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Outbreak of hypervirulent *Klebsiella pneumoniae* harboring *bla*$_{\text{VIM-2}}$ among mechanically ventilated drug poisoning patients with high mortality outcome in Iran

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Highlights:

- 9.4% (5/53) isolates of hypervirulent *K. pneumoniae* (hvKP) were obtained.
- The mortality rate among hvKP infected patients were 80% (4/5).
- Isolates were shown Pasteur's MLST ST23 and indistinguishable clone in PFGE typing.
- Class 1 integron harbored *bla*VIM-2 located on ~45 Kb plasmid with IncN incompatibility group.
- We described the emergence of new VIM-2-producing hvKP serotype with high mortality outcome.

Abstract

Objectives: Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infections are associated with increased rate of treatment failure and death. Several studies have reported isolates with combined hypervirulent and antibiotics resistance phenotype.

Methods: Herein, we studied molecular characteristics of hypervirulent *K. pneumoniae* isolated from mechanically ventilated patients admitted to toxicological ICU. String test, antibiotic susceptibility, virulence factors and plasmid replicon typing were carried out. Finally, clonal relatedness of isolates were analyzed by PFGE and MLST.

Results: In this study, hypervirulent *K. pneumoniae* (hvKP) accounted for 9.4% (5/53) of *K. pneumoniae* isolated from ventilator-associated pneumonia (VAP) among patients admitted to ICU with acute drug poisoning. The mortality rate were 7.54% (4/53) among KP infected patients. All fatal KP were hvKP isolates and resistant to imipenem and harbored *aacA7*, $bla_{\text{VIM-2}}$ and *dhfrI* cassettes arrangement in class 1 integron. Isolates were shown Pasteur's MLST ST23 and

exhibited similar PFGE patterns. Plasmid analysis revealed class 1 integron harbored *bla*$_{\text{VIM-2}}$ located on ~45 Kb plasmid with IncN incompatibility group.

Discussion: In the present study, we described emergence of VIM-2-producing hypervirulent *K. pneumoniae* serotype K1/ST23 in outbreak with high mortality in the hospital's toxicological ICU. It seems that we must alert and prepare our surveillance system for appearance, expansion and clinical importance of new clones of *K. pneumoniae* associated with highly antimicrobial resistance and robust virulence capabilities.

Keywords: *Klebsiella pneumoniae*, Hypervirulent, Ventilated associated pneumoniae, *bla*$_{\text{VIM-2}}$

1-Introduction

Klebsiella pneumoniae is causative agent of bloodstream and surgical site infections, catheter-associated urinary tract infection, and ventilator-associated pneumonia (VAP) worldwide [1]. Carbapenem-resistant *K. pneumoniae* (CRKP) infections is associated with increased rate of treatment failure and death [2].

Over the past two decades, a new hypervirulent *K. pneumoniae* (hvKP) with hypermucoviscosity has emerged as a significant pathogen [3]. The phenotype of hvKP is typically due to overproduction of capsular polysaccharide and certain serotypes, as well as presence of virulence genes including *rmpA* (regulator of mucoid phenotype A gene), *magA* (mucoviscosity-associated gene A) and aerobactin [4-7]. Unlike typical nosocomial infections of classic *K. pneumoniae*, hvKP can cause serious community-acquired infections even in healthy individuals [8,7].

Except for an intrinsic resistance to ampicillin, hvKP is rarely resistant to commonly used antibiotic [9,7]. However, hypervirulent carbapenem-resistant *K. pneumoniae* strains are

increasingly being reported in clinical settings [7]. The emergence of carbapenem-resistant hvKP gives rise to untreatable and severe infections in healthy individuals and poses a worrying crisis in public healthcare system [10,4,7].

A few studies have reported the isolates with combined hypervirulent and high resistance phenotype [11,9]. So far, research on antimicrobial-resistant hvKP strains has been limited and mainly based on case studies [9,12,13]. For the first time, in the present study, we described an outbreak of hvKP harboring *bla*$_{\text{VIM-2}}$ among mechanically ventilated drug poisoning patients with high mortality outcome.

