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RESEARCH ARTICLE OPEN ACCESS

Association between Virulence Factors and Antimicrobial Resistance of *Klebsiella pneumoniae* Clinical Isolates from North Kerala

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Abstract

***Klebsiella pneumoniae* is a common bacterial pathogen causes wide range of infections all over the world. The antimicrobial resistance of *K. pneumoniae* is a global concern and expresses several virulence factors contributing to the pathogenesis. The incidences of bacterial co-infection in viral pneumonia are common. Increased risk of *K. pneumoniae* co-infection in viral respiratory tract infection should be alerted in COVID-19 pandemic period. The study aims to detect the association between antimicrobial resistance and factors causing pathogenicity of *K. pneumoniae*. For the current study, 108 *K. pneumoniae* clinical isolates were included. Antimicrobial susceptibility test was done by Kirby-Bauer disc diffusion method according to CLSI guidelines. Virulence factors such as biofilm formation, haemagglutination, haemolysins, hypermucoviscocity, siderophore, amylase, and gelatinase production were determined by phenotypic method. In this study *K. pneumoniae* showed high level of antimicrobial resistance towards ampicillin (92.59%) followed by amoxicillin-clavulanic acid (67.59%) and cotrimoxazole (47,22%). An important association between biofilm formation and antimicrobial resistance was found to be statistically significant for cotrimoxazole (*P*-value 0.036) and amoxicillin-clavulanic acid (*P*-value 0.037). Other virulence factors like hypermucoviscocity, haemagglutination, amylase, and siderophore production were also showed a statistically significant relation (P-value <0.05) with antimicrobial resistance. Further molecular studies are necessary for the identification of virulence and antimicrobial resistance genes, for the effective control of drug-resistant bacteria.**

Keywords: Antimicrobial susceptibility, Biofilm, *Klebsiella pneumoniae,* Pathogenesis, Virulence factors

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INTRODUCTION

The burgeoning antimicrobial resistance in bacteria is a global challenge. Genus *Klebsiella* is one of the most important Gram-negative, non-motile, non-sporing, short stout bacilli, normally present as normal flora of the intestine of humans and animals.[1] *Klebsiella pneumoniae* is an opportunistic pathogen causing various diseases; however, urinary tract and respiratory tract infections are repeatedly reported. It also caused intra-abdominal infection, meningitis, wound infection, pyogenic liver abscess, and septicemia.[2] Rare cases of mycotic aneurysm, acute suppurative bacterial dacryoadenitis and community-acquired *Klebsiella pneumoniae* meningitis are reported in recent years.[3-5] Many reports have been published regarding the co-infection of *K. pneumoniae* in association with COVID-19 cases.[6-8]

Six multidrug-resistant bacterial pathogens are coming under the acronym ESKAPE such as *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanni, Pseudomonas aeruginosa* and *Enterobacter* species, they are escaping from the bactericidal activity of antimicrobial agents and mainly responsible for hospital acquired infection. *K. pneumoniae* as ESKAPE pathogens are highly resistant to antimicrobial agents and a principal source of hospital acquired infection in developed and low-income countries.[9,10] The pathogenesis of *K. pneumoniae* is because of the production of different virulence factors such as capsular polysaccharide, siderophore production, biofilm formation, hypermucoviscosity, haemagglutination, haemolysis and production of extracellular enzymes.[11] These factors aid in the establishment of *Klebsiella* infection and also act as a barrier to antimicrobial agents. Among the virulence factors, the capsular polysaccharide is the most important; it protects the microbes by inhibiting phagocytosis.[12] Due to the production of capsular polysaccharide, the colonies of *K. pneumoniae* becomes mucoid.

