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Original Research Article

Title: Genotypic validation of extended-spectrum β-lactamase and virulence factors in multidrug resistance *Klebsiella pneumoniae* in an Indian hospital.

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Abstract

The emergence of extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* has been increasing rapidly across the world. The presence of virulence factors in ESBL producers further adds to the pathogenicity and severity of infection, which often complicate empirical therapy and sometimes result in treatment failures. In the present study, 227 non-repeated clinical isolates of *K. pneumoniae* obtained from different clinical specimens from a tertiary care hospital in India were analyzed to detect the genes responsible for ESBL production (blaTEM, blaCTX-M, and blaSHV), virulence (fimH-1, mrkD, entB, irp-1), and capsule production (K1-K2). Phenotypically identified 72 ESBL producing *K. pneumoniae* isolates were further subjected to PCR based genotypic analysis but only 20 were found to have at least one of the ESBL producing genes. blaTEM was the most predominant gene (100%), followed by blaSHV (90%), and blaCTX-M (85%). Similarly, the most common virulence genes were fimH-1 (70%), entB (65%), markD (55%), irp-1 (25%), K1 (25%), and K2 (20%). REP-PCR profile separated them into five major clusters (I-V), indicating the existing heterogeneity among the isolates. The resistance profile data obtained from the present study can serve as the information base to understand the infection pattern prevailing in the hospital and for physicians to recommend suitable antibiotics for the patients.

Keywords: Extended-spectrum β-lactamase (ESBL); *Klebsiella pneumoniae;* virulence genes; REP-PCR.

Introduction

Klebsiella pneumoniae is the most important gram-negative pathogenic bacteria of the family Enterobacteriaceae, and it is frequently associated with several nosocomial infections. This bacteria has been reported to have developed resistance globally [1]. Hence, routine testing and reporting for this bacterium have been recommended by CLSI since 2006. The intensity of their pathogenicity and virulence depends on the presence of several other factors, including adhesion, lipopolysaccharide cell wall, serotype of the capsule, iron-scavenging mechanism, and biofilm-producing ability. The beta-lactam group of antibiotics is the most common treatment option worldwide for treating diseases caused by gram-negative bacterial isolates. However, frequent exposure of this group of antibiotics to bacterial isolates (including *K. pneumoniae*), have induced the diversification and production of the hydrolytic enzyme beta-lactamase. Beta-lactamase enzymes are generally plasmid-encoded and can hydrolyze the beta-lactam group of antibiotics. Only few bacteria can hydrolyze third-generation penicillins and cephalosporins [2], and they are called extended-spectrum beta-lactamase (ESBL)-producing bacteria.

ESBL-producing bacteria are commonly identified using the double disk diffusion test, but the efficacy of this test is currently challenged by inconsistencies in the results produced[3]. Thus, detection of the specific resistance genes (blaCTX-M, blaSHV, and blaTEM) using PCR and sequencing is now being followed commonly as powerful tools for the validation of ESBL-producing bacteria. In addition, the genes responsible for virulence (fimH-1, mrkD, entB and irp-1) [4, 5] and capsule production (K1 & K2) have been probed to understand their role in the severity of the infection.

Previous studies from different countries, including India, have reported the frequency of ESBL-producing genes to range from 8–80% [6]. However, there is a paucity of scientific

information available to correlate the prevalence of genes with the range of ESBL producers in the species *K. pneumoniae*. Accordingly, the present study was conducted to detect the predominance of ESBL producers among *K. pneumoniae* isolates at our university hospital and their molecular characterization.

Materials and methods

Materials

Antibiotic discs, growth media, and chemicals were purchased from HIMEDIA (India), Molecular biology reagents and PCR master mix kits were purchased from Thermo Fisher Scientific and Qiagen, India.

Methods

Sample collection

Samples were collected from our University hospital (Institute of Medical Sciences and SUM hospital) from patients of the outpatient department (OPD) and intensive care unit (ICU). Written informed consent was obtained from all enrolled patients or their guardians/family members as per the guidelines approved by the Indian Council of Medical Research (ICMR), Government of India.

