Development of imipenem resistance in an *Aeromonas veronii* biovar *sobria* clinical isolate recovered from a patient with cholangitis

Javier Sánchez-Céspedes,¹ Maria José Figueras,² Carmen Aspiroz,³ Maria José Aldea,³ Miguel Toledo,³ Anabel Alperí,² Francesc Marco¹ and Jordi Vila¹

Correspondence

Jordi Vila
jvila@ub.edu

¹Servei de Microbiologia, Centre de Diagnòstic Biomèdic, Hospital Clínic, IDIBAPS, Facultat de Medicina, Universitat de Barcelona, Villarroel 170, 08036 Barcelona, Spain

²Unitat de Microbiologia, Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, Sant Llorenç 21, 43201 Reus, Spain

³Unidad de Microbiología, Hospital Royo Villanova, Avda San Gregorio 30, 59915 Zaragoza, Spain

Several imipenem-susceptible and -resistant *Aeromonas veronii* biovar *sobria* isolates with different morphologies and antimicrobial susceptibilities recovered from bile samples of a patient with cholangitis were analysed. These isolates belonged to the same clone and the imipenem-resistant strains showed overexpression of the *imiS* gene, encoding a chromosomal carbapenemase. These results should make clinicians aware of the possible emergence of multidrug-resistant *A. veronii* biovar *sobria*, perhaps as a consequence of previous treatment of a urinary tract infection with amoxicillin plus clavulanic acid.

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INTRODUCTION

Members of the genus *Aeromonas* are causative agents of clinical diseases ranging from gastroenteritis and wound infections to severe septicaemia (Figueras, 2005; Figueras *et al.*, 2000a; Janda & Abbot, 1998). Despite the fact that 14 of the species included in the genus have been associated with human disease, *Aeromonas veronii* biovar *sobria*, *Aeromonas caviae* and *Aeromonas hydrophila* are the prevailing species, accounting for over 90 % of the clinical isolates (Figueras, 2005; Figueras *et al.*, 2000a; Janda & Abbot, 1998). *Aeromonas* species are an important cause of hepatobiliary infections in patients with impaired biliary drainage due to stone formation, malignancy or surgical procedures, including liver transplantation (Figueras, 2005; Janda & Abbot, 1998; Clark & Chenoweth, 2003; Mencacci *et al.*, 2003). *Aeromonas* species have been reported to be susceptible *in vitro* to a variety of antimicrobial agents (Ko *et al.*, 1996, 2003; Koehler & Ashdown, 1993; Maluping *et al.*, 2005; Sader & Jones, 2005; Vila *et al.*, 2002, 2003), including broad-spectrum cephalosporins, aminoglycosides (Rossolini *et al.*, 1996), chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole (Koehler & Ashdown, 1993), aztreonam and fluoroquinolones (Ko *et al.*, 1996). However, increases in resistance to broad-spectrum cephalosporins have been reported in clinical *Aeromonas* isolates (Ko *et al.*, 1996).

Resistance to β -lactams is a potential problem for the therapeutic management of extraintestinal diseases, especially when dealing with species such as *A. veronii* biovar *sobria*, which has been implicated in important life-threatening infections (Figueras, 2005; Ouderkirk *et al.*, 2004; Shiina *et al.*, 2004). The genetic profiles of β -lactamases of this species have been well characterized (Stunt *et al.*, 1998; Walsh *et al.*, 1997), as has its ability to produce three enzymes, a Bush group 2d penicillinase, a group 1 cephalosporinase and a metallo- β -lactamase (ImiS) (Tsai *et al.*, 2006), which are all chromosome-encoded, inducible (Ko *et al.*, 1998) and co-regulated by a common pathway (Alksne & Rasmussen, 1997; Walsh *et al.*, 1995). This ImiS metallo- β -lactamase belongs to the molecular class B or group 3 and is 98 % identical to CphA, a metallo- β -lactamase found in *A. hydrophila* (Hernandez Valladares *et al.*, 2000). ImiS is a monomeric enzyme able to hydrolyse carbapenems such as imipenem and meropenem but with poor activity against other β -lactams (Walsh *et al.*, 1996, 1998).