2-Material and methods

2.1. Collection of isolates

This cross-sectional study was carried out at university hospital in Tehran during a 6 month period (June to November 2012). Fifty-three unique patient isolates of *K. pneumoniae* were recovered from clinical specimens. Genus and species identification of *K. pneumoniae* was confirmed using API 20E strips (bioMérieux) [14]. Moreover, clinical characteristics of patients with a positive culture were collected. The clinical characteristics of patients including age, gender, antibiotic treatments, history and duration of hospitalizations, date of the first *K. pneumoniae* detection in hospital laboratory, site of infection, invasive procedures and outcomes were obtained.

2.2. Antimicrobial resistance testing

The isolates were tested for susceptibility by disk diffusion method according to the CLSI guidelines [15]. The following discs were used (micrograms per disc): Cefotaxime (CTX, 30), Ceftazidime (CAZ, 30), Cefepime (CPM, 30), Ceftriaxone (CRO, 30), Imipenem (IPM, 10),

Ertapenem (ETP, 10), Meropenem (MEM, 10), Aztreonam (ATM, 30), Amikacin (AN, 30), Ampicillin/sulbactam (AMS, 10), Cefoxitin (CFX, 30), Gentamicin (GM, 10), Trimethoprim-sulfamethoxazol (SXT, 10/10) and Ciprofloxacin (CIP, 5). Minimal inhibitory concentration (MIC) was determined for imipenem resistant isolates by E-test (bioMérieux) method. Susceptibility, interpretations were based on clinical CLSI breakpoints. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains.

2.3. Detection of antimicrobial resistance genes

PCR assays were carried out with primers specific for class A and B carbapenemase-encoding genes including bla_{KPC} and bla_{GES} (class A), bla_{IMP-1}, bla_{IMP-2}, bla_{VIM-1}, bla_{VIM-2}, bla_{SPM}, bla_{SIM} and bla_{NDM} (class B) as previously described [16]. Moreover, the antimicrobial resistance gene cassettes harboring in class 1 integrons were detected as described previously [17].

2.4. Phonotypic and genotypic identification of hvKP

Detection of hypermucoviscosity phenotype of *K. pneumoniae* was carried out by string method as described previously [6]. All *K. pneumoniae* isolates were screened for the presence of capsular type K1 and K2 associated genes using a PCR protocol as described previously [18]. Virulence associated factors including *rmpA*, *magA* genes and aerobactin were determined by specific primers and PCR condition described previously [19,20].

2.5. Clonal relatedness of hvKP isolates

Molecular typing of hvKP isolates was performed by pulsed-field gel electrophoresis (PFGE) using a CHEF DRIII (Bio-Rad) based on *Xba*I-digested total DNA as previously described [21].

Dendogram was generated by Dice coefficient based on the unweighted pair group method using arithmetic averages (UPGMA) according to the fingerprinting GelCompare II software version 5.0 (Applied Maths) and interpreted with 80% similarity cut-off. Moreover, multilocus sequence typing (MLST) of strains were done according to the protocol provided on the MLST website (www.pasteur.fr/mlst). Alleles and sequence types (STs) were determined according to the MLST database (www.pasteur.fr/mlst/Kpneumoniae.html).

2.6. Conjugation and Plasmid Analysis

Filter mating assays were carried out with *E. coli* K12 [F- lac+ Nal (r)] as the recipient strain. Five isolates harboring the $bla_{\text{VIM-2}}$ gene, were subjected to conjugation experiments by Filter mating method. Transconjugants were selected on MacConkey agar plates containing 32 μg/mL nalidixic acid and 1 μg/mL meropenem. The $bla_{\text{VIM-2}}$ gene was sought by PCR amplification. Plasmid DNA was extracted from both donor and transconjugant strains using the GeneJET Plasmid Maxiprep Kit (Thermo Scientific). The plasmid bands were observed on 0.7% agarose gel and their molecular weight was determined. Plasmid incompatibility was characterized by a PCR-based replicon typing scheme, as previously described [22].

2.7. Statistical Analysis

Statistical analysis was performed using SPSS software version 20.0 (IBM, Chicago, USA). P-Value of < 0.05 was considered statistically significance for two-tailed test.

