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Detection and Analysis of *Klebsiella pneumoniae* causing Liver Abscess

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Research Article

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ABSTRACT

Background: Compared with "classical" *K. pneumoniae*, hypervirulent variant of *Klebsiella pneumoniae* have the ability to cause serious, life-threatening community-acquired infection, including liver abscess, pneumonia, meningitis and endophthalmitis and the ability to metastatically spread. An objective diagnostic test suitable for routine use in the clinical microbiology laboratory is needed.

Methods: A retrospective study was conducted in 240 patients with cultures positive for *K. pneumoniae* hospitalized in the Chinese PLA General Hospital from May 2013 to August 2014. The clinical and molecular data of the hypervirulent *K. pneumoniae* (hvKP) causing liver abscess were analyzed.

Results: Among 240 strains of *K. pneumoniae,* hvKP accounted for 42.5% (102/240), hvKP causing liver abscess were 37 strains, accounting for 36.3% (37/102), patients with diabetes were 11 (11/37, 29.7%), 13 (13/37, 35.1%) patients were diagnosed as fever of unknown origin at first, 7(7/37, 18.9%) patients with tumor, the rest 6 (6/37, 16.2%) patients with postoperative infection or other site infection. Univariate analysis revealed the following risk factors for hvKP causing liver abscess: string test (odds ratio (OR), 11.306[95% confidence interval (CI), 3.579-35.711]), serotype K1 (OR, 3.109[95% CI, 1.338–7.222]) and fever of unknown origin (OR, 6.921[95% CI, 2.503–19.136]). The results detected by multiplex PCR were consistent with single PCR. 102 strains of hvKP were tested the sensitivity to 14-19 drug, 37 strains caused liver abscess were not found with ESBL.

Conclusions: The detection of string test combined with *rmpA* and *aerobactin* can better identify hvKP. Patients with liver abscess generally were diabetes, but some patients didn't have other disease. There is not much difference about the chance to be attacked by hvKP among male and female. ESBL was not detected among the hvKP causing liver abscess. Multiplex PCR assay could detect hvKP quickly.

INTRODUCTION

In recent years, *Klebsiella pneumoniae* strains have become the second largest bacteria after the *E. coli* in China causing community-acquired infections and nosocomial infection [1]. The *K. pneumoniae* strains commonly recognized by clinicians and microbiologists are termed classic *K. pneumoniae* (cKP). Such strains are notorious for their capacity to cause nosocomial infections

and acquire antimicrobial resistance [2,3]. A new variant of *K. pneumoniae*, designated as hypervirulent *K. pneumoniae* (hvKP), was first described in 1986 by a group of Taiwanese doctors reporting a clinical syndrome of community-acquired *K. pneumoniae* infections [4-6]. Compared with ordinary *pneumonia Klebsiella*, hvKP is able to effectively produce capsule, the formation of high mucoid phenotype with high virulence factors, the hvKP strains exhibit a striking capacity to cause serious infections such as pneumonia, liver abscess, encephalitis, endophthalmitis, biliary tract inflammation [7]. Hypervirulent *K. pneumoniae* colonies grown on agar exhibit hypermucoviscosity. This phenotype has been used as a standard laboratory test to distinguish hvKP from cKP and is defined as a positive "string test" [8]. Liver abscess is a serious disease of digestive system, single or multiple. From the beginning of 1990s, hvKP gradually become the main pathogenic bacteria of liver abscess from *Escherichia coli*, Streptococci and Enterococci. It is easy to cause infection of other parts by transfer and the time of therapy is long, sometimes leads to recurrence, sometimes combined with other serious disease even to death [9]. An increasing number of cases of liver abscess due to hvKP are present in various Western countries. Recently Romania found liver abscess associated with severe myopathy caused by *K. pneumoniae* serotype K1 [3]. In Brazil, there was a case of K1 type causing liver abscess and finally to death [5]. At present, hvKP is becoming more and more prevalent in China; there are a lot of reports about the isolation and identification, drug resistance [6]. But how to define hvKP is still lack of unified standards [9]. Most of the hvKP strains identified to date are susceptible to antimicrobials with the exception of ampicillin [6]. However, the enhanced hvKP strains now have acquired antimicrobial resistance in some hospitals in China [8]. Combined with the increased risk to susceptible populations, these issues have attracted calls for preemptive intervention to mitigate the possibility of the globally damaging effects of hvKP infections. We conducted a retrospective analysis of 240 patients with positive cultures for *K. pneumoniae* hospitalized in General Hospital of PLA (people's liberation army) from May 2013 to August 2014, detected the mucoid phenotype, the capsule serotypes and the main virulence genes by multiplex PCR, analysed the clinical characteristics with disease, hope it can provide reference to laboratory diagnosis and treatment of hvKP.

