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Antimicrobial Resistance, Capsular Serotypes and Virulence Associated-Genes of Hypervirulent and Classical *Klebsiella pneumoniae* in El-Mansoura, Egypt.

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ABSTRACT

Background: Hypervirulent *Klebsiella pneumoniae* (*K. pneumoniae)*, generally associated with the hypermucoviscous phenotype has emerged as a clinically significant pathogen responsible for serious disseminated infections, such as pyogenic liver abscesses, osteomyelitis, and endophthalmitis, in a generally younger and healthier population. The aim of this study was to investigate the molecular characterization of hypervirulent (hypermucoviscous) *K. pneumoniae* strains, which mainly emerged in EGYPT.
Methods: Bacterial species were identified by standard methods with a VITEK 2 compact system. The serotyping, biotyping and the String test for phenotypic detection of mucoviscosity were used. Antimicrobial susceptibility to several commonly used antibiotics were determined by the Kirby-Bauer's disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI, 2018). Polymerase chain reaction (PCR) was used to amplify virulence-associated genes (*rmpA*, *magA*, *entB*, and *iucA*).
Results: One-hundred isolates of each hypervirulent *K. pneumoniae* (hvKP) and classical *K. pneumoniae* (cKP) were screened over a 2-year period. The serotyping showed that 76 isolates had K1-type while 19 had K2-type of capsule and 5 non-K1/K2 isolates of hvKP while for cKP, only 3% was K1, 1% K2 and 96% were non k1/K2. Among the hvKP isolates, the prevalence of virulence associated-genes as *rmpA* gene was 93% and for *magA* gene was 40% and for *iucA* 92%. Otherwards, the prevalence of these genes in cKP isolates was very low 11%, 7% and 0%, respectively. However, there was no significant difference for *entB* (89 % and 92 %).
Conclusion: The hypervirulent isolates were infrequent among *K. pneumoniae* isolates causing bacteremia in our geographical area (El-Mansoura, Egypt). For good understanding the basic biology of hypervirulent *K. pneumoniae*, this study summarized and focused on epidemiology, hypervirulence-associated factors, and antimicrobial resistance mechanisms of such hypervirulent strains. This epidemiological study give alarm about the dissemination of hypervirulent *K. pneumoniae* strains. Therefore, an immediate response to recognize these hypervirulent strains with resistance determinants is an urgent priority.

***K*eywords:** Hypervirulent, Hypermucoviscous, Classical *Klebsiella pneumoniae*, antimicrobial resistance, String test, and Virulence factors.

1. Introduction

In recent past, the prevalence of *Klebsiella pneumoniae* infections has exceedingly raised in the clinical settings (Vuotto, Longo, Balice, Donelli, & Varaldo, 2014). Recently, it has emerged globally as a multidrug-resistant hospital pathogen for which there are few treatment options (Paczosa & Mecsas,2016). The investigations about its virulence factors have added newer insights to its self-protective pathogenic strategies, which comprise mainly of fimbriae, capsule, and lipopolysaccharide responsible for the attachment to host surface, protection against phagocytosis, desiccation, and complement evasion, respectively. Besides, type 1 and type 3 fimbriae are revealed to arbitrate its colonization on passive/inert abiotic (Piperaki, Syrogiannopoulos, Tzouvelekis, & Daikos, 2017). Recently, Ferry et al. have reported implant-associated ESBL producing *Klebsiella pneumonia* involved in bone and joint infection in a healthy 40-year-old man who underwent a bifocal fracture of the left leg (Ronde-Oustau, Lustig, Dupieux, & Ferry, 2017).

Several virulence factors have been identified in *K. pneumoniae* isolates, including a polysaccharide capsule, which is the basis of the serotype classification (at least 78 different serotypes) (Liao, Huang, Chang, Hsu, & Hsueh, 2014). Although the *K. pneumoniae* capsule acts as a determinant virulence factor, certain serotypes (K1, K2, K4, and K57) are known as hypervirulent clones (Pan et al., 2008). Besides the capsule, other virulence factors that are usually associated with the genotype have been described in *K. pneumoniae*. The most important clonal complex (CC) is CC23, which includes hypervirulent *K. pneumoniae* strains which have an easily recognizable hypermucoviscous phenotype (Struve et al., 2015).

