MOLECULAR EPIDEMIOLOGY

A cluster of cases of infections due to *Aeromonas hydrophila* revealed by combined RAPD and ERIC-PCR

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Two polymerase chain reaction (PCR)-based methods were used for epidemiological typing of *Aeromonas hydrophila*. Random amplification of polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) were applied to an outbreak involving seven patients. The epidemiological situation appeared complex; with the exception of two clinical isolates, all gave unique patterns with both techniques. These methods demonstrated nosocomial transmission in one unit and permitted the study to exclude a common environmental source in the hospital. The coincidental clustering of patients infected with *A. hydrophila* probably resulted from an increased prevalence of aeromonads in waters during summer, although no single RAPD or ERIC-PCR pattern was found among both clinical and environmental samples. RAPD and ERIC-PCR proved to be effective for the epidemiological study of *A. hydrophila* strains.

Introduction

Aeromonas species are gram-negative bacteria that can be isolated from water, soil and foodstuffs. Several species are responsible for severe haemorrhagic syndrome in a variety of fishes and multiple diseases in poikilothermic animals [1]. In man, all motile species are potentially pathogenic; half the aeromonads isolated from clinical specimens or hospital environment are *A. hydrophila* [2]. These motile species have been associated with several categories of human infections, including gastrointestinal infections and extra-intestinal infections, such as endocarditis, meningitis, septicaemia and urinary tract and wound infections. Abscesses or wound infections associated with exposure to soil or water represent the most prevalent extra-intestinal infections [3,4].

In most cases, the origin of the infection is suspected to be environmental, but the causative strain is only rarely isolated, despite intensive screening and the utilisation of highly discriminatory typing methods. Phenotypic methods used to characterise *Aeromonas* spp. include biotyping, serotyping, esterase electrophoresis, radiolabelled cell proteins and outer-membrane protein profiles [5–9]. Genotypic typing

methods have been applied to these species; plasmid analysis is unhelpful because plasmid carriage is infrequent (20–58%) in *A. hydrophila* [4]. In contrast, rRNA gene restriction patterns provide good discrimination within *A. hydrophila* [10, 11]. However, all the above techniques are time-consuming, expensive and labour intensive and are not able to provide reliable and rapid results on a routine basis. Rapid, simple and cheap methods are desirable for hospital-based laboratories and such PCR-based techniques have been applied successfully to the epidemiological typing of various bacterial species. Random amplification of polymorphic DNA (RAPD) is based on the amplification of random DNA segments with a single primer of arbitrary nucleotide sequence [12]. RAPD has been used widely for epidemiological investigation of numerous bacterial species [13]. Because of the low stringency inherent in this procedure, the patterns generated by RAPD may be affected by experimental parameters and standardisation is crucial. Previous investigations on the reproducibility of RAPD allowed the standardisation of parameters for routine use [14, 15]. An alternative approach based on families of short and repetitive sequences, such as enterobacterial repetitive intergenic consensus (ERIC) sequences, has been used by Versalovic *et al.* in a genomic fingerprinting method called ERIC-PCR [16]. ERIC sequences represent an extragenic, highly conserved and dispersed DNA sequence that has been observed in many bacterial species. Consensus primers com-

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plementary to each end of the repeated sequence are orientated such that PCR amplification of DNA sequences proceeds between adjacent repeated ERIC elements.

During a 2-month period, five cases of wound infections and two cases of bacteraemia due to *A. hydrophila* were observed in seven patients hospitalised in different units within the same hospital. This unusually high incidence prompted further investigations. *Aeromonas* spp. isolated from water sources in the hospital were characterised and compared with those from the infections to determine whether there was an association. All clinical strains and aeromonads isolated from water samples from different parts of the hospital distribution system were studied by RAPD and ERIC-PCR. Utilisation of a combination of molecular typing methods is important in order to increase the potential of discrimination by detecting a higher number of polymorphisms, and hence increases confidence in the results. RAPD analysis has been applied previously to the typing of *A. salmonicida* subsp. *salmonicida* and a few strains of *A. hydrophila* [17]. To our knowledge this is the first application of RAPD and ERIC-PCR in combination to epidemiological typing of *A. hydrophila*.

Materials and methods

Clinical and environmental isolates

Between 18 July and 30 Sept. 1995, 10 *A. hydrophila* isolates were obtained from seven patients hospitalised in five different units of the University Hospital in Marseille, France (Table 1). Patients 1 and 2 were hospitalised in the same ward for 32 days. Patients 1 and 3 received courses of hyperbaric oxygen in the same hyperbaric centre at the same time. Of the 10 isolates of *A. hydrophila*, eight were from wounds or post-operative scars in five patients, and two were responsible for bacteraemia in two patients. Of the seven infections, five were considered nosocomial, as clinical cultures were positive at least 48 h after hospitalisation. Swab specimens from wound abscesses were obtained with Culturette swabs (Becton Dickinson and Co., Paramus, NJ, USA). Colonies were isolated on blood agar (bioMérieux, Marcy l'Etoile, France) incubated in air at 37°C for 24 h. Blood cultures were performed in Bactec 6A culture medium (Becton Dickinson).

