{\text{SHV}}$ and $\textit{bla}_{\text{TEM}}$ genes. On the other hand, about 66.67 and 50% isolates in genotype 3 harboured $\textit{bla}_{\text{SHV}}$ and $\textit{bla}_{\text{TEM}}$ genes, respectively.

CONCLUSION

Klebsiella species harboured six virulence genes out of the ten in our study. About 16.67% of the isolates carried six virulence genes while about 33.33% isolates expressed four virulence genes. The *fimH1*, *ycfM* and *entB* genes were commonly detected in all the isolates studied. Twenty-five percent isolates carried both $\textit{bla}_{\text{TEM}}$ and $\textit{bla}_{\text{SHV}}$ genes. Antimicrobial resistance has emerged over last few decades due to abuse of antibiotic drugs. Antibiotic development is no longer considered to be economically feasible since antibiotics are used for relatively short periods and are not profitable to treat chronic conditions such as diabetes, psychiatric disorders, gastroesophageal reflux or asthma. Resistance regarding antibiotic abuse has been suggested by many microbiologists and infectious-disease specialists. Before prescribing a new antibiotic, physicians would be suggested to reserve it for only the worst cases. It is also suggested to prescribe the old antibiotics by analyzing the resistance pattern. So, new antibiotics can be considered as ‘last line’ drugs to combat serious infectious illnesses. This study helps to predict resistance based on genotype and aids in the selection of antibiotic would be the most effective for treating *Klebsiella* infections.

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