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The Determination and Correlation of Various Virulence Genes, ESBL, Serum Bactericidal Effect and Biofilm Formation of Clinical Isolated Classical *Klebsiella pneumoniae* and Hypervirulent *Klebsiella pneumoniae* from Respiratory Tract Infected Patients

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Abstract

Klebsiella pneumoniae strains that are commonly recognized by clinicians and microbiologists are termed as classical *K. pneumoniae* (c*KP*). A strain with capsule-associated mucopolysaccharide web is known as hypervirulent *K. pneumoniae* (hv*KP*) as it enhances the serum resistant and biofilm production. Aim is to determine and correlate various virulence genes, ESBL, serum bactericidal effect and biofilm formation of clinical isolated c*KP* and hv*KP* from respiratory tract infected patients. A total of 96 *K. pneumoniae* strains were isolated from sputum of respiratory tract infected patients. The isolates were performed string test, AST, ESBL virulence gene, serum bactericidal and biofilm assays. Out of 96 isolates, 39 isolates (40.6%) were identified with hypervirulent phenotypes. The number of c*KP* exhibiting resistance to the tested antimicrobials and ESBLs were significantly higher than that of the hv*KP* strains. The virulence genes of *K. pneumoniae* such as K1, K2, *rmp*A, *uge*, *kfu* and aerobactin were strongly associated with hv*KP* than c*KP*. However, no significant difference was found in FIM-1 and MrKD3 genes. ESBL producing c*KP* and hv*KP* were significantly associated with strong biofilm formation (both $P < 0.05$) and highly associated with bactericidal effect of serum (both $P < 0.05$) than c*KP* strains. However, neither biofilm formation nor bactericidal effect of serum was found with significant difference in between ESBL producing c*KP* and ESBL producing hv*KP* strains (both $P > 0.05$). Although the hv*KP* possess more virulence gene, but they didn't show any significant difference between biofilm formation and bactericidal effect of serum compared with ESBL producing c*KP* strains.

Key words: biofilm, ESBL, classical *K. pneumoniae vs* hypervirulent *K. pneumoniae*, serum resistance, virulence genes

Introduction

Klebsiella pneumoniae is an opportunistic pathogen of the *Enterobacteriaceae* family and principally causes pneumonia (Podschun and Ullmann, 1998), and also is associated with pyogenic liver abscesses over the past decade (Wang *et al*., 1998) and it has been implicated in 7–12% of hospital-acquired pneumonia in ICUs in the United States (1997). It typically expresses different virulence factor genes such as a smooth lipopolysaccharide (O antigen) and capsule polysaccharide (K antigen) on its surface (Kenne *et al*., 1983). There are at least 77 capsular serotypes defined, and serotype-related variation in the infection severity has been observed. Out of 77 capsular serotypes (K), the strains with capsular serotypes K1 and K2 have been identified as the predominant virulent strains, and their virulence has been confirmed in mouse models (Fung *et al*., 2002). The *rmp*A is a transcriptional activator of capsular polysaccharide (CPS) gene transcription, CPS synthesis and HV in *K. pneumoniae* K1/K2 (Lai *et al*., 2003). The *uge* gene encodes uridine diphosphate galacturonate 4-epimerase which expresses both smooth lipopolysaccharide with O antigen molecules and CPS with K antigen on the surface. Aerobactin is a siderophore that aids the bacterium in its competition with the host for iron (Quinn, 1994). The *kfu* gene encodes for an iron uptake system which is a significantly associated with the purulent tissue infections and HV phenotype (Ma *et al*., 2005). The *fim*H (or *fim*H-1) and *mrk*D genes which are relevant to type 1 and type 3 fimbriae respectively are responsible for attachment to host cells (Podschun and Ullmann, 1998). These all factors contribute to virulence and are important for colonization, invasion and pathogenicity.

