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Epidemiological relationships between *Aeromonas* strains isolated from symptomatic children and household environments as determined by ribotyping

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Abstract. Ribotyping was used to study the epidemiology of *Aeromonas* associated gastro-enteritis in young children. Ribotyping patterns of 29 *Aeromonas* strains (16 *Aeromonas caviae*, 8 *Aeromonas hydrophila*, 3 *Aeromonas eucrenophila*, 1 *Aeromonas veronii*, and 1 *Aeromonas encheleia*) isolated from primary stool cultures of sick children were compared using the GelCompare software with patterns of 104 strains (39 *Aeromonas eucrenophila*, 29 *Aeromonas caviae*, 11 *Aeromonas encheleia*, 10 *Aeromonas hydrophila*, 6 *Aeromonas bestiarum*, 3 *Aeromonas veronii*, 3 *Aeromonas popoffii* and 3 *Aeromonas media*) isolated from their household environment in order to investigate the route of transmission of these bacteria. Fifteen strains (≈47%) isolated from stool cultures of patients showed the same riboprofile as strains found in contacts or environment. In particular, three strains isolated from patients shared the same riboprofile with strains found in their domestic environment. The wide diffusion of potentially pathogenic *Aeromonas* strains in our household samples, and the high rate of asymptomatic carriers among family members, suggested that predisposing factors of the host could make children prone to an *Aeromonas*-related intestinal disease.

Key words: *Aeromonas*, Epidemiological typing, Ribotyping

Introduction

Members of the genus *Aeromonas* are ubiquitous in aquatic environments [1]. They have long been recognised as primary pathogens to a variety of both cold- and warm-blooded animals. The role of *Aeromonas* in human intestinal diseases has not been clearly established despite there appears to be sufficient evidence to consider at least some biotypes as putative enteropathogens [2].

The main cause of *Aeromonas* presence in the digestive tract of human beings is thought to be water, although previous works demonstrated that strains from human and environmental origin formed distinct groups [3, 4]. Direct epidemiological links between strains isolated from patients and strains isolated from the environment have been demonstrated only in the case of infections following injury [5]. On the other hand, person-to-person transmission of *Aeromonas* strains as well as infection following the consumption of contaminated food have been demonstrated by rRNA gene restriction fragment polymorphism (ribotyping) of such strains [6–8].

Ribotyping is widely used in bacteriology for epidemiological and taxonomic studies [9–11]. Its usefulness for studying *Aeromonas* has been extensively investigated by Moyer and co-workers [3, 8]. The rDNA patterns obtained after hybridisation with plasmid pKK3535 of *Sma* I – digested total genomic DNAs permitted an excellent discrimination between strains as well as their taxonomic classification [12–14].

Ribotyping was thus chosen to compare strains of *Aeromonas* associated with gastro-enteritis in young children (from birth to age six) with strains from their household environment, attempting to trace the route of transmission of these bacteria, whose frequency of isolation from stool cultures in our laboratory is of ≈2%.

Materials and methods

Sampling procedures, media and strains

Faecal samples of patients suffering from an intestinal illness were sent to our laboratory for microbiological investigation, and were analysed by using routine lab procedures [15] to determine the presence of rotavirus (only for stool samples from children up to the age of four), *Salmonella*, *Campylobacter*,

Shigella, *Plesiomonas* and *Aeromonas*. For the latter, Columbia Agar Base (Oxoid, Hampshire, UK) with 5% sheep erythrocytes supplemented with 10 mg l^{-1} ampicillin (Merck, Dietikon, Switzerland) was used both for the direct plating of stools, and plating after enrichment in alkaline Peptone Water (Oxoid), pH 8.6. Plates were incubated at 30 °C, and alkaline Peptone Water enrichments at room temperature for 24 hours.

When the presence of *Aeromonas* (haemolytic or slightly haemolytic colonies, oxidase positive) was found in stool samples of children up to the age of six as unique putative pathogen, the family was contacted by phone. A visit was arranged as quickly as possible (i.e. within a maximum of 7 days) to take samples for the isolation of *Aeromonas* strains, which might be related to the strain recovered in stool. Samples consisted of at least 4 l of tap water, and three swabs from wet surfaces, mainly in the kitchen and bathroom. Stool samples of other members of the family, irrespective of the presence or absence of diarrhoea, were also analysed, as well as a second stool specimen of the patient sent to our laboratory one week or more after the first one.