Due to the increased antimicrobial resistance and multidrug resistance, the genus is showing high morbidity and mortality. Presently, *K. pneumoniae* shows increased resistance to broad-spectrum antibiotics, including beta-lactam, aminoglycosides and fluoroquinolones.[13] Antimicrobial resistance is mainly because of the production of enzyme, virulence factors, efflux pumps and mutation.[14] The enzyme Extended-spectrum beta-lactamases (ESBLs) are present in various Gram-negative bacteria worldwide, of which the most common bacterial isolate is *K. pneumoniae*. ESBLs producing bacteria are resistant to penicillins, first-, second-and third-generation cephalosporins and monobactams (aztreonam), but not to cephamycin or carbapenems.[15] Thus, carbapenems are found to be the most reliable drug for the treatment of ESBL-producers; however, there have been a lot of research reports on increasing resistance to carbapenem antibiotics worldwide.[16] The resistance to the carbapenem is due to the acquisition of resistant gene and the presence of some virulent factors.[17]

As per recent researches worldwide, in majority of viral pneumonia infections, bacterial co-infections are found to be in significant proportion. *K. pneumoniae* is a major bacteria acting as a co-pathogen in viral respiratory tract infection, it should be alerted in this COVID-19 pandemic. Due to its pathogenic and antimicrobial resistance, *K. pneumoniae* is a well-established pathogen in health care system. Therefore, this study aims to detect the virulence factors and antimicrobial susceptibility of *K. pneumoniae*. We also focused on the association between the virulence factors and antimicrobial resistance.

MATERIALS AND METHODS

The study was done at Karuna Medical College, Palakkad during 2017 January to 2018 March, to assess the prevalence of antimicrobial resistance among *K. pneumoniae* and its virulence factor determination. A total of 650 samples were received to microbiology laboratory, in this 556 (85.54%) samples were showed growth. Of this One hundred and eight strains (n=108) of *K. pneumoniae*, were identified based on the cultural and biochemical profile described by Bergey's Manual for systematic Bacteriology.[18]

Antimicrobial resistance testing

The antimicrobial susceptibility test for all the isolates were performed by Kirby-Bauer disc diffusion test with the following commercially available discs (Himedia Laboratories

Pvt, Ltd., Mumbai, India) such as ampicillin (10µg), ampicillin-sulbactam (10 µg /10 µg), gentamicin (10 µg), cotrimoxazole (25 µg), levofloxacin (5µg), cefoxitin (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), amoxyclav (10 µg) and amikacin (30 µg). The results were interpreted as per CLSI guidelines.[19]

Detection of ESBLs production

Phenotypic screening test for the detection of ESBL production by *K. pneumoniae* was tested with third-generation cephalosporin such as ceftriaxone (30 µg), cefotaxime (30 µg), and ceftazidime (30 µg). All the isolate indicated resistant or intermediate sensitivity to any of these three discs, were suspected as ESBLs producers and is phenotypically confirmed by a combined disc diffusion test. The isolate was inoculated on to Muller Hinton Agar (Himedia Laboratories Pvt, Ltd., Mumbai, India) and susceptibility tested with ceftazidime or cefotaxime and with or without clavulanic acid. The plates were incubated at 37°C overnight. The ESBL production was confirmed phenotypically by the difference of zone diameter more than 5mm between the discs such as cefotaxime or ceftazidime and their respective clavulanic acid disc combination.[20]

Virulence factors determination

Biofilm formation

Quantitative estimation of biofilm formation of the isolate was performed according to the protocol by O" Toole and Kolter (1998) and Dusane et al.[21,22] The optical density was measured by 570nm Enzyme linked immunosorbent assay reader and graded the intensity of the biofilm as strong (>0.240), moderate (0.120-0.240) and weak (<0.120).

Hypermucoviscosity

K. pneumoniae hypermucoviscosity was phenotypically determined by a string test. The colonies were stretched by a wire loop, and the formation of a viscous string at a length of ≥5mm indicates hypermucoviscosity positive.

Haemagglutination

Bacterial fimbriae were detected by the clumping of erythrocyte narrated by Vagarali et al.[23] Human O^{+ve} blood was used for the test. The 3% of RBC solution is prepared in fresh saline and one drop of this suspension was added to one drop of bacterial suspension and rotates for 5 minutes at room temperature.

Haemolysin production

The isolates were streaked on the blood agar. Haemolysins are three types; Alpha, Beta and gamma.[24] Alpha haemolysis is partial lysis of RBC, shown as greenish discoloration. Beta haemolysis, complete lysis of red blood cells, is clearance around the colony. Gamma haemolysis doesn't exhibit haemolysis. It was detected after 24 hours of incubation at 37°C.[25]

Siderophore production

This was carried out by chromazerol sulphate agar disc diffusion assay.[26] Positive results show blue to orange colour change due to the chelation of iron by siderophore.