The first hypervirulent *K. pneumoniae* strains with a hypermucoviscous phenotype were described in Taiwan in 2004 (Liu et al., 2020). Since then, several reports worldwide have described these strains (Catalán-Nájera, Garza-Ramos, & Barrios-Camacho, 2017; Liu et al., 2020; Sellick & Russo, 2018). Although the most common focus of infection is pyogenic liver abscess (PLA) (Choby, Howard-Anderson, & Weiss, 2020), they have been implicated in other infections, including metastatic abscesses (Brisse et al., 2009; Lederman & Crum, 2005; Shi et al., 2018). Infections due to hypermucoviscous *K. pneumoniae* have been identified in patients with and without underlying diseases, and also in asymptomatic carriers (Clegg & Murphy, 2016).

The hypermucoviscous phenotype in *K. pneumoniae* is related to the presence of the chromosomally encoded hypermucoviscosity gene A (*magA*), which is characteristic of the K1 capsular operon, and/or to the plasmid gene regulator of the mucoid phenotype A (*rmpA*), which is a regulator of extracapsular polysaccharide synthesis (Holt et al., 2015). In addition, hypervirulent-community-associated (CA) strains that infect seemingly healthy individuals have emerged. These strains are particularly worrisome since they cause severe diseases such as pyogenic liver abscess (PLA), meningitis, and endophthalmitis (Y. Guo et al., 2017). Originating in the Asian Pacific Rim, these strains are now being reported globally, including in the United States (Fierer, Walls, & Chu, 2011).

In this study, we analyzed the prevalence of hypermucoviscous *K. pneumoniae* as a cause of bacteraemia in adult patients over a 2-year period in Mansoura University Hospitals in El-Mansoura, EGYPT. We also characterized the strains and analyzed the clinical characteristics of patients with bacteraemia according to phenotype, serotype and genotype.

2. Material and methods

2.1. Isolation and Identification of K. pneumonia Isolates:

A total of 100 non-duplicate of each HvKP and cKP isolates were collected from inpatients and outpatients at El-Mansoura University Hospitals, EGYP between January 2018 and December 2019. Bacterial species were identified by standard methods with a VITEK 2 compact system (Biomérieux, Marcy l'Etoile, France). All procedures performed were approved by the Ethical Committee of Faculty of Pharmacy, Mansoura University, Egypt.

2.2. String Test for Detection of Mucoviscosity:

The hypermucoviscous phenotype of *K. pneumoniae* isolates was confirmed by string test. The bacterial strains were grown on an agar plate at 37°C overnight and the formation of a mucoviscous string measuring >5 mm on a bacteriology inoculation loop was defined as a positive string test (Shon, Bajwa, & Russo, 2013).

2.3. Serological Identification of Capsular Antigen by Quelling Test:

The suspected isolates were serologically identified antigens K1 and K2 of *Klebsiella pneumonia*. Such specific antigens were purchased from Staten's Serum Institute, Copenhagen, Denmark. Accordingly, quelling test was carried out according to the producer instruction. The antigen-antibody reactions are observed microscopically. A positive quelling reaction is the result of the binding of the capsular polysaccharide with type specific antibody contained in the typing antiserum (Edmondson & Mary Cooke, 1979).

2.4. Antimicrobial Susceptibility Testing:

Antimicrobial susceptibility of all isolates to 12 commonly used antibiotics were determined by VITEK-2 System and the other 6 antimicrobial by Kirby-Bauer disk diffusion method on Mueller-Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI, 2018) guidelines. Antibiotic discs (Oxoid Ltd., Basingstoke, UK) used included Cefuroxime (CXM; 30 μg), Cefepime (FEP; 30 μg), Aztreonam (ATM; 30 μg), Levofloxacin (LEV; 5 μg), Imipenem (IPM; 10 μg), and Meropenem (MEM; 10 μg).

