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ORIGINAL ARTICLE

Seroepidemiology, Antimicrobial Susceptibility and Virulence Characteristics of Clinical *Klebsiella pneumoniae* Isolates in Mansoura University Hospitals

[1]Aya H. Elasmer, [1,2]Mohammed Y. Ibrahim, [1]Dina E. Rizk*

[1]Microbiology & Immunology Department, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt
[2]School of Biological Sciences, University of Cambridge

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Corresponding Author:
Dina E. Rizk.
Postal address: Department of Microbiology and Immunology, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt
Tel: +201002605440,
Fax: 002-0502247496
dena@mans.edu.eg

ABSTRACT

Background: *Klebsiella pneumoniae is one of the crucial causes of nosocomial and community-acquired infections that can result in various infections in human.* ***Objectives:*** *The present study aim to investigate the prevalence of capsular serotypes, antimicrobial susceptibility and virulence characteristics of K. pneumoniae isolated from different Mansoura University Hospitals.* ***Methodology:*** *K. pneumoniae isolates were collected from different clinical sources at Mansoura University Hospitals. The antimicrobial susceptibility to 14 different antibiotics was determined by disk diffusion method. The capsular serotypes were assessed by quelling test. Serum resistance, haemagglutination, biofilm, lipase, protease and lecithinase enzymes production were assessed phenotypically. Moreover, four virulence genes (rmpA, fimH, kfuBC and wabG) were detected by PCR. The genetic relatedness among isolates was investigated using ERIC-PCR molecular typing.* ***Results:*** *Seventy-three isolates were confirmed as K. pneumoniae. The vast majority of isolates demonstrated MDR patterns (72.6%) including a high resistance rate to the beta-lactam antibiotics (ampicillin: 98.6%, amoxicillin-clavulanic acid: 97.26 %, piperacillin: 97.26 %, amoxicillin: 93.15% and cefotaxime: 94.52%). K1 and K2 were the main serotypes found among the isolates, K1 serotype was the predominant (79.45%). It was found that serum resistance was the highest detected virulence factor among isolates (95.9%) and lipase was the lowest detected factor (19.2%). Haemagglutination was detected in 63% of the isolates especially from rectal swab (83.3%) and sputum (72.7%). The biofilm formation was detected mainly among urine and blood isolates. rmpA, fimH, kfuBC and wabG genes were harbored by 20.5%, 92%, 66% and 94.5% of isolates, respectively. ERIC- PCR showed high genetic diversity (100%, typability, Simpson's index of diversity= 1).* ***Conclusion:*** *The current study revealed the high antibiotic resistance levels, pathogenic potential, and genetic diversity among K. pneumoniae isolated from different clinical sources which is considered a serious health problem that necessitates interventions to control its spread.*

INTRODUCTION

Klebsiella pneumoniae is one of the world's leading causes of infections. It has been reported as a cause of both community-acquired and hospital-acquired infections. It results in increased patient morbidity and mortality[1]. It causes different kinds of infections in humans such as pneumonia, septicemia, urinary tract infection and pyogenic liver abscess [2]. *K. pneumoniae* is an extremely resistant bacterium as it has multiple mechanisms of resistance to different antibiotic classes such as β-lactams, aminoglycosides, quinolones and polymyxins [3,4]. It can produce a wide range of virulence factors including adhesins, capsular polysaccharides, lipopolysaccharide (endotoxins), iron sequestering systems (siderophores) and biofilm formation that are basically identified to be the main causes for its pathogenesis [5]. The most crucial pathogenic factor in *K. pneumoniae* is polysaccharide capsule. Based on the composition of its capsular polysaccharides, it can be categorized into 77 serological K antigen types. This capsule inhibits the activation or uptake of complement components especially C3b[6], so it provides protection against phagocytosis and bacterial serum factors [7]. Adhesins factors are responsible for the adherence of bacteria to the respiratory, the urinary and to the intestinal epithelial cells[8]. Biofilms are extracellular polymeric substances that shield bacteria from opsonization and phagocytosis. The development of biofilms by pathogenic bacteria plays an important role in facilitating evasion of host defense mechanisms, communication between bacterial cells and protection against antibiotic action[9]. One of the crucial elements

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for bacterial survival is iron. The organism synthesizes small iron-scavenging particles termed as siderophores that has the uppermost iron affinity. The pathogen produces additional siderophores, as yersiniabactin and salmochelin, that permit it to evade from the host defense [10].

