PATHOGENICITY

Association of *Aeromonas* spp. with travellers' diarrhoea in Finland

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Summary. The association of *Aeromonas* spp. with travellers' diarrhoea was studied among 978 Finnish tourists travelling to Morocco in winter ($n = 398$) and autumn ($n = 580$) in 1989. Fifty-five isolates from diarrhoeal patients with ($n = 16$) or without ($n = 39$) a recent travelling history in a developing country were also included. In Morocco, *Aeromonas* spp. were isolated from 8.7% of patients with diarrhoea and from 1.4% of non-diarrhoeal tourists ($p < 0.001$). *Aeromonas* spp. were found as the sole pathogen in 5.5% of patients ($p < 0.001$). Diarrhoea with multiple pathogens, including *Aeromonas* spp., was found in 3.1% of patients. Species identification by phenotypic and genotypic methods indicated that *A. veronii* biotype *sobria* (hybridisation group HG 8/10) and *A. caviae* (HG 4) were the most common *Aeromonas* spp. associated with travellers' diarrhoea. *A. hydrophila* (HG 1) and *A. caviae* (HG 4) were common in patients acquiring diarrhoea in Finland. Ribotyping of strains within a species showed that all strains had different ribotypes although the tourists were infected during the same trip. This study suggested that only certain *Aeromonas* spp. were commonly found in travellers' diarrhoea. However, the causative role of those species is unclear.

Introduction

An increasing number of epidemiological studies indicate that *Aeromonas* spp. may be aetiological agents in sporadic diarrhoeal illness in both developed and developing countries.^{1–7} In developed countries, *Aeromonas* spp. have been found in 0.8–7.4% of stool specimens submitted for examination for enteropathogens. Their prevalence in stool specimens from healthy persons has varied from 0.4% to 2.1%. In developing countries, the isolation rate from diarrhoeal and control samples seems to be higher than in developed countries. For example, in Thailand, *Aeromonas* spp. were isolated from 34% of stools from adult patients with diarrhoea and from 27.5% of those from healthy controls.⁸ *Aeromonas* spp. occurred in faecal specimens of Peace Corps workers in Thailand in 30.8% of episodes of diarrhoea but in only 8.5% of healthy controls.⁹ These indirect pieces of evidence suggest that *Aeromonas* spp. may have an aetiological role in travellers' diarrhoea.^{1,7}

The assessment of the role of mesophilic *Aeromonas* spp. in diarrhoeal illness has been hampered by the complicated taxonomy of the species.¹⁰ At least eight species are described on the basis of phenotypic

characteristics: *A. hydrophila*, *A. caviae*, *A. media*, *A. eucrenophila*, *A. veronii* biotypes *sobria* and *veronii*, *A. jandaei*, *A. schubertii* and *A. trota*.^{10,11} DNA-DNA hybridisation studies identify 13 genospecies (hybridisation groups, HGs).^{10,12}

The aim of the present study was to examine the role of *Aeromonas* spp. in travellers' diarrhoea in Finnish tourists travelling to Morocco in 1989. Some *Aeromonas* strains isolated from faecal samples of Finnish subjects with or without a history of recent travel to a developing country were also included. The phenotypic and genotypic characteristics of all isolates were identified carefully.

Materials and methods

Subjects and faecal specimens

The *Aeromonas* data were collected during studies on the aetiology, prevention and treatment of diarrhoea in Finnish tourists travelling to Morocco in 1989.^{13–15} Faecal samples were cultivated from 398 adult travellers in the winter (11 Jan.–8 Feb.) and from 580 adult travellers in the autumn (24 Oct.–21 Nov.). Of these 978 subjects, 389 had received the oral B subunit or whole cell cholera vaccine before the trip in

a double blind manner. Faecal samples from tourists who had diarrhoea during the trip were investigated in Morocco by Finnish experts at the temporary local laboratory for the common enteric pathogens.¹⁵ Travellers' diarrhoea was defined as four or more unformed stools within a 24-h period or at least three unformed stools in an 8-h period and at least one of the following additional symptoms: nausea, vomiting, abdominal pain or cramps.³

For comparison of possible regional differences in the distribution of *Aeromonas* spp., all *Aeromonas* strains that were isolated from diarrhoeal patients and submitted to the Laboratory of Enteric Pathogens, Finland during the years 1986–1989 were included in the study (a total of 55 faecal isolates); 16 were isolated after patients' travel abroad and 39 isolates were from subjects living in the southern part of Finland and without a known history of recent travel abroad.

