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FEMS Immunology and Medical Microbiology 32 (2002) 243–247

FEMS Immunology and Medical Microbiology

www.fems-microbiology.org

Potential virulence and antimicrobial susceptibility of *Aeromonas popoffii* recovered from freshwater and seawater

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Received 24 November 2001; accepted 28 November 2001

First published online 14 January 2002

Abstract

Aeromonas popoffii is the most recent species within the genus *Aeromonas* described from freshwater. In our study this species was also recovered from this habitat and for the first time from seawater. Most of the virulence factors known in *Aeromonas* spp. (aerolysin/hemolysin, serine protease, lipases and DNases) were highly prevalent in this species. Third-generation cephalosporins and quinolones were the most active antimicrobial agents against *A. popoffii*.

Keywords: *Aeromonas popoffii*; Virulence factor; Water; Antimicrobial susceptibility

1. Introduction

Aeromonas spp. are autochthonous inhabitants of aquatic environments and are considered causative agents of human gastrointestinal and, to a lesser extent, extraintestinal infections [1]. Currently, the genus *Aeromonas* comprises 14 species, although its taxonomy remains confusing [2]. *Aeromonas popoffii* is one of the most recently described species, and only a few strains have been recovered up to now [3,4]. In an extensive study carried out in our laboratory, some strains showed an atypical biochemical behavior [5] and could only be identified as *A. popoffii* by using a 16S rDNA restriction fragment length polymorphism (RFLP) technique [6]. However, due to the limited number of strains isolated to date [3,4], there are no data on the incidence and potential virulence of this new species. Pathogenic species of *Aeromonas* have been found to possess several virulence factors that may be involved in infection mechanisms [7]. Among them, aerolysin, a pore-forming cytolysin, is the most studied [7]. The glycerophospholipid:cholesterol acyltransferase (GCAT) is an extracellular lipase only so far investigated in fish furunculosis caused by *Aeromonas salmonicida* [8,9]. Both aerolysin and GCAT are secreted as proenzymes, and the latter has been found to be activated by serine protease [10], which is also considered a virulence factor. Other virulence factors common in *Aeromonas* are extracellular lipases (*lip*, *lip*H3, *pla* and *plc*), which may alter the plasma membrane of the host, and DNases, although little is known about their role in *Aeromonas* pathogenesis [7]. The aim of this study was to investigate the incidence of *A. popoffii* in freshwater and seawater samples from Catalonia (northeast Spain). Since antimicrobial susceptibility patterns for this species have only been investigated in seven strains [3], we considered it important to elucidate this aspect in all available strains, i.e. in those previously reported, along with eight strains from Switzerland [4] and in those isolated by us in Catalonia, adding 10 new antimicrobial agents never before investigated in *A. popoffii*. In addition, the presence of the above-mentioned virulence genes and their associated phenotypic activity were evaluated in the above-mentioned strains.

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0928-8244/02/$22.00
PII: S0928-8244(01)00303-0

2. Materials and methods

A total of 113 water samples, including freshwater and seawater, were investigated for the presence of *Aeromonas* spp. using the methodology described in a previous paper [5]. Of three typical colonies selected from ampicillin dextrin agar, only one isolate representing a species was considered from each sample for incidence calculation. Strains were identified genetically to species level (Table 1) by the 16S rDNA RFLP technique [6,11].

Sequences from GenBank (GB) and European Molecular Biology Laboratory (EMBL) were used to design primers (Primer Designer 3 software, Scientific Educational Software, Durham, NC, USA) for each group of genes (Table 2) after alignment with a CLUSTAL W program [12]. The gene accession numbers for aerolysin/hemolysin were those of the 10 sequences used by Kingombe et al. [13], while the others were: serine protease, GB AF159142 [14] and EMBL X67043 [15]; GCAT, EMBL X70686 [16] and X07279 [17]; lipases, GB U63543 [18], S65123 [19], AF092033 [20] and U14011 [21]; DNase, GB AF004392 (unpublished), L78266 [22] and M99491 [23]. PCR amplifications were performed with 1.2–2 μg of DNA as previously described [11] and using conditions shown in Table 2. Selected primers were compared to all sequences deposited in GB and EMBL databases to ensure their specificity. PCR products were confirmed by sequencing with an ABI-Prism™ 310.