K. pneumoniae strains usually recognized by microbiologists and clinicians are termed as c*KP*. Such strains are scandalous for their capability to cause acquired

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hospital infections and acquire multidrug resistant especially extended-spectrum beta lactamase (ESBL) that has led the treatment to limited options (Ko *et al*., 2002; Podschun and Ullmann, 1998). ESBLs are plasmid mediated enzymes and it inactivate β-lactam antibiotics such as oxyimino-cephalosporins and oxyimino-monobactam, except cephamycins and carbapenems (Paterson and Bonomo, 2005) and it is inhibited by clavulanic acid and placed it under Bush's functional class 2be (Bush *et al*., 1995). Till the date more than 200 different types of ESBLs have been found. There is a increase in the prevalence of TEM, SHV and CTX-M type of ESBLs among the *Enterobacteriaceae* in Europe and Asia (Bonnet, 2004). A new variant of *K. pneumoniae*, designated as HV *K. pneumoniae* due to the high production of mucopolysaccharide was first described in 1986 by a Taiwanese doctors. The HV phenotype is also known as hv*KP*, and it enhances the biofilm production and resistance to serum bactericidal activity. Biofilm is a complex polymer matrix composed of cells and matrix materials. The serum bactericidal activity is mediated by the complement proteins through the complement pathway. The complement pathways lead, *via* the activation of C3, for the formation of the opsonin C3b, which finally results in the formation of the terminal C5b-C9 complex and thus plays a key role in this defense system (Tomas *et al*., 1986). Many studies identified that hv*KP* strains produced more biofilm and are less susceptible to human serum than c*KP* strains (Li *et al*., 2014; Wu *et al*., 2011). Some studies have addressed that ESBL producing strain formed heavy biofilm than non-ESBL producing strains of *K. pneumoniae* (Yang and Zhang, 2008) and recently another study have been shown that the serum-resistant strains are significantly more general among ESBL-producing *K. pneumoniae* strains than among non-ESBL producers (Sahly *et al*., 2004). In view of previous findings, the goal of this study is to identify and correlate the various virulence genes, biofilm formation, bactericidal effect of serum and ESBL in between hv*KP* strain and c*KP* strain. To the best of our knowledge, the combine study of virulence genes, biofilm formation, bactericidal effect of serum and ESBL in between hv*KP* strain and c*KP* strain have not been previously reported.

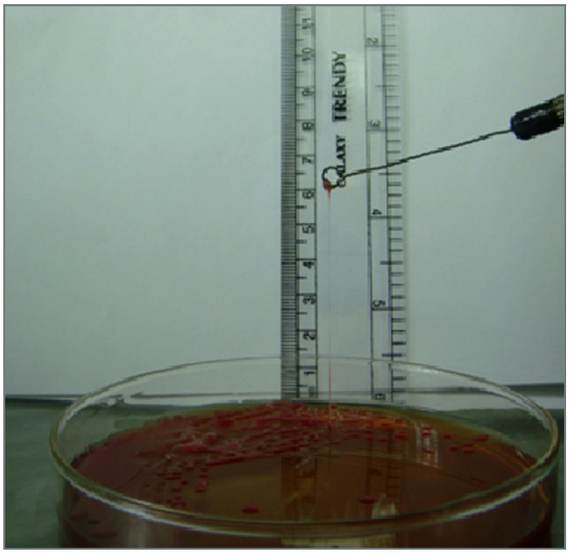

Fig. 1. Positive string test (Mucoviscous string > 5 mm on Agar plate).

Experimental

Materials and Methods

Bacterial strains and HV testing. A total of 96 *K. pneumoniae* were isolated from sputum of pneumonic patients and were collected from a period of March 2013 to October 2014 in Shenyang Hospital of Liaoning Province and Tongliao Hospital of Nei Menggu province in China. *K. pneumoniae* strains were isolated from sputum that were identified and confirmed by standard methods (Farmer, 2003).

HV testing was done by string test. The string test was performed to distinguish hv*KP* from c*KP* strains. The string test was defined as the positive when the formation of a mucoviscous string of > 5 mm was observed, by using a bacteriology inoculation loop to stretch a colony that was grown overnight on an agar plate at 37°C. A positive string test with *K. pneumoniae* strains were designated as hv*KP* in Fig. 1 (Fang *et al*., 2004).