3-Results

3.1. Clinical characteristics of patients and bacterial isolates

Fifty-three unique clinical isolates of *K. pneumoniae* were recovered from clinical specimens including trachea (n=11/53; 20.7%), bronchoalveolar lavage (18/53, 34%) urine (n=8/53; 15%), sputum (n=10/53; 18.86%), blood (n=6/53; 11.32%). Among 53 isolates of *K. pneumoniae* five isolates were recovered from tracheal (2/5) and bronchoalveolar lavage (BAL, 3/5) specimens of patients admitted to intensive care units (ICU) with acute drug poisoning. Data of antimicrobial susceptibility testing is shown in Table 1. The isolates showed resistance to all tested antibiotics especially expressed high-level resistance to carbapenem in disc diffusion method. Patients age were >40 and mean hospitalization and ICU length of stay in CRKP infected patients were 16 days and more than 5 days, respectively. After admission to ICU, they undergo dialysis and received antibiotics as prophylaxis (Table 2). Ageing, drug poisoning, ventilator-associated pneumonia, radiographic features suggestive of pneumonia (Fig. 1), body temperature >38°C, respiratory distress (oxygen saturation ranging from 91-94% and respiratory rate ranging from 41-46/minute) were the clinical characteristics of CRKP infected patients. Laboratory tests evidenced leukocytosis (WBC$\geq$12 $\times$ 10^3/μL) and serum C-reactive protein >39mg/dL and analysis of respiratory secretion in these patients revealed presence of white blood cells with a 95% neutrophil left shift. Additional laboratory tests include string test to determine the bacteria were hypermucoviscosity phenotype and the causative agent of fulminant pneumonia. The mortality rate was 7.54% (4/53) due to causes of infection and this increase was statistically significant ($p<0.05$). All fatal KP were hvKP isolates. The mortality risk ratio among patient's subjects with carbapenem-resistant *K. pneumoniae* were 5.486 (95% CI 2.43-12.37; P-value 0.01). The clinical and laboratory profile for CRKP isolates is summarized in Table 2; The MICs for imipenem in isolates were 16 μg/ml. Antimicrobial susceptibility profile in disk diffusion method for 53 isolates of *K. pneumoniae* is summarized in Table 1.

3.2. Molecular Detection of Carbapenemase and Class 1 Integrons

The PCR assays for beta lactamase genes including *bla*$_{KPC}$, *bla*$_{IMP-1}$, *bla*$_{IMP-2}$, *bla*$_{VIM-1}$, *bla*$_{VIM-2}$, *bla*$_{GES}$, *bla*$_{SPM}$, *bla*$_{SIM}$ and *bla*$_{NDM}$ only revealed the presence of *bla*$_{VIM-2}$ in five isolates of CRKP. Class 1 integron mapping of these isolates by PCR indicated the same cassette region were 2346 bp in size and contained *aacA7*, *bla*$_{VIM-2}$ and *dhfrI* from 5´ to 3´. Sequencing of the *bla*$_{VIM-2}$ PCR products was confirmed by BLAST tool at NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and sequences were deposited in GenBank database under accession numbers MG785026, MG775279, MG775280 and MG763934. The cassette arrangement of class 1 integron harboring *bla*$_{VIM-2}$ in CRKP was very similar to that of *P. aeruginosa* class I integron (GenBank: DQ984668.1, AM749810.1, AM296017.1), which carries the same genes.

3.3. Virulence-Associated Features and Molecular Typing

The PCR assays for detection of virulence associated genes revealed presence of *rmpA* and aerobactin in CRKP isolates and positive results in string test confirmed the hypervirulent/hypermucoviscosity phenotype. All CRKP strains were tested for genes encoding the capsule K antigens. K1 antigens were associated positively with CRKP/hvKP phenotype. In order to investigate the genetic relationships, PFGE analysis of 5 CRKP isolates identified the same pulsotype patterns designated A1 (data not shown). Moreover, MLST analysis showed all isolates were ST23. The same fingerprint pattern, indicating a clonal lineage and demonstrating CRKP isolates were originated from same clone (Table 2).

3.4. Plasmid Analysis

The plasmid replicon typing revealed that *bla*$_{\text{VIM-2}}$ carrying *K. pneumoniae* isolates contained plasmid types belonging to incompatibility group N plasmids. Conjugation assays showed that the plasmids carried *bla*$_{\text{VIM-2}}$ CRKP strains were successfully transferred to *E. coli* K12 as recipient strain with mean observed conjugation frequency 4.5×10^{-6}. PCR confirmed presence of the *bla*$_{\text{VIM-2}}$ in the transconjugants. Plasmid analysis indicated that *bla*$_{\text{VIM-2}}$ in transconjugants was located on a ~45 kb IncN conjugative plasmids (Table 2). Moreover, digestion pattern of plasmids by *Eco*RI showed identical profiles of acquired plasmids in transconjugants.