2.5. Detection of Virulence Genes and Capsular Serotypes Encoding Genes:

DNA of all isolates were extracted and the virulence-associated genes (*rmpA, magA. iucA and entB*) were amplified by polymerase chain reaction (PCR). The primers are listed in Table (1). PCR amplifications performed in a Thermal Cycler T100™ (Bio-Rad, USA) with a final volume of 50 μL (25 μL of Master mix, 15 μL of water, 4 μL of each primer with a final concentration of 0.8 μM (Biotechnology Industries, Canada) and 2 μL of template DNA), with an initial denaturation temperature of 98 °C for 10 s, extension at 72 °C for 15 s and a final extension at 72 °C for 1 min. The annealing temperatures was 57 °C. PCR products were loaded on a 1.5% (w/v) agarose gel and visualized by UV transillumination (Bio-Rad Chemi-Doc™MP System).

2.6. Statistical Analyses:

All statistical analyses were performed using IBM-SPSS version 22.0 for Windows (SPSS Inc., Chicago, IL, USA). A *P*-value < 0.05 was considered to be statistically-significant.

2.7. Ethical Considerations:

The design of this study was approved by the local institutional review board. Informed consent was obtained from all participants included in this study.

Results

3.1. Bacterial Isolates:

During the period of two years a total of 100 (8.2 %) consecutive, non-duplicate (single isolate/patient) isolates of hvKP out of 1460 patient samples, were identified. The distribution of these isolates according to the source of isolation was illustrated in table (2). In addition, 100 isolates were selected as cKP to be used in comparison.

3.2. Hypermucoviscous Phenotype, and Clinical Features of hvKp vs. cKp:

The results the of string test according to (Y. Guo et al., 2017) showed that 100 isolates were hypermucoviscous *K. pneumonia* (hvKP). However, the 100 isolates of classical *K. pneumonia* (cKP) were negative string test. The most affected group of patients were male (65 %) ($P < 0.05$) as compared to female (35%). The mean age of the hvKp group was younger than the cKp group (63.5 ± 7.35 vs. 68 ± 8.5 years, P=0.029). Comparison between the age of the hvKp and cKp patients was showed in Table (3), which revealed that the maximum number of cases was found in male in the age group of (61–70) years (24 samples, 24%, P = 0.0335). The prevalence of hvKP isolated from male inpatients infections were found higher than the isolated samples from male outpatients. While isolated from female patients were mainly from outpatients (Figure 1).

3.3. Antimicrobial Susceptibility Pattern of all K. pneumonia Isolates to Antimicrobials:

The rate of antibiotic resistance of cKP isolates was much higher than that of hvKP isolates, although the majority of clinical isolates showed an elevated rates of resistance to most tested antimicrobial agents. The cKP isolates were resistant to antimicrobial agents such as Ampicillin (100%), Amoxicillin/clavulanic acid (91%), Trimethoprim/Sulfamethoxazole (95%), Cefotaxime (93%), Ceftriaxone (85%), Ceftazidime (87%), Cefuroxime (69%), Cefepime (48 %), Aztreonam (88%), Nitrofurantoin (86%), Gentamicin (70%), Amikacin (60%), Ciprofloxacin (65%), Levofloxacin (37%), Chloramphenicol (49%), Doxycycline (15%), Imipenem (59%), and Meropenem (42%). Except for Ampicillin and Chloramphenicol, hvKP had much lower resistance rates to all antimicrobial agents than cKP. The results were expressed as frequency (n, %) in Table (4) and Fig. (2),

3.4. Biotypes and Capsular Serotypes Identification.

In this study the results of serotype detection revealed that, only 19% of hvKP isolates carried K2 serotypes, while 76% of hvKP isolates were related with K1 serotypes and 5% of the serotypes were not K1/K2. However, for cKP serotype the data showed that only one isolate was K2, 3% of isolates were K1 and 96% showed non K1/K2 serotype. However, the findings of biotyping for all isolates of *K. pneumoniae* divided into five different biotypes, with B1 being the most prevalent (56%) followed by B3 and B4 that are almost identical (15%) and (14%) and B2 that is least prevalent (6%) (Fig.3).

