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Molecular Analysis of a Prolonged Spread of *Klebsiella pneumoniae* Co-producing DHA-1 and SHV-12 β-Lactamases

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The study investigated molecular mechanisms for prolonged nosocomial spread of multidrug-resistant *Klebsiella pneumoniae* co-producing plasmid-mediated AmpC β-lactamase DHA-1 and extended-spectrum β-lactamase SHV-12. Forty-eight clinical isolates of *K. pneumonia,* resistant to the extended-spectrum cephalosporins, were collected in a 750-bed university hospital over a year. The isolates were characterized for PCR-based β-lactamase genotypes, isoelectric focusing and pulsed-field gel electrophoresis (PFGE) profiles. Resistance transfer was performed by plasmid conjugation and confirmed by a duplex-PCR and Southern hybridization. On β-lactamase typing, the strains producing only the DHA-1 enzyme (n=17) or co-producing DHA-1 and SHV-12 enzymes (n=15) were predominant. Judging from a one year-distribution of PFGE profiles, the co-producer was spread primarily with single clonal expansion of the PFGE-type A with subtypes (n=14), whereas the strains producing only DHA-1 enzyme were spread simultaneously with the PFGE-type A (n=11) and other PFGE types (n=6). Transconjugants of the co-producers were confirmed to harbor either both *bla*$_{DHA-1}$ and *bla*$_{SHV-12}$ or only the *bla*$_{DHA-1}$. In conclusion, this study indicated that the persistent nosocomial spread of multidrug-resistant *K. pneumoniae* strains was primarily associated with expansion of a clone harboring both the *bla*$_{DHA-1}$ and *bla*$_{SHV-12}$ or the *bla*$_{DHA-1}$ only, and to a lesser extent with the horizontal transfer of the resistant plasmids. Our observations have clinical implication for the control and prevention of nosocomial dissemination of multidrug-resistant *K. pneumoniae* strains.

***Keywords*:** *Klebsiella pneumoniae*, spread, plasmid, DHA-1, SHV-12

Resistance to extended-spectrum β-lactam antibiotics is becoming an ever-increasing, worldwide problem with regard to Gram-negative pathogens since it was first described in *Klebsiella penumoniae* in 1983 (Knothe *et al.*, 1983). Instances of extended-spectrum β-lactamase (ESBL)-producing *K. pneumoniae* and *Escherichia coli* have been increasing in Korea (Kim *et al.*, 2005; Ryoo *et al.*, 2005). Identification of TEM-52, SHV-2a, SHV-12, and CTX-M β-lactamases has been commonly reported (Pai *et al.*, 1999; Jeong *et al.*, 2004; Ryoo *et al.*, 2005). In addition, the emergence and spread of plasmid-ediated AmpC β-lactamases (PABLs) such as DHA-1, CMY-1, and CMY-2 among *K. pneumoniae* and *E. coli* isolates have been reported in Korean hospitals since 2003 (Yum *et al.*, 2005; Lee *et al.*, 2006; Song *et al.*, 2006a). AmpC β-lactamases confer resistance to both cephamycins and oxyimino-β-lactams and are resistant to inhibition by clavulanic acid. They are inducible and can be expressed at high levels in some bacterial infections during antibiotic therapy (Jacoby, 2009).

Multidrug-resistant *K. pneumoniae* is an important nosocomial pathogen that causes severe mortality and morbidity in critical patients. Several outbreaks of infection caused by such strains producing novel plasmid-mediated β-lactamases have been widely reported (Komatsu *et al.*, 2001; Brinas *et al.*, 2004; Mamlouk *et al.*, 2006; Damjanova *et al.*, 2007). More recently, studies have reported the emergence and spread of *K. pneumoniae* strains producing multiple plasmid-mediated β-lactamases (Moland *et al.*, 2007; Corvec *et al.*, 2009; Shi *et al.*, 2009). Dissemination of the PABL and ESBL co-producers is of particular concern, as only a couple of antimicrobial agents including carbapenems remain as effective drugs. Two outbreaks caused by *K. pneumoniae* strains co-producing AmpC β-lactamase DHA-1 and an ESBL have already been reported (Song *et al.*, 2006b; Roh *et al.*, 2008). However, molecular studies on the prolonged nosocomial spread of *K. pneumoniae* coproducing PABLs and ESBLs are still limited.