4-Discussion

Carbapenem-resistant acquired *K. pneumoniae* isolates were first seen in April 2000, being recovered from the respiratory secretions of hospitalized patients in ICUs [23]. During the last decade, infections due to CRKP has been reported around the world [24, 25]. On the other hand, a new highly invasive hvKP has emerged as a clinically significant pathogen causing infections in both healthy and immunocompromised individuals, such as liver abscesses, [7]. However, along with the global dissemination of mobile genetic elements, antibiotic-resistant hvKP isolates are increasingly being reported [13,7]. Herein, we documented occurrence of five strains of CRKP isolated which concurrently had hypervirulent phenotype (CR/hvKP).

In this study, hvKP accounted for 9.4% (5/53) *K. pneumoniae* induced VAP in mechanically ventilated patients. In the study conducted by Yan *et al*. among 49 mechanically ventilated patients, 28.6 % (14/49) were infected by hvKP. Antimicrobial resistant rate was significantly higher in cKP than that in hvKP (26). Also, in two studies in Taiwan, prevalence of hvKP infections among hospitals which acquired *K. pneumoniae* were reported 14.8% and 15.2%, respectively [27,28]. hvKP is characterized by causing severe community acquired infections such as liver abscesses in healthy people, with the ability to cause metastatic dissemination [29]. In our study, the mortality

rate among hvKP infected patients was 80% (4/5) and this increase was statistically significant ($p<0.05$). However, the pathogenesis of these isolates should be seriously considered.

The hvKP strains are rarely resistant to commonly used antibiotics. However, in this study, for the first time, we observed the CR/hvKP isolates with outbreak incidence in an Iranian hospital. The reason for low prevalence of antibiotic resistance-hvKP remains unclear; however, reports of these isolates are increasingly being given worldwide, often, in countries with an epidemic dissemination of hvKP [7]. With a global prevalence of hvKP, several studies in China have showed emergence of hvKP with extensive antibiotic resistances, including carbapenem resistance. In 2015, among 28 isolates of carbapenem-resistant *K. pneumoniae*, five carbapenem-resistant hvKP isolates were found [26].

Our results showed that the CR/hvKP isolates harboring the $bla_{\text{VIM-2}}$ gene was located on a novel class 1 integron in *K. pneumoniae*. These isolates were identified from intubated patient's tracheal specimens in toxicological ICU. Regarding the results of genotyping, PFGE typing indicate that the outbreak of $bla_{\text{VIM-2}}$ producing hvKP infection was monoclonal and a clonal relationship was confirmed among these isolates. All CR/hvKP isolates were identified as MLST sequence type (ST) 23 and confirmed to be serotype K1 by PCR. These homogeneities suggest that $bla_{\text{VIM-2}}$ producing strains of hvKP circulatied in our hospital. The hvKP/ST23 isolated from ventilated patients has been previously reported [29] but this is the first description of VIM-2-producing CR/hvKP/K1/ST23.

Our data showed selection of a single VIM-2-producing *K. pneumoniae* epidemic genotype assigned to PFGE type A1 and ST23, which was isolated only from patients in the toxicological ICU but not in other wards of the hospital, thus suggesting that cross transmission among respiratory carrier patients may have favored spread of VIM-2 producing *K. pneumoniae* clones.

In addition, carbapenem-resistant transconjugants were obtained from isolates of the CR/hvKP/ST23 clone and carried a single IncN plasmid of ~45 kb. Plasmids belonging to IncN group represent one of the most frequently encountered resistance and self-conjugative plasmid types in Enterobacteriaceae [30]. However, IncN plasmid detected in CR/hvKP could be the vehicles for the spread of the *bla*$_{VIM-2}$ gene in hospitalized patients. Several studies revealed that the horizontal gene transfer through transposons and conjugative plasmids contributes to the spread of resistance to carbapenems [11,31]. Accordingly, we showed that resistance to imipenem was transferred from CR/hvKP/ST23 isolates to susceptible *E. coli* along with a *bla*$_{VIM-2}$ positive plasmid of IncN incompatibility group. So, we postulate that imipenem resistance in these strains depends mainly on the expression of VIM-2 carbapenemase carried by IncN plasmids.