The associated phenotypic activities investigated were: β-hemolysis, assayed on TSA agar (Difco) containing 5% sheep or human blood agar, at 20 and 37°C; DNase activity, assessed at 37°C on DNase agar; lipase activity, tested at 37°C on TSA plates containing 0.5% tributyrin emulsified in the presence of 0.2% Triton X-100; and the serine protease activity, detected at 30°C by the azocasein method as previously described [24].

Antibacterial susceptibility testing was performed using Combo Urine 1S panels containing 26 antibiotics (Dade-MicroScan).

3. Results and discussion

A total of 102 (90.3%) water samples were positive for *Aeromonas* spp., 72 from freshwater (reservoirs and rivers) and 30 from seawater. The species *A. popoffii* was present in 8.8% of the samples studied. From a total of 145 strains of *Aeromonas* recovered, 11 belonged to *A. popoffii* (eight from freshwater and three from seawater at bathing areas), representing 8% of strains isolated from freshwater and 6.8% from seawater (Table 1). In freshwater the most frequently recovered species were *A. veronii*, *A. hydrophila* and *A. caviae*, while in seawater *A. caviae* was the most abundant (Table 1). Although it has been argued that *A. popoffii* is unable to grow in the presence of NaCl [3], three of the isolates studied were from seawater with a high salinity rate. This is the first report of the species in this habitat.

Most of the known genes encoding virulence factors in *Aeromonas* were also present in the strains of *A. popoffii* tested (Table 3). All strains had the genes for DNases, GCAT and lipases and showed DNase and lipase activity. Although the role of DNases is unknown in *Aeromonas* pathogenicity, these genes are involved in *Streptococcus* infections [25], and are considered important for bacterial nutrition [7]. GCAT had only been previously investigated by PCR in isolates of *A. salmonicida* [9]. Practically all *A. popoffii* strains (96%) presented the serine protease genes, and 69% showed protease activity with the azocasein test. All strains isolated in Switzerland and Scotland–Belgium had the genes encoding aerolysin/hemolysin, while they were only present in 73% of the isolates from Spain. Aerolysin/hemolysin is commonly found in *A. hydrophila* strains that cause bacteremia [26]. Additionally, it has been observed that deletion mutants for the *aer*A gene (encoding aerolysin) are less virulent than parental strains [27]. Our study demonstrated that the occurrence of the aerolysin/hemolysin genes in *A. popoffii* (92%) is similar to that in common clinical species (e.g. *A. veronii* bt sobria), and also in the fish pathogen *A. salmonicida* [13]. In the original description, the isolates of *A. popoffii* were tested in sheep blood at 37°C and considered non-β-hemolytic [3]. However, in our study strains of *A. popoffii* were clearly β-hemolytic, although dependent on the temperature and the type of blood used (Table 3), which agrees with data on other *Aeromonas* spp. [28].

To date, the patterns of antibiotic susceptibility of *A. popoffii* have only been investigated in the seven strains used to describe the species [3,29]. In this study, we have tested all available strains against a total of 26 antibiotics, 10 of which have never been tested against this species (piperacillin–tazobactam, cefalotin, ceftibuten, merope-

Table 1
Distribution of *Aeromonas* species isolated from freshwater and seawater

	A. hydrophila	*A. bestiarum*	*A. salmonicida*	*A. caviae*	*A. media*	*A. sobria*	*A. veronii*	*A. jandaei*	*A. popoffii*	*A. schubertii*	Total
Freshwater	14[a]	8	3	13	7	1	44	3	8	–	101
	(13.9)[b]	(7.9)	(2.9)	(12.9)	(6.9)	(1.0)	(43.6)	(2.9)	(8.0)		(100)
Seawater	2	6	5	21	4	–	1	1	3	1	44
	(4.5)	(13.6)	(11.4)	(47.7)	(9.1)		(2.3)	(2.3)	(6.8)	(2.3)	(100)

[a]Number of strains.
[b]Percentage (incidence).