MATERIALS AND METHODS

Patient Information: A retrospective study was conducted on 240 consecutive *K. pneumoniae* culture–positive patients hospitalized at General Hospital of PLA from May 2013 to August 2014. Clinical and laboratory data were gathered and analyses were made among patients from which hvKP strains were isolated.

The protocol for this study was approved by General Hospital of PLA Ethics Committee, and the Guidelines for Human Experimentation (PRC) were followed throughout. All patients gave written informed consent upon admission for their information to be stored and used for research.

Clinical isolates of *K. pneumonia*: 240 KP strains were from blood infection, respiratory tract infection, urinary tract infection, liver abscess patients, isolated from blood, sputum, bronchoalveolar lavage fluid, urine, liver abscess and other abscess puncture fluid, bile, sterile site of puncture fluid (pleural effusion and ascites and cerebral spinal fluid and synovial fluid), drainage liquid, central catheter. All of the strains were identified as *K. pneumonia* by using an automated bacterial identification system (VITEK® 2, bioMe rieux, USA), some bacteria were confirmed through mass spectrometer (VITEK MS, bioMerieux, USA) [10].

Clinical microbiologic characterization of the *K. pneumoniae* strains: All *K. pneumoniae* isolates were frozen and stored at -80°C. Susceptibility testing (Phoenix 100 automated microbiology system, BD, Franklin Lakes, New Jersey) to amikacin, amoxicillin-clavulanate, ampicillin, ampicillin-sulbactam, aztreonam, ceftizoxime, cefepime, cefotaxime, ceftazidime, ciprofloxacin, gentamicin, levofloxacin, piperacillin, tetracycline, trimethoprim-sulfamethoxazole, chloramphenicol, imipenem, meropenem, and piperacillin-tazobactam was conducted for every strain. Extended spectrum β-lactamase (ESBL) production was also determined using the Phoenix 100 system. A string test, *arobactin* and *rmpA* were performed to distinguish hvKP from cKP. *K. pneumoniae* strains with a positive string test or *arobactin* or *rmpA* were designated as hvKP. Virulence plasmid acquisition may be an important mechanism for the increased virulence of hvKP. Genes that encode a number of virulence factors, including those that are responsible for the hypermucoviscous phenotype (*rmpA*) and iron-acquisition factors (*aerobactin*) [9]. Another assay is species-specific for *K.pneumoniae*, detecting the *Klebsiella* hemolysin gene (*khe*). K1, K2, K5, K16, K20, K54 and K57 were detected to establish the capsular serotypes.

String test: A string test was performed to distinguish hvKP from cKP. A positive string test was defined as the formation of a mucoviscous string of >5 mm, when using a bacteriology inoculation loop to stretch a colony grown overnight on an agar plate at 37°C. *K. pneumoniae* strains with a positive string test were designated as hvKP.

Polymerase Chain Reaction–mediated detection of *RmpA, Arobactin*, and capsular serotype-specific genes: Genomic DNA was extracted from all *K. pneumoniae* strains (Thermal cracking method) and the *rmpA*, *arobactin*, and serotype-specific genes for the K1, K2, K5, K16, K20, K54, and K57 capsular serotypes were amplified by polymerase chain reaction(ABI Veriti® Thermal Cycler, Applied Biosystems Asia Pte Ltd., Singapore) Capsule type genes and virulence genes amplification primers synthesized by Shanghai sheng gong. The total volume of the reaction is 25 µl and contains 10 kinds of primers. Ten kinds of primers were K1, K2, K54, K57, *rmpA*. In another group ten kinds of primers were K5, *Khe*, *arobactin*, K16, K20. 0.5 µl dNTPS (each dNTP final concentration of 0.2 mmol/L). Primers F (0.6 µmol/L) and primer R (0.6 µmol/L) 1µl, Taq enzyme is 0.5 µl, 2 µl templates. The primers used are listed in **Table1**.