The aim of this study was to investigate molecular mechanisms for the prolonged spread of extended-spectrum cephalosporin-resistant *K. pneumoniae* (ESC-R-KPN) over the period of a year, especially focusing on the isolates co-producing DHA-1 and SHV-12 β-lactamases.

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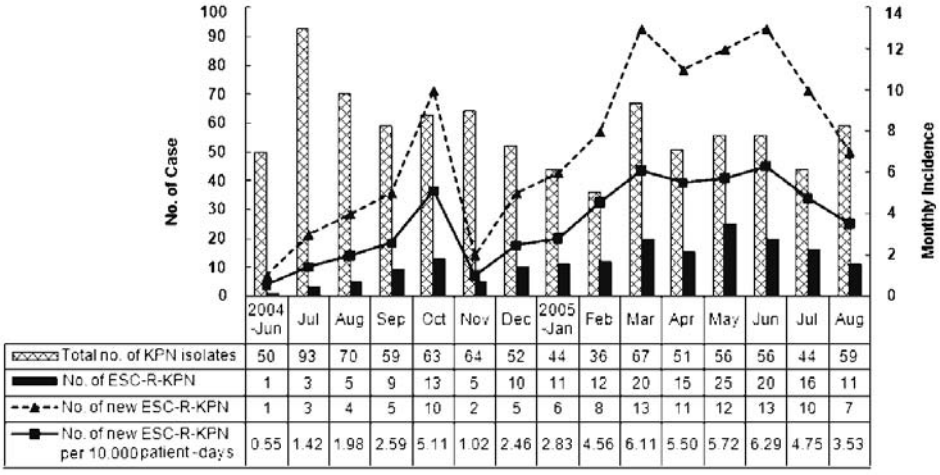

	2004 -Jun	Jul	Aug	Sep	Oct	Nov	Dec	2005 -Jan	Feb	Mar	Apr	May	Jun	Jul	Aug
Total no. of KPN isolates	50	93	70	59	63	64	52	44	36	67	51	56	56	44	59
No. of ESC-R-KPN	1	3	5	9	13	5	10	11	12	20	15	25	20	16	11
No. of new ESC-R-KPN	1	3	4	5	10	2	5	6	8	13	11	12	13	10	7
No. of new ESC-R-KPN per 10,000 patient-days	0.55	1.42	1.98	2.59	5.11	1.02	2.46	2.83	4.56	6.11	5.50	5.72	6.29	4.75	3.53

Fig. 1. Monthly incidence of ESC-R-KPN clinical isolates (one isolate per patient) during the study period.

Materials and Methods

Hospital setting

This study was conducted at a 750-bed academic tertiary care hospital in Seoul, Korea. The hospital has 50-bed medical and surgical intensive care units (ICUs). The hospital runs a monthly microbial surveillance system and a computerized antibiotic prescription and approval system.

Bacterial isolates and patients

A total of 59 ESC-R-KPN isolated strains were collected during the study period from August 2004 through July 2005. Forty-eight isolates from 43 hospitalized patients were analyzed for the study. One or more isolates from each patient were included if different β-lactamase types or pulsed-field gel electrophoresis (PFGE) profiles were obtained.

The Vitek 1 system using the GNS-120 card (bioMérieux Inc., USA) was used in accordance with the guidelines of the manufacturer for ESBL detection and antibiotic susceptibilities. The interpretation of the results was based on the reduction of growth due to cefotaxime/clavulanic acid or ceftazidime/clavulanic acid compared to either cefotaxime or ceftazidime alone. The results were reported as EBSL-positive or ESBL-negative. MICs were evaluated for the 48 ESBL-producing *K. pneumoniae* strains according to the new breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI, 2010). Demographic and clinical data were collected from the patients' medical records, including the potential risk factors for the acquisition of ESC-R-KPN.