Environmental isolates were collected from tap water during epidemiological screening carried out in the different units of the hospital. Thirteen *A. hydrophila* isolates were obtained from the water supply system at four different times (15 May, 26 June, 26 July and 5 Sept. 1995) from four different units (Dermatology, Vascular surgery, Neurosurgery and Haematology) during the study period (Table 1). Water samples (100 ml) were collected and filtered through a 0.45-

Table 1. Details of patients associated with the outbreak investigated

Unit	Patient no.	Age/sex	<i>A. hydrophila</i> isolate no.	Date of isolation (day/month)	Disease	Positive sample	Hyperbaric oxygen therapy	Contamination	Antibiotype*	Aeromonads isolated from water in the corresponding unit (number of strains isolated)
D	1	73/M	1-4	22/07-30/08	Chronic leg ulcer	Slough	Y	Community acquired	RSSS	<i>A. hydrophila</i> (1)
D	2	49/F	5	17-19/08	Melanoma (ablation)	Abscess	N	Nosocomial	RSSS	<i>A. hydrophila</i> (6)
V	3	68/M	6	18-20/08	Thrombectomy	Necrosis on cicatrisation	Y	Nosocomial	SRSS	<i>A. hydrophila</i> (3)
N	4	62/M	7	18-22/07	Lacerated head after fall in a ditch	Suppuration	N	Community acquired	SRSS	
NP	5	79/F	8	31/08-02/09	Septic syndrome	Blood culture	N	Nosocomial	SRSS	
H	6	65/F	9	09/08	Leukaemia	Blood culture	N	Nosocomial	RRSS	
H	7	57/F	10	30/09	Cervix carcinoma	Abscess	N	Nosocomial	SRSR	

D, dermatology; V, vascular surgery; N, neurosurgery; NP, nephrology; H, haematology.
* Antibiotype according to susceptibility to cefalothin, ticarcillin, colistin and imipenem, respectively.

μm membrane that was then placed on Trypticase-soya Agar (bioMérieux). All isolates were identified as below and stored in conservation vials (AES, Combourg, France) at -80°C before epidemiological analysis.

Two isolates of *A. hydrophila* from rectal swabs from patients during the same period, and four strains of *A. hydrophila* from our culture collection were included as controls for comparison.

Finally, epidemiological information concerning *A. hydrophila* isolated from clinical specimens during the last 4 years was obtained for comparison.

Identification

Clinical and environmental isolates were identified to the species level according to oxidase reaction, growth at 42°C , and biochemical characteristics by the API 20 E identification system (bioMérieux), used according to the manufacturer's instructions. Identifications were completed by biochemical reactions according to the schema of Janda [18] together with aesculin hydrolysis and β -haemolysis.

Susceptibility testing

Susceptibility to 43 antimicrobial agents and six combinations of agents was determined by a standard disk diffusion method on Mueller-Hinton Agar (bioMérieux) [19].

Epidemiological typing

The isolates were characterised by RAPD and ERIC-PCR. For RAPD, the primers were AP12H (5'-CGGCCCTGT-3') and HLW74 (5'-ACGTATCTGC-3') [14, 15]. For ERIC-PCR, the primers were ERIC1R (5'-ATGTAAGCTCCTGGGGATTAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') [16]. For DNA preparation, isolates were grown overnight at 37°C on Mueller-Hinton agar. Total cellular DNA was extracted by the Chelex technique [20]. Five bacterial colonies were washed in a 1.5-ml eppendorf tube containing 1 ml of TE buffer (10 mM Tris, pH 8.0, 10 mM EDTA) by centrifugation for 5 min at 14 000 *g*. The bacterial pellet was resuspended in 100 μl of TE buffer and 150 μl of lysis solution (Chelex 100 (BioRad) 15%, SDS 1%, Nonidet P40 1% and Tween 20 1%) were added. After mixing, the sample was incubated at 90°C for 30 min, centrifuged for 10 min at 14 000 *g* and the supernate was transferred to a new tube. DNA concentrations were estimated by comparison with standards on agarose gels. Amplification reactions were performed in a total volume of 47 μl containing 100 μM dATP, 100 μM dCTP, 100 μM dGTP and 100 μM dTTP, plus 0.2 μM primer, 25 ng of template DNA and 1.25 units of *Taq* polymerase (Perkin-Elmer/Cetus, Norwalk, CT, USA) in 1 $\times$ PCR