Antimicrobial susceptibility testing and ESBLs detection. Susceptibility testing for the 96 *K. pneumoniae* strains was carried out with disc diffusion methods. The control strains were used as *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, and *Escherichia coli* ATCC 25922. The interpretation of results were recorded according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2015). Antimicrobial agents tested included ampicillin, piperacillin, cefazolin, cefuroxime, cefoxitine, cefoperazon, ceftriaxone, cefotaxime, ceftazidime, cefepime, meropenem, imipenem, aztreonam, amikacin, gentamicin, trimethoprim-sulfamethoxazole, and ciprofloxacin. These all antibiotics were purchased from Oxoid company, UK.

The combination-disk synergy tests using ceftazidime (30 μg) ± clavulanic acid (10 μg) and cefotaxime (30 μg) ± clavulanic acid (10 μg) were performed to detect the phenotype of ESBLs for all the collected isolates. The phenotype of ESBLs was confirmed by 5 mm or more increased zone diameter for the combination of clavulanic acid with either cefotaxime or ceftazidime versus its zone when tested alone. The ESBL negative

and positive strains were used as *E. coli* (ATCC 25922) and *K. pneumoniae* (ATCC 700603) respectively.

PCR for amplification of ESBL and virulence associated genes. The template DNA was prepared from bacterial colonies. The colonies of bacteria were picked and suspended in 100 µl of mili-Q water. The suspensions of sample were boiled for 15 min and rapid cool at –20°C for 5 min. The bacterial cell debris was separated by centrifugation for 10 min at 15,000 rpm and the supernatant was collected and used as a template DNA. The DNA concentration was measured by using Epoch Gen5 CHS 2.01. The PCR specific primers and cycling condition used to detect the target gene are shown in Table I and II respectively. The PCR product from each of the detected genes was sequenced and compared with *K. pneumonie* DNA sequences on BLAST of NCBI (http://blast.ncbi.nlm.nih.gov/). Each of the target gene sequenced showed a high level of identification (> 98% homology) with the published target sequence (Gen Bank accession number: KF77780.1 for *mrk*D; FJ483592.1 for *fim*H-1; AB355924.1 for *mag*A (K1); AB362367.1 for *wzy* (K2); X17518.1 for

Table I
Primers used to detect the target gene sequences.

Target gene	Sequences of Primer (5'-3')	Size of amplified product (bp)
FimH-1	F: ATG AAC GCC TGG TCC TTT GC R: GCT GAA CGC CTA TCC CCT GC	**688**
*mrk*D	F: CCA CCA ACT ATT CCC TCG AA R: ATG GAA CCC ACA TCG ACA TT	240
*mag*A (K1)	F: GGT GCT CTT TAC ATC ATT GC R: GCA ATG GCC ATT TGC GTT AG	**1 282**
wzy (K2)	F: GAC CCG ATA TTC ATA CTT GAC AGA G R: CCT GAA GTA AAA TCG TAA ATA GAT GGC	**641**
*rmp*A	F: ACT GGG CTA CCT CTG CTT CA R: CTT GCA TGA GCC ATC TTT CA	**535**
uge	F: TCT TCA CGC CTT CCT TCA CT R: GAT CAT CCG GTC TCC CTG TA	**534**
kfu	F: GAA GTG ACG CTG TTT CTG GC R: TTT CGT GTG GCC AGT GAC TC	**797**
aerobactin	F: GCA TAG GCG GAT ACG AAC AT R: CAC AGG GCA ATT GCT TAC C T	**556**
TEM	F: ATA AAA TTC TTG AAG ACG AAA R: GAC AGT TAC CAA TGC TTA ATC	**1 080**
SHV	F: GGG TTA TTC TTA TTT GTC GC R: TTA GCG TTG CCA GTG CTC	**930**
CTX-M	F: SCS ATG TGC AGY ACC AGT AA R: ACC AGA AYV AGC GGB GC	**585**

Table II
Cycling condition for PCR.