Molecular typing is an important method to reveal the genetic relatedness between isolates which may cause outbreaks in health care settings. Enterobacterial Repetitive Intergenic Consensus (ERIC) is considered one of the widely used fingerprinting methods to characterize the genetic diversity of this pathogen [11].

The present study aims to shed light on the prevalence of different serotypes, virulence determinants, antibiotic susceptibility and molecular typing of *K. pneumoniae* isolated from different clinical sources at different Mansoura hospitals.

METHODOLOGY

Bacterial Isolation and Identification:

One hundred and ninety-one clinical specimens were obtained during the period from March to November 2016 from the Infection Control Unit at Faculty of Medicine, Mansoura University. Different clinical sources at Mansoura hospitals were included: Mansoura University Hospitals (MUH), Mansoura Emergency Hospital (MEH), Burns and Cosmetic Center (BCC), Pediatric University Hospital (PUH) and Urology and Nephrology Center (UNC). The specimens were collected in sterile containers and transported as promptly as possible to the laboratory for further identification. This study was approved by the Ethics Committee in Faculty of Pharmacy, Mansoura University, Egypt (Code Number: 2020-116).

The collected specimens were identified as *K. pneumoniae* according to laboratory biochemical standards[12]. The genomic DNA was extracted as previously stated using the rapid DNA extraction method [13]. The phenotypically detected *K. pneumoniae* isolates were confirmed by PCR using primers of 16S rRNA gene sequence outlined in table 1[14]. Confirmed *K. pneumoniae* isolates were preserved at −80°C for further study.

Antimicrobial Susceptibility Testing:

The antimicrobial susceptibility test was performed by the Kirby-Bauer disk diffusion method according to the criteria set by the Clinical and Laboratory Standards Institute (CLSI, 2016). Fourteen antibiotics (Oxoid) were tested: Ampicillin (10), Amoxicillin (20), Amoxicillin /clavulanic acid (20/10), Amikacin (30), Cefotaxime (30), Chloramphenicol (30), Ciprofloxacin (5), Imipenem (10), Gentamicin (10), Streptomycin (10), Piperacillin (100), Piperacillin/tazobactam (100/10), Tobramycin (10), Trimethoprim/ sulfamethoxazole (1.25/23.75).

Serological identification of capsular antigen:

Serological identification of antigens K1 and K2 of *Klebsiella pneumoniae* were performed by Quellung test[15] using specific antibodies purchased from Statens Serum Institute, Copenhagen, Denmark. The antigen-antibody reactions were observed microscopically.

Phenotypic Detection of Virulence Factors:

- ***Haemagglutination assay*:**

The isolates were examined for their ability to agglutinate human erythrocytes by their fimbria by the slide method[16].

- ***Assay of biofilm formation*:**

The biofilm production was quantified in 96 well flat bottomed polystyrene microtitre plate [17]. The mean OD of each tested isolates (OD_T) was calculated and the formed biofilm was categorized according to Stepanovic *et al.,* [18]

- ***Detection of lipase, protease and lecithinase enzymes production*:**

Isolates were tested for lipolytic activity by using nutrient agar plates containing 1% tween 80 [19]. After incubation for 7 days at 37°C, the isolate was considered positive when an opaque zone was observed around the grown colonies.

The proteolytic activity was detected according to Hassan *et al* [20]. Clear zone around the growth indicating positive proteolysis.

For lecithinase enzyme, the isolates were spotted onto agar plate containing 2.5% egg yolk [19]. An opaque zone surrounding the spot was considered a positive result.

- ***Serum resistance*:**

Serum resistance of the tested isolates was assessed using the turbidmetric assay as previously stated by Gharrah *et al.* [21].