Surface swabs were processed as described above for stool samples (direct plating and enrichment) while water samples (1 l each) were filtered through 0.45 μm membranes (MicronSep^{TM}, MSI, Westboro, MA) and incubated on m-*Aeromonas* Selective Agar (Biolife, Milan, Italy) with 10 mg l^{-1} ampicillin at 30 °C for 24 hours [16].

The *Aeromonas* strains (Table 1) were isolated from the following samples: 29 stool samples of children up to the age of six suffering from diarrhoea in whose stool cultures only *Aeromonas* was isolated as putative pathogen, 28 stool samples from the same children taken one week or more after the first one, 92 stool samples of family members, 123 samples of tap water, and 105 surface or pipe swabs. Ten stool samples of healthy children, 22 stool samples of family members, 36 samples of tap water, and 35 surface or pipe swabs were also analysed in control families with healthy children of the same age group as our patients, to investigate the presence of *Aeromonas* strains in home environments of healthy people.

The following reference strains were also included in the study: *Aeromonas hydrophila* ATCC 7966 (HG 1), *Aeromonas bestiarum* CDC 9533-76 (HG 2), *Aeromonas caviae* ATCC 15468 (HG 4), *Aeromonas media* ATCC 33907 (HG 5B), *Aeromonas eucrenophila* NCMB 74 (HG 6), *Aeromonas jandaei* ATCC 49568 (HG 9), *Aeromonas veronii* ATCC 35624 (HG 8/10), *Aeromonas encheleia* ATCC 35941 (HG 11) and *Aeromonas schubertii* ATCC 43700 (HG 12).

Table 1. Cases description, strains of *Aeromonas* used in the study and ribotype numbers

Case number	Sex (M/F)	Age in months	Clinical symptoms	Origin and designation of the *Aeromonas* strains. In brackets the corresponding ribotype number
1	F	17	Intermittent diarrhoea for two months	Stools of patient: 1A (96) Household environment: 1B (4), 1C (2), 1D (5)
2	M	34	Gastroenteritis	Stools of patient: 2A (87)
3	F	66	Diarrhoea with vomiting and fever	Stools of patient: 3A (7) Household environment: 3B (2), 3C (11)
4	M	69	Diarrhoea with vomiting and fever	Stools of patient: 4A* (33) Stools of family members: 4D (6) Household environment: 4B (6), 4C (6)
5	F	17	Enteritis	Stools of patient: 5A* (17) Household environment: 5B (101)
6	F	20	Gastroenteritis	Stools of patient: 6A* (54) Household environment: 6B (77), 6C (59), 6D (69), 6E (61)
7	M	49	Diarrhoea	Stools of patient: 7A* (98) Household environment: 7B (75), 7C (49), 7D (44), 7E (63), 7F (65)
8	M	28	Diarrhoea with fever	Stools of patient: 8A* (6)
9	M	45	Diarrhoea with vomiting and fever	Stools of patient: 9A (97)
10	F	21	Diarrhoea with vomiting and fever	Stools of patient: 10A* (11) Household environment: 10B (72), 10C (71)
11	F	42	Diarrhoea with vomiting and fever	Stools of patient: 11A (9), 11B* (99) (control)[a] Household environment: 11C (70), 11D (66), 11E (65), 11F (8)
12	M	15	Diarrhoea	Stools of patient: 12A* (98), 12B* (23) (control) Household environment: 12C (76), 12D (48), 12E (44), 12F (63), 12G (89), 12H (68), 12I (44), 12L (23), 12M (13)

Table 1. (*Continued*)