Target gene	Cycling Conditions					
FimH-1	95°C 4 min	95°C 1 min	57°C 1 min	72°C 1 min	72°C 10 min	Repeated for 35 cycles
*mrk*D	95°C 4 min	95°C 45 sec	55°C 45 sec	72°C 45 sec	72°C 7 min	Repeated for 30 cycles
*mag*A (K1)	95°C 4 min	95°C 45 sec	59°C 45 sec	72°C 2 min	72°C 9 min	Repeated for 30 cycles
wzy (K2)	95°C 4 min	95°C 45 sec	63°C 45 sec	72°C 1 min	72°C 9 min	Repeated for 35 cycles
*rmp*A	95°C 4 min	95°C 45 sec	52°C 45 sec	72°C 1 min	72°C 7 min	Repeated for 30 cycles
uge	95°C 4 min	95°C 45 sec	55°C 45 sec	72°C 1 min	72°C 7 min	Repeated for 30 cycles
kfu	95°C 3 min	95°C 45 sec	59°C 45 sec	72°C 1 min	72°C 7 min	Repeated for 35 cycles
aerobactin	95°C 5 min	95°C 1 min	54°C 1 min	72°C 1 min	72°C 7 min	Repeated for 35 cycles
SHV	95°C 4 min	95°C 45 sec	56°C 45 sec	72°C 1 min	72°C 7 min	Repeated for 35 cycles
TEM	94°C 4 min	94°C 45 sec	55°C 45 sec	72°C 1 min	72°C 7 min	Repeated for 35 cycles
CTX-M	95°C 4 min	95°C 45 sec	58°C 45 sec	72°C 1min	72°C 7 min	Repeated for 35 cycles

*rmp*A; 633804.1 for *uge*; KJ633800.1 for *kfu*; X98099.1 for SHV, HM131427.1 for TEM; HQ214044.1 for CTX-M). These genes were chosen as the positive control for the consequent PCR experiments.

Serum bactericidal assays. Normal human serum was obtained from the healthy adult volunteers. The sera were stored as aliquots at –70°C. *K. pneumonia* strains were determined by an established method (Podschun *et al*., 1993). An inoculum of 2.5×10^4 CFU, prepared from the mid-log phase, was mixed at a 1:3 vol/vol ratio with normal human serum. The final mixture, comprising 75% serum by volume, was incubated at 37°C for 3 hours, and 100 µl from each well was taken out for CFU determination before and after incubation at 37°C. The rate of survival was expressed as the number of viable bacteria treated with serum compared to the number of pretreatment. The assay was performed in triplicate and repeated three times.

Interpretation of results: following the criteria used by Benge (1988). The isolates were regarded as serum sensitive if at 3 h the viable counts were reduced to < 1% of the initial counts and resistant if > 90% of the organisms were still viable. Isolates having survival rates of 1–90% were regarded as intermediate sensitive.

Biofilm formation assays. Microtiter plate method was carried out according to Stepanovic *et al.* (2007) with a few modifications. Briefly, *K. pneumoniae* strains were grown overnight at 37°C in Mueller Hinton broth. The culture was adjusted to 0.5 McFarland then diluted 1:100 in the Mueller Hinton broth. Three wells of a sterile 48-well plastic tissue culture plate with a lid were filled with 1000 µl of diluted bacterial culture each. The negative control wells contained MH broth only. The plates were covered and incubated at 37°C for 24 h. The content of each well was aspirated, and was washed three times with normal saline (to remove free-floating "planktonic" bacteria). Biofilms formed by bacteria adherent "sessile" to the wells were heat-fixed by exposing them to hot air at 60°C for 3 hours and stained with 0.5% of Crystal Violet for 15 min. Excess stain was rinsed off with running tap water and the plates were dried. To quantify biofilm biomass, the crystal violet dye bound to the adherent cells was dissolved by adding 1000 µl of 33% acetic acid solution to each well and after 10 min, the OD of each well was measured at 595 nm. Each assay was performed in triplicate and repeated at least three times.