Identification and antibiotic susceptibility test

A total of non-repeated 227 clinical isolates were obtained from different clinical specimens (urine, blood, and pus) of patients of varying age (5–80 years) during a two month-period in 2018 from the OPD and ICU of our university hospital. Isolates were identified using routine biochemical analysis. Phenotypical screening of ESBL producers was performed using

the double disc synergy test with cefotaxime (30 µg), cefotaxime/clavulanic 20 acid (30/10 µg), ceftazidime (30 µg), and ceftazidime/clavulanic acid (30/10 µg) discs [7].

Antibiogram was performed using antibiotic discs from Himedia Laboratories Pvt. Ltd. based on Kirby Bauer's Method [8]. The antibiotics used were as follows: AK, amikacin (30 µg); AMC, amoxicillin with clavulanic acid (30 µg); CAZ, ceftazidime (30 µg); CFM, cefixime (30 µg); COT, co-trimoxazole (25 µg); CXM- cefuroxime (30 µg); CTR, ceftriaxone (30 µg); CL, colistin (10 µg); CTX, cefotaxime (30 µg); MRP, meropenem (10 µg);,LE- levofloxacin (5 µg); NX, norfloxacin (5 µg); NET, netilimicin (30 µg); OF, ofloxacin (5 µg); PI, piperacillin (100 µg); and PIT, piperacillin/tazobactam (100/10 µg).

Resistance and virulence determinants detection

Genomic DNA extraction was carried out using a modified ROSE method (Rapid one-step extraction) [9]. The concentration and purity of DNA was measured using UV-VIS spectrophotometer (Thermo Scientific, USA). ESBL positive isolates were tested for the presence of blaTEM, blaCTX-M, and blaSHV genes using gene-specific primers (Table S1) through a PCR-based method. About 25 ng of template DNA was mixed with PCR master mix, which contained 12.5 µL of 2X Taq PCR master mix (QIAGEN, India); 1 µL each forward and reverse primers and 9.5 µL of nuclease-free water. PCR amplification reactions were performed with the BIORAD thermal cycler (T100) using 30 cycles of 94˚C for 1 min, 55˚C for 45 s, and 72˚C for 1 min, with initial denaturation at 94˚C for 5 min and a final extension at 72˚C for 10 min. Similarly, virulence-associated genes encoding type 1, type 3 adhesins (fimH-1, mrkD), enterobactin biosynthesis (entB), yersiniabactin biosynthesis (irp), and capsule serotypes (K1 and K2) were screened through PCR assays. The PCR conditions were similar to those of ESBL genes except the annealing temperature described in Table S1. The amplified products were run with 1% (w/v) agarose gel and visualized under UV trans-illuminator.

Molecular typing

The genetic relatedness among the ESBL-producing *K. pneumoniae* isolates was determined using REP-PCR (Repetitive element palindromic-PCR) [10]. PCR reactions were performed with 35 cycles of 94°C (1 min), 45°C (1 min), 72°C (2 min) with initial denaturation at 95°C (7 min) and a final extension at 65°C (8 min).

The PCR amplified bands were scored as '1' for the presence and '0' for the absence of bands. Using the binary data obtained from REP-PCR, a dendrogram was constructed using the distance matrix obtained by the Unweighted Pair-Group Method with Arithmetic Means (UPGMA) with 1000 bootstrap resampling using the Darwin 6.0 software [11].

Biofilm testing

The biofilm production test was performed using the microtiter plate method as described by Singh et al. [12]. Two hundred microliters of diluted (100 times dilution) cultures were poured in each of the microtiter plates and incubated at 37°C for 48 h. Next, the cultures were removed from each well and 25 µL of crystal violet (0.1% crystal violet in 90% ethanol) was added and incubated at 25-30ºC for 30 min. The plate was dried after removing the crystal violet solution. A volume of 200 µL of 33% acetic acid was then added to each well and the absorbance was measured at 595 nm. The control experiment was performed without bacteria. The outcomes were categorized as strong (OD>0.5), moderate (OD<0.5–0.1), and weak (OD<0.1) biofilm producers.