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Table 1. Primers.

Primer Name	Sequence
khe	
Forward	5-TGATTGCATTCGCCACTGG-3
Reverse	5-GGTCAACCCAACGATCCTG-3
Arobactin	
Forward	5-GCATAGGCGGATACGAACAT-3
Reverse	5-CACAGGGCAATTGCTTACCT-3
rmpA	
Forward	5-ACTGGGCTACCTCTGCTTCA-3
Reverse	5-CTTGCATGAGCCATCTTTCA-3
K1	
Forward	5-GTAGGTATTGCAAGCCATGC-3
Reverse	5-GCCCAGGTTAATGAATCCGT-3
K2	
Forward	5-GGAGCCATTTGAATTCGGTG-3
Reverse	5-TCCCTAGCACTGGCTTAAGT-3
K5	
Forward	5-GCCACCTCTAAGCATATAGC-3
Reverse	5-CGCACCAGTAATTCCAACAG-3
K16	
Forward	5-GTGCTTAACGGAGAACTGAAC-3
Reverse	5-CCTCACCTGGAAGAAGTGTA-3
K20	
Forward	5-CCGATTCGGTCAACTAGCTT-3
Reverse	5-GCACCTCTATGAACTTTCAG-3
K54	
Forward	5-CATTAGCTCAGTGGTTGGCT-3
Reverse	5-GCTTGACAAACACCATAGCAG-3
K57	
Forward	5-CGACAAATCTCTCCTGACGA-3
Reverse	5-CGCGACAAACATAACACTCG-3

Statistical analysis: SPSS software (version 15.0) was used for data analysis. Logistic regression was used to analyze risk factors for hvKP. P values of <0.05 were considered dependent risk factors for hvKP.

RESULTS

Patient characteristics: From May 2013 to August 2014, a total of 240 patients admitted to General Hospital of PLA were identified as having cultures positive for *K. pneumoniae* at 1 or more sites. Isolates of hvKP and cKP were obtained from 102 (43.3%) and 138 (57.5%) of patients, respectively. The strains were isolated from blood (cKP 71.7%, hvKP 28.3%), urine (cKP 86.2%, hvKP 13.8%), sputum (cKP 53.7%, hvKP46.3%), ascites (cKP 50%, hvKP 50%), bile (cKP63.2%, hvKP36.8%), and abscess fluid (cKP39.1%, hvKP60.9%). A significantly higher number of patients with hvKP in abscess fluid were detected. No significant differences were detected in the other sample types.

37 cases of liver abscess were caused by hvKP. 43.3% (102/240) of *K. pneumoniae* were hvKP, 37 strains caused liver abscess, accounting for 35.6% (37/102). CKP accounted for 57.5% (138/240), only 1 strain caused liver abscess, accounting for 0.07% (1/138). Among liver abscess patients caused by 37 hvKP strains, 11 patients were diabetic, 13 patients were diagnosed as fever of unknown origin at first, 7 were tumor patients, and 4 patients were with postoperative infection or other site infection.

Genetic characteristics of hvKP: String test was positive in 79 strains hvKP, accounting for 77% (79/102); the rmpA positive strains were 93, accounting for 91% (93/102); the *aerobactin* positive strains were 92, accounting for 86% (88/102). 34 strains were K1, accounting for 33.3% (34/102); type K2 29 strains, accounting for 28.4% (29/102); type K5 9 strains, accounting for 8.8% (9/102); type K16 0 strains; 3 strains of K20, accounting for 2.9% (3/102); type K54 8 strains, accounting for 7.8% (8/102); 6 strains of type K57, accounting for 5.9% (6/102); the remaining 6 strains with virulence factors but were not identified as related type strains, accounting for 5.9% (6/102). K1, K2, K5, K54 were positively correlated to hvKP. All *K. pneumoniae* were positive in Khe test. Non-toxic factor of K5 were 40% (6/15), K57 strain without virulent factor were 14.3% (1/7), 6.5% (2/31) strains of K2 have no virulence factors, 12.7% (13/102) with virulence factor but the type were not identified as related type. In the 37 hvKP strains caused liver abscess, string test positive strains were 31, accounted for 83.8% (31/37); the rmpA positive strains of 35, accounting for 94.6% (35/37); the aerobactin positive strains of 32, accounting for 86.5% (32/37); 21 strains of type K1, accounting for 56.8% (21/37); type K2 10 strains, accounting for 29.7% (11/37); type K5 4 strains, accounting for 10.8% (4/37); type K54 1 strains, accounting for 2.7% (1/37), 1 strain without type.