3.5. Molecular Detection of Virulence-Associated Genes.

The data in this paper revealed that hvKP isolates were positive for most virulence-associated genes with high percentage especially for *rmpA* (93%**)**, *iucA* (92%), e*ntB* (89%), but at low percentage of *magA* (40%) (Table 5). However, cKP strains exhibited very low percentages of Virulence-Associated Genes with just; 11% for *rmpA*, 7% for *magA*, and for the *iucA* 0%. But the cKP isolates showed a high prevalence of the e*ntB* gene with 92%.

Discussion:

During the last 3 decades, hypervirulent *K. pneumoniae* (hvKP) isolates have emerged, causing severe community-acquired infections primarily in the form of pyogenic liver abscesses. This syndrome has so far primarily been found in Southeast Asia, but increasing numbers of cases are being reported worldwide, indicating that the syndrome is turning into a globally emerging disease (Arato, Raso, Gasperini, Scorza, & Micoli, 2021).

The hypermucoviscosity phenotype was detected in 8.2% of all *K. pneumoniae* isolates. These phenotypes were more significantly detected in hvKP. In previous studies, the prevalence of hvKP varied from 7.8% to 25% (Choby et al., 2020; C. R. Lee et al., 2017; Li et al., 2014). whereas another study conducted in Egypt reported that about 40% of *K. pneumoniae* isolates were hypermucoviscous (Ahmed, Ibrahim, Abdelhaliem, & Elariny, 2022). The hvKP prevalence of our study was lower than those Chinese reports mentioned above, but significantly higher than the reports from a teaching hospital in Spain between 2007 and 2013 (5.4%, 53/878; Cubero et al. 2016) and a surveillance study in Alberta, Canada (8.2%; Peirano et al. 2013). Sun et al. reported 81.6% (31/38) of *K. pneumoniae* causing PLA were hvKP determined by the string test (Sun et al., 2019), which was significantly higher than that in our investigation. A report from Taiwan also showed that 90% of *K. pneumonia* associated PLA were hvKP (Yan, Zhou, Zou, & Liu, 2016).

These variations in prevalence can be partly attributed to the geographical location. For example, previous studies showed that the prevalence of the hvKP in European countries is lower than East Asian countries (Baron et al., 2021; Gorrie et al., 2017; Heiden et al., 2020; Wyres et al., 2019).

Although hvKP isolates are usually more susceptible to clinically often used antimicrobial agents relative to cKP isolates, more and more hvKP isolates were found to be multi-resistant to antimicrobial agents, even to carbapenems (S. Guo et al., 2016; C.-H. Lee, Chang, Liu, Chen, & Yang, 2014; Yao et al., 2015). In the present study, four hvKP isolates were found to be resistant to most antibiotics tested but less than cKP isolates. In addition, the results of this study were in line with another study where, a high resistance rate that reported Cefuroxime (69%), Gentamicin (70%), Amikacin (60%), and Ciprofloxacin (65%), (Shinu et al., 2020). In contrast, Zaki et al. (2019) showed high susceptibility to Sulfamethoxazole /trimethoprim and Ciprofloxacin (Catalán-Nájera et al., 2017).

The capsules of K1 and K2 serotypes essentially protect the bacteria from phagocytosis (Ku, Chuang, & Yu, 2008). Hypermucoviscosity has been related to the capsular serotype K1, and in a lower proportion with the serotype K2 (Cubero et al., 2016; Remya, Shanthi, & Sekar, 2018). The majority of hvKP isolates were associated with K1 serotypes (76%), and only 19% of hvKP isolates had K2 serotypes. However, 5% were non-K1/K2 serotypes. Although the K1/K2 capsular serotypes are common among hvKP isolates, some studies have shown that a considerable proportion of the hvKP strains may have a non-K1/K2 serotype (Ma et al., 2018; Yao et al., 2015).