buffer (20 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM MgCl_2 , gelatin 0.001% w/v). A negative control without template DNA was included in each experiment. The reaction mixtures were overlaid with mineral oil and subjected to amplification in a DNA thermal cycler (TR2, Cera-Labo, Aubervilliers, France) programmed for 45 cycles of 1 min at 94°C , 1 min at 45°C and 1 min at 74°C . Amplification products (10 μl samples) were electrophoresed in agarose 1.2% gels in Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.2), and then stained with ethidium bromide and photographed on a UV light transilluminator. A molecular size standard (Marker V, Boehringer-Mannheim, Germany) was included on each gel. Fingerprint patterns were interpreted and compared without knowledge of epidemiological data. Heterogeneity with respect to the intensity and shape of bands was not considered to be a difference. Strains were considered different if their profiles differed by two or more bands as in previous studies [21].

For each of the PCR-based techniques, reproducibility was determined by testing at least two independent DNA preparations extracted from single-colony cultures at different times, amplified on at least two occasions.

Results

During the last 6 years, 27 isolates of *A. hydrophila* were obtained from clinical specimens in the laboratory (of which eight were isolated in 1994 and 10 in 1995); in most cases these were associated with wound infections or bacteraemia. Twenty of these 27 isolates (74%) were isolated during the summer period (June–Sept.).

All 10 clinical isolates from the summer of 1995 were obtained in pure culture except isolates 1, 2 and 3 from patient 1, which were associated with a *Proteus mirabilis* strain. Four antibiotic susceptibility patterns which varied in susceptibility to cefalothin, ticarcillin, colistin and imipenem were detected amongst the isolates. No variations in antimicrobial resistance patterns were observed between isolates 1–4 from patient 1. All isolates were resistant to penicillin, ampicillin, pristinamycin and erythromycin.

Twenty-six tap water specimens were collected from the five hospital units concerned. In half of them, between 1 and 30 000 colonies of *A. hydrophila* were isolated. Representative colonies of *A. hydrophila* isolates from each of the 13 positive water sources were studied.

RAPD and ERIC-PCR with single primers successfully typed all the isolates. Patterns obtained with independent extracts from one isolate and amplified in two

independent experiments were equivalent except for fainter intensity of bands (data not shown). RAPD and ERIC fingerprints for all strains studied are represented in Figs. 1 and 2. A perfect correlation was found between the two PCR typing methods. The results from the PCR typing methods for each strain are presented in Table 2. Among the 10 clinical isolates, six different PCR-patterns were identified. The four isolates from patient 1 and the isolate from patient 2 had identical RAPD and ERIC fingerprints. All other clinical isolates and strains used as controls gave different patterns. The 13 *A. hydrophila* isolated from the different water sources gave seven different patterns with each primer; in two cases, four strains belonged to the same RAPD and ERIC type. No fingerprints were common to both environmental and clinical isolates.

Discussion

From 1989 to 1994, 27 cases of *A. hydrophila* infections were observed in Sainte Marguerite Hospital (Marseille, France), i.e., about four cases each year. During a 3-month period in 1995, seven patients from different units were infected by *A. hydrophila*, corresponding to a notable increase in the prevalence of this species.

Two molecular typing methods based on PCR, RAPD and ERIC-PCR, were used to compare isolates from water samples with those from patients to answer rapidly the following questions: (1) is the hospital

water supply the origin of clinical infections and is there any relationship between patient strains and (2) could patients have been contaminated during common clinical procedures such as dermatological therapy and hyperbaric oxygen therapy?

A great heterogeneity was demonstrated in both clinical and environmental strains of *A. hydrophila*. Typing results revealed that five isolates from two patients were identical. In contrast, other clinical strains and those used as controls showed different patterns. Therefore, analysis of the PCR fingerprinting patterns confirmed that patients 1 and 2 were infected by a single *A. hydrophila* strain. These patients were hospitalised in the same dermatological unit during a common period of 32 days. Moreover, they resided in the same room 15 days apart. The medical files revealed that patient 1, who was obese and rowdy, had broken his bathtub. He was put in another room so that repairs could be made. Fifteen days after the repairs, patient 2 was hospitalised in the same room and used the same bathtub. Although no *Aeromonas* strains were found in any sample from the bathtub water, it is possible that patient 2 was infected through the bathtub or water. In previous reports of *A. hydrophila* infection, it is generally thought that the strain causing wound infections is of environmental origin, but identical environmental strains are rarely found. As patients 1 and 3 were treated in the same hyperbaric centre at the same time, this suggested possible transmission during their treatment. However, the typing results excluded this possibility.

Fig. 1. Representative RAPD fingerprints of clinical and environmental isolates of *A. hydrophila*, obtained with primer AP12H (a) or HLW74 (b). The numbers at the top are the isolate numbers listed in Table 2. Lane M: size marker V.