Despite the extensive work performed on carbapenem resistance mediated by chromosomally located carbapenemases in *Aeromonas* species, carbapenem-resistant *A. veronii* biovar *sobria* isolates selected in clinical practice have not been described to our knowledge. In the present study, we report the *in vivo* development of imipenem resistance in isolates of *A. veronii* biovar *sobria* recovered from bile samples of a patient with cholangitis. The

Abbreviation: ERIC-PCR, enterobacterial repetitive intergenic consensus PCR.

emergence of imipenem resistance was associated with overexpression of the *imiS* gene.

METHODS

Patient. The patient was an 88-year-old woman with a history of diabetes mellitus, hypertension and Alzheimer's disease, admitted to hospital from 13 to 18 June 2007 due to a severe urinary tract infection. She was directly treated intravenously (i.v.) with ciprofloxacin (two doses of 200 mg). Treatment was changed to amoxicillin-clavulanic acid (1 g every 8 h i.v.) on days 2 and 3, and an oral treatment (875/125 g) with the same antibiotic was established on days 4 and 5. No cultures were performed. The patient was released and instructed to complete the same treatment up to 15 days (until 30 June). The patient was again admitted on 4 July with signs of general malaise, vomiting, fever and altered mental state. Physical examination revealed jaundice and hypotension. Blood tests showed hyperglycaemia, leukocytosis, hyperbilirubinaemia and increased alkaline phosphatase concentration (490.6 IU l^{-1} , normal rate $45\text{--}122 \text{ IU l}^{-1}$). Abdominal ultrasonography revealed the presence of stones in the bile ducts and cholecystitis. A laparoscopic cholecystectomy and bile duct clearance were performed and a pig-tail catheter was placed. The patient was diagnosed with acute cholelithiasis secondary to choledocolithiasis, and remained in the hospital for 20 days. She was initially treated for 2 days with amoxicillin plus clavulanic acid, but treatment was changed for 10 days to piperacillin-tazobactam on confirmation of the diagnosis. A first bile sample was obtained on placement of the pig-tail catheter and the second was collected from the biliary drainage 5 days later. Both samples were processed on solid and in liquid media. Nine oxidase-positive isolates with different colony morphologies and phenotypic characteristics (three from the first sample and six from the second) were identified with API 20E (bioMérieux) as *A. hydrophila*.

The isolates were sent to two different reference laboratories for further studies. The nine isolates were identified on the basis of the 16S rDNA-RFLP (Figueras *et al.*, 2000b).

Susceptibility testing. Antimicrobial susceptibility was determined twice at two different laboratories using different methods: (i) MicroScan Combo Gramnegative panels (Dade MicroScan); (ii) the Kirby-Bauer method to test the susceptibility to ampicillin and cephalothin following the Clinical and Laboratory Standards Institute criteria (CLSI, 2006). The MIC of imipenem was also determined in the absence and the presence of 10 mM EDTA by Etest (Biodisk).

Molecular typing. To elucidate the epidemiological relationship of these strains, the nine isolates were genotyped at two different laboratories using chromosomal DNA analysis by digestion with low frequency restriction enzymes and then PFGE and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) following the conditions previously described (Gallardo *et al.*, 1999; Soler *et al.*, 2003).

RT-PCR. The RNA was purified free of DNA and RT-PCR was performed following the procedure previously described (Ruiz *et al.*, 2007). The primers used to amplify a 400 bp region of the *imiS* gene were ImiS-1 (5'-GTCTATTCGGGGCCAAGGGAGTG-3') and ImiS-2 (5'-GCTGGTCGGGAAGTAAACAAAGA-3'). As an internal control for the reaction, the 16S rRNA gene was used. The primers used to amplify this gene were 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 806R (5'-GGACTACCAAGGGTATCTAAT-3') amplifying a region of 798 bp. After several trials, the final number of cycles was 16 for each of the two genes.