Gelatinase production

Gelatinase production was detected by using a Nutrient gelatin agar medium. Isolates were inoculated on the test tube contain medium by a straight wire and incubate at 37°C for 48hrs. After incubation, keep the tubes at 4°C overnight. The Liquefaction of gelatin shows a positive result.[27]

Table 1. Distribution of *Klebsiella pneumoniae* isolates in different samples

No.	Particulars	No. of isolates
1.	Urine	41 (37.96%)
2.	Sputum	39 (36.11%)
3.	Pus	23 (21.3%)
4.	Blood	1 (0.93%)
5.	Secretion tip	1 (0.93%)
6.	Anal fistula	1 (0.93%)
7.	Wound swab	1 (0.93%)
8.	Bronchial wash	1 (0.93%)

Table 2. incidence of MDR isolates in different sample

No.	Sample	No: (%) of MDR isolates
1.	Urine	19/41 (46.34)
2.	Sputum	6/39 (15.38)
3.	Pus	5/23 (21.74)
4.	Blood	0
5.	Secretion tip	1 (100)
6.	Anal fistula	0
7.	Wound swab	1(100)
8.	Bronchial wash	1(100)

Amylase production

Nutrient gar with 1% starch was used to detect amylase production. After 48hrs of incubation, plates flooded with an Iodine solution. The zone of clearance indicates a positive reaction.[28]

Statistical analysis

All the data was analyzed statistically by using SPSS version 21(IBM Corporation/Armonk, New York/USA). The association between drug resistance and the factors related to pathogenicity of *K. pneumoniae* isolates and ESBLs producers and virulence determinants were evaluated by the chi-square test. 'P' value less than or equal to 0.05 was considered as statistically significant.

RESULTS

For the current study, *K. pneumoniae* isolates (n= 108) were collected from various clinical samples such as urine (41), sputum (39), pus (23), blood, secretion tip, anal fistula, wound swab and bronchial wash (1 each) (Table 1). Among this, more than 90% of isolates were resistant to ampicillin (92.59%; 100/108) followed by,

Table 3. Distribution of ESBL producing *K. pneumoniae* in different samples

No.	Sample	No. of ESBL) producers (N=49; 45.37%)	No. of Non ESBL producers (N=59; 54.63%)
1.	Urine	26 (53.06%)	15 (25.42%)
2.	Sputum	11 (22.45%)	28 (47.46%)
3.	Pus	8 (16.33%)	15 (25.42%)
4.	Blood	0	01 (1.70)
5.	Secretion tip	1 (2.04%)	0
6.	Anal fistula	1 (2.04%)	0
7.	Wound Swab	1 (2.04%)	0
8.	Bronchial wash	1 (2.04%)	0

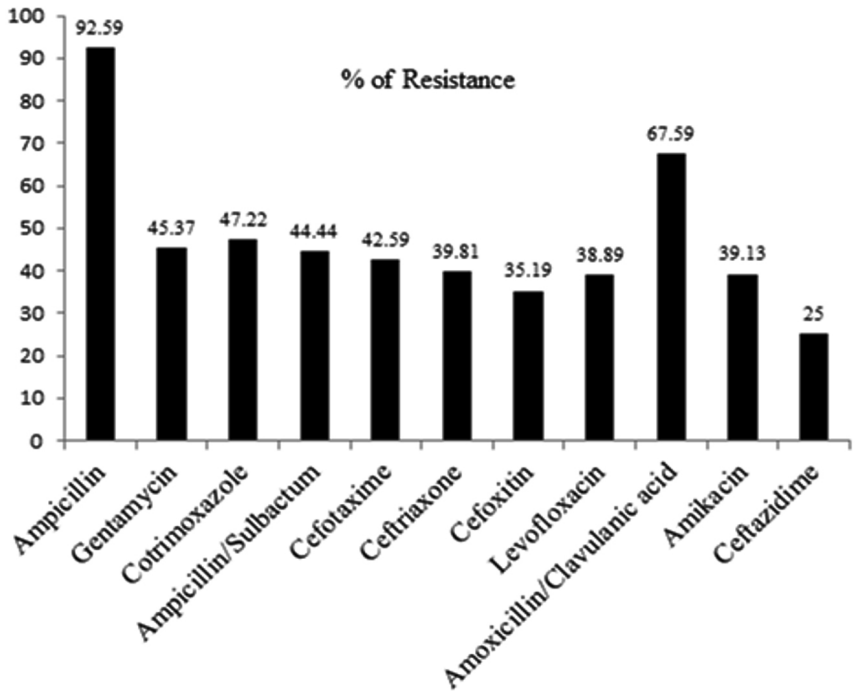

Fig. 1. Antimicrobial susceptibility pattern of *K. pneumoniae* isolates.