Detection of Virulence factors genes:

Genes encoding virulence factors: *fim*H (type I fimbriae), *kfu*BC (iron acquisition system-related gene), *wab*G (endotoxin-related genes) and *rmp*A (regulator of mucoid phenotype A) were investigated by PCR. The primer sequences and the sizes of the amplified products are listed in table 1. The PCR program followed was denaturation at 94ºC for 5 min, 35 cycles at 94ºC for 30 sec; annealing temperature as specified in table 1 for 1 min.; and 72ºC for 90 sec, then a final elongation at 72ºC for 10 min[22]. A negative control without DNA template was also included. The PCR products were electrophoresed using 1.5% agarose gel and visualized by ethidium bromide staining and UV transillumination.

Table 1: The sequence of primers used in the current study

Target gene	Primer	Nucleotide sequence (5` to 3`)	Annealing temperature	Band size(bp)	reference
16S rRNA	F	ATTTGAAGAGGTTGCAAACGAT	58 ºC	130	[13]
	R	TTCACTCTGAATTTTCTTGTGTTC			
*rmp*A	F	ACTGGGCTACCTCTGCTTCA	57ºC	516	[22]
	R	CTTGCATGAGCCATCTTTCA			
*fim*H	F	TACTGCTGATGGGCTGGTC	64ºC	640	[22]
	R	GCCGGAGAGGTAATACCCC			
*kfu*BC	F	GAAGTGACGCTGTTTCTGGC	59ºC	797	[22]
	R	TTTCGTGTGGCCAGTGACTC			
*wab*G	F	CGGACTGGCAGATCCATATC	57ºC	683	[22]
	R	ACCATCGGCCATTTGATAGA			
ERIC-PCR	ERIC-1R F	ATGTAAGCTCCTGGGGATTCA	48 ºC	Multiple bands	[23]
	ERIC-2R R	AGTAAGTGACTGGGGTGAGCG			

F: forward, R: reverse, bp: base pair

ERIC-PCR

Genotyping of *K. pneumoniae* isolates was carried out by ERIC-PCR using the primers described in table 1. The cycling program was followed as described by Wu *et al.* [23]. The PCR products were visualized by agarose gel electrophoresis (1.2%), and scanned by gel documentation system (Model Gel Doc 1.4, 1189; AccuLab®). DNA patterns were analyzed and dendrogram was constructed using GelJ software version 2.0.

Comparison of different typing methods

The discriminatory power of antibiogram, capsular serotyping, virulence patterns and ERIC-typing were analyzed by calculating the Simpson's discriminatory index (D) according the following equation:

$$D = 1 - \frac{1}{N-1} \cdot \sum_{j=1}^{s} nj(nj-1)$$

Where N: the total number of isolates, S: the total number of types, Nj: the number of isolates belonging to the jth type[24].

Statistical analysis

Graph-pad Prism version 5 was used to statistically analyze the results. The chi-square test was performed to compare between groups. Significant difference was considered when P value ≤ 0.05.

RESULTS

Bacterial Isolation and Identification

Seventy-three isolates were phenotypically and genotypically confirmed as *K. pneumoniae* from blood (n=17), wound swab (n=13), urine (n= 11), sputum (n= 11) catheter and rectal swab (n= 6), swabs (throat, oral and nasal swab), tube and endotracheal aspirate were grouped as miscellaneous (n= 15).

Antimicrobial Susceptibility:

The antimicrobial susceptibility of the tested strains was performed. A high resistance to ampicillin (98.6%), amoxicillin-clavulanic acid (97.26 %), piperacillin (97.26 %), amoxicillin (93.15%) and cefotaxime (94.52 %) was observed. About 71.23% and 61.64% of isolates were resistant to trimethoprim/sulfamethoxazole and tobramycin, respectively. The tested isolates were susceptible to imipenem (94.52%), amikacin (73.97%), ciprofloxacin (57.53%), Gentamicin (50.68%), streptomycin (50.68%), Chloramphenicol (47.94%) and piperacillin/tazobactam (42.46%). A percent of 72.6 (53/73) of isolates was multidrug resistant (resistant to ≥ three different antibiotic classes)

Concerning the resistance patterns, isolates were distributed into 59 antimicrobial resistance patterns (fig 2). Seven patterns were revealed by more than one isolate. A55 and A35 patterns were the most predominant being shown by five isolates and four isolates, respectively. While A16, A21 patterns appeared three times, A33, A50, A53 patterns appeared twice.