Case number	Sex (M/F)	Age in months	Clinical symptoms	Origin and designation of the *Aeromonas* strains. In brackets the corresponding ribotype number
13	M	12	Diarrhoea with cramps	Stools of patient: 13A (19) Household environment: 13B (95)
14	F	13	Diarrhoea	Stools of patient: 14A (25) Stools of family members: 14B (25) Household environment: 14C (21)
15	M	72	Colics with diarrhoea vominting and fever	Stools of patient : 15A (91), 15C* (91) (control) Stools of family members: 15B* (91)
16	F	11	Diarrhoea	Stools of patient: 16A (3)
17	M	59	Abdominal colics	Stools of patient: 17A (11)
18	F	18	Diarrhoea	Stools of patient: 18A* (10)
19	M	19	Diarrhoea with vomiting and fever	Stools of patient: 19A* (10)
20	F	39	Diarrhoea	Stools of patient 20A (93) Stools of family members: 20I* (92) Household environment: 20B (28), 20C (28), 20D (28), 20E (31), 20F (78), 20G (62), 20H (43)
21	M	16	Enteritis with vomiting	Stools of patient: 21A* (80) Stools of family members: 21B (102)
22	F	19	Diarrhoea with vomiting	Stools of patient: 22A (17) Stools of family members: 22D* (82) Household environment: 22B (1), 22C (22)
23	M	12	Enteritis	Stools of patient: 23A* (19) Household environment: 23B (85)
24	F	39	Diarrhoea with vomiting	Stools of patient: 24A (52)
25	M	35	Diarrhoea	Stools of family members: 25O (91), 25P (19), 25Q* (90), 25R (93) Household environment: 25B (45), 25C (46), 25D (32), 25E (45),25F (30), 25G (41), 25H (38), 25I (27), 25L (60), 25M (79), 25N (53)
26	M	25	Enteritis	Stools of patient: 26A* (14) Household environment: 26B (56), 26C (58), 26D (57)
27	M	26	Diarrhoea with vomiting	Stools of patient: 27A* (24) Stools of family members: 27B* (81)
28	M	4	Blood in stools and tummy-ache	Stools of patient: 28A* (26)
29	M	45	Diarrhoea with vomiting	Stools of patient: 29A (100) Household environment: 29B (18), 29C (18), 29D (28), 29E (28), 29F (28), 29G (37), 29H (74), 29I (29), 29L (47), 29M (28), 29N (34), 29O (29), 29P (67)
NC1[b]	F	46		Household environment: NC1A (2), NC1B (16), NC1C (6)
NC2	M	14		Household environment: NC2A (10), NC2B (10)
NC3	F	59		Household environment: NC3A (84)
NC4	M	32		Household environment: NC4A (39), NC4B (39), NC4C (23)
NC5	F	10		Household environment: NC5A (12)
NC6[c]	M/F	60/16		Household environment: NC6A (55), NC6B (35), NC6C (86), NC6D (42), NC6E (42), NC6F (40)
NC7	M	51		Household environment: NC7A (2), NC7B (17), NC7C (15)

* Strains isolated after enrichment in alkaline Peptone Water.
[a] Strains isolated from the stool samples of the patients sent to our laboratory one week or more after the first one.
[b] Negative Control: family environments without children suffering from *Aeromonas* gastroenteritis.
[c] Negative control 6 consisted of a family environment where two brothers lived.

Biotyping

All strains were analysed using classical tests to confirm the genus *Aeromonas*: Gram stain, oxidase, catalase, oxidation and fermentation of glucose, resistance to the vibriostatic compound 0/129 [17]. The following reactions were used to differentiate the strains: gas from glucose, aesculin hydrolysis, VP, cephalothin resistance, and the commercial identification system ID 32 GN (bioMerieux, Charbonnières-les-Bains, France).

Ribotyping and analysis of patterns

Genomic DNA was prepared according to the method described by Ausubel et al. [18]. Briefly, *Aeromonas* cultured on blood agar were resuspended in TE buffer (10 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EDTA, pH 8.0) and the cells were lysed at 37 °C for 1 hour with proteinase K (50 μg ml^{-1}; Boehringer Mannheim, GmbH, Mannheim, Germany) and SDS (1%). Polysaccharides and other contaminant macromolecules were removed by a 10-min incubation at 65 °C with CTAB (1% hexadecyl-trimethylammonium bromide in 0.7 mol l^{-1} NaCl), followed by extraction with chloroform and phenol/chloroform. DNA was precipitated in isopropanol, dried and resuspended in TE buffer. RNA was completely digested by 1 hour incubation at 37 °C with 20 μg ml^{-1} of RNase (Boehringer Mannheim), and purified DNA was subsequently obtained using NaAc (3 mol l^{-1}) and ethanol precipitation.