RESULTS AND DISCUSSION

The isolates were genetically and biochemically identified as *A. veronii* biovar *sobria*. Both typing methods, PFGE and ERIC-PCR, demonstrated that the nine isolates had identical patterns, confirming that they belonged to the same clone (Fig. 1). The antimicrobial susceptibility patterns are illustrated in Table 1. The nine clinical isolates were susceptible to ciprofloxacin, showing a MIC lower than $0.125 \mu\text{g ml}^{-1}$. All the isolates except 767-4 and 767-6 with MICs lower than $4 \mu\text{g ml}^{-1}$ were resistant to ampicillin. This is an interesting finding since most *Aeromonas* species, with the exception of a few strains and *Aeromonas trota*, are resistant to ampicillin (Maluping *et al.*, 2005; Abbott *et al.*, 2003). Overall, a low

Fig. 1. PFGE (a) and ERIC-PCR (b) of the isolates. Lanes: 1, 459-1; 2, 459-2; 3, 459-3; 4, 767-1; 5, 767-2; 6, 767-3; 7, 767-4; 8, 767-5; 9, 767-6; M, DNA molecular mass markers.

Table 1. MICs ($\mu\text{g ml}^{-1}$) of different antibacterial agents for *A. veronii* biovar sobria

Antimicrobial agent	459-1	459-2	459-3	767-1	767-2	767-3	767-4	767-5	767-6
Cefazolin	16	>16	≤ 8	>16	>16	>16	≤ 8	>16	≤ 8
Cefoxitin	≤ 8	≤ 8	≤ 8	≤ 8	≤ 8	≤ 8	≤ 8	>16	≤ 8
Ceftriaxone	16	≤ 8	≤ 8	16	16	≤ 8	≤ 8	≤ 8	≤ 8
Sulbactam–ampicillin	>16	>16	>16	>16	>16	>16	16	>16	<8
Gentamicin	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4
Trimethoprim–sulfamethoxazole	$\leq 2/38$	$\leq 2/38$	$\leq 2/38$	$\leq 2/38$	$\leq 2/38$	$\leq 2/38$	$\leq 2/38$	$\leq 2/38$	$\leq 2/38$
Ticarcillin	>64	>64	>64	>64	>64	>64	>64	>64	≤ 16
Tobramycin	≤ 4	≤ 4	8	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4
Amikacin	≤ 8	>16	>16	≤ 8	≤ 8	≤ 8	≤ 8	≤ 8	≤ 8
Piperacillin	>64	>64	>64	>64	>64	>64	64	>64	≤ 16
Piperacillin–tazobactam	64	>64	>64	>64	>64	>64	64	>64	≤ 16
Amoxicillin–clavulanic acid	8	16	16	>16	>16	>16	$\leq 4/2$	>16	$\leq 4/2$
Ciprofloxacin	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12
Ofloxacin	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5
Ampicillin	>16	>16	>16	>16	>16	>16	≤ 4	>16	≤ 4
Cefotaxime	≤ 0.5	≤ 0.5	≤ 0.5	1	1	1	≤ 0.5	≤ 0.5	≤ 0.5
Ceftazidime	2	8	≤ 1	2	2	2	≤ 1	≤ 1	2
Cefepime	≤ 1	2	≤ 1	8	8	8	≤ 1	≤ 1	≤ 1
Aztreonam	2	2	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
Imipenem*	<1	32	<1	32	32	32	32	32	32
Imipenem + EDTA*	<1	<1	<1	<1	<1	<1	ND	ND	ND
Cephalothin†	R	R	S	R	R	R	S	R	S
Ampicillin†	R	R	R	R	R	R	S	R	S

ND, Not determined.

*MIC of imipenem determined by Etest.

