Case Report

Emergence of CTX-M-3, TEM-1 and a new plasmid-mediated MOX-4 AmpC in a multiresistant *Aeromonas caviae* isolate from a patient with pneumonia

Ying Ye,^{1,2} Xi-Hai Xu¹ and Jia-Bin Li^{1,2}

Correspondence

Jia-Bin Li

lijabin948@vip.sohu.com

¹Department of Infectious Diseases, First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, PR China

²Bacterial Resistance Monitoring Center of Anhui Province, Hefei, Anhui, PR China

Aeromonas species rarely cause pulmonary infection. We report, for what is believed to be the first time, a case of severe pneumonia in a cancer patient caused by *Aeromonas caviae*. Detailed microbiological investigation revealed that this isolate carried three β -lactamase-encoding genes (encoding MOX-4, CTX-M-3 and TEM-1) conferring resistance to all β -lactams but imipenem. The β -lactamase with a pl of 9.0 was transferred by conjugation and associated with a 7.3 kb plasmid, as demonstrated by Southern blot hybridization. Analysis of the nucleotide and amino acid sequences showed a new *ampC* gene that was closely related to those encoding the MOX-1, MOX-2 and MOX-3 β -lactamases. This new plasmid-mediated AmpC β -lactamase from China was named MOX-4. This is believed to be the first report of MOX-4, CTX-M-3 and TEM-1 β -lactamases in a multiresistant *A. caviae*.

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Introduction

Plasmid-mediated AmpC β -lactamases have been reported in clinical strains of various Gram-negative bacilli in the last decade that confer resistance to oxyimino-cephalosporins and cephamycins, as well as to β -lactamase inhibitors. For some of them, the amino acid and nucleotide sequences are very similar to those of the chromosome-encoded AmpC β -lactamases of *Enterobacter cloacae* (ACT-1 and MIR-1), *Citrobacter freundii* (CMY-2, CMY-4, CMY-5 and LAT-1), *Morganella morganii* (DHA-1 and DHA-2) and *Hafnia alvei* (ACC-1). The phylogeny of MOX-type and FOX-type enzymes is unclear as they show lower sequence similarities (<74 %) to those of the chromosomally encoded AmpC β -lactamases of *Aeromonas sobria*, *Pseudomonas aeruginosa* and *Serratia marcescens* (Philippon *et al.*, 2002), but Fosse *et al.* (2003) showed that FOX is almost certainly descended from CAV-1 from *Aeromonas caviae*. The *A. caviae* strains isolated in the past were found to be resistant to several antibiotics, such as ticarcillin, cefalotin, quinolone and streptomycin. The spread of these resistances is mainly due to chromosomally encoded FOX AmpC β -lactamase (Fosse *et al.*, 2003) or to mutations in the QRDRs (quinolone-resistance-determining regions) of *gyrA* (Alcaide *et al.*, 2009) or to the presence of class 1 integrons (Pérez-Valdespino

et al., 2009). To date, plasmid-mediated AmpC β -lactamase has not been reported in *A. caviae*. Our results showed that *A. caviae* GN07160 isolated from a pneumonia patient, which was resistant to a broad spectrum of β -lactam antibiotics, harboured a new plasmid-mediated AmpC β -lactamase.

Case report

A 68-year-old man with a squamous cell carcinoma of the oesophagus in May 2007, was admitted to the Department of Infectious Diseases of the Anhui Medical University Hospital, China, on 29 October 2007, with a fever of 39 °C, dyspnoea, chest discomfort and a productive cough with purulent sputum. He lived in Hefei city, with relatives, and didn't receive any therapy for his oesophagus carcinoma. His vital signs on admission were the following: a heart rate of 116 beats min⁻¹, blood pressure of 140/75 mmHg and a respiration rate of 26 breaths min⁻¹. Examination of the chest revealed the presence of bronchial breath sounds and rales over the bilateral lower lung fields. A lung computed tomography scan showed bilateral multiple patchy infiltrates. Admission laboratory values included: a haematocrit level of 41.2 %; 12.3×10^9 white blood cells l⁻¹, with 89 % polymorphonuclear elements; 87×10^9 platelets l⁻¹; 84 mmol serum creatinine l⁻¹; and 32 g albumin l⁻¹. Sputum, protected specimen brush material of bronchial secretions and two sets of blood specimens were taken. The patient was started on empirical antibiotic treatment with

Abbreviation: ESBL, extended spectrum β -lactamase.