Table 2
Primers designed and PCR conditions for the detection of virulence genes

Gene	Primers[a]	PCR conditions		Cycles
		°C	min	
Aerolysin/hemolysin	aer-f: 5′-CCTATGGCCTGAGCGAGAAG-3′	95	3	35
	aer-r: 5′-CCAGTTCCAGTCCCACCACT-3′	94	1	
		56	1	
		72	1	
		72	5	
GCAT	GCAT-f: 5′-CTCCTGGAATCCCAAGTATCAG-3′	95	3	35
	GCAT-r: 5′-GGCAGGTTGAACAGCAGTATCT-3′	94	1	
		56	1	
		72	1	
		72	5	
Serine protease	Serine-f: 5′-CACCGAAGTATTGGGTCAGG-3′	95	3	35
	Serine-r: 5′-GGCTCATGCGTAACTCTGGT-3′	94	1	
		60	1	
		72	1	
		72	5	
DNase	Exu-f: 5′-(A/G)GACATGCACAACCTCTTCC-3′	95	3	35
	Exu-r: 5′-GATTGGTATTGCC(C/T)TGCAA(C/G)-3′	94	1	
		54	1	
		72	1	
		72	5	
Lipases	lip-f: 5′-CA(C/T)CTGGT(T/G)CCGCTCAAG-3′	95	3	35
	lip-r: 5′-GT(A/G)CCGAACCAGTCGGAGAA-3′	94	1	
		56	1	
		72	1	
		72	5	

[a]The selected primers produced an amplicon of 431 bp for aerolysin/hemolysin; 350 bp for serine protease; 237 bp for GCAT; 247 bp for lipases; and 323 bp for DNase.

nem, fosfomycin, pipemidic acid, colistin, ampicillin–sulbactam, amoxycillin–clavulanic acid and trimethoprim–sulfamethoxazole). As expected, all *A. popoffii* strains were resistant to ampicillin, a characteristic trait for most of the species of the genus [30]. By contrast, all the strains were susceptible to piperacillin, piperacillin–tazobactam, cefalotin, cefuroxime, ceftibuten, cefotaxime, ceftazidime, aztreonam, imipenem, meropenem, gentamicin, amikacin, tobramycin, fosfomycin, pipemidic acid, ciprofloxacin, norfloxacin, colistin and trimethoprim–sulfamethoxazole. 65% of the strains were resistant to ampicillin–sulbactam, 54% to cefazolin, 42% to ticarcillin, 27% to amoxycillin–clavulanic acid, 8% to cefoxitin, and 4% to tetracycline. The combination of penicillin and a β-lactamase inhibitor proved to be useful for reducing the *A. popoffii* resistance to ampicillin. The resistance to β-lactam antibiotics in the genus *Aeromonas* has been considered dependent on chromosome-mediated β-lactamases [31–33]. However, it is not clear if clavulanic acid shows intrinsic activity against *Aeromonas*, or if it inhibits the ac-

Table 3
Presence of virulence genes and associated phenotypic activity in 26 strains of *A. popoffii*

