ORIGINAL ARTICLE

Molecular Characterization of Carbapenamases in Hypervirulent *Klebsiella pneumoniae* Isolates among Pediatric Patients

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ABSTRACT

Background: *Carbapenem resistant hypervirulent Klebsiella pneumoniae (CR-hvKp) have led to fatal outbreaks. The rapidly increasing detection rate of CR-hvKp in pediatric patients makes the diagnosis of CR-hvKp crucial to overcome the indiscriminate use of antibiotic.* ***Objective:*** *The present study aims to isolate and antibiotype of CR-hvKp from clinical samples in Mansoura University Children Hospital. The study also directs to characterize carbapenemases in CR-hvKp isolates.* ***Methodolgy:*** *Eighty five K. pneumoniae isolates were tested for both carbapenems resistance and hypervirulence through detection of peg-344 gene by PCR. Antimicrobial susceptibility testing was done for CR-hvKp isolates by means of Kirby Bauer disc diffusion method. Multiplex polymerase chain reaction (multiplex PCR) was done to detect cabapenemase genes in CR-hvKp isolates.* ***Results:*** *Within the 85 K. pneumoniae isolates, 27strains (27/85; 31.76) were CR-Kp. Among CR-Kp isolates, 20 strains (20/27; 74.07%) were CR-hvKp. All CR-hvKp isolates were resistant to co-amoxiclav, piperacillin-tazobactam, cephalosporines and carbepenams, with variable resistant pattern to aminoglycosides, aztreonam, ciprofloxacin and levofloxacin. Respiratory samples were the main sample type of CR-hvKP isolates (60%), and tracheal intubation was the higher risk factor (50%). Cabapenemase genes were detected in only 13 isolates (13/20; 65%) . The identified carbapenemase genes included bla KPC-2 gene which was the predominant gene (6/13; 46.15%), bla VIM gene was present in 3 isolates (23.08%), and bla IMP and bla NDM genes each was present in 2 isolates (15.38% for each gene).* ***Conclusion:*** *It is critical to improve the clinical responsiveness of healthcare personnel and management of CR-hvKP infections, especially amongst pediatric patients.*

INTRODUCTION

Klebsiella pneumoniae is an opportunistic bacteria that frequently causes different types of infections. Hypervirulent *K. pneumonia* (hvKp), which is a virulent variant of classical *K. pneumoniae* (cKp), has turned out to be a worldwide health problem. Unlike cKp which is a principal cause of global nosocomial infection, hvKp variant is more destructive as it can cause community acquired infection in healthy individual with affection of distant site (such as central nervous system, lungs, eyes), leading to high morbidity and mortality[2].

Multiple genetic biomarkers like iuc (aerobactin synthesis), prmpA/A2 (capsule production regulator), iro (salmochelin biosynthesis), or peg344 (a transporter located on the inner membrane) can identify hvKp[3]. *Peg-344* gene is significantly considered hvKp specific and consequently can differentiate hvKp from cKp rapidly[4].

Resistance of CR-hvKp to the only remaining antibiotic option carbapenems, is a serious alarm. Notably, both hypervirulence and carbapenem resistance in CR-hvKp have led to fatal outbreaks, and have represented a recently dangerous worldwide health problem[6]. Mechanisms of CR-hvKP evolution can be briefly explained by 2 principal models: (i) gaining of carbapenem resistance by hvKP strain; or (ii) gaining of hypervirulence by CRKP strain[7].

Klebsiella pneumoniae carbapenemase is predominantly related to carbapenemase (KPC) family which is encoded by the *bla*KPC gene; a class A ß-lactamase[8]. Further carbapenemases, produced by epidemics-associated *Klebsiella pneumonia,* include VIM (Verona integron-encoded metallo β-lactamase), NDM (New Delhi metallo β-lactamase), IMP (Imipenemase metallo β-lactamase), and GIM (German

imipenemase) enzymes; class B ß-lactamases or metallo-enzymes[9].

To date, hvKp studies have mostly focused upon adults but recently surveillance done from 2017 to 2019 reported that there is a rising risk of hvKp infection in pediatric patients, mainly school-age children and adolescents[10].