Purified DNA samples were digested with restriction endonuclease *Sma* I (Boehringer Mannheim) as recommended by the manufacturer. Fragments were separated by electrophoresis on a 0.8% agarose gel in TBE buffer 1× (Tris–Borate–EDTA, pH 8.0) at 25 V for 14 hours. The fragments were transferred to a positively charged nylon membrane (Boehringer Mannheim), fixed to the membrane for 30 min at room temperature and for 15 min at 80 °C.

The plasmid pKK3535 [19], labelled with digoxigenin (DIG DNA labelling Kit, Boehringer Mannheim), was used as probe. Prehybridisation, hybridisation (both performed at 68 °C), and the immunological detection of the fragments were performed according to the protocol of the DIG Nucleic Acid Detection Kit (Boehringer Mannheim).

All strains associated with the same case were analysed together on a membrane including a set of molecular weight markers (DNA Molecular Marker II, DIG-labelled, Boehringer Mannheim), used as internal reference during each electrophoresis run. The molecular weight markers permitted also the comparison of patterns on different membranes. The reproducibility of riboprofiles was checked with 20 strains which were analysed twice.

Membranes were scanned by using the Sharp JX-330 densitoscanner. Transmission image data were stored in TIFF files and were further processed by using the GelCompare software, version 3.1 (applied Maths, Kortrijk, Belgium). Levels of similarity between ribotyping profiles were calculated by using the band-matching Dice coefficient. Cluster analysis was performed by using the unweighted pair group method with arithmetic averages.

Results

The 29 *Aeromonas* strains isolated as sole putative pathogen from stool samples of children from birth to the age of six suffering from an intestinal illness (Table 1) were identified at the species level as *Aeromonas caviae* (16 strains), *Aeromonas hydrophila* (eight strains) *Aeromonas eucrenophila* (three strains), *Aeromonas veronii* (one strain), and *Aeromonas encheleia* (one strain). For 28 patients a second stool specimen was analysed one week or more after the first one, and three (10.7%) were again positive for *Aeromonas* spp. after enrichment in alkaline Peptone Water. Only for one patient, the two *Aeromonas* strains, identified as *Aeromonas hydrophila*, showed the same riboprofile. Of 92 stool cultures taken from the patient's families, 11 were positive for *Aeromonas* (6 after direct plating and 5 after enrichment). No *Aeromonas* positive stools were found in the negative control families, with healthy children ($p = 0.083$; 1-tailed Fisher exact test). Thirty-one *Aeromonas* strains were isolated from surface or pipe swabs, and 59 from tap water. The prevalence of positive environmental samples in patient's families was not significantly higher than that in the negative controls ($p = 0.21$; Yates corrected test). Approximately 30% of the environmental samples were negative in regard to the isolation of *Aeromonas* in both groups.

A total of 142 *Aeromonas* strains were compared by ribotyping. The use of the GelCompare software allowed the analysis of all the riboprofiles simultaneously, and the construction of the dendrogram shown in Figure 1. The intragel-specific correlation levels for the molecular weight markers were higher than 95%. The minimal differences between the two independent profiles obtained for the 20 strains did not affect their position in the dendrogram. Our strains, together with the reference strains, showed 103 different ribotypes and formed nine main clusters.

Following the scheme proposed by Martinetti Lucchini and Altwegg [12] for the identification of the HGs on the basis of the low-molecular-weight DNA patterns, and our biochemical reactions, we could characterise the clusters of the dendrogram in Figure 1 at the genospecies level.