Isoelectric focusing

Isoelectric focusing (IEF) of sonicated extracts from bacterial cells was performed by the method of Mathew *et al.* (1975) using a Mini IEF cell system (Bio-Rad, USA). An inhibition assay was performed by overlaying the gels with 0.5 mM nitrocefin, with or without 0.3 mM cloxacillin or 0.3 mM CA in 0.1 M phosphate buffer, pH 7.0, as described previously (Pai *et al.*, 2004). Strains carrying plasmids encoding the β-lactamases DHA-1, CMY-2, CMY-1, TEM, SHV, ACT-1, and CTX-M-14 served as IEF standards.

Table 1. Characteristics and risk factors in 43 patients with ESC-R-KPN

Characteristics	ESC-R-KPN n=43 (%)
Age (mean±SD, years)	63.01±13.79 (range, 1-89; median, 69)
Male (%)	24 (57.8)
Underlying disease	
Neurologic disease	18 (41.9)
Cardiovascular disease	13 (30.2)
Diabetes	12 (27.9)
Renal failure	9 (20.9)
Others[a]	10 (23.3)
Clinical infection	24 (55.8)
Pneumonia	9 (20.9)
Urinary tract infection	7 (16.3)
Skin and skin structure infection	3 (7.0)
Intra-abdominal infection	4 (9.3)
Musculoskeletal infection	1 (2.3)
Hospital stay, days	75.02±73.3 (range, 3-874; median, 34)
ICU stay, days	32.6±18.8 (range, 0-159; median, 4)
In hospital mortality	33 (76.7)
Surgery	7 (16.3)
Utilization of device	
Central catheter	5 (11.6)
Nasogastric tube	14 (32.6)
Foley catheter	22 (51.2)
Endotracheal tube	1 (2.3)
Antibiotics exposure[b]	27 (62.8)
Quinolones	13 (30.2)
3rd cephalosporins	6 (14.0)
Aminoglycosides	8 (18.6)
Glycopeptides	10 (23.3)
Carbapenems	9 (20.9)

[a] Pulmonary, hepatobiliary, gastrointestinal, and genitourinary diseases.
[b] Antibiotic exposure two weeks prior to isolation of the ESC-R-KPN.
ESC-R-KPN, extended-spectrum cephalosporin-resistant *K. pneumoniae*; ICU, intensive care unit.

Polymerase chain reaction for the resistance genes

The DHA-1-, TEM-, SHV-, ACT-1, and CTX-M-14-related genes were amplified from the clinical isolates by polymerase chain reaction (PCR), as described previously (Pai *et al.*, 2004).

Conjugal transfer

Plasmid was extracted from *K. pneumoniae* isolates by rapid alkaline lysis and visualized on a 0.7% agarose electrophoresis gel. Plasmid conjugative transfer was performed by the agar mating method using sodium azide-resistant *E. coli* J53. For the clinical isolates with DHA-1 or the DHA-1 and SHV-12 co-producer, transconjugants were selected on a Miller Hinton agar plate, supplemented with sodium azide (100 mg/L) and cefoxitin disc (30 μg) (Oxoid, UK). *E. coli* transconjugants were confirmed by API 20 E testing (bioMérieux, France), followed by a duplex-PCR with the bla_{DHA-1}- and bla_{SHV}-allele-specific primers and Southern hybridization with two specific probes (DHA-1, 5′-TGCGTCTGTATGCAAACAGCAG-3′-DIG; SHV, 5′-GCCGCCATTACCATGAGCGATA-3′-DIG).

Pulsed-field gel electrophoresis

Genomic DNA from clinical isolates was digested with *Xba*I (New England Biolabs, UK) and separated using the GeneNavigator system (Amersham Biosciences Ltd., Sweden) using 130 V at 16°C; 5 sec pulse time for 4 h, 25 sec pulse time for 6 h, 45 sec pulse time for 20 h and 75 sec pulse time for 6 h. Banding patterns were analyzed using the Molecular Imager Gel Doc XR system (Bio-Rad) and Quantity One analysis software version 4.6.1 (Bio-Rad). The unweighted pair group method, using arithmetic averages (UPGMA), was used to construct a dendrogram. Band patterns of the isolates were compared using criteria for bacterial strain typing (Tenover *et al.*, 1995).