Isolation and identification of *Aeromonas* spp.

Aeromonas spp. were detected on *Aeromonas* Selective Medium (Difco). Initially, *Aeromonas* spp. were identified by standard methods by the oxidase test and API 20E (bioMérieux SA, Marcy d'Etoile, France). The selective medium was incubated at 30°C and biochemical tests were first done at 37°C. After isolation the strains were stored in skimmed milk at –70°C.

Aeromonas spp. were identified to species level by the criteria of Altwegg *et al.*,¹ Popoff¹¹ and Carnahan *et al.*¹⁶ The most important criteria used in their identification were: aesculin hydrolysis; amino acid (arginine, lysine and ornithine) decarboxylation or dehydrogenation; gas production from glucose; acid production from arabinose, sorbitol, saccharose, salicin or rhamnose; acetylmethyl-carbinol production (V-P); elastase production; cephalothin sensitivity and haemolysin production. For the phenotypic identification of the three hybridisation groups (HG 1, HG 2 and HG 3) of *A. hydrophila*, the following methods were used: utilisation of DL-lactate, citrate or urocanic acid as a sole source of carbon and production of acid from sorbitol and rhamnose.^{12, 16} Similarly, for the identification of HG 4, HG 5A and HG 5B (*A. caviae*), utilisation of DL-lactate or citrate and haemolysis were used.^{12, 17} In these identifications, both conventional media and API 20E and ID 32GN were used (bioMérieux).

Genotypic methods

Ribotyping was used for genetic identification of a genospecies (HG) and for subtyping of strains beyond species level.¹⁸ Chromosomal DNA was purified by phenol extraction and isopropanol precipitation.¹⁹ The purity and concentration of DNA were determined spectrophotometrically and in agarose gels. Chromosomal DNA (2–5 µg) was digested with

restriction endonuclease *Sma*I under recommended conditions (Boehringer Mannheim, GmbH Mannheim, Germany). DNA from representative strains of *A. caviae* (HG 4) and *A. sobria* (HG 8/10) were also digested with *Pst*I and *Bgl*II (Boehringer Mannheim). The digests were analysed in agarose 1.2% gels (Sea Kem ME agarose, FMC BioProducts, Rockland, USA), with half-strength TBE (45 mM Tris, 1 mM EDTA, pH adjusted to 8.0 with boric acid) as running buffer. The DNA was transferred to a positively charged nylon membrane (Hybond[®]-N+; Amersham International plc) by vacuum-blotting (Vacugene[®] XL vacuumblotting system; Pharmacia LKB Biotechnology, Uppsala, Sweden). The probe was plasmid pKK3535 harbouring the 5S, 16S, and 23S rRNA genes. The membranes were pre-hybridised for 2 h and hybridised overnight at 60°C in sealed plastic bags. DNA restriction fragments which hybridised with the digoxigenin-labelled probe were detected colorimetrically by alkaline phosphatase-labelled anti-digoxigenin as recommended in the DIG DNA Labeling and Detection Kit (Boehringer Mannheim). Reference strains of each hybridisation group were used for comparison of ribotyping patterns.

Statistical analysis

Fisher's exact test was used when the groups with or without diarrhoea were compared.

Results

Aeromonas spp. in tourists to Morocco

Aeromonas spp. were isolated from 32 (3.3%) of 978 adult tourists travelling to Morocco in the winter and autumn of 1989 (fig. 1). Of these, 22 isolates (8.7%) were from 254 tourists with diarrhoea ($p < 0.001$, compared with 10 isolates from 724 non-diarrhoeal participants). The total isolation frequency in winter (2%) did not differ statistically from that of autumn (4%). In 14 (5.5%) tourists with diarrhoea, *Aeromonas* spp. were isolated as the sole pathogen ($p < 0.001$, compared with similar findings in eight tourists without diarrhoea). In 10 tourists with or without diarrhoea, *Aeromonas* spp. were found in association with some other enteric pathogen (enterotoxigenic *Escherichia coli*, *Campylobacter jejuni* or *Salmonella enterica* serovar Enteritidis) (fig. 1).

A. sobria (*A. veronii* biotype *sobria*) and *A. caviae* were the most common *Aeromonas* spp. identified (fig. 2). More than half of *A. veronii* biotype *sobria* (6 of 11 strains) and *A. caviae* (6 of 10 strains) isolations were from diarrhoeal patients with no other identified pathogens in their stools ($p < 0.001$, compared with the corresponding findings in non-diarrhoeal participants) (fig. 2). In the samples from 19 non-diarrhoeal participants, all three common *Aeromonas* spp.—*A. hydrophila* (four isolates), *A. caviae* (two) and

Fig. 1. *Aeromonas* spp. in faecal samples from 978 adult Finnish tourists who travelled to Morocco in 1989: ▨, sole pathogen isolated; □, with another enteric pathogen; * $p < 0.001$ compared with non-diarrhoeal tourists, Fisher's exact test.