In this study the results revealed that, the genetic factors associated with the increased virulence of hvKP clinical isolates. A specific virulence plasmid was found to be associated with hypervirulence. Our findings in this study proved that, the *K. pneumoniae* isolates with hypermucoviscosity phenotype (hvKP) were positive for most virulence-associated genes with high percentage especially for *rmpA* (93%), *iucA* (92%), e*ntB* (89%), and at low percentage of *magA* (40%). These findings were similar to a multicenter investigation from China (97.7%; (Ye et al., 2016)). In addition, these data were consistent with previous

reports regarding the virulence genetic profiles of clinical *K. pneumonia* isolates (Sellick & Russo, 2018; Yao et al., 2015) which also like our data found that the prevalence of virulence-associated genes *rmpA* and aerobactin (*iucA*) were strongly associated with hvKp than cKp strains.

Conclusion

As the majority of *K. pneumoniae* strains even cKP or hvKP were multidrug resistant and these need development of novel antibiotics and implementation of infection control measures and antimicrobial stewardship programs to limit its spread. Also, the prevalence of hvKP was considered high with presence of virulent genes that make more dangerous pathogen and for protecting patients from hvKp requires action from all levels of the healthcare system.

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Disclosure

There is no conflict of interest to disclose.tables ***and graphs***

Table (1): Oligonucleotide primers targeted K. pneumoniae virulence related genes were utilized throughout the research.

Target gene	Type	Nucleotide sequence (5` to 3`)	Product size	Annealing temperature	Reference
Set of primers used for amplification of virulence genes					
rmpA	**F**	**5-ACTGGGCTACCTCTGCTTCA-3**	**536**	**56 ° C**	(Wang, Shen, Wu, & Ma, 2017)
	R	**5-CTTGCATGAGCCATCTTTCA-3**			
magA	**F**	**5-GGTGCTCTTTACATCATTGC-3**	**1149**	**55 ° C**	(Y. Guo *et al.*, 2017)
	R	**5-GCAATGGCCATTTGCGTTAG-3**			
iucA	**F**	**5-AGGATAAATGGCCACATTG-3**	**556**	**54.5 ° C**	(Yan *et al.*, 2016)
	R	**5-ATGAACGCCTGGTCCTTTGC-3**			
entB	**F**	**5-GTCAACTGGGCCTTTGAGCCGTC-3**	**400**	**54.5 ° C**	(Yan *et al.*, 2016)
	R	**5-TATGGGCGTAAACGCCGGTGAT-3**			
FimH	**F**	**5-AGGATAAATGGCCACATTG-3**	**688**	**57 ° C**	(Y. Guo *et al.*, 2017)
	R	**5-GCT GAACGCCTATCCCCTGC-3**			
iroB	**F**	**5-ATCTCATCATCTACCCTCCGCTC-3**	**739**	**54 ° C**	(Yan *et al.*, 2016)
	R	**5-GGTTCGCCGTCGTTTTCAA-3**			

Table (2): Distribution of clinical isolates collected from different Mansoura Hospitals

Geographical distribution	No of hvKP (n=100)	Percentage (%)	No of cKP (n=100)	Percentage (%)
UNC	4	4 %	19	19 %
MUH	65	65 %	30	30 %
MUCH	2	2 %	4	4 %
CDH	29	29%	47	47 %

- (UNC) Urology and Nephrology Center - (MUH) Mansoura University Hospital
- (MUCH) Mansoura University Children Hospital - (CDH) Chest Diseases Hospital