amoxicillin-clavulanic acid (67.59%; 73/108) and Co-trimoxazole (47.22 %; 51/108) (Fig. 1). Out of total 108 isolates 30.55% of *K. pneumoniae* isolates were resistant to 3-4 functional group of antimicrobial agents, which is considered as MDR (multidrug resistance) (Table 2). The present study, ESBLs production was found in 49 isolates (45.37%, 49/108) and non ESBLs producers 59 (54.63%) were detected by combined disc diffusion test (Table 3).

The biofilm and siderophore production was found to be the most common virulence factors among the isolates, but the incidence of Haemolysin and Amylase production was not often (Fig. 2). In the present study most of the *K. pneumoniae* isolates were biofilm producers. The majority of isolates produced a moderate intensity of biofilm in both ESBL producing and non-ESBLs producing *K. pneumoniae* and different categories of biofilm production are present in (Fig. 3). The current study reveals prevalence of haemagglutination (*P*-value 0.033) and siderophore production (*P*-value 0.001) was higher among ESBL producing *K. pneumoniae* (Table 4).

A significant association was noticed between antimicrobial resistance and some virulence factors. Table 5 shows the prevalence of virulence factors and antimicrobial susceptibility of *K. pneumoniae*. The siderophore production exhibited a statistically significant association in the antimicrobial resistance with all antimicrobial agents used in the current study (*P*-value <0.05). Moreover, significant associations were observed between biofilm formation and cotrimoxazole (*P*-value 0.036) and amoxicillin-clavulanic acid resistance (*P*-value 0.037); hypermucoviscosity with gentamicin (*P*-value 0.034) and ceftriaxone (*P*-value 0.026) and cefotaxime (*P*-value 0.044); haemagglutination (*P*-value 0.045) and amylase production (*P*-value 0.044) with cefotaxime.

Table 4. Association of ESBL producing *K. pneumoniae* with respect to virulence factors

	Virulence factors					
	Hypermucoviscocity	Haemolysin	Haemagglutination	Amylase	Gelatinase	Siderophore
Positive	4.1	6.1	24.5	12.2	16.3	77.6
Negative	95.9	93.9	75.5	87.8	83.7	22.4
P-value	0.061	0.726	0.033	0.137	0.472	0.001

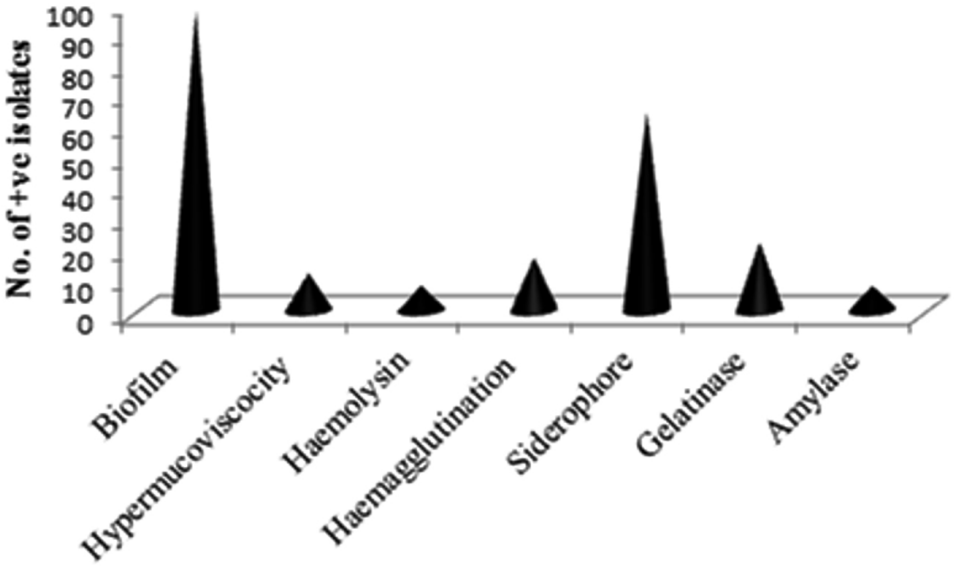

Fig. 2. Distribution of various virulence factors in *K. pneumoniae* isolates.