†Antimicrobial susceptibility determined using the disc diffusion method.

reproducibility and changeable susceptibility were observed for almost all β -lactam antibiotics with the exception of aztreonam and cefotaxime. The susceptibility of the isolates to cephalothin was also variable, with isolates 459-1, 459-2, 767-1, 767-2, 767-3 and 767-5 showing resistance to this antimicrobial agent. In fact, susceptibility to the latter antibiotic is one of the specific characteristics of *A. veronii* biovar sobria (Abbott *et al.*, 2003); therefore, the variability observed compromises the classical use of this phenotypic character for species delineation (Abbott *et al.*, 2003). Seven isolates were also resistant to imipenem (459-2, 767-1, 767-2, 767-3, 767-4, 767-5 and 767-6) with MICs of $32 \mu\text{g ml}^{-1}$. In the presence of 10 mM EDTA, the MIC of imipenem decreased to $<1.0 \mu\text{g ml}^{-1}$, suggesting the implication of a metallo- β -lactamase in the resistance to imipenem. Resistance to the latter drug is not uncommon. In a recent study on *Aeromonas* bacteraemia in patients with haematological malignancies, 35.6% of the strains were found to be resistant to imipenem (Tsai *et al.*, 2006).

Isolates 459-1 and 767-5 were selected to analyse the role of the *imiS* gene in the resistance to imipenem. The imipenem-resistant isolate 767-5 showed a greater expression of the *imiS* gene when compared to the expression in the imipenem-susceptible isolate 459-1 (Fig. 2). Meanwhile, the expression of the 16S rRNA gene was equivalent in both isolates. This imipenem-resistant isolate

demonstrated overproduction of ImiS and showed a resistant phenotype also characterized by resistance to cephalotin, suggesting an overexpression of the chromosomal cephalosporinase. In fact, the three chromosomally located β -lactamases found in *A. veronii* biovar sobria are simultaneously overexpressed in mutants (Walsh *et al.*, 1995).

Aeromonas species have been involved in a broad spectrum of human infections (Figueras, 2005; Janda & Abbot, 1998). It has been reported that this group of bacteria has been implicated in 12% of hepatobiliary infections, among which the most common is cholangitis (Figueras, 2005; Janda & Abbot, 1998; Clark & Chenoweth, 2003). Previous cases of acute cholecystitis produced by *A. veronii* without septicemia, as in our patient, as well as bacteraemia secondary to biliary tract infection have been described (Figueras, 2005; Maluping *et al.*, 2005). It has been speculated that primary undiagnosed biliary infection may induce *Aeromonas* septicemia (Maluping *et al.*, 2005).

The emergence of resistance among *Aeromonas* species has been accelerated by the clinical use of antibiotics. To our knowledge, carbapenem-resistant *A. veronii* biovar sobria strains selected in clinical practice have not been reported to date. In the present study, we have shown the presence of several imipenem-susceptible and -resistant *A. veronii*

Fig. 2. RT-PCR of the *imiS* gene from isolates 767-5 (lane 1) and 459-1 (lane 2). Lane 3, PCR of the RNA as a control of DNA contamination. Lanes 4 and 5, RT-PCR of the 16S rRNA gene from isolates 767-5 and 459-1, respectively. Lane 6, PCR of the RNA as a control of DNA contamination. Lane M, DNA molecular mass marker.

biovar *sobria* isolates that belonged to the same clone in bile samples of a patient with cholangitis. This fact led us to consider that the development of resistance to imipenem in this patient was probably due to the antimicrobial pressure over the original imipenem-susceptible bacteria. As in our study, acquired resistance to tetracycline and fluoroquinolone has recently been demonstrated in strains of *Aeromonas*, possibly associated with antimicrobial therapy (Maluping *et al.*, 2005). These results should make clinicians aware of the possible emergence of multidrug-resistant *A. veronii* biovar *sobria*, which, in our study, is probably a consequence of previous treatment of urinary tract infection with amoxicillin plus clavulanic acid.

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