Serological identification of capsular antigen.

It was found that 93.2%(68/73) of isolates were serotyped as K1or K2. K1 serotype was significantly prevalent as it was harbored by 79.45% (58/73) of isolates (P = <0.0001), while 13.7% (10/73) of isolates were K2 serotype. Five isolates (6.85%) were non K1/K2 serotype. The distribution of serotypes among different clinical sources is shown in table 3. K1 serotype was mostly found among blood (22.4%) as shown in (table 2).

Table 2: Distribution of capsular serotypes of *K. pneumoniae* isolates among different clinical sources

Clinical source	Serotype Number of isolates (%)			P-value
	K1	K2	Non K1/K2	
Blood	13(22.4%)	2(20%)	2(60%)	0.6482
Wound swab	10(17.2%)	2(20%)	1(20%)	0.9695
Urine	8(13.8%)	3(30%)	-	0.2589
Sputum	8(13.8%)	2(20%)	1(20%)	0.8358
Rectal swab, catheter	5(8.6%)	-	1(20%)	0.4010
Miscellaneous	14(24.2%)	1(10%)	-	0.2963
total	58	10	5	**<0.0001**

Phenotypic Detection of Virulence Factors

Most isolates (95.9%) revealed serum resistance. Haemagglutination was detected in 63% of the isolates, especially among those obtained from rectal swab and sputum. The protease enzyme was produced by 52% of the isolates mainly from wound swab and miscellaneous group. Lecithinase enzyme was produced by 42.5% of the isolates. Regarding lipase enzyme, only 19.2% of the tested isolates were positive lipase producers. The results of biofilm formation were as follow: 1.37% strong adherent, 16.43% moderately adherent, 58.9% weakly adherent and 23.28% non-adherent.

Genotypic Detection of Virulence Factors:

The virulence genes *kfu*BC, *wab*G and *fim*H were amplified in 66% (48/73), 94.5% (53/73) and 92% (67/73) of isolates, respectively. *rmp*A gene was the least detected, found in 20.5% (15/73) of isolates only (fig. 1).