Results and Discussion

Identification and antibiotic susceptibility profile

In this study, out of 227 clinical isolates, 72 samples were found to be ESBL producers using the disc diffusion method. These 72 isolates also exhibited resistance to more than three classes

of antibiotics including third-generation cephalosporins. Out of 72 phenotypically confirmed ESBL producers, *K. pneumoniae* constituted the highest percentage of bacteria (43.06%), followed by 13.9% *Escherichia coli* (n=10), 12.5% *Pseudomonas aeruginosa* (n=9), 11.11% *Proteus mirabilis* (n=8), 6.94% *Proteus vulgaris* (n=5), 5.55% *Acinetobacter baumannii* (n=4), 2.77% *Enterobacter aerogenes* (n=2), 2.77% *Citrobacter freundi* (n=2), and 1.38% *Citrobacter koseri* (n=1). *K. pneumoniae* has been reported to be the most common infectious agent in hospital-acquired as well as health-associated community infections. Therefore, *K. pneumoniae* isolates were subjected to further analysis.

The distribution patterns of 31 ESBL-producing *K. pneumoniae* among different pathological specimens were as follows: urine (n=10), blood (n=15), and pus (n=6) as obtained from IMS and SUM Hospital, Bhubaneswar. The antibiotic susceptibility patterns of 31 MDR *K. pneumoniae* isolates showed the highest percentage (100%) of resistance to ceftazidime, followed by cefuroxime (83.87%), ofloxacin (83.87%), amoxicillin with clavulanic acid (70.96%), piperacillin (70.96%), and levofloxacin (64.51%). The lowest percentage of resistance was observed in meropenem and colistin (6.4%) (Fig.1). Percentage occurrence of ESBL *K. pneumoniae* isolates has been found to vary among different countries; Canada (4.90%) and United States (44%) [13], Algeria (20%) [14], Spain (20.80%) [15], Taiwan (28.40%) [16], China (51%) [17], and Turkey (78.60%) [18] whereas the highest percentage range (4–83%) [19,20] was reported from India. This shows the widespread occurrence of ESBL producers across the globe.

Molecular detection of ESBL genes (blaTEM, blaCTX-M, blaSHV)

All the 31 phenotypically confirmed ESBL-producing *K. pneumoniae* isolates were subjected to molecular detection of ESBL genes (blaTEM, blaCTX-M, and blaSHV) and 20 isolates (urine, n=7; blood, n=8 and pus, n=5) were identified as ESBL positive. The lack of correlation

between phenotypic and genotypic ESBL detection was evident in this study, which claims genotypic analysis as a prerequisite method for the detection of ESBL. Therefore, we believe it should be incorporated into all routine diagnostic tests. Among all these 20 *K. pneumoniae* isolates, blaTEM was the most predominant gene (100%), followed by blaSHV (85%), and blaCTX-M (50%). Similarly, the blaTEM genes were predominantly found in Portugal (40.9%) [21], Turkey (72.7%) [22], and Italy (45.4%) [23]. The co-existence of blaTEM+blaSHV was observed in 17 isolates (85%), blaTEM+blaCTX-M in 10 isolates (50%), blaCTX-M+blaSHV in 9 isolates (45%), and blaTEM+blaCTX-M+blaSHV in 9 isolates (45%). In this study, the co-occurrence of TEM and SHV was higher than that in a previous report from Lucknow, India, where the authors observed blaTEM and blaSHV in only 26.5% of *K. pneumoniae* isolates [24]. The co-existence of three ESBL genes was also higher (45%) than that in the reports by other authors who conducted similar studies [25,26]. The co-existence of the genes blaTEM+blaSHV was the highest in blood samples (Fig. 2). Similarly, blaTEM+blaCTX-M and blaCTX-M+blaSHV were equally distributed in both urine and blood samples. The co-existence of blaTEM+blaCTX+blaSHV was the highest in urine samples, followed by blood, and pus samples. However, the predominance (100%) of the blaTEM type β-lactamase gene in *K. pneumoniae* in the present study concurs with those of previous studies [27-29]. ESBL-producing *K. pneumoniae* infection results in ineffective therapy, treatment failure due to lack of alternate antimicrobial agents, and increased mortality.