Origin	Number of strains	Extracellular lipases			Serine protease		Extracellular nucleases		Aerolysin/hemolysin				
		GCAT presence	*lip*, *plc*1, *lip*H3, *pla* presence	Tributyrin assay	Gene presence	Azocasein test	Gene presence	DNase assay	Gene presence	β-Hemolysis			
										Sheep blood		Human blood	
										20°C	37°C	20°C	37°C
Scotland–Belgium	7	7[a] (100)[b]	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)	2 (28.6)	0 (0)	6 (85.7)	4 (57.1)
Switzerland	8	8 (100)	8 (100)	8 (100)	8 (100)	3 (37.5)	8 (100)	8 (100)	8 (100)	0 (0)	0 (0)	7 (87.5)	4 (50)
Spain	11	11 (100)	11 (100)	11 (100)	10 (90.9)	8 (72.7)	11 (100)	11 (100)	8 (73)	2 (18)	0 (0)	6 (55)	5 (45)
Total	26	26 (100)	26 (100)	26 (100)	25 (96)	18 (69)	26 (100)	26 (100)	23 (88)	4 (15)	0 (0)	19 (73)	13 (50)

[a]Number of strains positive for the test.
[b]Percentage.

tivity of some of the chromosome-mediated β-lactamases. The resistance to cephalosporins shown in our study agrees with that reported for the species *A. jandaei*, *A. schubertii*, *A. trota* and *A. veronii* bt veronii [30] and also with that previously reported for *A. popoffii* [3,29]. Our results confirm that third-generation cephalosporins are the most active against *A. popoffii*, followed by second- and first-generation cephalosporins [29]. Resistance to quinolones has been described in clinical isolates of *Aeromonas* [34], however they were active against *A. popoffii*.

We can conclude that, although *A. popoffii* has never been reported from clinical samples, it has been found both in drinking water and in freshwater that is a source of production of drinking water. Since drinking water is considered a source of infection [35], some European countries, The Netherlands [36] and recently also the United States Environmental Protection Agency have added *Aeromonas* to the list of emerging pathogens of concern. The fact that *A. popoffii* possesses most of the virulence genes present in other *Aeromonas* pathogenic species indicates that its potential pathogenesis should not be underestimated. However, commercial identification systems are unable to identify this species, which may hamper establishing its true incidence.

Acknowledgements

This work has been supported by Grants FIS 99/0944 and FIS 96/0579 from the Spanish Ministry of Health, CIRIT (SGR 1999/00103), Generalitat Valenciana (GV98-21-05), Fundació Ciència i Salut and a fellowship from the Universitat Rovira i Virgili. We would like to thank the Colección Española de Cultivos Tipo (CECT), the Belgium Co-ordinated Collection of Micro-organisms (LMG) and Dr. Demarta for kindly providing isolates.