The rapidly increasing detection rate of CR-hvKp in pediatric patients makes the diagnosis of CR-hvKp is critical to improve the clinical utilization of antibiotics[11]. This study aims at isolation and antibiotyping of CR-hvKp from clinical samples at Mansoura University Children Hospital. The study also directs to characterize carbapenemases among CR-hvKp isolates.

METHODOLOGY

Study design

A cross sectional descriptive study was done on 795 pediatric patients who had evidence of sepsis and antibiotic treatment failure, within 7 months from January to July, 2024. All patients in the study were admitted to Mansoura University Children Hospitals, Egypt. Clinical data was thoroughly taken out from patient medical records. In this study, nosocomial infection was defined as infection that developed after hospital admission by more than 48 hours. Community acquired infection was defined as infection that developed within 48 hours of admission[12].

Ethical approval

The study was accepted by Institutional Review Board of the Faculty of Medicine, Mansoura University; code number: R.24.08.2764.

Isolation of *K. pneumoniae*

The collected samples such as urine, blood, pus, sterile body fluids (pleural fluid, CSF), catheter (intravenous and urinary) and respiratory samples (nasopharyngeal swab, sputum, endotracheal aspirate, bronchoalveolar lavage), or other samples were cultured on chocolate agar, blood agar, and MacConkey's agar, while urine samples were cultured on CLED agar. Blood cultures were taken in special pediatric blood culture bottles with standard incubation time of 5 days. Growth detection of manual blood culture bottles depends on visible changes in the broth such as hemolysis, turbidity, puff ball or gas production. *K. pneumoniae* isolates were identified by Gram stained smear, colonial morphology and biochemical reactions using Kligler iron agar, oxidase, Lysine iron agar, methyl red, Voges-Proskauer, citrate, motility indole ornithine tests[13].

Antimicrobial susceptibility testing

Antimicrobial susceptibility test was done by Kirby Bauer disc diffusion method. Bacterial suspension was adjusted in reference to the 0.5 McFarland's turbidity standard. The suspension was streaked on the Mueller Hinton agar plate (Bio-Rad, USA). The antibiotic discs of amikacin (30 µg), gentamicin (10 µg), co-amoxiclav (30 µg), cefotaxime (30 µg), ceftriaxone(30 µg), ceftazidime(30 µg), cefepime (30 µg), cefuroxime (30 µg), cefoxitin (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), aztreonam (30 µg), piperacillin-tazobactam (100/ 10 µg), ertapenem (10 µg), meropenem (10 µg) and imipenem (10 µg) (Oxoid, England) were used for susceptibility testing. The diameters of each inhibition zone were measured after overnight incubation at 35-37°C. Result of each isolate were recognized as resistant, intermediate or sensitive to the antibacterial disc corresponding to CLSI guidelines[14].

Extraction of DNA

To extract the isolated *K. pneumoniae* DNA, bacterial colonies were boiled in sterile distilled water for 10 minutes. Following centrifugation, the supernatant was used as DNA template[15].

Detection of hv-Kp by identifying *peg-344* gene

Conventional PCR was done to detect *peg-344* gene. Strains positive for *peg-344* were considered as hv-Kp[3]. One µg of the extracted DNA was amplified in 50 µL of the reaction mixture. Each PCR reaction contained Taq Polymerase (Promega, USA), 2 mM MgCl2, 0.2 mM dNTP (Roche Diagnostics, Germany) and 20 µl of *peg-344* specific oligonucleotide primers (Promega, USA). Forward and reverse primers for *peg-344* gene and amplicon size are illustrated in table 1. The samples were overlaid with 100 µL of mineral oil, and subjected to 30 cycles of amplification in the DNA thermal cycler (Bio-Rad Laboratories Inc., USA). Parameters for amplification cycles were denaturation for 2 minutes at 94°C, annealing of primers at 55°C for 1 minute, and primer extension at 72°C for 1 minute. After the last cycle, the PCR tubes were incubated at 72°C for 7 minutes. The reaction products were visualized by ultraviolet light transilluminator (Bio-Rad Laboratories Inc., USA)[4].