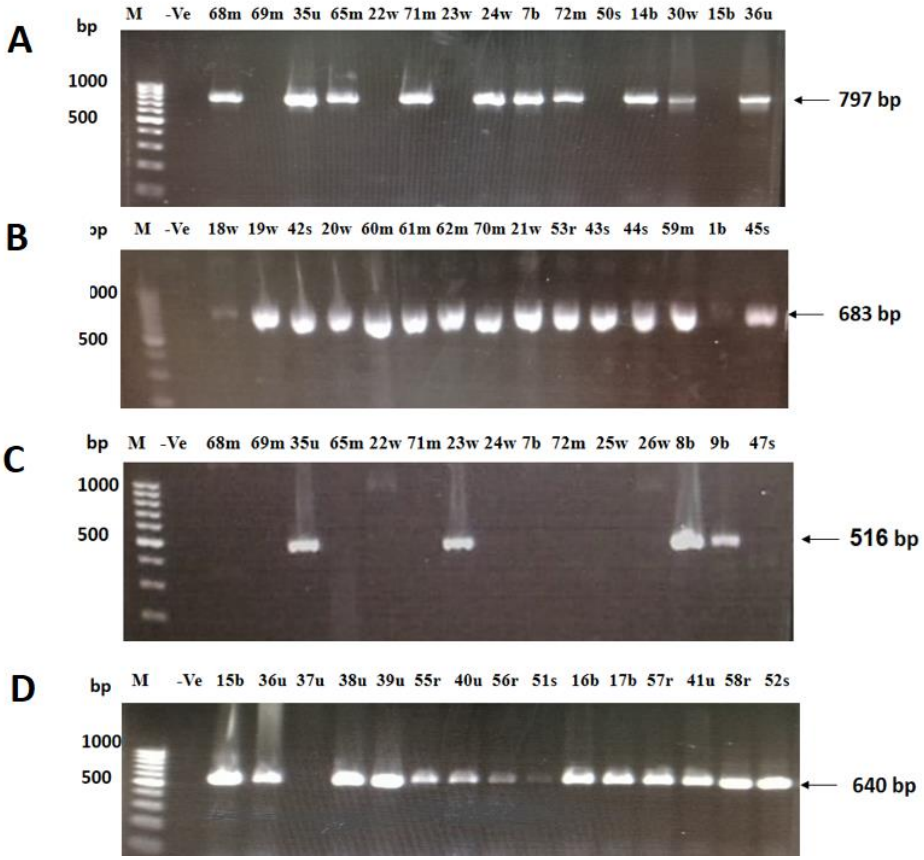

Fig. 1: Agarose gel (1.5%) electrophoresis of A: *kfuBC* gene, B: *wabG* gene, C: *rmp*A gene and D: *fimH* gene. M: DNA ladder 100 bp, Lane 1: Negative control.

Table 3 illustrates the distribution of the studied virulence factors among different serotypes. As compared to K2 serotype, it was found that K1 was significantly equipped with numerous virulence factors (P< 0.05). K1 serotype exhibited the highest prevalence of haemagglutination, biofilm formation, protease production and *kfu*BC gene existence. While K2 showed the highest lecithinase production and *wab*G gene existence.

Regarding the isolation source, it was found that isolates obtained from blood and wound were equipped with a range of virulence factors. This source exhibited the highest prevalence of biofilm, protease production. While isolates from sputum and rectal showed the highest haemagglutination and lecithinase production and showed the highest prevalence of *fim*H gene. Moreover, isolates obtained from urine and miscellaneous showed the highest prevalence of *rmp*A and *kfu*BC genes (table 4).

Table 3: Distribution of the virulence factors and virulence factor encoding genes among *K. pneumoniae* isolates of different serotypes.

Virulence factor	Serotype Percent of isolates			P-value
	K1 (n=58)	K2 (n=10)	Non K1/K2 (n=5)	
Serum resistance	94.8%	100%	100%	0.0062**
Haemagglutination	67.24%	30%	80%	< 0.0001***
biofilm formation	67.24%	50%	60%	0.0489.*
Protease	55.17%	40%	40%	0.0483.*
Lipase	19%	20%	20%	0.9791.
Lecithinase	39.65%	60%	40%	0.0047.**
*rmp*A gene	20.68%	10%	40%	< 0.0001***
*Fim*H gene	89.65%	100%	100%	< 0.0001***
*kfu*BC gene	70.68%	60%	20%	< 0.0001***
*wab*G gene	94.8%	100%	80%	< 0.0001***

Table 4: Distribution of the virulence factors among *K. pneumoniae* isolated from different clinical sources

Clinical Source	Virulence factors and Virulence associated genes Number of isolates (%)									
	Serum resistance	Haem-agglutination	biofilm	protease	lipase	lecithinase	*rmpA*	*fimH*	*kfuBC*	*wabG*
Blood	17 (100.0)	10 (58.8)	14 (82.3)	10 (58.8)	0 (00.0)	8 (47.0)	3 (17.6)	16 (94.0)	10 (58.8)	14 (82.3)
Wound swab	12 (92.3)	7 (53.9)	9 (69.2)	8 (61.5)	3 (23.1)	6 (46.2)	1 (7.6)	10 (77.0)	7 (53.8)	13 (100.0)
Urine	11 (100.0)	6 (54.5)	11 (100.0)	5 (45.5)	3 (27.3)	4 (36.4)	5 (45.4)	10 (91.0)	9 (81.8)	11 (100.0)
Sputum	10 (91)	8 (72.7)	7 (63.6)	4 (36.4)	2 (18.2)	6 (54.5)	2 (18.2)	11 (100)	5 (45.4)	10 (91)
Rectal swab, catheter	6 (100.0)	5 (83.3)	4 (66.7)	2 (33.3)	3 (50.0)	3 (50.0)	1 (16.7)	6 (100.0)	4 (66.7)	6 (100.0)
Miscellaneous	14 (100.0)	10 (66.7)	11 (73.3)	9 (60.0)	3 (20.0)	4 (26.7)	3 (20.0)	14 (93.3)	13 (86.7)	15 (100.0)
P-value	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**< 0.0001**	**<0.0001**	**< 0.0001**