Age group In Years All patients (%)		**hvKP**				**cKP**			
		Male (%)	**Female (%)**	**In-patient (%)**	**Out-patient (%)**	**Male (%)**	**Female (%)**	**In-patient (%)**	**Out-patient (%)**
≤20 years old	12 (6%)	1 (1%)	0 (0%)	1 (1%)	0 (0%)	7 (7%)	4 (4%)	10 (10%)	1 (1%)
21–30 years old	8 (4%)	4 (4%)	3 (3%)	6 (6%)	1 (1%)	0 (0%)	1 (1%)	1 (1%)	0 (0%)
31–40 years old	21 (10.5%)	9 (9%)	7 (7%)	12 (12%)	4 (4%)	2 (2%)	3 (3%)	3 (3%)	2 (2%)
41–50 years old	28 (14%)	7 (7%)	6 (6%)	5 (5%)	8 8%)	8 (8%)	7 (7%)	11 (11%)	4 (4%)
51–60 years old	37 (18.5%)	11 (11%)	7 (7%)	16 (16%)	2 (2%)	9 (9%)	10 (10%)	12 (12%)	7 (7%)
61–70 years old	48 (24%)	23 (23%)	4 (4%)	24 (24%)	3 (3%)	10 (10%)	11 (11%)	19 (19%)	2 (2%)
71–80 years old	36 (18%)	8 (8%)	6 (6%)	11 (11%)	3 (3%)	17 (17%)	5 (5%)	16 (16%)	6 (6%)
>80 years old	10 (5%)	2 (2%)	2 (2%)	4 (4%)	0 (0%)	2 (2%)	4 (4%)	5 (5%)	1 (1%)
Total	200 (100%)	65 (65%)	35 (35%)	79 (79%)	21 (21%)	55 (55%)	45 45%	77 (77%)	23 (23%)

Table (3): Age-related clinical features of hvKP and cKP isolates.

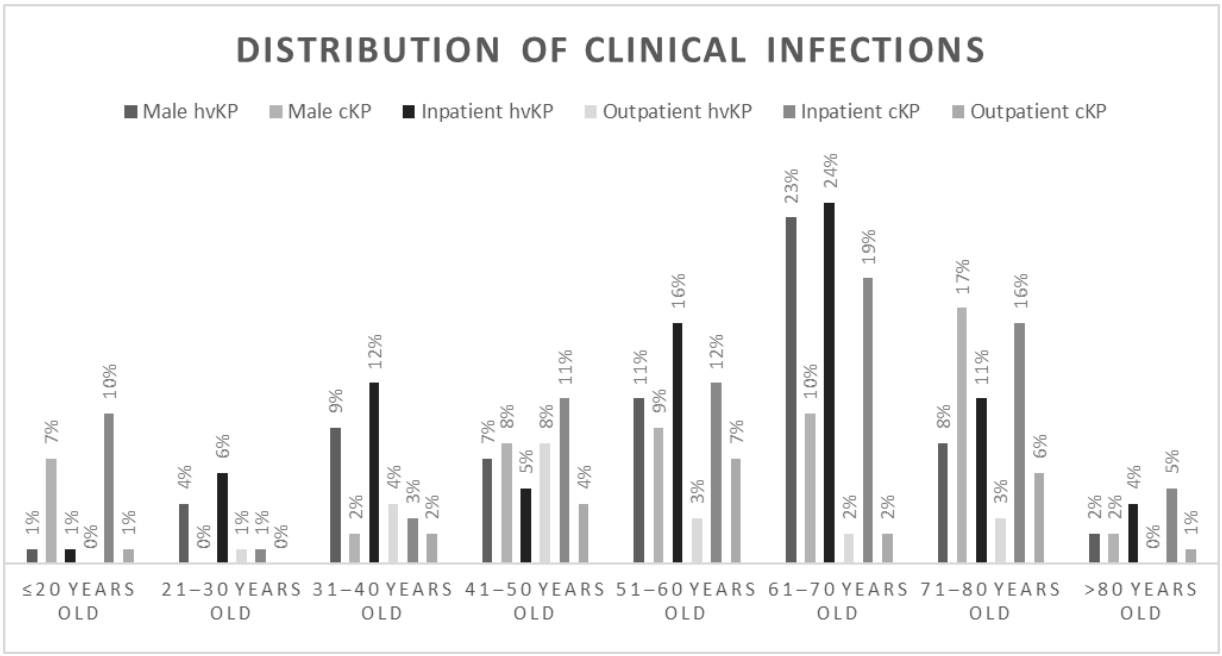

Fig.(1): hvKp and cKP clinical isolates according to the Gender and the Source of isolation.