Multiplex PCR analysis of carbapenemase genes in CR-hvKp

The *bla* KPC-2, *bla* VIM, *bla* NDM and *bla* IMP genes were detected through multiplex PCR by using gene-specific primers (Promega, USA). Primer sequences and amplicon sizes are listed in table 1. Dream Taq™ Green PCR Master Mix (Fermentas, USA) and specific-group primers were added to the DNA template supernatant. PCR steps were: initial denaturation at 95°C for 5 minutes; 40 cycles at 94°C for 20 seconds, 60°C for 30 seconds and 72°C for 45 seconds and final extension at 72°C for 7 minutes. From each reaction, an aliquot was evaluated on a 1.2% agarose gel stained with ethidium bromide and compared with 100 bp DNA ladder (Thermo Fisher Scientific, USA)[16].

Table 1: PCR primers

Primer	Sequence (5′–3′)	Amplicon size (bp)	References
Peg-344	F: CTTGAAACTATCCCTCCAGTC R: CCAGCGAAAGAATAACCCC	508	3
bla KPC-2	F: TCCGTTACGGCAAAAATGCG R: CGGCATAGTCATTTGCCGTG	462	17
bla VIM	F: TGGTGTTTGGTCGCATATCG R: AATCTCGTTCCCCTCTACCTC	298	18
bla NDM	F: CAACTGGATCAAGCAGGAGA R: TCGATCCCAACGGTGATATT	291	19
bla IMP	F: GAAGCTTGGCCAAAGTCCG R: TGTAAGTTTCAAGAGTGATGCGTC	108	19

RESULTS

Eighty five *K. pneumoniae* strains were isolated from 795 clinical processed samples, (85/795; 10.69%). Within the 85 *K. pneumoniae* isolates, 27strains (27/85; 31.76) were CR-Kp as detected through resistance to carbapenems (imipenem, ertapenem and meropenem) according to CLSI guidelines. Among CR-Kp isolates, 20 strains (20/27; 74.07%) were CR-hvKp, through detection of *peg-344* gene by PCR, table 2 & figure 1. CR-hvKp were isolated mainly from children (60%), and medical records indicated that nosocomial infections were 75% of CR- hvKp infections. Out of 20 CR-hvKp isolates, 12 (60%) isolates were obtained from respiratory samples, 6 (30%) isolates from urine and 2 (10%) isolates from pus. No metastatic infections were reported in CR-hvKp infected children. Tracheal intubation, diabetes mellitus and hepatobiliary disorders were risk factors, table 3. All the 20 CR-hvKp isolates (100%) were resistant to co-amoxiclav, piperacillin-tazobactam, cephalosporines and carbepenams, with variable resistant pattern to aminoglycosides, aztreonam, ciprofloxacin and levofloxacin, table 4. Of 20 CR-hvKp isolates, cabapenemase genes were detected in only 13 isolates (13/20; 65%). The identified carbapenemase genes included *bla* KPC-2 gene which was the predominant gene (6/13; 46.15%), *bla* VIM gene was present in 3 isolates (23.08%), and *bla* IMP and *bla* NDM genes each was present in 2 isolates (15.38% for each gene), table 5 & figure 2.

Table 2: Frequency of CR-hvKp

Total no. of samples	*K. pneumoniae* isolates		CR-Kp		CR-hvKp		Carbapenamase genes detection by multiplex PCR	
	No	%	No	%	No	%	No	%
795	85/795	10.69	27/85	31.76	20/27	74.07	13/20	65

Table 3: Clinical characteristics of patients with CR-hvKp (No=20)

Category	CR-hvKp	
	No	%
Age		
-Neonates	2	10
-Infants	6	30
-children	12	60
Infection setting		
-Hospital acquired	15	75
-Community acquired	5	25
Site of infection		
-Pneumonia	12	60
-Urinary tract infection	6	30
-Wounds	2	10
Coexisting condition		
- Diabetes mellitus	9	45
-Hepatobiliary disorders	7	35
- Malignancy	4	20
Invasive device		
-Endotracheal intubation	10	50
-Urinary catheter	6	30
-Central venous catheter	4	20

Table 4: Antimicrobial resistance profile of CR-hvKp (No=20)