Resistant bacteria are circulated in hospitals environment by clonal expansion and horizontal gene transfer (32). The similarities of cassette arrangement of class 1 integron in the present study to that of *P. aeruginosa* class I integron reported previously (GenBank: DQ984668.1, AM749810.1, AM296017.1), suggests that probably *bla*$_{VIM-2}$ carrying conjugative plasmid in hvKP is acquired from *P. aeruginosa* through horizontal gene transfer and is expanding among these strains.

Treatment of serious infections associated with carbapenemase-producing *K. pneumoniae* is difficult because these bacteria have frequently multi-drug resistant phenotype. Moreover, the concomitant hypervirulent phenotype makes this problem more complicated. In the present research, we described emergence of VIM-2-producing hypervirulent *K. pneumoniae* serotype K1/ST23 in outbreak in a hospital's toxicological ICU. The susceptibility profile of CR/hvKP isolates was recovered during the present study underscoring extremely limited antibiotic choices available for the treatment of infected patients. Our data showed that outbreak strains rapidly disseminated among clinical setting through patient to patient spreading and caused a severe

pneumonia contributing to high mortality rate among poisoned patients. It seems that we must alert and prepare our surveillance system for appearance, expansion and clinical importance of the new clones of *K. pneumoniae* associated with highly antimicrobial resistance and robust virulence capabilities.

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Declarations

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Ethical Approval: Not required

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Legends

Fig. 1. Chest radiograph of an intubated patient in the ICU with clinical signs and symptoms of ventilator-associated pneumonia (VAP).

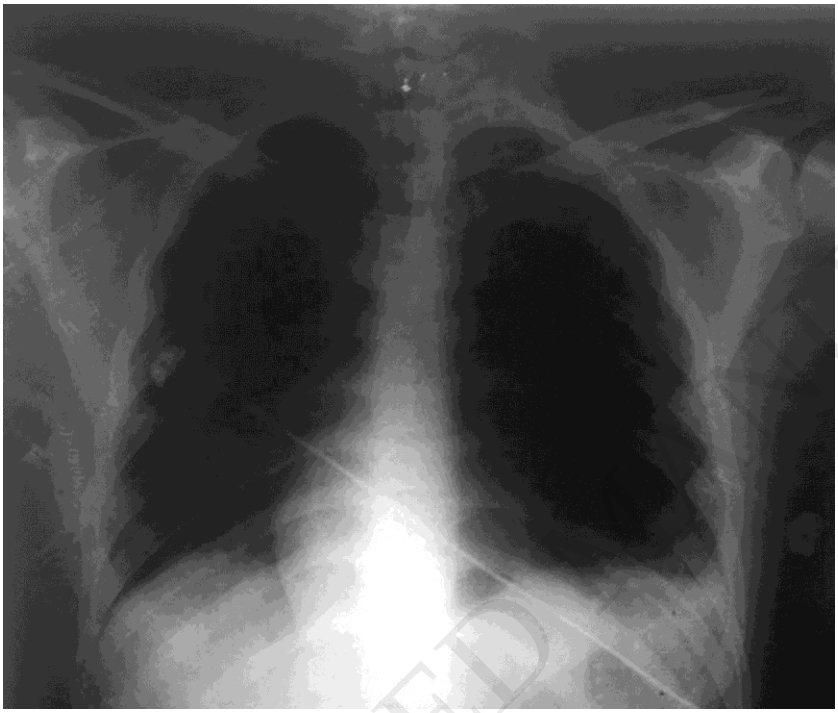

Table 1. Antimicrobial susceptibility profile of 53 isolates of *K. pneumoniae*.

	Antibiotic resistance profile % (No.)													
	CTX	CFX	CAZ	CPM	CRO	AMS	IPM	ETP	MEM	ATM	AN	SXT	GM	CIP
Klebsiella pneumoniae	73.6 (39)	84.9 (45)	69.8 (37)	54.7 (29)	69.8 (37)	56.6 (30)	9.4 (5)	9.4 (5)	9.4 (5)	24.5 (13)	20.8 (11)	58.5 (31)	67.9 (36)	69.8 (37)

Abbreviations: CTX, cefotaxime; CFX, cefoxitin; CAZ, ceftazidime; CPM, cefepime; CRO, ceftriaxone; AMS, ampicillin/sulbactam; IPM, imipenem; ETP, ertapenem; MEM, meropenem; ATM, aztreonam; AN, amikacin; SXT, trimethoprim-sulfamethoxazol; GM, gentamicin; CIP, ciprofloxacin.