The interpretation of biofilm formation was done according to the criteria of Stepanovic *et al.* (2007).

Interpretation of biofilm formation results

Average OD value	Biofilm production
≤ ODc / ODc < ~ ≤ 2x ODc	Non/weak
2x ODc < ~ ≤ 4x ODc	Moderate
> 4x ODc	Strong

Note: ODc = average OD of negative control + 3x standard deviation of negative control optical density cut-off value (ODc)

Statistical analysis. The statistical analyses were performed using Statistical Package for Social Science 21.0. The descriptive data were reported as mean ± SD and percentage. The normally distribution of the data was performed by Kolmogorov-Smirnov test. For the differences in the outcomes between various groups, categorical variables were compared using chi-square analysis. When the number of cases was smaller than 5, the Fisher's exact test was used. The P value < 0.05 was defined as a significance.

Ethics statement. For collection of normal human serum from healthy adult volunteers and sputum from pneumonic patients, the method and the respective consent of documents were approved by the Ethics Committee of the Norman Bethune Health Science Center, Jilin University, China. The written informed consent is provided by all volunteers.

Results

Bacterial strains and HV of *K. pneumoniae* strains. The samples were collected from March 2013 to October 2014, and a total of 96 patients were diagnosed as suffering pneumonia with the culture-positive *K. pneumoniae*. Out of them, 71 (74.0%) were males and 25 (26.0%) were females. The mean age was 64.8 ± 15.4 years. Based on the results of the modified string test HV phenotypes were identified in 39 (40.6%) of the 96 isolates. The isolation of c*KP* and hv*KP* strains were obtained as 57 (59.4%) and 39 (40.6%), respectively. A significantly higher number of patients with c*KP* (P = 0.009) was detected. Neither age nor sex was associated with positive string test (both P > 0.05).

Antimicrobial resistance and ESBL. The number of c*KP* strains exhibiting resistance to the tested antimicrobials was significantly higher than that of the hv*KP* strains, with the exception of ampicillin, piperacillin, cefuroxime, cefoxitine, cefoperazone, imopenem, meropenem and ciprofloxacin, shown in Table III. The results of the combined disk test confirmed that 37 (38.5%) isolates were ESBL-producing strains. ESBL were identified in more number of c*KP* strains (28/57 [49.1%]) than in hv*KP* strains (11/39 [28.2%]) (P = 0.040). These results indicate a significant negative association between the ESBL producer and the HV phenotype in these isolates. In addition, single and multiple types of ESBL genes were present in c*KP* strains, whereas, only single gene was present in hv*KP* strains, shown in Table IV.

Virulence genetic characteristics of *K. pneumoniae*. The pneumonia is caused by *K. pneumoniae* strains

Table III
The percentage of antimicrobial resistance of *c*KP strain and *hv*KP strain.

	*c*KP (n = 57) No. (%)	*hv*KP (n = 39) No. (%)	P – value
Ampicillin	51 (89.5)	37 (94.9)	0.347
Pipracillin	27 (47.4)	14 (35.9)	0.264
Cefazolin	33 (57.9)	10 (25.6)	0.002*
Cefuroxime	31 (54.4)	15 (38.5)	0.125
Cefoxitin	14 (24.6)	6 (15.4)	0.277
Cefoperazon	12 (21.1)	4 (10.3)	0.163
Ceftriaxone	31 (54.4)	8 (20.5)	0.001*
Cefotaxime	28 (49.1)	9 (23.1)	0.010*
Ceftazidime	23 (40.4)	7 (17.9)	0.020*
Cefepime	24 (42.1)	2 (5.1)	0.001*
Aztreonam	25 (43.9)	4 (10.4)	0.001*
Imipenem	3 (5.3)	1 (2.6)	0.644
Meropenem	4 (7.0)	0 (0)	0.144
Amikacin	18 (31.6)	2 (5.1)	0.002*
Gentamycin	26 (45.6)	10 (25.6)	0.047*
Trimethoprim-sulfamethoxazole	28 (49.1)	8 (20.5)	0 .004*
Ciprofloxacin	15 (26.3)	9 (23.1)	0.719
ESBL total	28 (49.1%)	11 (28.2%)	0 .040*

* P < 0.05 is significant

that were encoded following percentage of virulence genes as FIM-H 85 (88.5%), *mrk*D 80 (83.4%), *mag*A 22 (22.9%), K2 25 (26.0%), *rmp*A 62 (64.6%), *uge* 74 (77.0%), *kfu* 33 (34.3%), and aerobactin 63 (65.6%).