Antibiotics	Resistant		Intermediate		Susceptible	
	No	%	No	%	No	%
Co-amoxiclav	20	100	0	0	0	0
Piperacillin-tazobactam	20	100	0	0	0	0
Cefoxitin	20	100	0	0	0	0
Cefuroxime	20	100	0	0	0	0
Cefotaxime	20	100	0	0	0	0
Ceftriaxone	20	100	0	0	0	0
Ceftazidime	20	100	0	0	0	0
Cefepime	20	100	0	0	0	0
Ciprofloxacin	16	80	2	10	2	10
Levofloxacin	15	75	2	10	3	15
Gentamicin	17	85	3	15	0	0
Amikacin	15	75	1	5	4	20
Aztreonam	15	75	0	0	5	25
Imipenem	20	100	0	0	0	0
Meropenem	20	100	0	0	0	0
Ertapenem	20	100	0	0	0	0

Table 5: Distribution of carbapenamase genes among CR-hvKp (No=13)

Carbapenamase genes	Number of isolates (%)
bla KPC-2	6 (46.15%)
bla VIM	3 (23.08%)
bla IMP	2 (15.38%)
bla NDM	2 (15.38%)

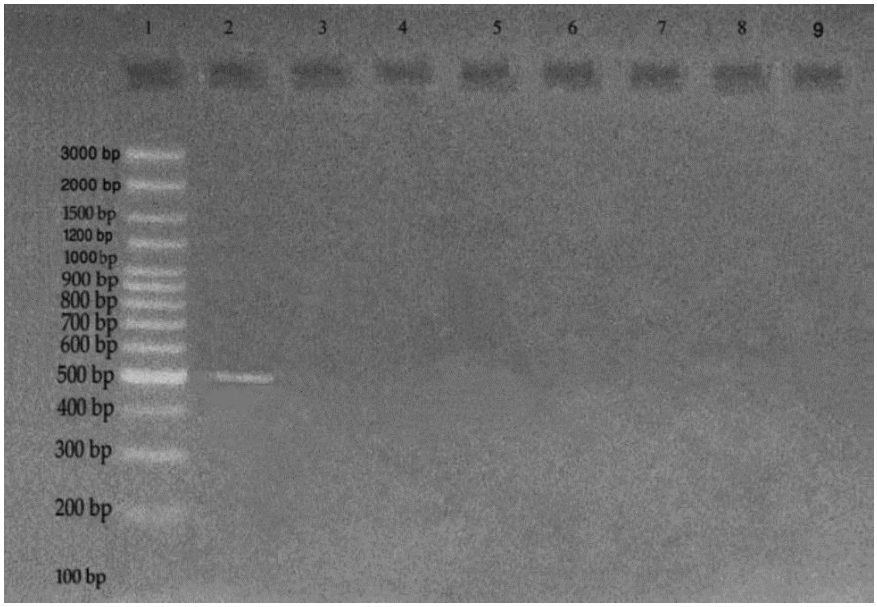

Fig. 1: Detection of hypervirulent *Klebsiella pneumonia* by identifying *peg-344* gene
Lanes 1: 100 bp DNA size marker, lane 2: *Peg-344* gene (508 bp).