Results

Descriptive epidemiology for the spread of the ESC-R-KPN

The hospital microbial surveillance system first noticed an increase in ESC-R-KPN isolates on July 2004. The monthly incidence of new ESC-R-KPN was 4.08±1.80 cases per 10,000 patient-days during the study period from August 2004 to July 2005 (Fig. 1). The 48 isolates (43 patients) characterized at the molecular levels were collected most commonly from urine (n=22), followed by sputum (n=10) and pus (n=5). Clinical characteristics and risk factors were summarized in Table 1. Longer hospitalization and ICU stay (>2 weeks) occurred for 30 and 15 patients, respectively. More than half of the patients were exposed to antibiotics before the isolation of the strains. High hospital mortality reflected the critical status of the patients.

β-Lactamase typing, PFGE typing and antibiotic susceptibility

Results of β-lactamase typing, PFGE typing and antibiotic susceptibility patterns are summarized in Table 2. All of the 48 strains had one to three β-lactamases in various combinations: The identified ESBL genotypes included SHV-12 (pI

Table 2. β-Lactamase types, PFGE types and antibiotic susceptibility patterns of 48 isolates of ESC-R-KPN

No. of isolate	Specimen	PFGE type(n)	Type of enzyme	pI value	MIC ranges, mg/L (no. of the susceptible isolate)[b]								
					ctx	fox	caz	fep	cip	gm	atm	ipm	tzp
n=15	urine(9) wound(3) sputum(2)	A0(2),A1(5), A2(1),A4(1), A5(1),A6(2), A7(1),A11(1), B0(1)	DHA-1, SHV-12	7.8 8.0	≤1- ≥64 (1)	4- ≥64 (2)	≥8 (0)	≤1-16 (14)	1- ≥4 (1)	≤1- ≥16 (4)	8- ≥32 (0)	≤0.5- 4 (12)	≤4- ≥128 (12)
n=17	urine(6) sputum(6) others[a] (5)	A0(4),A4(1), A6(2),A7(1), A8(1),A10(1), A12(1),C(1), E(1),G(1), H(1),I(1),K(1)	DHA-1	7.8	≤1- 64 (1)	16-≥64 (0)	≥8 (0)	≤1 (17)	≤0.25-≥4 (6)	≤1-≥16 (8)	16-≥32 (0)	≤0.5-16 (16)	≤4- ≥128 (8)
n=4	urine(3) sputum(1)	A0(1),A1(1), A3(1),A9(1)	DHA-1, CTX-M-14 pI 7.6	7.8 8.0 7.6	2- ≥64 (0)	4- ≥64 (1)	≥8 (0)	≤1-32 (3)	≥4 (0)	2- ≥16 (2)	≥32 (0)	≤0.5-4 (3)	8- 64 (2)
n=4	urine(2) bile(2)	A6(1),B0(1), B1(1),E(1)	SHV-12, pI 7.6	8.2 7.6	4- ≥64 (0)	≤2- ≥64 (2)	≥8 (0)	≤1-≥64 (3)	≤0.5- ≥4 (1)	4- ≥16 (1)	≥32 (0)	≤1- 4 (3)	≤4- ≥128 (3)
n=3	urine(1) sputum(1) pus(1)	A1(1),A9(2)	SHV-2a	7.6	4- ≥64 (0)	4- ≥64 (1)	≥8 (0)	≤1-32 (2)	≥4 (0)	≤0.5-≥16 (2)	8- ≥32 (0)	≤0.5- 4 (2)	≤4- ≥128 (2)
n=1	blood	F(1)	ACT-1 pI 5.4	>8.2 5.4	≥64 (0)	≥64 (0)	≥8 (0)	≤1 (1)	1 (1)	≥16 (0)	≥32 (0)	≤1 (1)	≥128 (0)
n=1	blood	J(1)	ACT-1 CTX-M-14 pI 5.4	>8.2 8.0 5.4	≥64 (0)	≥64 (0)	≥8 (0)	≤1 (1)	2 (0)	8 (0)	≥32 (0)	≤1 (1)	≤4 (1)
n=1	urine	D(1)	CTX-M-14	8.0	≥64 (0)	≥64 (0)	≥8 (0)	≤1 (1)	4 (0)	4 (1)	≥32 (0)	≤0.5 (1)	8 (1)
n=2	urine(1) pus(1)	B1(1), untyped(1)	TEM-like	5.4	8 (0)	≤2- ≥64 (1)	≥8 (0)	≤1 (2)	≤0.5- ≥4 (0)	≤0.5-16 (0)	≥16 (0)	≤0.5- 4 (1)	≤4- ≥128 (1)