The GenBank/EMBL/DBJ accession number for the MOX-4-encoding sequence of *A. caviae* is FJ262599.

intravenous cefoperazone (1 g, every 8 h) and ciprofloxacin (200 mg, every 12 h). On day 5, his respiratory function worsened (severe dyspnoea, tachypnoea, paCO_2 (partial pressure of CO_2 in arterial blood) of 62 mmHg and oxygen saturation of 70 %). A repeated lung computed tomography scan showed development of the bilateral middle and lower lobe infiltrate with pleural inflammatory reaction. He was intubated and mechanically ventilated, and then given oxygen, aminophylline and 0.5 g imipenem every 8 h. But despite the therapy, he died on the eighth day of hospitalization because of severe respiratory insufficiency.

Gram stain of the sputum sample showed moderate numbers of leukocytes and a few short, Gram-negative rods. Two blood cultures were negative. At 72 h after protected specimen brush sample inoculation on sheep blood agar, pure growth of smooth entire and non-haemolytic colonies of about 2 mm in diameter appeared on the plates. The concentration of bacteria was calculated to be $>10^5$ colonies ml^{-1} . One purified isolate was deposited in the Bacteria Collection System as GN07160. GN07160 was identified as either *A. caviae* or *Vibrio fluvialis* by MicroScan W/A 40 identification strip (Dade Behring). Next, PCR amplification of the complete 16S rRNA gene was performed with genomic DNA in accordance with a published protocol (Al-Benwan *et al.*, 2007). The amplification product was subjected to direct sequencing, and a 100 % match to GenBank sequence

accession number X60408.1 (<http://www.ncbi.nlm.nih.gov>) of *A. caviae* was noted.

The antimicrobial susceptibility of GN07160 was studied with Mueller–Hinton agar (Oxoid) using the disc diffusion method according to Clinical and Laboratory Standards Institute recommendations (Al-Benwan *et al.*, 2007). The isolate was resistant to ciprofloxacin, gentamicin, piperacillin–tazobactam, cefotaxime, ceftriaxone, ceftazidime, cefepime, cefoxitin and aztreonam, but susceptible to imipenem. The β -lactam antibiotic resistance phenotype suggested the presence of an extended-spectrum β -lactamase (ESBL) and/or an AmpC β -lactamase. Isoelectric focusing was performed as described by Wei *et al.* (2005). It revealed that three β -lactamases with pI values of 5.4, 8.4 and 9.0 were present. To amplify the sequences of TEM, SHV and CTX-M β -lactamase related genes, PCR was carried out with primers specific for these genes as described by Rotimi *et al.* (2008). Two types of β -lactamases were detected by PCR and confirmed as TEM-1 and CTX-M-3 by sequence analysis. The pI 5.4 protein corresponded to the TEM-1 β -lactamase, and the pI 8.4 protein corresponded to CTX-M-3 ESBL. The detection of the plasmid-mediated AmpC β -lactamase was carried out using a multiplex PCR technique for the amplification of six sets of genes as described by Pérez-Pérez & Hanson (2002). A MOX group AmpC-encoding gene was found. Subsequently, the primer pair AmpF (5'-TTGAATTC-ATGCAACAACGACAATCC-3') and AmpR (5'-TTCT-

MOX-1	MQQRQSILWG	AVATILMWAGL	AHAGEASVPD	PLRPVVDASI	QPLLKEHRIP	50
MOX-2			T		R	
MOX-3			T			
MOX-4			DT A		R	
MOX-1	GMAVAVLKDG	KAHYFNYGVA	NRESGASVSE	QTLFEIGSVS	KTLTATILGAY	100
MOX-2		D	RAVG		P	
MOX-3		D	RAVG		P	
MOX-4		D	RAVG		P	
MOX-1	AVVKGAMQLD	DKASRRHAPWL	KGSVFDSITM	GELATYSAGG	LPLQFPPEEVD	150
MOX-2			A			
MOX-3			A			
MOX-4			A			
MOX-1	SSEKMRAYYR	QWAPVYSPGS	HRQY YSN PSIG	LFGLHAASSL	KQPPFAQLMEQ	200
MOX-2	L Q	T A			M	
MOX-3	L Q	T A				
MOX-4	L Q	T A			M	
MOX-1	TLLPGLGMHH	TYVNVPKQAM	ASYAYGSKE	DKPIRVNPGM	LADEAYGIKT	250
MOX-2	L			S		
MOX-3	L			S		
MOX-4	L			S		
MOX-1	SSADLLAFVK	ANIGGVDDKA	LQQAISLTHK	GHYSVGGMTQ	GLGWESYAYP	300
MOX-2	R	S H				
MOX-3	R	S				
MOX-4	R	S				
MOX-1	VTEQTLLAGN	SAKVILEANP	TAAPRESGSQ	VLFN KTG SSN	GFGAYVAFVP	350
MOX-2	S			M	TS	
MOX-3				M	TS	
MOX-4	S	E	DL	M	TS	
MOX-1	ARGIGIVMLA	NRNYPPIPARV	KAHAAILAQL	AG		382
MOX-2	K		T	R		
MOX-3	K		T	R		
MOX-4	K		T	R		

Fig. 1. Amino acid sequences of the MOX enzymes. Amino acid replacements are indicated. The β -lactamase active site SVSK, the conserved triad KTG, and the typical class C motif YSN are indicated in bold.