Virulence profile pattern:

The profiles of the virulence factors and virulence factor ecoding genes were extremely diverse. Fifty-eight different virulence profiles were obtained including 49 unique profiles (figure 2). V3, V9 and V25 patterns were demonstrated by four (1b, 12b, 13b and 57r) and three isolates (7b, 64m and 71m), respectively. Eight profiles (V4, V8, V12, V17, V20, V27, V44 and V45) were shown twice. Such diversity prompted us to further investigate the association of the virulence factors and virulence factors associated genes with the isolation source. This was achieved through estimation of virulence score and mean virulence score. Virulence score (VS) was defined as the number of all virulence factors detected in each isolate and mean virulence score was calculated by the sum of all VS of the isolates of each source divided by number of isolates of the same source. Most of the tested isolates (90%) were highly virulent (VS≥ 5). It was found that isolates from rectum had the highest mean virulence score 6.73. In contrast, isolates from wound recorded the lowest mean virulence score 5.48. Isolates from blood and sputum recorded mean virulence score of 6, 5.9, respectively.

ERIC-PCR

ERIC genotyped the tested isolates into 72 different patterns (typability 98.6%), only one isolate was untypable. According to the generated dendrogram, the isolates were clustered into 10 clusters (A- K) with largest one (J) comprising 12 isolates. There were no isolates with 100% similarity.

Discriminatory power of typing techniques for *K. pneumoniae* isolates

The performance of antibiogram, capsular serotyping, virulence patterns and ERIC-typing in discrimination between isolates was assessed by calculating Simpson's index of diversity (table 5). It was found that ERIC-typing gave the highest discrimination (D = 1) followed by phenotypic methods, virulence pattern and antibiotype.

Table 5: Discriminatory power of typing techniques for *K. pneumoniae* isolates.

Typing method	No. of patterns	Simpson's index of diversity (D)
Antbiogram	59	0.990
serotyping	3	0.350
Virulence pattern	58	0.992
ERIC-PCR	73	1.000