DISCUSSION

Klebsiella pneumoniae is one of the frequently isolated, antimicrobial resistant organisms from different clinical samples. It is also associated with hospital acquired infection and a co-pathogen in viral diseases. A study from China reported 91.9% 0f COVID-19 patient had bacterial co-infection, where it was predominant with Streptococcus pneumoniae followed by *K. pneumoniae*.[29] In the present study, the

Table 5. Association between antibiotic resistance and virulence factors of *K. pneumoniae*

Virulence markers		Antibiotics							
		AMP	GEN	COT	A/S	CTX	CTR	LE	AMC
Biofilm formation	Strong	19	18.4	23.5	18.8	26.1	27.9	26.2	19.2
	Moderate	57	67.3	54.9	62.5	54.3	53.5	59.5	54.8
	Weak	13	6.1	17.6	14.6	15.2	14	11.9	17.8
	Negative	11	8.2	3.9	4.2	4.3	4.7	2.4	8.2
	P-value	0.126	0.309	0.036	0.298	0.069	0.065	0.072	0.037
Hypermucoviscosity	Positive	11	4.1	9.8	6.2	4.3	2.3	7.1	9.6
	Negative	89	95.9	90.2	93.8	95.7	97.7	92.9	90.4
	P-value	0.897	0.03	0.683	0.15	0.044	0.026	0.295	0.467
Haemolysin	Positive	7	4.1	5.9	8.3	6.5	9.3	7.1	8.2
	Negative	93	95.9	94.1	91.7	93.5	90.7	92.9	91.8
	P-value	0.568	0.229	0.567	0.74	0.762	0.541	0.933	0.642
Haemagglutination	Positive	17	14.3	15.7	20.8	23.9	20.9	16.7	19.2
	Negative	83	85.7	84.3	79.2	76.1	79.1	83.3	80.8
	P-value	0.204	0.705	0.988	0.194	0.045	0.228	0.833	0.157
Amylase	Positive	6	8.2	11.8	10.4	13	9.3	11.9	9.6
	Negative	94	91.8	88.2	89.6	87	90.7	88.1	90.4
	P-value	0.48	0.785	0.102	0.285	0.044	0.541	0.155	0.211
Gelatinase	Positive	19	18.4	19.6	18.8	19.6	18.6	23.8	19.2
	Negative	81	81.6	80.4	81.3	80.4	81.4	76.2	80.8
	P-value	0.211	0.638	0.852	0.708	0.858	0.711	0.479	0.657
Siderophore	Positive	60	77.6	70.6	75	80.4	74.4	83.3	69.9
	Negative	40	22.4	29.4	25	19.6	25.6	16.7	30.1
	P-value	0**	0**	0.023	0.003	0**	0.009	0**	0.001**

P-Value * -Significant ** highly significant; AMP- Ampicillin, GEN- Gentamicin, COT- co-trimoxazole, A/S- Ampicillin Sulbactum, CTX- Cefotaxime, CTR- Ceftriazone, LE- Levofloxacine, AMC- Amoxicillin clavulanic acid.