Detection of virulence genes

Since the presence and expressional ability of the virulence factors in any bacteria add to the severity of infection, a myriad of genes contributing to virulence have been mined. Type 3 fimbriae (mrkD) play a crucial role in the binding of infecting bacteria to the surface of collagen molecules of the host cells [30]. The gene for type 3 fimbriae (mrkD) was found to be the highest in blood (30%), followed by pus (20%), and urine (5%) samples (Table 1). In the

present analysis, type 1 fimbriae (fimH-1) adhesions were detected in 70% isolates and were most prevalent in urine samples (Table 1). A similar predominance in urinary tract infections has also been reported in a previous study [31]. Expression of both Type 1 and type 3 fimbriae genes was normally found together in clinical isolates of *K. pneumoniae* in different specimens [32,33]. In our analysis, the siderophore genes (entB and irp-1 genes) were found among 65% and 25% of MDR *K. pneumoniae*, respectively, whereas their percentages were 85% and 28% for entB and irp-1 genes, respectively in an Egyptian hospital [32]. These siderophore genes of *K. pneumoniae* are responsible for the uptake of iron from the host for inhibition of T cell proliferation [34,35]. Such irp-1 genes are also located in high-pathogenicity island (HPI) in *Yersinia* strains [36] and also in other members of the family Enterobacteriaceae, such as *E. coli*, *Enterobacter* spp., and *Citrobacter* spp. [37, 38]. The virulence of *K. pneumoniae* is associated with capsular serotypes K1 and K2 [39]. We found 9 out of 20 ESBL-producing *K. pneumonia* isolates to be typable; 25% (n = 5) exhibited K1 type whereas 20% (n = 4) of them were K2 type. A varying percentage of K1 capsule types were observed among all different sample categories, but K2 capsule types were completely absent in isolates collected from pus samples. In a previous study, Feizabadi et al. depicted the percentage of K1 and K2 serotypes to be 11.2% and 14.6%, respectively out of the total *K. pneumoniae* isolates studied [40].

Phenotypic validation of biofilm production

Biofilm formation is one of the most important virulence properties of *K. pneumoniae*, which help their attachment to live or abiotic surface, thereby protecting them from antimicrobial agents, phagocytosis, and opsonization by antibodies [41]. *In vitro*, experimental verification of biofilm formation by these isolates encourages phenotypic validation. Microplate crystal violet assay revealed 85% of our isolates had the biofilm forming ability out of 20 *K. pneumoniae*. A variable potential in biofilm formation was observed among all the ESBL K. *pneumoniae* members of the present study, ranging from weak (n=3) (15%), to moderate (n=8)

(40%), and to strong (n=6) (30%). *K. pneumoniae* isolates from urine and blood samples showed strong biofilm forming ability than the isolates from pus samples (Table 1). Our present results are consistent with those of previous studies, wherein the rates of biofilm production by *K. pneumoniae* were 96.2% and 77.8% [42], 77.7% [43], and about 50% [44].

Our results showed a correlation between the ability of these isolates to form biofilm and the presence of genes contributing to biofilm formation, K1 and K2, and entB [40,45]. From table 1, it is clear that almost all samples obtained from urine **(**n=7) and blood (n=8**)** do have either one or all of the three genes that contribute to the formation of biofilm and formed either strong or moderate amount of biofilm *in vitro* unlike samples collected from pus. The high potential of biofilm formation also enhances virulence and finally the severity of infection of the infecting agent.

Molecular typing

REP-PCR has been widely used as a well-accepted tool for molecular genotyping for understanding the heterogeneity among the ESBL-producing *K. pneumoniae* strains [46]. The dendrogram, obtained from REP-PCR fingerprints with amplicons ranging from 50–1500 bp of 20 ESBL positive *K. pneumoniae* isolates, formed five clusters (I-V) (Fig. 3). Cluster I consisted of eight isolates, out of which five isolates were from urine samples, two from blood, and one from pus sample. Cluster II consisted of two from urine samples, cluster III consisted of all two isolates from blood samples, and cluster V consisted of seven isolates including four from blood and three from pus. Cluster IV was separated from other clusters having one sample from pus. Existing genetic diversity among 20 *K. pneumoniae* isolates as observed from the multiple clustering patterns (figure 3) could be due the differences in their source and origin. Therefore, from this study, the possibility that the prevalence of ESBL-producing *K. pneumoniae* strains in different sample types was due to nosocomial infection may be ruled

out. However, to further confirm this, a highly precise, but costly, multi locus sequence typing of housekeeping genes of all the 20 isolates needs to be performed. Multiple clustering observed in our analysis also coincides with the highly heterogeneous nature of *K. pneumoniae* reported by Lai et al. [47].