ANTIBIOTIC Class	ANTIBIOTIC Name	No. of CKP	No. of hvKP	Total Percentage (N=200)	*P** Value
Aminoglycosides	Amikacin	60	22	41%	0.002*
	Gentamycin	70	38	54%	0.012*
Penicillin	Ampicillin	100	100	100 %	0.645
	Amoxicillin+ Clavulanic acid	91	89	90%	0.845
Cephalosporins	Cefotaxime	93	53	73%	0.021*
	Ceftriaxone	85	45	65%	0.025*
	Ceftazidime	87	37	62%	0.001*
	Cefuroxime	69	25	47%	0.012*
	Cefepime	48	20	34 %	0.02*
Carbapenems	Imipenem	59	19	39%	0.001*
	Meropenem	42	20	31%	0.003*
Monobactam	Aztreonam	88	48	68%	0.015*
Quinolones	Ciprofloxacin	65	15	40%	0.001*
	Levofloxacin	37	13	25%	0.014*
Sulphonamides	Trimethoprim/ Sulphamethoxazole	95	47	71%	0.02*
Tetracyclines	Doxycycline	19	11	15%	0.325
Chloramphenicol	Chloramphenicol	49	29	39%	0.027*

Synthetic drug	**Nitrofurantoin 300**	**86**	**44**	**65 %**	**0.003***	

Table (4): Antimicrobial susceptibility test results of K. pneumonia isolates.

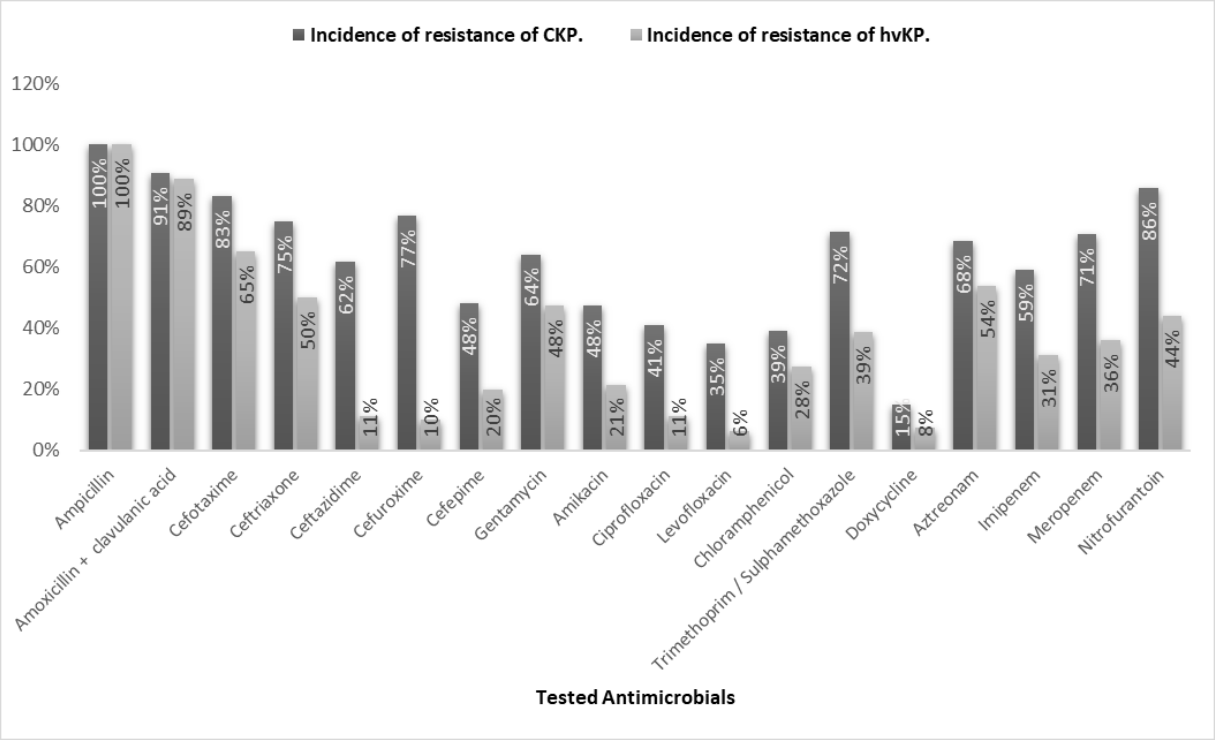

Fig. (2): Incidence of resistance of hvKP and cKP isolates to different antimicrobial agents.