[a] Tissue, bile, synovial fluid and peritoneal fluid.
[b] Antibiotic susceptibility was evaluated using the new interpretative criteria of the CLSI 2010 document.
PFGE, pulsed-field gel electrophoresis; MIC, minimum inhibitory concentration; ESC-R-KPN, extended-spectrum cephalosporin-resistant *K. pneumoniae*.

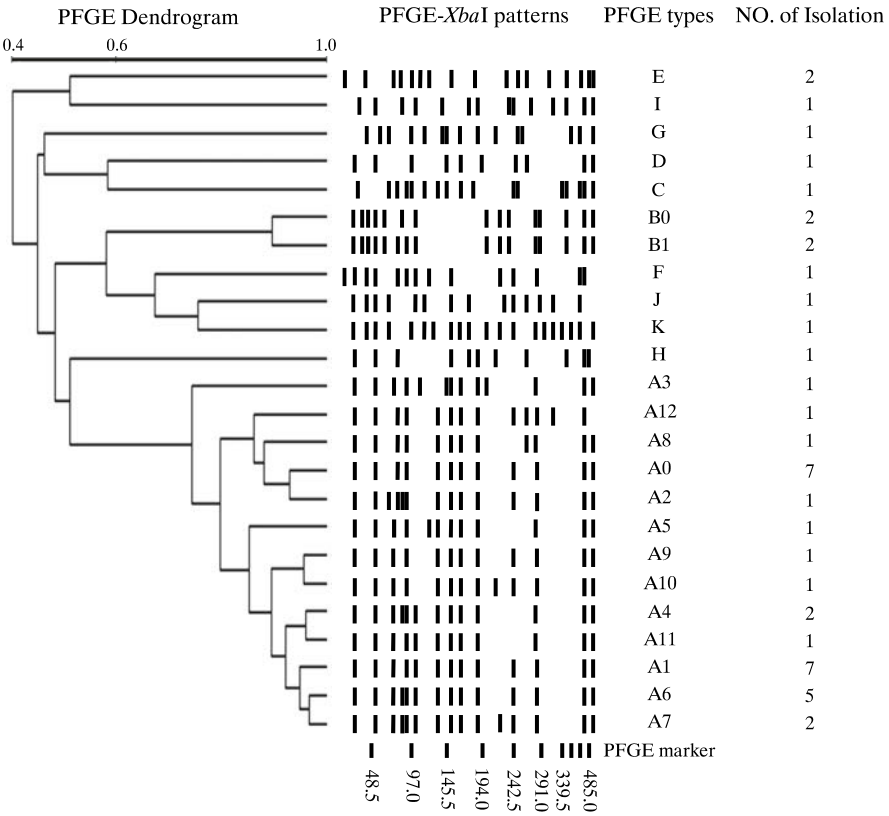

Fig. 2. Dendrogram of the 48 ESC-R-KPN isolates showing 11 different PFGE types. Values indicate the coefficient of similarity between the distinct clonal type clusters. PFGE types (A to K) are presented on the right of each lane. The Lambda Ladder Standard was used as a size marker.

8.2), CTX-M-14 (pI 8.0), TEM-like (pI 5.4), and SHV-2a (pI 7.6), and the PABLs included DHA-1 (pI 7.8) and ACT-1 (pI >8.2). Some isolates shared β-lactamases of pI 5.4, 7.6, 8.0, and >8.2 which were not detected by PCR for ESBL- or PABL- related genes. The DHA-1 enzyme was detected in 36 (75%) isolates. The strains producing only DHA-1 (n=17) and co-producing DHA-1 and SHV-12 enzymes (n=15) were identified as the two major circulating β-lactamase types. The co-production of DHA-1 and CTX-M-14 (n=1) or other undetermined ß-lactamases (pI=7.6, n=3) was also detected. PFGE profiles identified 11 PFGE types (A, B, C,…, K) and an untypable strain: type A and its subtypes (A1,…, A12) accounted for 33 (68.8%) isolates, as a major clone. The similarity with coefficients was 0.73-0.96 within type A, whereas it was 0.32-0.58 among the non-A types (Fig. 2).