Fig. 2. DNA hybridization with a MOX-specific probe. Plasmid DNAs were extracted from the *A. caviae* isolate (lane 2) and the transconjugant (lane 3), run on a 1.0 % agarose gel, and compared to standard plasmid molecular sizes of *E. coli* V517 (lane 1). On the Southern blot a hybridization signal was shown for the (7.3 kb) plasmid band. Lanes 6 and 7 correspond to lanes 2 and 3, respectively.

GCAGTTACCTGGCCAGTTGCGT-3') were used for amplification of the whole ORF. The purified ORF amplicon was linked into the vector pHSG398 by T4 DNA ligase after cleavage by *EcoRI* and *PstI* restriction enzymes. And then, the recombinant plasmid was introduced into *Escherichia coli* JM109 made competent by the calcium chloride method. Transformants were selected on Mueller–Hinton agar plates containing 2 µg cefoxitin ml⁻¹, 50 µg chloramphenicol ml⁻¹, 20 mg X-Gal ml⁻¹ and 0.5 mM IPTG. Finally, an ORF of 1149 nucleotides encoding a protein of 382 amino acids was identified by direct sequencing. The deduced peptide sequence contained the common conserved motifs SXSK, YXN and KTG, which are found in all AmpC β-lactamases. The differences in the amino acid sequence of MOX-4 are shown in Fig. 1 with respect to: *bla*_{MOX-2}, differences in 5 positions – 25 (D→E), 28 (A→P), 313 (E→K), 328 (D→G)

and 329 (L→S); *bla*_{MOX-3}, differences in 8 positions – 25 (D→E), 28 (A→P), 41 (R→Q), 190 (M→L), 302 (S→T), 313 (E→K), 328 (D→G) and 329 (L→S); and *bla*_{MOX-1}, differences in 30 positions – 25 (D→E), 26 (A→T), 28 (A→P), 41 (R→Q), 71 (D→N), 74 (R→S), 75 (A→G), 76 (V→A), 77 (G→S), 92 (P→T), 124 (A→V), 152 (L→S), 156 (Q→R), 163 (T→A), 165 (A→V), 190 (M→L), 208 (L→M), 237 (S→N), 257 (R→A), 264 (S→G), 302 (S→T), 313 (E→K), 328 (D→G), 329 (L→S), 331 (M→V), 339 (T→S), 340 (S→N), 352 (K→R), 378 (T→A) and 382 (R→G). This ORF was identified as encoding MOX-4 AmpC β-lactamase by the Jacoby/Bush repository for β-lactamase nomenclature. The ORF has been submitted to the GenBank database and assigned accession no. FJ262599.

Conjugation was performed with overnight cultures of GN07160 and *E. coli* C600, which were mixed in a ratio of 1:2 in brain heart infusion broth, and grown for a further 16 h (Li *et al.*, 2008). Transconjugants were successfully selected at a frequency of 10⁻⁴ to 10⁻⁵ cells per recipient cell on tryptone soy agar supplemented with streptomycin (500 mg l⁻¹) and cefoxitin (20 µg ml⁻¹). The presence of the MOX-4-encoding gene in the transconjugant was confirmed by PCR. Southern blotting was performed as described by Li *et al.* (2008). The 1149 bp MOX-4-specific detection probe was a PCR-generated amplicon labelled with digoxigenin (DIG) by the random priming technique. After agarose gel electrophoresis, DNA fragments of GN07160 and the transconjugant in the gel were transferred onto Hybond-N+ nylon membranes (Amersham Biosciences) and probed with the DIG-labelled MOX-4 probe. Fig. 2 shows a positive hybridization signal was detectable in the 7.3 kb band in GN07160 and the transconjugant. MICs of antimicrobial agents for *A. caviae*, as well as its transconjugant and its transformant, are listed in Table 1.