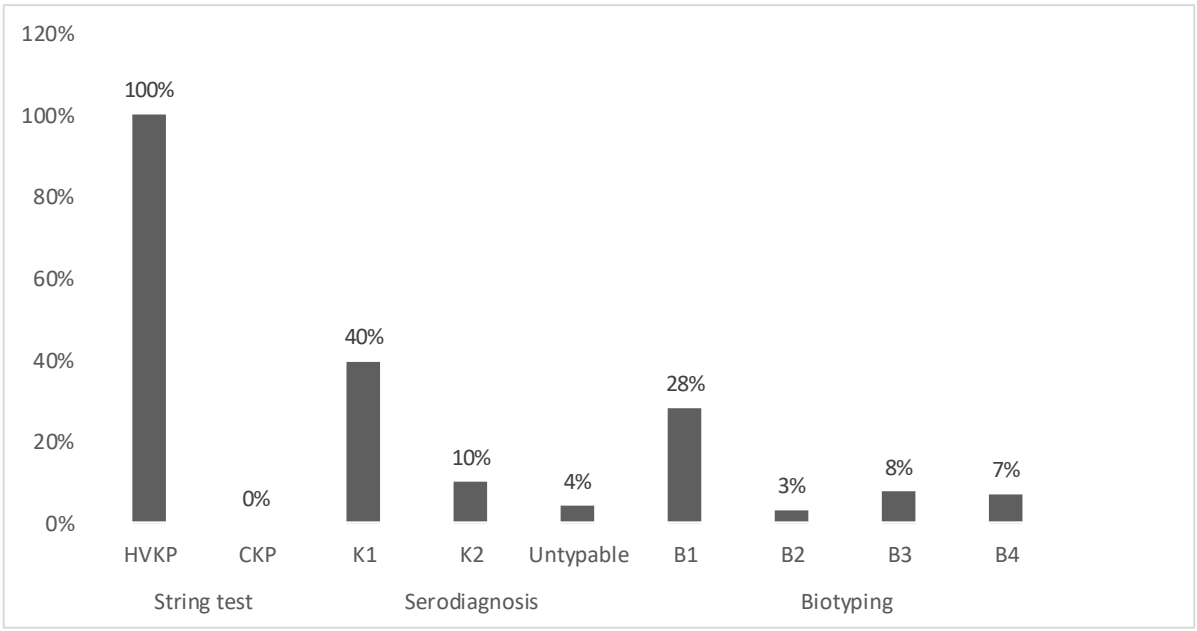

Figure (3): Prevalence of hypermucoviscosity phenotype of all K. pneumonia isolates by String test, Serodiagnosis and Bio-typing.

Table (5): Occurrence of hypermucoviscosity phenotypes, capsular serotypes, biotypes, and genetic characterization of virulence related genes.

Characteristic	hvKp (n=100) (%)	cKp (n=100) (%)	*P*-Value
String test			
Hypermucoviscosity	**100 (100%)**	**(0%)**	**0.001**
Serodiagnosis			
K1	**76 (76%)**	**3 (3%)**	**0.006**
K2	**19 (19%)**	**1 (%)**	**0.045**
Untippable	**5 (5%)**	**96 (96%)**	**0.347**
Biotyping			
B1	**56 (56%)**	**0%**	**0.007**
B2	**6 (6%)**	**0%**	**0.015**
B3	**15 (15%)**	**0%**	**0.010**
B4	**14 (14%)**	**0%**	**0.025**
Genotyping			
rmpA	**93 (93%)**	**11 (11%)**	**0.006**
magA	**40 (40%)**	**7 (7%)**	**0.012**
iucA	**92 (92%)**	**0 (0%)**	**0.001**
entB	**89 (89%)**	**92 (92%)**	**0.403**

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