Virulence genetic characteristics of hv*KP vs* c*KP*. The prevalence of K1 and K2 gene in hv*KP* isolates was significantly increased (P = 0.024 and P = 0.039, respectively), than that in c*KP* isolates. Moreover, hv*KP* strains were strongly associated with *rmp*A (P < 0.001), than c*KP* strains. In addition, *uge*, *kfu* and aerobactin were also strongly associated with hv*KP* strains (P = 0.015, P = 0.014 and P = 0.001, respectively) than c*KP* strains. However, no significant difference was found in FIM-1 and *mrk*D3 genes (P = 0.107 and P = 0.403, respectively) in between hv*KP* and c*KP* strains .

Biofilm and serum resistance characteristics of c*KP vs* ESBL producing c*KP* strains . ESBL producing c*KP* strains were highly associated with strong biofilm formation (P < 0.001) than c*KP* strains. But no significant difference (P = 0.208) was found with moderate biofilm formation in between ESBL producing c*KP* strains and c*KP* strains. In the serum bactericidal test, similar results were found as biofilm formation that ESBL producing c*KP* strains were significantly associated with serum resistance (P < 0.001) than c*KP* strains and no significant difference was found with intermediate sensitive results (P = 0.490) in Table V.

Biofilm and serum resistance characteristics of c*KP vs* hv*KP* strains. The hv*KP* strains were significantly more increased association with moderate and strong biofilm formation (P < 0.001 and P = 0.039 respectively) than c*KP* strains. In addition, the hv*KP* strains were also more associated with intermediate sensitive and resistance of the serum bactericidal test (P = 0.002 and P = 0.004 respectively) than c*KP* strains in Table VI.

Biofilm and serum resistance characteristics of ESBL producing c*KP vs* hv*KP* strains. The hv*KP* strains were only significantly more associated with moderate biofilm formation and intermediate sensitive of serum bactericidal test (P = 0.005 and P = 0.016 respectively) than ESBL producing c*KP* strains. Whereas, no significant difference was found with strong biofilm formation (P = 0.105) and with serum resistance (P = 0.420) of serum bactericidal test in between ESBL producing c*KP* strains and hv*KP* strains, Table VII. Biofilm and serum resistance characteristics of ESBL producing c*KP vs* ESBL producing hv*KP* strains. No significant

Table IV
The distribution of types of ESBL genes in *c*KP strain and *hv*KP strain.

Types of ESBL	*c*KP (Total no. of strains = 28) No. of strains (%)	*hv*KP (Total no. of strains = 11) No. of strains (%)
TEM	1 (3.6%)	2 (18.2%)
SHV	6 (21.4%)	5 (45.4%)
CTX-M	4 (14.3%)	4 (36.3%)
TEM+SHV	1 (3.6%)	–
TEM+CTX-M	11 (39.3%)	–
SHV+CTX-M	1 (3.6%)	–
TEM+SHV+CTX-M	4 (14.3%)	–

Table V
The comparision of biofilm and serum resistance in between *c*KP and ESBL producing *c*KP strains.