References

[1] Janda, J.M. and Abbott, S.L. (1998) Evolving concepts regarding the genus *Aeromonas*: An expanding panorama of species, disease presentations, and unanswered questions. Clin. Infect. Dis. 27, 332–344.
[2] Figueras, M.J., Guarro, J. and Martínez-Murcia, A. (2000) Clinically relevant *Aeromonas* species. Clin. Infect. Dis. 30, 988–989.
[3] Huys, G., Kämpfer, P., Altwegg, M., Kersters, I., Lamb, A., Coopman, R., Luthy-Hottenstein, J., Vancanneyt, M., Janssen, P. and Kersters, K. (1997) *Aeromonas popoffii* sp. nov., a mesophilic bacterium isolated from drinking water production plants and reservoirs. Int. J. Syst. Bacteriol. 47, 1165–1171.
[4] Demarta, A., Tonolla, M., Caminada, A.-P., Ruggeri, N. and Peduzzi, R. (1999) Signature region within the 16S rDNA sequences of *Aeromonas popoffii*. FEMS Microbiol. Lett. 172, 239–246.
[5] Borrell, N., Figueras, M.J. and Guarro, J. (1998) Phenotypic identification of *Aeromonas* genomospecies from clinical and environmental sources. Can. J. Microbiol. 44, 7–12.
[6] Figueras, M.J., Soler, L., Chacón, M.R., Guarro, J. and Martínez-Murcia, A.J. (2000) Extended method for discrimination of *Aeromonas* spp. by 16S rDNA-RFLP. Int. J. Syst. Evol. Microbiol. 50, 2069–2073.
[7] Pemberton, J.M., Kidd, S.P. and Schmidt, R. (1997) Secreted enzymes of *Aeromonas*. FEMS Microbiol. Lett. 152, 1–10.
[8] Lee, K.K. and Ellis, A.E. (1990) Glycerophospholipid:cholesterol acyltransferase complexed with lipopolysaccharide (LPS) is a major lethal exotoxin and cytolysin of *Aeromonas salmonicida*: LPS stabilizes and enhances toxicity of the enzyme. J. Bacteriol. 172, 5382–5393.
[9] Høie, S., Dalsgaard, I., Aase, I.L., Heum, M., Thornton, J.M. and Powell, P. (1999) Polymerase chain reaction (PCR)-based typing analysis of atypical isolates of the fish pathogen *Aeromonas salmonicida*. Syst. Appl. Microbiol. 22, 403–411.
[10] Howard, S.P., MacIntyre, S. and Buckley, J.T. (1996) Toxins. In: The Genus *Aeromonas* (Austin, B., Altwegg, M., Gosling, P.J. and Joseph, S., Eds.), pp. 267–286. John Wiley and Sons, Chichester.
[11] Borrell, N., Acinas, S.G., Figueras, M.J. and Martínez-Murcia, A. (1997) Identification of *Aeromonas* clinical isolates by restriction fragment length polymorphism of PCR-amplified 16S rRNA genes. J. Clin. Microbiol. 35, 1671–1674.
[12] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
[13] Kingombe, C.I.B., Huys, G., Tonolla, M., Albert, M.J., Swings, J., Peduzzi, R. and Jemmi, T. (1999) PCR detection, characterization, and distribution of virulence genes in *Aeromonas* spp. Appl. Environ. Microbiol. 65, 5293–5302.
[14] Cascon, A., Fregeneda, J., Allen, M., Yugueros, J., Temprano, A., Hernanz, C., Sanchez, M., Rodriguez Aparicio, L. and Naharro, G. (2000) Cloning, characterization and insertional inactivation of a major extracellular serine protease gene with elastolytic activity for *Aeromonas hydrophila*. J. Fish Dis. 23, 49–59.
[15] Whitby, P.W., Landon, M. and Coleman, G. (1992) The cloning and nucleotide sequence of the serine protease gene (*aspA*) of *Aeromonas salmonicida* ssp. *salmonicida*. FEMS Microbiol. Lett. 99, 65–72.
[16] Nerland, A.H. (1996) The nucleotide sequence of the gene encoding GCAT from *Aeromonas salmonicida* ssp. *salmonicida*. J. Fish Dis. 19, 145–150.
[17] Thornton, J., Howard, S.P. and Buckley, J.T. (1988) Molecular cloning of a phospholipid-cholesterol acyltransferase from *Aeromonas hydrophila*. Sequence homologies with lecitin-cholesterol acyltransferase and other lipases. Biochim. Biophys. Acta 959, 153–159.
[18] Chuang, Y.C., Chiou, S.F., Su, J.H., Wu, M.I. and Chang, M.C. (1997) Molecular analysis and expression of the extracellular lipase of *Aeromonas hydrophila* MCC-2. Microbiology 143, 803–812.
[19] Anguita, J., Rodriguez-Aparicio, L.B. and Navarro, G. (1993) Purification, gene cloning, amino acid sequence analysis, and expression of an extracellular lipase from an *Aeromonas hydrophila* human isolate. Appl. Environ. Microbiol. 59, 2411–2417.
[20] Merino, S., Aguilar, A., Nogueras, M.M., Regue, M., Swift, S. and Tomás, J.M. (1999) Cloning, sequencing, and role in virulence of two phospholipases (A1 and C) from mesophilic *Aeromonas* sp. serogroup 0:34. Infect. Immun. 67, 4008–4013.
[21] Ingham, A.B. and Pemberton, J.M. (1995) A lipase of *Aeromonas hydrophila* showing nonhemolytic phospholipase C activity. Curr. Microbiol. 31, 28–33.
[22] Dodd, H. and Pemberton, J.M. (1999) The gene encoding periplasmic deoxyribonuclease for *Aeromonas hydrophila*. FEMS Microbiol. Lett. 173, 41–46.
[23] Chang, M.C., Chang, S.Y., Chen, S.I. and Chuang, S.M. (1992) Cloning and expression in *Escherichia coli* of the gene encoding an extracellular deoxyribonuclease (DNase) from *Aeromonas hydrophila*. Gene 94, 175–180.
[24] Swift, S., Lynch, M.J., Leigh, F., Kirke, D.F., Tomás, J.M., Stewart, G. and Williams, P. (1999) Quorum sensing-dependent regulation