Conclusion

The rapid emergence of ESBL-producing *K. pneumoniae* in a tertiary health care set up adds to the complex treatment of patients as well as the escalation of treatment costs. Routine surveillance is required for understanding the prevalence of resistance patterns at the genetic level to help monitor the pattern of dissemination of nosocomial or community-acquired infections in hospitals, as well as in recommending a better empirical drug regimen. However, long-term routine surveillance is desirable in hospital settings where the rate of emergence of resistance genes is expected to be considerably high.

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Figure Captions

Fig.1: Percentage of resistance by *K. pneumoniae* isolates against different antibiotics. AK-Amikacin, AMC- Amoxicillin with clavulanic acid, CFM- Cefixime, COT- Co-trimoxazole, CXM- Cefuroxime, CTR- Ceftriaxone, CIP- Ciprofloxacin, CTX- Cefotaxime, MRP-Meropenem, LE- Levofloxacin, NX- Norfloxacin, NET- Netilmicin, OF-Ofloxacin, PI-Piperacillin, PIT- Piperacillin/Tazobactam.

Fig. 2: Prevalence of ESBL producing gene types among different samples.

Fig. 3: Dendrogram based on REP-PCR profile of *Klebsiella pneumoniae.*

Figure 1

Click here to access/download;Figure;Figure 1.tif

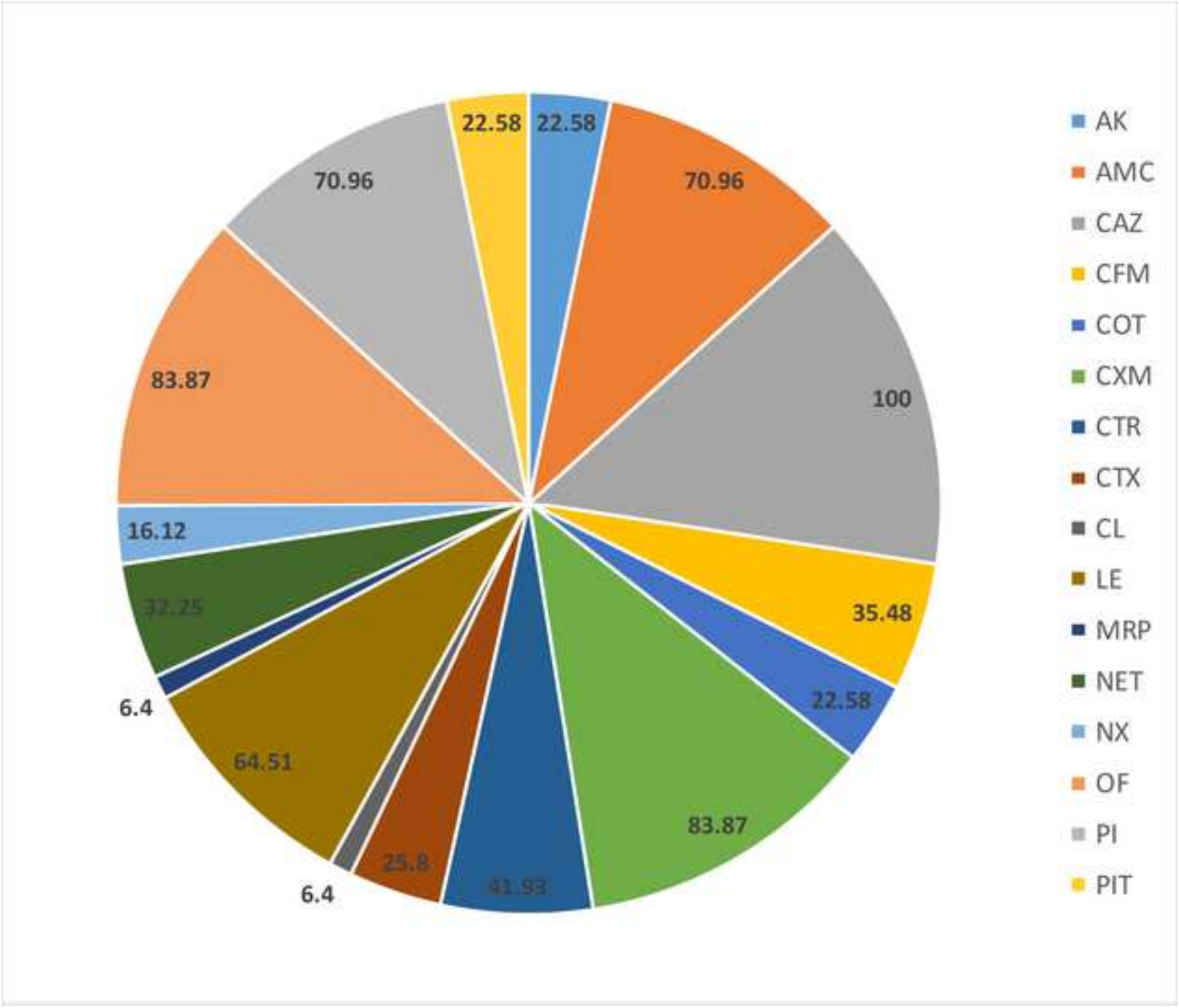


Figure 2

Click here to access/download;Figure;fig2.tif

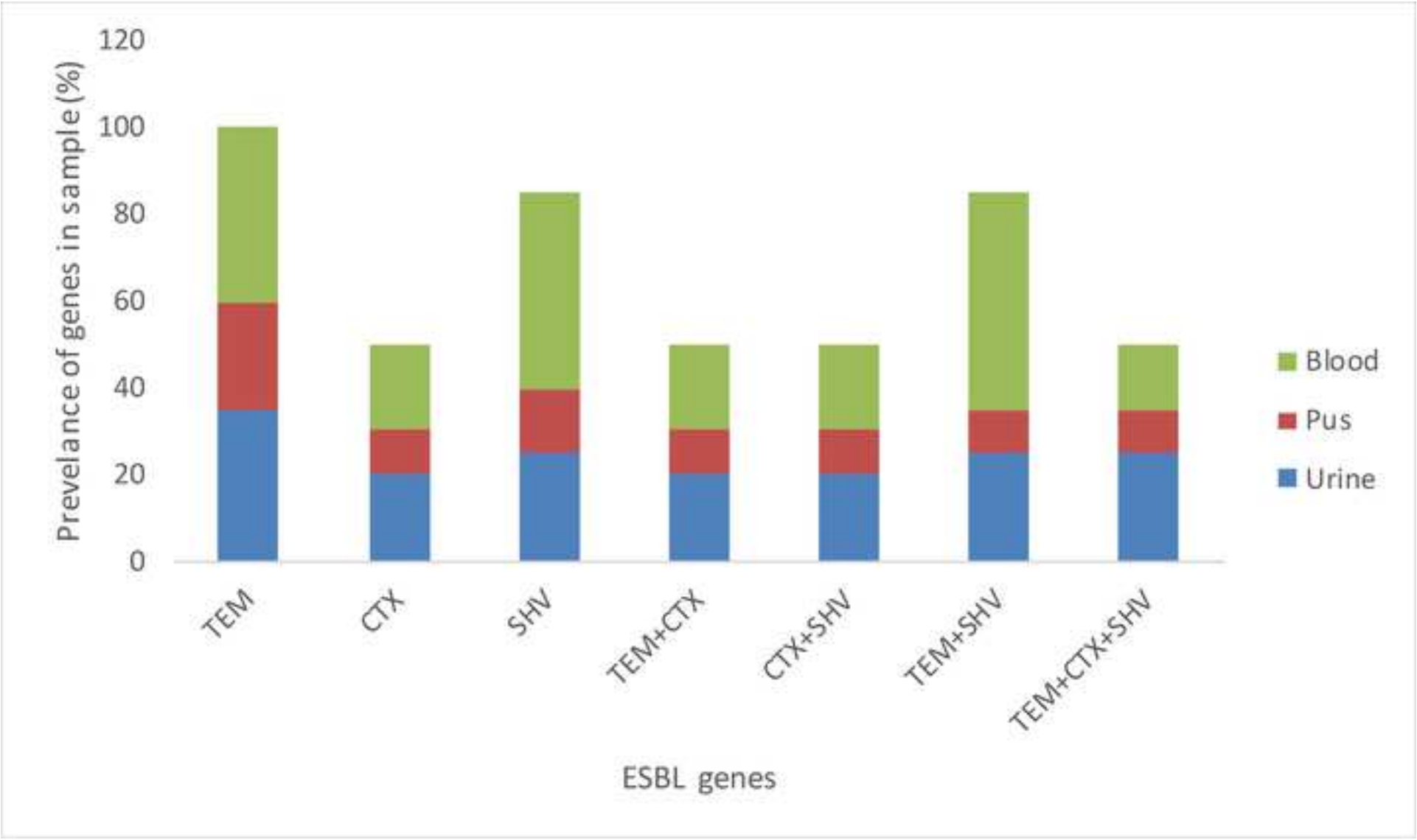
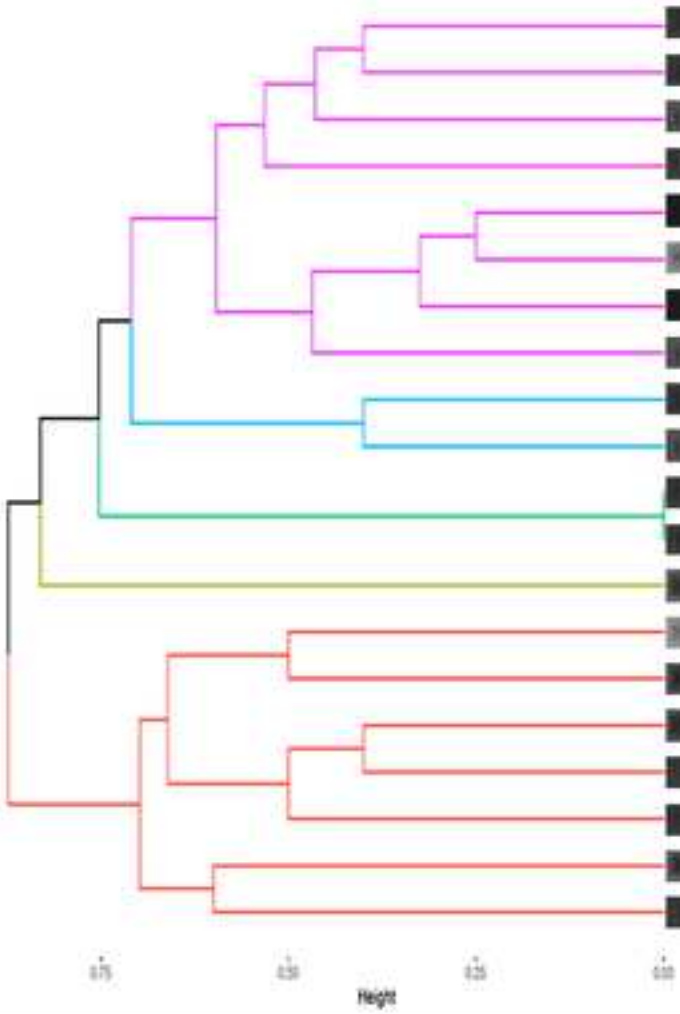