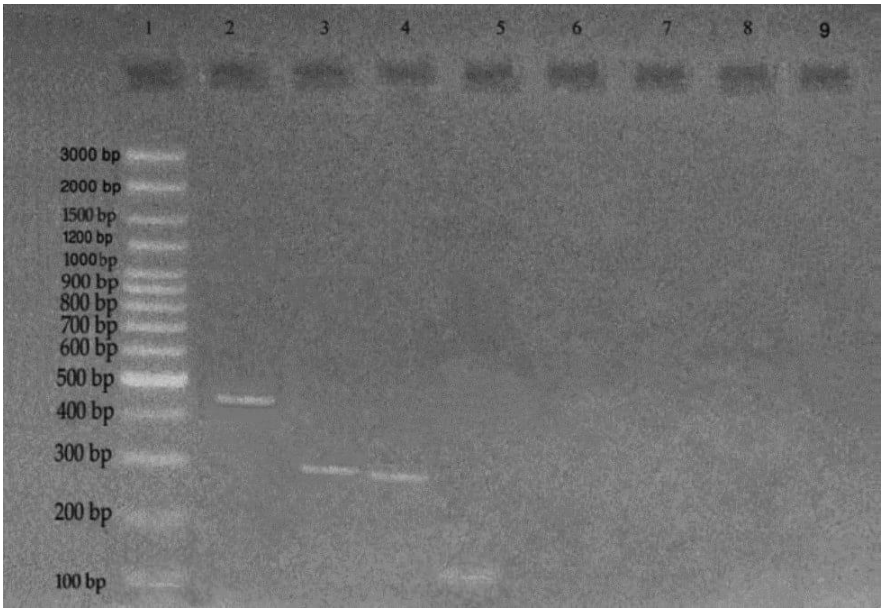

Fig. 2: Multiplex PCR analysis of carbapenemase genes in CR-hvKp
Lanes 1: 100 bp DNA size marker, lane 2: *bla* KPC-2 gene (462 bp), lanes 3: *bla* VIM gene (298 bp), lane 4: *bla* NDM gene (291 bp), lane 5: *bla* IMP gene (108 bp).

DISCUSSION

Serious infections caused by *K. pneumonia*e in pediatric patients have been reported[20]. Recently, the detection rate of hvKP in pediatric population had been increasingly to record 1.8%, 5.2% and 11.3% in 2017, 2018 and 2019 respectively[10]. Additionally, En et al reported a ten years age case with brain abscess with fatal outcome caused by hvKp[21].The combination of hypervirulence, multidrug resistance and elevated transmissibility makes CR-hvKP has a considerable threat toward human health [22,23]. Therefore, CR-hvKP has considered to be a next superbug[23].

There are many traditional techniques used to identify hvKp such as colony morphology, string test, serum killing assay or mouse lethality assay. However, these methods are time consuming and not accurate to detect the virulence[24,25]. It was found that biomarkers present on virulence plasmids can differentiate hvKp from cKp strains accurately. So, the biomarkers *iucA*, *iroB*, *peg-344*, *prmpA*, *prmpA2* and siderophore production more than 30 mg/mL have found to distinguish hvKp from cKp strains accurately. Fortunately, peg-344 gene has the highest sensitivity (0.99), specificity (0.96) and accuracy (0.97) as a marker to assess hvKp[3]. Importantly, *peg-344* gene has considered hvKp specific and, so can be used as an accurate and rapid molecular diagnostic test to differentiate hvKp from cKp[4].

The cKp is considered to be less virulent than the evolving pathovariant hvKp in clinical research. Infections caused by hvKp can occur in healthy individuals at any age and tend to infect patients at various infection sites with subsequent metastatic infections[26]. To decrease the morbidity and mortality due to hvKp infection, rapid, accurate and sensitive diagnostic method is an urgent need. In this study, among 27 CR-Kp isolates, 20 strains (20/27; 74.07%) were CR-hvKp, through detection of *peg-344* gene by PCR. In a previous study done at a hospital in China, the prevalence of CR-hvKP infections was 39.1%[27]. Another study done at 9 hospitals in 7 provinces in China reported that strains belonging to both CRKP and hvKp accounted for 8.3%[28]. This difference in prevalence of hvKP infections could be attributed to the demographical and geographical variations, and selected characters to identify hvKP.

In our study, CR-hvKp were isolated mainly from children (60%), and hospital-acquired infection also contributed to 75% of CR- hvKp infections. Another study done in China revealed that infections caused by hvKp were mostly affecting school-age children and adolescents (57.7%)[10]. Furthermore, another study in Shanghai found that hvKp infection were significantly more in older children without considerable sex bias[29]. In agreement with our findings, CR-hvKP is steadily becoming a dominant nosocomial microbe causing fatal outbreaks[22,27]. However others reported that the majority of infections caused by hvKp was reported to be community-acquired, because of hvKp colonization within the nasopharynx and gastrointestinal tract of healthy individuals in community [30].