The ESC-R-KPN isolates were highly resistant to ceftazidime (100%, 48/48), aztreonam (100%, 48/48), cefoxitin (85.4%, 41/48), cefotaxime (95.8%, 46/48), and ciprofloxacin (79.2%, 37/48), whereas they were highly susceptible to cefepime (91.7%, 44/48) and imipenem (83.3%, 40/48). The overall susceptibilities to piperacillin/tazobactam and gentamicin were 62.5% (30/48) and 39.6% (19/48), respectively.

Plasmid analysis and transfer of the resistance genes bla_{DHA-1} and bla_{SHV-12}

At least eight, large-plasmid profiles were identified from the 48 ESC-R-KPN isolates (data not shown). In transconjugation experiments, transfer of the bla_{DHA-1} gene was successful in *E. coli* transconjugants of the two *K. pneumoniae* strains producing DHA-1 only. In cases of the two selected *K. pneumonia* strains co-producing DHA-1 and SHV-1, 5 of the 8 transconjugants acquired both bla_{DHA-1} and bla_{SHV-12} genes simultaneously, but 3 transconjugants acquired the bla_{DHA-1} only, as detected by the duplex-PCR and Southern hybridization (Fig. 3).

Molecular characterization of the spread of the ESC-R-KPN

Time distribution of the 48 ESC-R-KPN isolates with their β-lactamase types and PFGE profiles is shown in Fig. 4.

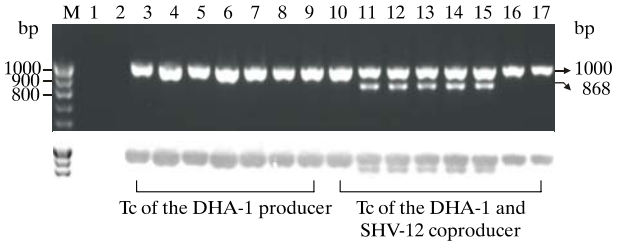

Fig. 3. Electrophoresis (upper) and Southern blot (lower) showing the 1,000 bp- and 868 bp-PCR amplicons of the bla_{DHA-1} and bla_{SHV-12} genes among *E. coli* transconjugants, respectively. Lanes: M, 100 bp ladder marker; 1, negative control; 2, *E. coli* J 53; 3, transconjugant (Tc)44-1; 4, Tc47-1∼6; 5, Tc11-1; 11-17, Tc56-1∼7.

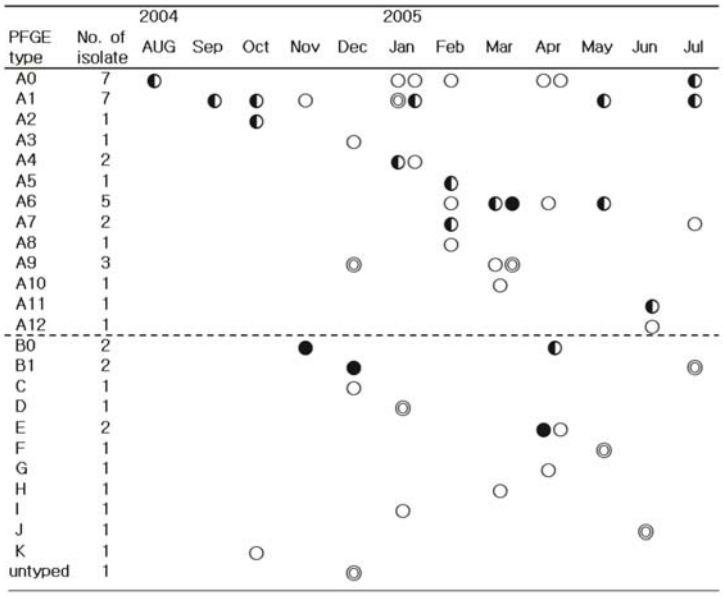

Fig. 4. Spread of ESC-R-KPN isolates analyzed by β-lactamase types and PFGE types over the time course from August 2004 to July 2005. ◐, DHA-1 and SHV-12 co-producer; ○, DHA-1-producer; ◈, DHA-1 and non-SHV-12 co-producer; ●, SHV-12; ◎, others.