Fig. 2. Distribution of identified *Aeromonas* spp. and corresponding hybridisation groups in faecal samples from 254 diarrhoeal and 724 non-diarrhoeal (□) Finnish tourists who travelled to Morocco in 1989; ▨, diarrhoeal cases with *Aeromonas* sp. as sole pathogenic isolate; ▩, diarrhoeal cases with *Aeromonas* sp. plus other enteric pathogens.

A. sobria (two)—were isolated. The remaining two isolates were *A. trota* and *A. veronii* biotype *veronii*.

All patients with *Aeromonas* spp. as the sole patho-

gen had macroscopically watery or slimy diarrhoea lasting 2–20 (mean 5.9) days. Fever, nausea, headache or abdominal pain were present in some patients. No

Fig. 3. *Aeromonas* spp. isolated from diarrhoeal patients living in southern part of Finland with (▨, 16 cases) or without (□, 39 cases) association to recent travel abroad.

Fig. 4. Ribotyping patterns of representative strains *A. caviae* HG 4 (lanes 1–8) and *A. veronii* biotype *sobria* HG 8/10 (10–19) isolated from tourists with diarrhoea in Morocco. DNA was digested with *Sma*I and the Southern blot was probed with digoxigenin-labelled cDNA of plasmid pKK3535 harbouring the 5S, 16S and 23S rRNA genes. Mol. wt marker (λ DNA *Hind*III digest; 21) and ribotypes of reference strains of HG 4 (CDC 9083-79, 9) and HG 8/10 (CDC 0337-80, 20).

clear association of particular symptoms with a specific *Aeromonas* sp. was found.

Species identification and typing of isolates

The strains were first identified by phenotypic methods as *A. hydrophila*, *A. caviae*, *A. sobria*, *A. trota* or biochemically atypical *Aeromonas* spp. Ribotyping was used for the identification of genospecies and for subtyping of strains within a genospecies. The genospecies most commonly identified were HG 8/10 (*A. veronii* biotype *sobria* and *A. veronii* biotype *veronii*-like), HG 4 (*A. caviae*) and HG 1 (*A. hydrophila*)

(fig. 2). Of 10 *A. caviae* strains, nine belonged to HG 4 and one to HG 5B. Ribotyping confirmed some atypical strains as *A. hydrophila* HG 1 and some strains resembling *A. veronii* biotype *veronii* as members of HG 8/10. All *A. veronii* biotype *veronii*-like strains gave positive results in arginine dihydrolase and negative in ornithine decarboxylase tests. In fig. 4, the ribotyping patterns of genomic DNA of some typical strains of the predominating *Aeromonas* spp. in Morocco, *A. caviae* HG 4 (lanes 1–8) and *A. sobria* biotype *veronii* HG 8/10 (lanes 10–19) digested with *Sma*I are presented. The mol. wt area (lane 21) of c. 0.8–4 kb identified a HG and the mol. wt area of

c. 4–23 kb was used for the comparison of identity within a HG.¹⁸ Ribotype patterns of DNA digested with *Sma*I, *Pst*I or *Bgl*II indicated that all strains within a genospecies had different ribotypes (results of digests with *Pst*I and *Bgl*II are not shown).

Analysis of stool samples collected in Finland

In faecal samples collected in Finland, either from people without any history of travelling or from people with a recent travelling history, *A. veronii* biotype *sobria*, *A. caviae*, *A. hydrophila* and *A. veronii* biotype *veronii* were isolated from 20, 18, 14 and three patients, respectively (fig. 3). Half (10 of 20) of the *A. veronii* biotype *sobria* isolates were associated with diarrhoea acquired during travelling abroad (Spain, Tanzania, Turkey, Egypt, Kenya or Italy). In four of 14 cases, *A. hydrophila* was isolated from faecal samples taken after travel to Russia, Estonia or India. Only one of 18 *A. caviae* isolates was associated with travelling abroad. Ribotypes of the strains were all different within a species and they also differed from the respective ribotypes of the strains isolated from subjects vacationing in Morocco.