Out of 20 CR-hvKp isolates, 12 (60%) isolates were obtained from respiratory samples, 6 (30%) isolates from urine and 2 (10%) isolates from pus. No metastatic

infections were reported in CR-hvKp- infected children. Parallel results were obtained from other researches in which most of patients infected by CR-hvKP had pneumonia (77.8%) in one study[27] and hvKP infected more than half of the children (55.6%) in another one[29]. To date, hvKP was considered to be a major cause of suppurative liver abscess in the past 3 decades[30]. What's more, hvKP is not only the main pathogen of pyogenic liver abscess, but it also causes primary extra-hepatic infections, as pneumonia, bacteremia, and soft tissue infection[31]. Tracheal intubation, diabetes and hepatobiliary disorders were the predominant risk factors in this research. Parallel to our findings, Li et al reported that tracheal intubation was significantly lower in CR-non-hvKP than CR-hvKP infections, signifying that tracheal intubation is a predisposing factor to develop CR-hvKP infections[27]. They also found that malignancy, hematological disorders and hepatobiliary disorders were underlying conditions associated with CR-hvKP infections. Additionally, El-Mahdy et al[32] detected a significantly higher number of hvKP isolated from a diabetic group.

Klebsiella pneumoniae carbapenemases are ß-lactamases that capably hydrolyze penicillins, all cephalosporins, monobactams, carbapenems, and also β-lactamase inhibitors[8]. In our work, all CR-hvKp isolates (100%) were resistant to carbepenams, co-amoxiclav, piperacillin-tazobactam, and cephalosporines, with variable resistant pattern to aminoglycosides, aztreonam, ciprofloxacin and levofloxacin. Correspondingly, antimicrobial susceptibility testing confirmed resistance to 3rd, 4th, and 5th-generation cephalosporins, aminopenicillins, aminoglycosides, and carbapenems, with intermediate resistance to levofloxacin[11]. Furthermore, antimicrobial sensitivity testing showed that all the CR-KP strains had the same pattern of resistance, but sensitivity pattern of ceftazidime/avibactam was relatively high[27]. Previous studies reported that tigecycline and colistin were still an option to treat CR-KP infection[33,34]. However, side effects of both drugs should be considered seriously, and must be taken with caution. Additionally, emergence of colistin and tigecycline-resistant strains and unfavorable pharmacokinetics have been reported with subsequent limitation of their use[35].

Resistance to carbapenems in *K. pneumoniae* is due to either production of carbapenemase enzymes, alteration of porin or upregulation of efflux pump[17]. Detection of *K. pneumonia* carbapenemases may be difficult and inaccurate based on routine antibiotic susceptibility testing[36]. So, the present work used multiplex PCR to detect carbapenamase genes among CR-Kp isolates. Multiplex PCR is one of the most significant diagnostic means as it can recognize the most prevalent genes. PCR is an accurate, rapid with low-cost. Moreover, it facilitates epidemiological screening of genetic appearance as well as spread of resistant strains[20]. In our work, of 20 CR-hvKp isolates, cabapenemase genes were detected in only 13 isolates (13/20; 65%) . The identified carbapenemase genes included *bla* KPC-2 gene which was the predominant gene (6/13; 46.15%), *bla* VIM gene which was detected in 3 isolates (23.08%), and *bla* IMP and *bla* NDM genes each was present in 2 isolates (15.38% for each gene). Similarly, it was found that KPC-type carbapenemase is a predominant cause of resistance in *K. pneumoniae*[17]. Interspecies transmission of *KPC* genes has been detected during epidemics in hospitalized patients. So, rapid and competent KPC diagnostic testing together with professional infection control measures and accurate use of antibiotic, all should be applied to minimize spread of KPC harboring *K. pneumoniae*[37].

Another study done by Fazeli and coauthors reported that the most common acquired MBLs include IMP, VIM, NDM-1, SPM (Sao Paulo metallo-β-lactamase), GIM (German imipenemase) and SIM (Seoul imipenemase) enzymes[19]. MBL genes are sited within different integron structures. Horizontal transfer of transposons or plasmids carrying MBL genes in between bacteria, has a significant role in occurrence of β lactam antimicrobial resistance in hospitals or community [38]. So, early detection of MBL-producing bacteria and prevention of nosocomial outbreak are very essential because these resistant organisms are poorly treated with subsequent increased mortality rate.

CONCLUSION

Prevalence and management of CR-hvKP have represented a big challenge. It is important to improve the clinical responsiveness and management of CR-hvKP infections, especially amongst pediatric patients. Additionally, it is essential to apply antibiotics properly to prevent the emergence or spread of microbial resistant strains in hospitals or community.

Conflicts of interest

The authors declare that they have no financial conflicts of interest related to the work done in the manuscript.

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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