Table 2. The clinical information, antibiotics resistance profile, molecular detection and typing of hypervirulent *K. pneumoniae* isolated from mechanically ventilated patients admitted to toxicological ICU.

	Clinical data						**Laboratory data**									
Strain No.	Sex /Age	Clinical presentation	Mechanical ventilation, days	Therapeutic actions	Antibiotic prescription	Outcome of patient	Source of isolation	Resistance profile (disk diffusion)	MIC of IMP (µg/ml)	Carbapenemase gene	Class 1 integron context	Virulence factors	Capsular serotype	Pulsotypes/ Sequence type	Transconjugation test	Plasmid molecular weight (kb) and Inc. group
CRKP-1	F/52	Pneumonia, Intoxication, Pleural effusion, Renal failure and Edema	18	Intubation, Haemodialysis, Double chest tube, Tracheostomy and Bronchoscopy	SAM, AN, IMP	Died	BAL/C	CTX, CAZ, CPM, CRO, IPM, ETP, MEM, ATM, AN, SXT, GM, CIP	16	*bla*$_{VIM-2}$	*aacA7*, *bla*$_{VIM-2}$, *dhfrI*	*rmpA*, *magA*, aerobactin	K1	A1/ ST23	Positive	~45kb/IncN
CRKP-2	M/54	Pneumonia, Intoxication, Renal failure, Addiction, HIV positive and Edema	14	Intubation, Hemodialysis and Tracheostomy	SAM, AN, IMP, CAZ	Died	T/C	CTX, CAZ, CPM, CRO, IPM, ETP, MEM, ATM, AN, SXT, GM, CIP	16	*bla*$_{VIM-2}$	*aacA7*, *bla*$_{VIM-2}$, *dhfrI*	*rmpA*, *magA*, aerobactin	K1	A1/ ST23	Positive	~45kb/IncN

CRKP-3	M/40	Bronchial infection, Intoxication and Edema	20	Intubation and Tracheostomy	IMP, CIP SAM, CAZ	Survived	T/C	CTX, CAZ, CPM, CRO, IPM, ETP, MEM, ATM, AN, SXT, GM, CIP	16	*bla*$_{VIM-2}$	*aacA7*, *bla*$_{VIM-2}$, *dhfrI*	*rmpA*, *magA*, aerobactin	K1	A1/ ST23	Positive	~45kb/IncN
CRKP-4	F/60	Pneumonia, Intoxication, Renal failure, COPD and Heart failure	16	Intubation. Tracheostomy, Hemodialysis and Bronchoscopy	IMP, CIP SAM, CAZ	Died	BAL/C	CTX, CAZ, CPM, CRO, IPM, ETP, MEM, ATM, AN, SXT, GM, CIP	16	*bla*$_{VIM-2}$	*aacA7*, *bla*$_{VIM-2}$, *dhfrI*	*rmpA*, *magA*, aerobactin	K1	A1/ ST23	Positive	~45kb/IncN
CRKP-5	M/63	Pneumonia, Intoxication, Renal failure, Pleural, Effusion and Edema	12	Intubation, Tracheostomy, Hemodialysis, Double chest tube and Bronchoscopy	SAM, AN IMP,	Died	BAL/C	CTX, CAZ, CPM, CRO, IPM, ETP, MEM, ATM, AN, SXT, GM, CIP	16	*bla*$_{VIM-2}$	*aacA7*, *bla*$_{VIM-2}$, dhfrI	*rmpA*, *magA*, aerobactin	K1	A1/ ST23	Positive	~45kb/IncN

Abbreviations: CTX, cefotaxime; CAZ, ceftazidime; CPM, cefepime; CRO, ceftriaxone; IPM, imipenem; ETP, ertapenem; MEM, meropenem; ATM, aztreonam; AN, amikacin; SXT, trimethoprim-sulfamethoxazol; GM, gentamicin; CIP, ciprofloxacin; SAM, ampicillin-sulbactam; BAL/C, Bronchoalveolar lavage Culture; T/C, Tracheal fluid Culture.