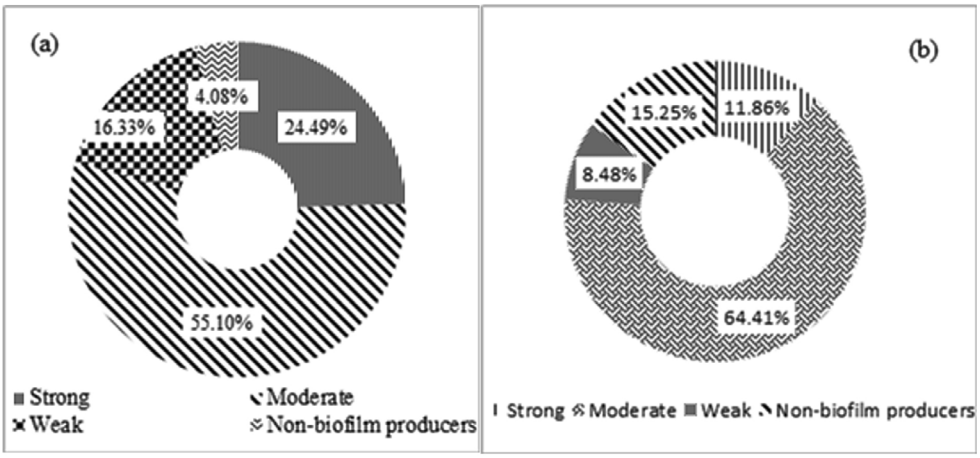

Fig. 3. Categories of biofilm on ESBL producing and non-ESBL producing *K. pneumoniae.*

predominant isolation of *K. pneumoniae* was found from urine samples followed by sputum and pus, which corroborates the observation of Phamba and Domanic.[30]

Antimicrobial resistance is a severe threat to the human for the successful treatment of health care infection. The current study revealed that 92.59 % (100/108), 67.59% (73/100) and 47.22% (51/108) of *K. pneumoniae* were resistance to ampicillin, amoxicillin-clavulanic acid and co-trimoxazole respectively. The prolonged usage of antibiotics like ampicillin and cotrimoxazole, the bacteria developed resistance against them.[31] As study done by Ahanjan et al.[32] reported that *K. pneumoniae* showed 100% resistance against cefotaxime and 47% to gentamycin. In our study, the resistance against gentamycin was 45.37% (49/108), but the cefotaxime resistance (42.59%; 46/108) was less than the mentioned study. In this survey 73/108 (67%) isolates of *K. pneumoniae* were resistant to amoxicillin-clavulanic acid. This report supported by Derakhshan et al.[33] that found 121/200 (60.5%) of resistance against amoxicillin-clavulanic acid. Meanwhile, another study revealed that 36.69% of resistance, which is lower than our result.[34]

In different studies reported, the incidence of ESBL producers in India ranging from 60%-80 %.[35] The incidence of ESBL producers in the present study was 45.37%, which supports the study of Wettal et al.[36] Nevertheless, Thenkhiwale et al.,[37] Mumtaz et al.[38] and Chander and Shestha[39] had reported a much lower rate of ESBL *Klebsiella* spp. The increased occurrence of multi drug resistant bacteria, influenced by various risk factors such as prolonged hospitalization, indiscriminate use of antibiotics and self-medication. In the present study, the rate of isolation of ESBL producers were more in urine 53.06% (26/49) followed by sputum 22.45% (11/49) and pus 16.33% (8/49). The similar results were reported by Akila et al.,[40] which is dependent with the sample size.

Virulence factors are contributing to the pathogenesis and antimicrobial resistance of the bacteria. The high density of bacterial population present in biofilm contributes to antimicrobial resistance. A significant association between biofilm formation and co-trimoxazole (*P*-value 0.036) and amoxicillin-clavulanic (*P*-value 0.037) was revealed in this study. Siderophore iron uptake is one of the major virulence determinants. In the current study, siderophore production showed a significant association with antimicrobial resistance. Karam et al.[41] reported a significant correlation between the presence of iron acquisition gene chuA and resistance of antibiotics such as co-trimoxazole and ceftazidime. Pramodhini et al.[42] reported that other bacteria showed a significant association between certain virulence factors and antimicrobial resistance. However, they suggested that all virulence factors are may not be significantly associated with antimicrobial resistance.

CONCLUSION

The bacterial virulence and antimicrobial resistance are not independent factors, the association between these factors may play a crucial role in pathogenesis of invasive infections. The present study indicated certain virulence factors of *K. pneumoniae* are associated with antimicrobial resistance. The antimicrobial resistant *K. pneumoniae* possess virulence characteristics, which lead to complications and treatment failure. Therefore the strict monitoring of virulence factors and antimicrobial resistance can be recommended in clinical laboratories with an emphasis on surveillance and infection control activities. Furthermore, molecular studies are required for the good understanding of the genetic relation between virulence factors and drug resistance of *K. pneumoniae*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

AUTHORS' CONTRIBUTION

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

FUNDING

None.

DATA AVAILABILITY

All datasets generated during the study are included in the manuscript

ETHICS STATEMENT

Not applicable.

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