and blockade of exoprotease production in *Aeromonas hydrophila*. Infect. Immun. 67, 5192–5199.
[25] Podbielski, A., Zarges, I., Flosdorff, A. and Weber-Heynemann, J. (1996) Molecular characterization of a major serotype M49 group A Streptococcal DNase gene (*sda*D). Infect. Immun. 64, 5349–5356.
[26] Vadivelu, J., Puthucheary, S.D., Phipps, M. and Chee, Y.W. (1995) Possible virulence factors involved in bacteraemia caused by *Aeromonas hydrophila*. J. Med. Microbiol. 42, 171–174.
[27] Chakraborty, T., Huhle, B., Hof, H., Bergbauer, H. and Goebel, W. (1987) Marker-exchange mutagenesis of the aerolysin determinant in *Aeromonas hydrophila* demonstrates the role of aerolysin in *A. hydrophila*-associated systemic infections. Infect. Immun. 55, 2274–2280.
[28] Santos, J.A., Gonzalez, C.J., Otero, A. and García-López, M.L. (1999) Hemolytic activity and siderophore production in different *Aeromonas* species isolated from fish. Appl. Environ. Microbiol. 65, 5612–5614.
[29] Kämpfer, P., Christmann, C., Swings, J. and Huys, G. (1999) In vitro susceptibilities of *Aeromonas* genomic species to 69 antimicrobial agents. Syst. Appl. Microbiol. 22, 662–669.
[30] Overman, T.L. and Janda, J.M. (1999) Antimicrobial susceptibility patterns of *Aeromonas jandaei*, *A. schubertii*, *A. trota*, and *A. veronii* biotype veronii. J. Clin. Microbiol. 37, 706–708.
[31] Hayes, M.V., Thomson, C.J. and Amyes, S.G.B. (1994) Three β-lactamases isolated from *Aeromonas salmonicida*, including a carbapenemase not detectable by conventional methods. Eur. J. Clin. Microbiol. Infect. Dis. 13, 805–811.
[32] Alksne, L.E. and Rasmussen, B.A. (1997) Expression of the AsbA1, OXA-12, and AsbM1 beta-lactamases in *Aeromonas jandaei* AER 14 is coordinated by a two-component regulon. J. Bacteriol. 179, 2006–2013.
[33] Walsh, T.R., Stunt, R.A., Nabi, J.A., MacGowan, A.P. and Bennett, P.M. (1997) Distribution and expression of β-lactamase genes among *Aeromonas* spp. J. Antimicrob. Chemother. 40, 171–178.
[34] Vila, J., Marco, F., Soler, L., Chacón, M.R. and Figueras, M.J. (2001) In vitro antimicrobial susceptibility of clinical isolates of *Aeromonas caviae, Aeromonas hydrophila* and *Aeromonas veronii* biotype sobria. J. Antimicrob. Chemother. (submitted).
[35] Gavriel, A.A., Landre, J.P.B. and Lamb, A.J. (1998) Incidence of mesophilic *Aeromonas* within a public drinking water supply in north-east Scotland. J. Appl. Microbiol. 84, 383–392.
[36] Havelaar, A.H., Versteegh, J.F.M. and During, M. (1990) The presence of *Aeromonas* in drinking-water supplies in the Netherlands. Zbl. Hyg. 190, 236–256.