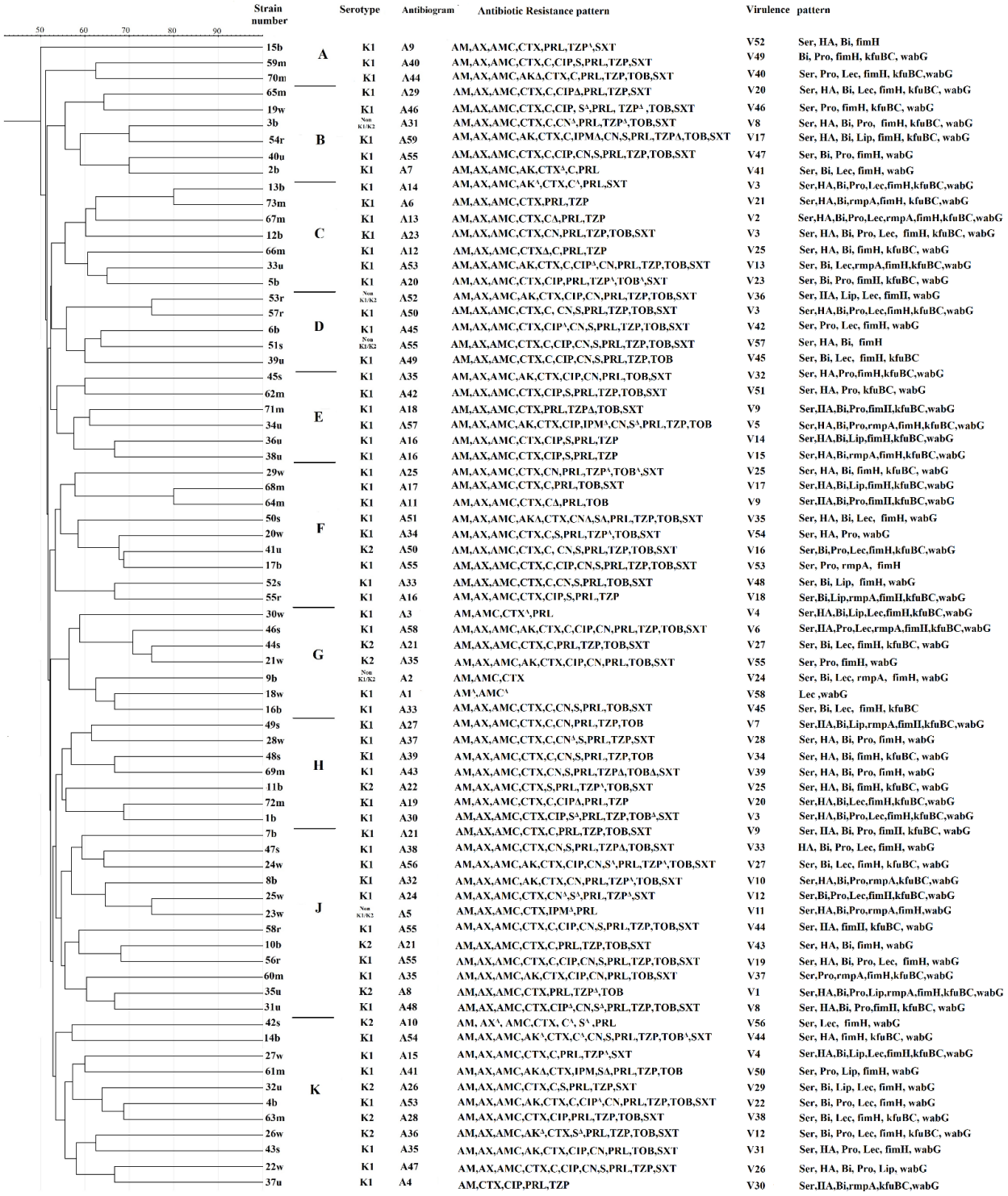

Fig. 2: Dendrogram constructed by the UPGMA clustering method showing the genetic similarity among 73 *K. pneumoniae* isolates using ERIC genotyping.

Δ: intermediate resistant. AM: Ampicillin, AMC: Amoxicillin/Clavulanic acid, AK: Amikacin, AX: Amoxicillin,b: blood, C: Chloramphenicol, CIP: Ciprofloxacin, CN: Gentamicin, CTX: Cefotaxime, IPM: Imipenem, m: miscellaneous, PRL: Piperacillin, r: rectum, s: sputum, S: Streptomycin, SXT: Trimethoprim/Sulphamethoxazole, TOB: Tobramycin, TZP: Piperacillin/tazobactam, u: urine, w: wound.

DISCUSSION

K. pneumoniae is an important etiological agent of several different healthcare-associated infections. Its pathogenicity has been contributed to various virulence factors [25]. Capsules are key virulence factors which are associated with the lethality of infection. In the present study, two capsular serotypes (K1 and K2) accounted for 93% of isolates. The previous studies also reporting the highest prevalence of K1 and K2 that results in poorer disease outcome[26]. K1 serotypes was the predominant followed by K2 serotypes (79.45% and 13.7%, respectively).

The antibiotic resistance level of *K. pneumoniae* has been changed rapidly in the last years involving the successive generation of beta lactam antibiotics [5]. Accordingly, *K. pneumoniae* isolates in this study showed a high resistance level to ampicillin, amoxicillin, amoxicillin-clavulanic acid and piperacillin. The noticed high levels of antimicrobial resistance may be due to the lack of strict policies that control the use of antibiotics in Egypt. Hudson *et al.* [27] have reported that resistance to amoxicillin and ampicillin is naturally expressed by chromosomal class-A β-lactamases in the whole of *K. pneumoniae* isolates. In contrast to Wasfi et al.[28] who noted higher resistance levels to imipenem (61.1%, 85.7%, respectively) and aminoglycosides, most of our isolates were susceptible to imipenem (94.52%) and amikacin (73.97%) that could be effective in treatment. This is in accordance with that reported by Amer et al.[29].