	*c*KP (n = 29) No. (%)	*c*KP producing ESBL (n = 28) No. (%)	P – value
Biofilm formation: Non**	21 (72.4%)	4 (14.3%)	0.001*
Moderate	2 (6.9%)	5 (17.9%)	0.208
Strong	6 (20.7%)	19 (67.9%)	0.001*
Serum Res. test: Sensitive	21 (72.4%)	6 (21.4%)	0.001*
Intermediate sensitive	6 (20.7%)	8 (28.6%)	0.490
Resistance	2 (6.9%)	14 (50%)	0.001*

** Non and weak biofilm formation is kept in non biofilm formation result.

Table VI
The comparison of biofilm and serum resistance between *c*KP and *hv*KP strains.

	*c*KP (n = 29) No. (%)	*hv*KP (n = 28) No. (%)	P – value
Biofilm Formation: Non**	21 (72.4%)	0 (0%)	0.001*
Moderate	2 (6.9%)	15 (53.6)	0.001*
Strong	6 (20.7%)	13 (46.4%)	0.039*
Serum Res. test: Sensitive	21 (72.4%)	0 (0%)	0.001*
Intermediate sensitive	6 (20.7%)	17 (60.7%)	0.002*
Resistance	2 (6.9%)	11 (39.3%)	0.004*

** Non and weak biofilm formation was kept in non biofilm formation result.

Table VII
The comparison of biofilm and serum resistance between ESBL producing *c*KP and *hv*KP strains.

	ESBL producing *c*KP (n = 28) No. (%)	*hv*KP (n = 28) No. (%)	P – value
Biofilm Formation: Non**	4 (13.4%)	0 (0%)	0.111
Moderate	5 (17.9%)	15 (53.6%)	0.005*
Strong	19 (67.9%)	13 (46.4%)	0.105
Serum Res. Test : Sensitive	6 (21.4%)	0 (0%)	0.023*
Intermediate sensitive	8 (28.6%)	17 (60.7%)	0.016*
Resistance	14 (50%)	11 (39.3%)	0.420

** Non and weak biofilm formation is kept in non biofilm formation result.

Table VIII
The comparison of biofilm and serum resistance between ESBL producing *c*KP and ESBL producing *hv*KP strains.

	ESBL producing cKP (n = 28) No. (%)	ESBL producing hvKP (n = 11) No. (%)	P – value
Biofilm formation: Non**	4 (14.3%)	0 (0%)	0.309
Moderate	5 (17.9%)	5 (45.5%)	0.080
Strong	19 (67.9%)	6 (54.5%)	0.435
Serum Res. test: Sensitive	6 (21.4%)	0 (0%)	0.158
Intermediate Sensitive	8 (28.6%)	6 (54.5%)	0.128
Resistance	14 (50%)	5 (45.5%)	0.798

** Non and weak biofilm formation is kept in non biofilm formation result.

difference was found in either biofilm formation or bactericidal effect of serum in between ESBL producing c*KP* and ESBL producing hv*KP* strains (both P > 0.05) in Table VIII.

Discussion

Although *K. pneumoniae* is known to be a common pathogen responsible for community and hospital-acquired pneumonia as well as blood and urinary tract infections (Lin *et al*., 2010; Podschun and Ullmann, 1998). Our data demonstrated a negative association between pneumonia and hv*KP* (P = 0.009). This implies that c*KP* strains predominantly are associated with respiratory infections. These data are consistent with previous reports (Li *et al*., 2014). In this study, we compared the drug resistance and ESBL characteristics of hv*KP* and c*KP* isolates. Previous studies have indicated that hv*KP* strains are stronger resistant to antibiotics than c*KP* strains, whereas more recent studies have not only indicated that such strains are less associated with antibiotic resistance (Li *et al*., 2014), but also shown that ESBL were significantly lesser than c*KP* strains (Su *et al*., 2008). Consequently, these data are inconclusive. In the present study, hv*KP* strains were shown less resistant than c*KP* strains, for 10 out of 17 antimicrobial drugs tested. Moreover, ESBL were shown a negative association with hv*KP* (P = 0.40). In addition, the c*KP* strains possessed one to three types of ESBL gene but hv*KP* strains have only one type. The reason for this difference remains unknown. It can be speculated that hv*KP* strains cannot acquire resistance-related plasmids, or that some drug-resistant genes are lost when they become hypervirulent (Li *et al*., 2014). Virulence genetic characteristics of hv*KP vs* c*KP* show That the hv*KP* was more virulent in a model of subcutaneous abscess of rat than c*KP* (Pomakova *et al*., 2012). Serotype K1 and K2 of *K. pneumoniae* cause pyogenic liver abscess and is also repeatedly reported in community acquired pneumonia (Decre *et al*., 2011). The *kfu* which mediates uptake of ferric iron, is more common in hv*KP* compared with c*KP* strains and was shown to be a virulence factor in mice after IG (intragestinal) but not after IP (intraperitonium) (Ko *et al*., 2002). Aerobactin production was more common in hv*KP* strains than c*KP* strains, which was demonstrated by a cross-feeding assay (Yu *et al*., 2007). This analysis suggested that the hv*KP* strains might have the capability to acquire iron more readily than the c*KP* strains.