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Multiple PCR detection results were consistent with single PCR shown in **Table 2**.

Table 2. Characteristics of *K. pneumoniae* strains.

Characteristic	String test No.	*rmpA* No.	Abc No.	No Virulence No.	Liver Abscess, No.
K serotype					
K1	30/34 (88%)	34/34 (100%)	32/34(94%)	0	21/37 (56.8%)
K2	25/31 (81%)	25/31 (81%)	28/31(90%)	2/31	11/37(29.7%)
K5	7/15 (47%)	8/15 (53%)	6/15(40%)	6/15	4/37 (10.8%)
K20	2/3 (67%)	3/3 (100%)	3/3(100%)	0	0
K54	3/8 (38%)	6/8 (75%)	8/8(100%)	0	1/37 (2.7%)
K57	6/7 (86%)	6/7 (86%)	6/7(86%)	1	0
K-non typable	6/13 (46%)	13/13(100%)	5/13(38%)	0	1/37 (2.7%)
Total	79/102 (77%)	93/102(91%)	88/102(86%)	9/102(9%)	

Risk factors: Univariate analysis showed that K1 (odds ratio (OR)=3.109), positive string test (OR=11.306) and fever of unknown origin (OR=6.921) were statistically significant risk factors for liver abscess. *RmpA* (OR=0.571), K2 (OR=0.821), *aerobactin* (OR=3.632), diabetes (OR=2.263), K5 (OR=1.041) appeared to have not much difference between hvKP caused liver abscess and hvKP caused other diseases shown in **Table 3**.

Table 3. Risk Factors for hvKP causing liver abscess vs hvKP causing other disease.

Risk	Factor Univariate Analysis	
	OR (95% CI)	P Value
rmpA	0.571 (0.035–9.415)	0.695
Aerobactin	3.632 (0.759–13.787)	0.106
Capsule antigen K1	3.109 (1.338–7.222)	0.008
Capsule antigen K2	0.821 (0.325–2.077)	0.678
Capsule antigen K5	1.041 (0.234-4.630)	0.958
Fever of unknown origin	6.921 (2.503–19.136)	0
Diabetes mellitus	2.263 (0.823–6.224)	0.113
Positive string test	11.306(3.579-35.711)	0

Antimicrobial resistance among hvKP isolates: All hvKP strains were resistant to ampicillin, which is consistent with previous studies [9,11]. However, resistance to all the tested antimicrobials, except carbapenems and amikacin, was observed in a proportion of hvKP strains, none of which expressed ESBL in the 37 hvKP causing liver abscess.

DISCUSSION

It is well known that *K. pneumoniae* is a common pathogen responsible for pneumonia as well as blood and urinary tract infections, easy to form resistance to antibiotics [12,13]. Recently, life-threatening liver abscess caused by *K. pneumoniae* has been paid more and more attention, the therapy time is longer, sometime easy to attack again, significant morbidity and mortality occurs [14]. Liver abscess caused by *K. pneumonia* this mostly were highly mucoid phenotype in biological classification, has some unique capsular types and virulence factors, known as hvKP [8]. Usually the hvKP identification is based on the string test, the identification of hvKP need a certain standard [9]. More experiments are needed to establish whether a virulence factor or property present in both cKP and hvKP is equally important for their pathogenesis or whether it accounts for the increased virulence of hvKP strains compared with cKP strains. Standard need to be make clear to make a clinical laboratory diagnosis as soon as possible to help doctors diagnose as quickly as they can so that they can make correct treatment and prevent metastasis and drug resistance. This retrospective study was conducted in 240 *K. pneumoniae* culture–positive patients hospitalized during the period from May 2013 to August 2014 in General Hospital of PLA. Anyone positive of *rmpA, arobactin* and string test can be diagnosed as hvKP. The clinical and biological characteristics analysis indicated that this strain identification method can be a better method to identify hvKP. No ESBL was found in the 37 hvKP which caused liver abscess. On the other hand, the study showed that multiplex PCR to detect hvKP by virulent type and capsule type can be more quickly to make the laboratory identification of clinical specimens than traditional methods.