MDR strains are considered of great public health importance, as they may result in further complication in treatment of *K. pneumoniae* infections in human with increased morbidity and mortality rates[30,31]. The MDR isolates account for 72.6% of isolates that represented by 41/ 59 antibiotic resistance patterns which increases the risk of failure of antimicrobial treatment in humans. This result is similar to that previously reported by other studies [32].

K. pneumoniae possess a wide range of virulence factors. Serum resistant bacteria have a higher survival rate when invade hosts and establish diseases [33]. In contrast to previous studies that recorded lower rates of serum resistance [21], serum resistance rate in this study (95.9%) is a solid indicator of Klebsiella higher pathogenicity. The biofilm production in our study was as follows: strong adherent (1.37%), moderately adherent (16.43%), weakly adherent (58.9%) and Non-adherent (23.28%). In contrast with Seifi *et al.* who indicated that a large proportion (> 93%) of isolates were biofilm-producers [34].

Different kinds of hydrolytic enzymes are produced by *K. pneumoniae* such as protease, lipase, lecithinase that contributed in increasing the pathogenesis [35]. In this study, lipase enzyme was produced by only 19.2% (14/73) of total isolates. Unlike our results, El-Mahdy *et al.*,[36] detected lipase production in 41% of *K. pneumoniae* isolates. The lecithinase enzyme production was scored in this study in 42.5% (31/73) of isolates. A different result was reported in a study conducted by Hassan et al.[20] all isolates showed no lecithinase production. While 52% (38/73) of the isolates tested in our study were positive protease producers, Anielski et al.[37] noted non-significant production of protease enzyme.

The most common adhesive organelles in *Enterobacteriaceae* are type 1 fimbriae that can result in urinary tract infections[38]. A gene cluster (*fim*) containing all the genes encodes type 1 fimbriae[39]. In the current study, we found that 92% (67/73) of total isolates harbored *fim*H gene. Fimbrial adhesins (hemagglutinins) in bacteria are crucial for bacterial adherence to human epithelial cells. In this study, only 63% of isolates exhibited haemagglutination phenotype. This result disagreed with a previous study of El-Mahdy *et al.* [34], in which all of the tested *K. pneumoniae* strains were positive to human blood haemagglutination and *fim*H gene.

Previous studies have reported the usual association of capsular serotypes K1 and K2 with the *rmp*A gene [40]. However, we detected *rmp*A gene in only 20.5% (15/73) mainly in urine isolates. *kfu*BC gene was detected in 66% (48/73) of total isolates mainly in (81.8%) urine isolates. In the current study, we found that the production of *wab*G gene is 94.5% (69/73) of total isolates. *wab*G gene was detected in all wound, urine, rectal swab and catheter isolates.

Investigation of the virulence factors indicated that the *fim*H and *wab*G genes were commonly distributed among isolates, which is in accordance with what reported by Calhau *et al.*, [41]. The existence of these genes in isolates proposed the pathogenicity and a possible risk of these isolates to human health.

Molecular typing is a powerful tool for studying genetic diversity of pathogens. ERIC typing shows high diversity of the studied isolated as it revealed 72 different genotypes which coincides with what Lai *et al.* report [42] who stated that pathogenic *K. pneumoniae* is highly heterogeneous. The high values of discrimination index of antibiotyping, virulence pattern typing and molecular typing methods used in the current study indicates that the tested isolates are greatly diverse

CONCLUSION

The current study indicated that *K. pneumonia*e strains isolated from different clinical sources are highly pathogenic and resistant to most antibiotics used. So, more prevention strategies and control guidelines have to be applicable to control the emergence of these strains.

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- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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