The taxonomic identification of the 43 strains isolated from stool samples, indicated that the majority of them belonged to the hybridisation groups 1 and 4. The half of the *Aeromonas caviae* HG 4 from faecal

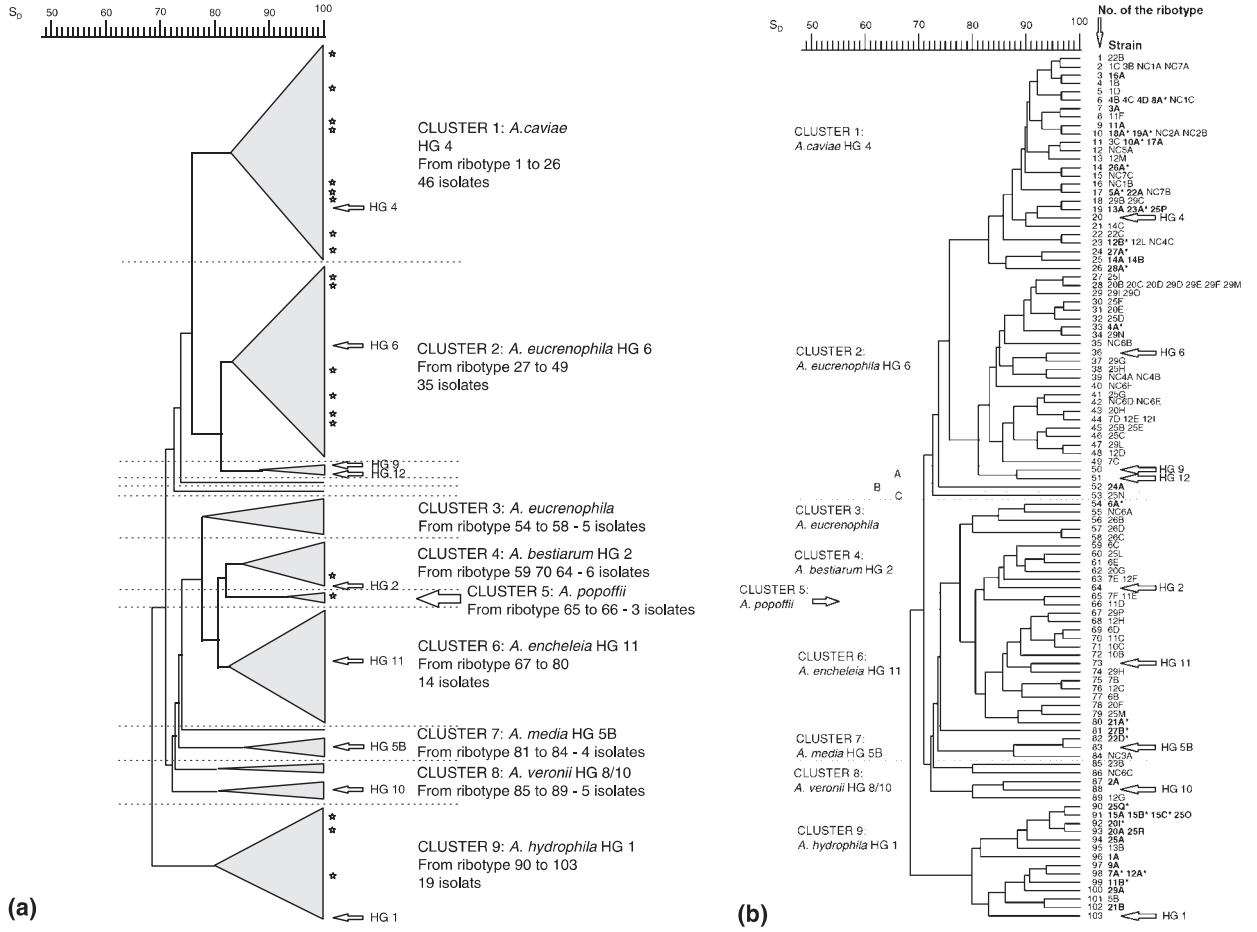

Figure 1. (a) Dendrogram derived from the analysis of the ribotyping patterns of 133 *Aeromonas* strains and 9 reference strains. "⇨" Position of the ribotyping patterns of the reference strains which are designed by their hybridization group. *Designes the position of the ribotyping patterns shared by more than one *Aeromonas* strain. (b) Dendrogram derived from the analysis of the ribotyping patterns of 133 *Aeromonas* strains and 9 reference strains. Strains of faecal origin are in bold and *designes those isolated after enrichment. "⇨"Position of the ribotyping patterns of the reference strains which are designed by their hybridization group.

origin was isolated from stool samples of symptomatic children after enrichment in alkaline Peptone Water.