HvKP detection in pus specimens is higher than other specimens. Other samples

For hvKP detection from high to low in turn is ascites, sputum, blood, urine, bile. Some studies have shown that infections caused by hvKP are more invasive and serious in healthy young people [10]. This study found 37 cases of liver abscess patients aged for a minimum of 20 years, the oldest was 79 years, the average age is 55 years; 18 of 37 cases were female, 19 were male,

the data showed age and gender have less correlation with liver abscess. 13 of 37 patients with liver abscess at first diagnosed as unknown origin fever, 11 were diabetes and 7 were tumour patients and 4 patients with postoperative infection or other site infection. The 13 patients with no other concurrent disease diagnosed as unknown origin fever finally was identified as liver abscess. As a result, liver abscess can be independently caused by hvKP and indicated that patients with diabetes mellitus is a relatively high risk factor, this is consistent with the majority of research [13,15].

Genes that encode a number of virulence factors, including those that are responsible for the hypermucoviscous phenotype (*rmpA*) and iron-acquisition factors (*aerobactin*). Among the 37 hvKP caused liver abscess, positive string test were 31, accounted for 83.8% (31/37); *rmpA* positive strains accounting for 94.6% (35/37); *aerobactin* positive strains accounting for 86.5% (32/37). It is clear that not all hvKP are hypermucoviscous. The experiments showed that the combination of *rmpA, arobactin*, string test can be a better method to identify hvKP infection compared to just depend on string test. Once some specimens were laboratory diagnosed as hvKP, clinicians could make timely treatment to prevent further spread of infection.

37 strains of *K.pneumoniae* caused liver abscess were all hvKP, 21 were K1, accounting for 56.8% (21/37); 10 were K2, accounting for 29.7% (11/37); 4 were K5, accounting for 10.8% (4/37); 1 is K54, accounting for 2.7% (1/37) and 1 was not the type to be detected. 35 strains expressed *rmpA*, accounting for 94.6% (35/37), among the 37 strains of hvKP, *rmpA* all were expressed in 21 K1. These data are consistent with previous reports [16-18]. Single factor regression analysis showed that K1, string test positive and unexplained fever is the risk factors to liver abscess compared to hvKP causing other disease.

Multiple PCR detection results were consistent with single PCR. Then the detection of hvKP can be quicker by using multiple PCR and can be used as a rapid method to identify hvKP in clinical laboratory.

Compounding an already challenging clinical situation is the recent propensity for cKP to become multi-, extreme or pan-drug-resistant, including the acquisition of extended-spectrum β-lactamases and carbapenemases, such as the recently described NDM-1[19]. To date, most strains of hvKP have been very susceptible to antimicrobials except ampicillin [20]. Nonetheless, some cases of infection due to MDR-hvKP have already been described [8,21]. The confluence of hypervirulence and extreme or pan-drug resistance in hvKP has the potential to create a “post-antibiotic” scenario. In this study, we compared the characteristics of drug resistance of isolated hvKP. In the present study of the 14-19 drug resistance tests, the acquisition of antimicrobial resistance to some drugs was existed, but there was no ESBL resistant strain existed in the 37 hvKP causing liver abscess.

In conclusion, the research of hvKP is susceptible to most antibiotics, the string test and multiplex PCR to virulence genes, capsular phenotype can quickly make the rapid identification of hcKP. This will help to make clinical diagnosis quickly and avoid the further development of the disease and reduce drug-resistant strains.

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