Fig. 2. Representative ERIC-PCR fingerprints of clinical and environmental isolates of *A. hydrophila*, obtained with primer ERIC 1R (a) or ERIC 2 (b). The numbers at the top are the isolate numbers listed in Table 2. Lane M: size marker V.

In two cases, isolates were considered to be community-acquired. Patient 1 was suffering from chronic ulcers of the leg with scabs and was infected on admission by *A. hydrophila*. He was working in naval repairs and was in contact with the marine environment. Patient 4 was found lying in a ditch after a fall. His head was lacerated and immersed in sludge.

Although aeromonas bacteraemia is probably endogenous in origin (from the gastrointestinal tract or infected wounds), there are few reports documenting the source of the original gastrointestinal or wound infection [4]. In the present study, patients 5 and 6 were suffering from diarrhoea, acquired >48 h after hospitalisation, before the onset of their nosocomially

Table 2. Origin and genotyping results of *A. hydrophila* isolates studied

<i>A. hydrophila</i> isolate no.	Origin	RAPD type with primer		ERIC type with primer	
		AP12h	HLW74	ERIC 1R	ERIC 2
1	Clinical (patient 1)	1	1	1	1
2	Clinical (patient 1)	1	1	1	1
3	Clinical (patient 1)	1	1	1	1
4	Clinical (patient 1)	1	1	1	1
5	Clinical (patient 2)	1	1	1	1
6	Clinical (patient 3)	2	2	2	2
7	Clinical (patient 4)	3	3	3	3
8	Clinical (patient 5)	4	4	4	4
9	Clinical (patient 6)	5	5	5	5
10	Clinical (patient 7)	6	6	6	6
11	Control (rectal swab)	7	7	7	7
12	Control (rectal swab)	8	8	8	8
13	Control (collection)	9	9	9	9
14	Control (collection)	10	10	10	10
15	Control (collection)	11	11	11	11
16	Control (collection)	12	12	12	12
17	Environmental (unit N)	13	13	13	13
18	Environmental (unit N)	14	14	14	14
19	Environmental (unit N)	15	15	15	15
20	Environmental (unit H)	16	16	16	16
21	Environmental (unit H)	17	17	17	17
22	Environmental (unit H)	13	13	13	13
23	Environmental (unit V)	18	18	18	18
24	Environmental (unit V)	13	13	13	13
25	Environmental (unit V)	18	18	18	18
26	Environmental (unit V)	19	19	19	19
27	Environmental (unit V)	13	13	13	13
28	Environmental (unit V)	18	18	18	18
29	Environmental (unit D)	18	18	18	18

D, dermatology; V, vascular surgery; N, neurosurgery; NP, nephrology; H, haematology.

acquired bacteraemia. No faecal carriage was investigated during the diarrhoeal episodes, but a relationship between *A. hydrophila* enteric carriage and the septicaemias cannot be excluded. In a review of the literature Altwegg *et al.* noted that *A. hydrophila* invades the bloodstream from the gastrointestinal tract through a defective intestinal mucosa although only a few stool cultures are found to be positive for *Aeromonas* strains at the same time [1].

The present study revealed the co-existence of multiple *A. hydrophila* strains in water samples collected in the same unit and the existence of a single strain isolated over a period of 4 months from water samples from different units. The finding of common types (type 13 and type 18) of *A. hydrophila* in three separate hospital units suggested that they were present in the water distribution system. Kuhn *et al.* [6] have shown that a single strain could be found in several sampling sites and at different times in the public drinking water distribution system. The results of the present study demonstrate colonisation of public water supplies with some widespread clones of *A. hydrophila*. The high number of *A. hydrophila* environmental strains collected correlated with the increase in clinical cases, as observed elsewhere [8, 10]. However, no relationship between clinical and environmental aeromonads could be demonstrated by RAPD or ERIC-PCR. Colonisation or infection of hospitalised patients by *Aeromonas* species from hospital water supplies has been suggested [2, 8, 22, 23], but environmental sources are rarely detected. It seems probable that an association of hot weather and increased water usage (drinks, baths and showers etc.) may have led to the increase in *A. hydrophila* infections. Moreover, the clinical isolates of *A. hydrophila* were all obtained during June–Oct. and the abundance of *A. hydrophila* in fresh water has been found to be seasonally distributed, with a maximum occurring during the summer [23, 24]. In this study, strains from patients and environmental sources were different, but it seems likely that water sources other than those investigated were involved in contaminating patients.

The typing methods, RAPD and ERIC-PCR, showed that the apparently large outbreak was due to the superposition of a horizontal transmission, involving only two patients, and a series of sporadic cases, probably due to an increase in the contamination of the water supply by *A. hydrophila*.

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