In this study, we found that the prevalence of virulence associated genes viz., K1, K2, *rmp*A, *uge*, *kfu* and aerobactin were strongly associated with hv*KP* than c*KP* strains. There was no any significant difference of FIM-1, *mrk*D genes between both. These data are consistent with previous reports, which describe an association between K1 and K2 expression and associated with rmpA in between hv*KP* and c*KP* strains (Li *et al*., 2014). Also an agreement with some another studies suggested that the K1 and K2 capsular genes are common in hv*KP* strains, c*KP* strains may also pos-

sess these genes (Brisse *et al*., 2009) and hv*KP* strains may have a non-K1/K2 genes (Fang *et al*., 2007). Some studies have considered the sources of the specimen and found that the proportion of ESBL-producing strains in the isolates from sputum and urine except blood and wound was significantly higher in biofilm-forming strains than in non-biofilm-forming strains (Watnick and Kolter, 2000; Yang and Zhang, 2008). In the present study, we showed that the ESBL producing isolated strains had a more ability to form biofilm in comparison with non ESBL producing strains in the source of sputum specimen. It may be due to: (i) the biofilm is a multispecies microbial community and these species can share at a high rate of their genetic material (ii) the ESBLs can be induced by low concentration of antibiotic, which is a cause of decrease penetration into biofilm (Wacharotayankun *et al*., 1993; Yang and Zhang, 2008). Another recent study identified that the hv*KP* strain produces higher biofilm than c*KP* strains (Wacharotayankun *et al*., 1993). Even our study showed similar correlation between hv*KP strain* and c*KP strain*, and it has been suggested that this ability increases colonization. Studies have shown that serum-resistant strains are significantly more increased among ESBL-producing *K. pneumoniae* strains than among non-ESBL producer strains (Sahly *et al*., 2004) and hv*KP* strains are more resistant to serum than compared with c*KP* strains (Wacharotayankun *et al*., 1993). We investigated a relationship between ESBL production and the serum resistance of hv*KP* strains and c*KP* strains. The ESBL-producing *K. pneumoniae* strains showed significantly more resistant to the serum bactericidal effect than their non-ESBL-producing strains. Moreover, hv*KP* strains were shown significantly high resistant to the serum bactericidal effect than compared with c*KP* strains. However, no any significant difference was found in between ESBL producing c*KP* strains and ESBL producing hv*KP* strains. It has been suggested that (I) the production of R-plasmid-coded ESBLs are increased when associated with adhesive (Darfeuille-Michaud *et al*., 1992), (ii) and the increase production of extra-capsular polysaccharide of hv*KP* strains (Wacharotayankun *et al*., 1993).

Conclusion

The hv*KP* strains produce increased biofilm, less susceptible to serum and possess more virulence gene compared with c*KP* strains. Whereas, the ESBL producing c*KP* strains didn't show any significant difference between biofilm formation and bactericidal effect of serum compared with hv*KP* strains and ESBL producing hv*KP* strains. Thus hv*KP* strains and ESBL producing c*KP* strains are highly pathogenic to compare c*KP* strains.

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Conflicts of interests

We declare that we do not have conflicts of interest

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