Discussion

Aeromonas species are Gram-negative, motile, facultatively anaerobic bacilli. At present, the family *Aeromonas* comprises 14 species, among which only five species (*A. caviae*, *Aeromonas hydrophila*, *Aeromonas veronii*,

Table 1. MICs of antimicrobial agents for *A. caviae*, its transconjugant and its transformant

Strain	MIC (µg ml ⁻¹)												
	AMP	PIP	TZP*	CTX	CRO	CAZ	FEP	FOX	ATM	CIP	AMK	LVX	IMP
<i>A. caviae</i>	>256	>128	>128	>256	>256	>64	>128	>128	128	16	>256	16	1
<i>E. coli</i> C600	4	1	1	0.12	0.12	0.25	<0.06	0.25	0.5	0.06	2	0.12	0.12
Transconjugant	>256	>128	32	64	128	32	0.5	128	128	8	16	8	1
<i>E. coli</i> JM109	4	1	1	0.12	0.12	0.25	<0.06	0.25	0.5	0.06	2	0.12	0.12
Transformant	>256	>128	32	128	>256	32	0.5	>128	128	8	16	8	1

AMK, Amikacin; AMP, ampicillin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; IMP, imipenem; LVX, levofloxacin; PIP, piperacillin; TZP, piperacillin–tazobactam.

*The concentration of TZP was tested at a fixed concentration of 4 µg ml⁻¹.

Aeromonas jandaei and *Aeromonas schubertii*) are potentially pathogenic for humans. Over the past decade, gastroenteritis, primary bacteraemia and wound infections are the three major clinical manifestations of *Aeromonas* infections. *A. hydrophila* was the most common pathogen (52 %), followed by *A. sobria* (24 %) and *A. caviae* (23 %) (Wu *et al.*, 2007). *Aeromonas* sp. organisms rarely cause respiratory tract infections. To date, there have been only nine cases reported in English-language articles, all caused by *A. hydrophila* (Janda & Abbott, 1998). *A. caviae* was first identified in 1974 in a patient with diarrhoea as *Aeromonas punctata*, and was then renamed as *A. caviae* in 1988. The *A. caviae* isolate in this study was identified by using a MicroScan W/A system and also by 16S rRNA gene sequencing. To the best of our knowledge, the present report is the first case of pulmonary infection due to *A. caviae*. *Aeromonas* have been isolated from freshwater, and tap water, soil and marine animals. We suspected that ingestion of seafood may have been the source of infection in this case. The patient denied ever going into the sea or other direct exposure to estuarine water or seawater; but his relatives stated he enjoyed eating seafood and often bought seafood from the local supermarket. It is not clear how the seafood was stored and what actual freeze-drying process was used, although it seems that it was commercially prepared.

Data presented in our study document that *A. caviae* produced three β -lactamases (TEM-1 β -lactamase, CTX-M-3 ESBL and MOX-4 AmpC β -lactamase). ESBLs are plasmid-encoded, constitutively expressed enzymes that are mostly derived from the older, narrower-spectrum β -lactamases TEM-1, TEM-2 and SHV-1 (Winokur *et al.*, 2001). In our study, CTX-M-3 ESBL was detected in the clinical isolate but was not detected in the transconjugant with specific PCR primers. Plasmid-mediated AmpC β -lactamases have been discovered most frequently in naturally AmpC-negative species, but recently they also have been found in other AmpC-producing species, such as *Enterobacteriaceae*, *Aeromonas* and *Pseudomonas*. It is believed that such β -lactamases arose through the transfer of chromosomal AmpC genes to plasmids. In this study, the MOX-4 β -lactamase from *A. caviae* with a pI of 9.0 was transferred by conjugation and associated with a 7.3 kb plasmid detected by Southern blot hybridizations. Its transconjugant and transformant revealed typical AmpC enzyme properties, especially resistance to cefoxitin and susceptibility to cefepime (Table 1). Higher MICs were obtained with the transformant, probably due to more copies of the MOX-4 AmpC-encoding gene. In addition, the clinical strain was resistant to fluoroquinolones and amikacin, while lower MICs were shown in the transconjugant and transformant, suggesting the presence of other resistance genes co-transferred with the MOX-4-encoding gene. Plasmid-mediated AmpC enzymes may confer broad resistance to all β -lactams other than carbapenems, and hence pose a major therapeutic challenge. Carbapenems are active against strains that harbour these enzymes, especially

in isolates that coproduce AmpC and ESBL (Park *et al.*, 2005). In our study, *A. caviae*, which harboured β -lactamase genes encoding MOX-4, CTX-M-3 and TEM-1 simultaneously, showed broad resistance to all β -lactams other than imipenem.

In summary, this report is interesting and important because it describes what we believe to be the first case of severe pneumonia caused by *A. caviae*. Moreover, to the best of our knowledge, this is the first time that genes encoding MOX-4, CTX-M-3 and TEM-1 β -lactamases have been detected simultaneously in an *A. caviae* isolate from a patient.

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