Figure 3

Click here to access/download;Figure;Fig3.tif



Samples	Source	Age (Year)	Sex	AK	AMC	CFM	CFS	CIP	COT	CPT	CTR	CTX	CXM	LE	MRP	NET	NX	OF	PI	PIT
KP10	Blood	24	M	-	+	+	-	-	-	-	+	+	+	+	-	+	-	+	+	-
KP07	Urine	48	M	-	-	-	-	-	-	-	+	-	+	+	-	-	-	+	+	-
KP03	Urine	64	F	+	-	+	-	-	+	-	-	-	+	+	-	+	-	+	+	-
KP11	Blood	74	M	-	-	-	-	-	-	-	+	-	+	+	-	-	+	+	+	-
KP05	Urine	50	F	-	+	+	-	-	+	-	-	-	-	-	-	-	+	+	+	-
KP17	Pus	19	F	-	+	-	-	-	-	-	-	+	+	+	-	-	-	+	+	-
KP04	Urine	5	F	-	+	+	-	-	-	-	+	-	+	-	-	-	-	+	+	-
KP01	Urine	42	F	+	+	+	-	-	+	-	-	-	+	+	-	+	-	+	-	-
KP06	Urine	50	M	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	+	+
KP02	Urine	81	M	+	+	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-
KP09	Blood	36	F	-	+	-	-	-	-	-	+	-	+	+	-	-	-	+	+	-
KP08	Blood	55	M	-	I	-	-	-	-	-	-	I	I	-	-	I	-	-	I	-
KP20	Pus	42	M	-	+	+	-	-	-	-	+	-	-	+	-	-	-	+	-	+
KP18	Pus	5	F	-	+	+	-	-	-	-	+	-	+	-	-	-	-	+	+	-
KP15	Blood	32	M	-	+	-	-	+	-	-	+	-	+	-	+	-	-	-	-	+
KP16	Pus	20	F	-	+	-	-	-	+	-	-	-	+	+	-	-	-	+	-	-
KP12	Blood	70	M	-	-	-	-	-	-	-	+	-	+	-	-	+	-	+	+	-
KP13	Blood	55	M	-	+	-	-	-	-	-	-	+	+	+	-	-	-	+	+	-
KP19	Pus	40	M	+	-	-	-	-	-	+	+	+	-	+	-	+	-	+	-	+
KP14	Blood	50	M	-	+	-	-	-	-	-	-	+	+	-	-	-	+	-	+	-

Table

Table 1: Prevalence and distribution pattern of virulence factors among clinical specimen in *K. pneumoniae* (n=20).

Samples	Virulence factors												Biofilm formation					
	FimH		mrkD		entB		irp		K1		K2		weak		moderate		strong	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Urine (N=7)	5	25	1	5	5	25	3	15	2	10	1	5	0	0	4	20	2	10
Blood (N=8)	5	25	6	30	5	25	1	5	1	5	3	15	1	5	3	15	4	20
Pus (N=5)	4	20	4	20	3	15	1	5	2	10	0	0	2	10	1	5	0	0
Total	14	70	11	55	13	65	5	25	5	25	4	20	3	15	8	40	6	30

Supplementary information

Genotypic validation of extended-spectrum β-lactamase and virulence factors in multidrug resistance *Klebsiellae pneumoniae* in an Indian hospital.

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Table S1: List of primers used in this study

Target Region	Primer Sequence	Annealing Temperature (°C)	Reference
TEM	F-5'-ATG AGT ATT CAA CAT TTC CGT G-3' R-5'-TTA CCA ATG CTT AAT CAG TGA G-3'	55	Essack et al. 2001
SHV	F-5'-TTA TCT CCC TGT TAG CCA CC-3' R-5'-GAT TTG CTG ATT TCG CTC GG-3'	55	Essack et al. 2001
CTX-M	F-5'-SCS ATG TGC AGY ACC AGT AA-3' R-5'-CCG CRA TAT GRT TGG TGG TG-3'	55	Saladin et al. 2002
K1	F-5'-GGT GCT CTT TAC ATC ATT GC-3' R-5'-GCA ATG GCC ATT TGC GTT AG-3'	47	Fang et al. 2007
K2	F-5'-GGA TTA TGA CAG CCT CTC CT-3' R-5'-CGA CTT GGT CCC AAC AGT TT-3'	45	Fang et al. 2007
mrkD	F-5'-CCA CCA ACT ATT CCC TCG AA-3' R-5'-ATG GAA CCC ACA TCG ACA TT-3'	52	El Fertas-Aissani et al. 2013
fimH-Type 1	F-5'-ATG AAC GCC TGG TCC TTT GC-3' R-5'-GCT GAA CGC CTA TCC CCT GC-3'	55	El Fertas-Aissani et al. 2013
irp1	F-5'-TGA ATC GCG GGT GTC TTA TGC-3' R-5'-TCC CTC AAT AAA GCC CAC GCT-3'	57	Pelludat et al. 2002
entB	F-5'-CTG CTG GGA AAA GCG ATT GTC-3' R-5'-AAG GCG ACT CAG GAG TGG CTT-3'	57	Wasfi et al. 2016
REP	F-5'-III ICG ICG ICA TCI GGC-3' R-5'-ICG ICT TAT CIG GCC TAC-3'	47	Versalovic et al. 1991

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