Discussion

At present, about 500 000 Finnish people travel yearly to holiday destinations in Mediterranean countries or Asia. Gastrointestinal symptoms, including diarrhoea during or soon after the trip, affect more than 30 000 of them.^{13, 20} In our extensive study of travellers' diarrhoea among Finns vacationing in Morocco, specific enteropathogens were isolated from c. 60% of diarrhoeal patients. *Aeromonas* spp. were the fourth most frequent enteropathogens isolated.^{13–15}

In the present study, the role of different species of *Aeromonas* in diarrhoea was examined by phenotypic and genotypic identification of all strains isolated during two seasons. Although some studies on the aetiology of travellers' diarrhoea report on the isolation of *Aeromonas* spp. among other enteropathogens, they do not report on the phenospecies or genospecies distribution.^{4, 7, 8, 21} *Aeromonas* spp. as the sole pathogen were isolated with significantly higher frequency (5.5%) from diarrhoeal patients than from subjects without diarrhoea (1.4%). No seasonal variation in the overall isolation rate of *Aeromonas* spp. was seen, in contrast to *Campylobacter jejuni*, which was more common in winter and to enterotoxigenic *E. coli* (ETEC), which was more common in autumn.¹³ *Aeromonas* spp. were also isolated from 1.1% of faecal samples from 724 people without diarrhoea. The carriage rate of *Aeromonas* spp. in the general population is reported to vary from 0.5 to 20% depending on the population groups studied and the methods used for the detection of the

organisms.^{1, 22} Symptomatic infections with multiple pathogens are rather common in travellers' diarrhoea.^{13, 21} In the present study, *Aeromonas* spp. were isolated from eight patients (36%) from a total 22 with diarrhoea, together with either ETEC, *C. jejuni* or *Salmonella*. One patient had *A. veronii* biotype *sobria*, *A. veronii* biotype *veronii*-like and ETEC in his stool sample.

A. caviae (HG 4) and *A. veronii* biotype *sobria* (HG 8/10) were the predominant species in diarrhoeal patients and they also predominated in the patients who had an *Aeromonas* spp. as the sole pathogen. The analysis on the distribution of *Aeromonas* spp. among another group of tourists who had diarrhoea after returning from various developing countries to Finland also suggested that *A. veronii* biotype *sobria* was associated with travelling. In contrast, *A. caviae* and *A. hydrophila* isolates were not frequently associated with travelling but the infection was acquired in Finland. In Europe and the USA,^{1, 6, 22} *A. caviae* has been the predominant species identified in diarrhoeal patients, although *A. hydrophila* and *A. sobria* were also isolated. *A. hydrophila* (HG 1) and *A. trota* (HG 13) were isolated from asymptomatic travellers in Morocco or in association with some other pathogen in symptomatic patients. *A. trota* is probably a typical species for subtropical or tropical regions because the original 12 *A. trota* strains described by Carnahan *et al.*²³ also were isolated from southern or southeastern Asia.

Identification of *Aeromonas* spp. to the genospecies level may produce new information on the epidemiology and pathogenesis of aeromonad-associated infections. This study confirmed the results of the earlier studies of Kuijper *et al.*,⁶ Altwegg *et al.*¹² and Abbott *et al.*¹⁷ on the predominance of HG 8/10 (*A. veronii* biotype *sobria* and biotype *veronii*), HG 4 (*A. caviae*) and HG 1 (*A. hydrophila*) in faecal samples. HG 3 (*A. hydrophila*) was isolated from one diarrhoeal patient (Russia) and HG 5B (*A. caviae*) from one sample from a patient with diarrhoea (Morocco). HG 13 (*A. trota*) is probably associated with infections acquired in temperate or tropical areas.

Ribotyping of strains was used to investigate possible epidemiological links between strains. All strains within a HG had a unique ribotype with all three restriction enzymes used. No epidemiological link was found between the strains of a species although they were isolated during the same trip to Morocco or other parts of the world. The wide distribution of ribotypes indicated heterogeneity within a HG and suggested that a wide variety of strains within a HG can colonise the human intestine. More specific studies are needed to prove the role of *Aeromonas* spp. in diarrhoea. *Aeromonas* spp. are common in food and water,²⁴ and eating food or drinking water contaminated with *Aeromonas* strains is not uncommon.

In conclusion, this study has suggested that *Aeromonas* spp., especially *A. veronii* biotype *sobria* (HG 8/10) and *A. caviae* (HG 4), were common in

travellers' diarrhoea although their causative role is not proven. No evidence was shown of a possible

epidemiological link between the strains because all the strains within a species had different ribotypes.

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