Throughout the study period, the strains co-producing DHA-1 and SHV-12 enzymes were spread mainly with clonal expansion of the PFGE-type A. The strains producing only DHA-1 enzyme were distributed widely by the PFGE-type A and other PFGE types, suggesting the presence of clonal expansion of PFGE type A as well as horizontal transfer of the plasmid harboring the $bla_{\text{DHA-1}}$.

Discussion

In the present study, we analyzed the molecular characteristics of a prolonged spread of *K. pneumoniae* resistant to the extended-spectrum cephalosporins in a university hospital over a one year period. We identified the co-existence of two major circulating strains producing either both DHA-1 and SHV-12 enzymes or the DHA-1 only. We were able to transfer the resistant genes $bla_{\text{DHA-1}}$ and $bla_{\text{SHV-12}}$ to transconjugants by plasmid conjugation. From the molecular aspect, the prolonged spread was primarily associated with the expansion of the clone harboring both the $bla_{\text{DHA-1}}$ and $bla_{\text{SHV-12}}$ or the $bla_{\text{DHA-1}}$ only, and to a lesser extent with the horizontal transfer of the resistant plasmids into different *K. pneumoniae* strains.

In contrast to the reported outbreaks caused by single epidemic clone of *K. pneumoniae* strains co-producing DHA-1, AmpC, and an ESBL enzyme (Song *et al.*, 2006b; Roh *et al.*, 2008), our study disclosed the co-existence of multiple clones harboring the same resistant trait, suggesting persistent transfer of the resistant plasmid over several months. In addition, small clusters of the identical PFGE subtypes might result from small scattered outbreaks during the study period.

The risk factors for a PABL or a ESBL acquisition in patients have been studied widely, and include prolonged hospitalization, prolonged ICU or neonatal ICU stay, exposure to third-generation cephalosporins or ciprofloxacin, use of medical devices such as central venous catheter or indwelling urinary catheter, severity of illness, etc. (Jacoby, 2009). In our study, the patients with ESC-R-KPN isolates had various risk factors such as prolonged hospital stays, indwelling urinary catheterization and prior exposure to antibiotics, which might make them susceptible to horizontal transfer of the strains or the resistant plasmids they harbor.

The strain with a PABL often carries several β-lactamases, including ESBLs (Hanson *et al.*, 1999). Both ESBLs and PABLs are associated with broad multidrug resistance because other antibiotic resistance existed on the same plasmid (Hanson *et al.*, 1999). In our study, when compared to strains with only the DHA-1 enzyme, the strains co-producing DHA-1 and SHV-12 enzymes had lower *in vitro* susceptibility to ciprofloxacin (6.7% vs. 35.3%), gentamicin (26.7% vs. 47.1%) and imipenem (80.0% vs. 94.1%). These findings support the idea that the co-producers might be more resistant to antibiotics. Although the co-producers showed 80.0% susceptibility to piperacillin/tazobactam, the clinical outcome of the drug might be unfavorable for bloodstream infections (Qureshi *et al.*, 2011). Carbapenem has been used as the most effective agent, but its use was followed by the emergence of carbapenem-resistant *K. pneumoniae* (Kaczmarek *et al.*, 2006). Therefore, dissemination of the co-producers can cause serious problems.

There is a potential limitation regarding this study. We characterized some ESC-R-KPN isolates that were randomly collected during the study period. Therefore, it might not represent the actual proportion of the strains with PABLs, ESBLs or in combinations. However, the isolates were collected prospectively during the monthly hospital microbial surveillance monitoring, and this might point to an ongoing spread.

In conclusion, our study describes the molecular mechanisms of the prolonged spread of *K. pneumoniae* strains co-producing DHA-1 and SHV-12 β-lactamases or the DHA-1 enzyme only, and indicates the presence of persistent transmission of the causative clone as well as the horizontal transfer of resistant

plasmids among the patients. We point out that these observations have clinical implication for the control and the prevention of dissemination of these strains.

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