Twenty ribotypes grouped more than one strain (Figure 1). From an epidemiological point of view, the most interesting ribotypes grouping strains of faecal and environmental origin were found in cluster 1 (*Aeromonas caviae*) and were numbered 6, 10, 17, and 23. Ribotype No. 6 was shared by strains 4B, 4C and 4D (Table 1), isolated from surface swabs and a stool specimen of a family member in case No. 4, as well as by strain 8A isolated from stools of patient No. 8. Ribotype No. 10 was composed by two strains of faecal origin (cases No. 18 and 19) as well as two household strains found in negative control case no. 2. Ribotype No. 17 grouped two strains isolated from the stool samples of patients No. 5 and 22 and a strain isolated from the household of negative control case No. 7. Ribotype No. 23 grouped the strains isolated from the stools of control of patient No. 12, from a pipe swab in his household environment and from a surface swab in the negative control case No. 4.

Discussion

In the present study, a total of 133 *Aeromonas* isolated from symptomatic children (birth to age six) and from their household environments, as well as from control cases, were analysed by ribotyping together with nine reference strains, with the aim of finding the specific source of contamination.

The prevalence of *Aeromonas* isolation in the 29 families investigated and in the then control groups was not significantly different.

Using the GelCompare software to analyse riboprofiles we were able to compare all the strains. The 133 *Aeromonas* analysed and the nine reference strains showed 103 different profiles, reflecting the extreme diversity of the genus, as well as the discriminatory power of the method used. Fifty-nine strains were grouped into 20 ribotypes. In disagreement with results of other authors [3, 4], identical ribotypes could be found in epidemiologically related but also in unrelated strains, and strains of faecal origin shared the same riboprofile as household en-

vironmental strains. It has to be noted that these studies considered *Aeromonas* strains isolated from environments, which were not as close to the patients as our samplings.

Our results indicated that the contamination of the patient via his household environment was highly probable. The stool cultures of family members showed an *Aeromonas* strain identical, at ribotyping level, to that of the patient in two situations only out of 11. The person-to-person transmission, already described by other authors [8], seemed thus not to be a major route of infection in our study. The case No. 4, where the strain isolated from the stool samples of a family member was the same as two strains isolated in his household environment (ribotype 6), strengthened the hypothesis that the patients could be infected via their domestic environments. Furthermore, the results obtained among the negative control cases showing that potential pathogenic strains could also be isolated in domestic environments where healthy people lived, showed that the contamination of the environment by the patient is very little probable. Finally, wet surfaces or tap water are known to act as source and/or reservoirs of enteric pathogens, and can thus be transmitted to the patients [20].

Strains originating from stool samples did not cluster in any specific group, and only clusters 4 and 5 did not comprise strains of faecal origin. Surprisingly, in this study the environmental strains were grouped according to the kind of environmental sample, namely water or swab. As expected, most of our *Aeromonas* of faecal origin (85.7%) were clustered within strains showing the biochemical characteristics of *Aeromonas caviae* (cluster 1), *Aeromonas veronii* (cluster 10), and *Aeromonas hydrophila* (cluster 11), i.e. species which are generally recognised as potential human pathogens. Cluster 1 grouped also 80.6% of *Aeromonas* strains originating from surface and pipe swabs, whereas 95% of strains isolated from tap water belonged to clusters 2 trough 6.

Even though potentially pathogenic *Aeromonas* were rarely isolated from drinking water, their sporadic presence might be sufficient to colonise other ecological niches, as, for example, pipe surfaces. Contamination of patients might thus occur also from this secondary source [20].

In conclusion, the use of ribotyping has allowed to point out that strains of *Aeromonas* which may be associated to intestinal illnesses in children can have the same riboprofile as the strains isolated from samples taken in their household environment. These strains could be found in the drinking water of our region, and seem to be able to colonise wet surfaces. Their presence in the human gastrointestinal tract can therefore also be due, among other causes, to the swallowing of tap water or contact with a secondary source in household environments. The large distribution of potential pathogenic *Aeromonas* strains in domestic environments considered in our study, as well as the rate of asymptomatic carriers in family members, indicate that host determinants could make children more prone to acquiring an *Aeromonas*-related intestinal infection.

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