Phenotypic and genotypic identification of *Aeromonas* spp. isolated from a chlorinated intermittent water distribution system in Lebanon

Sima Tokajian and Fuad Hashwa

ABSTRACT

Aeromonas spp. were detected in samples collected from both untreated groundwater and treated drinking water in Lebanon. *Aeromonas* spp. levels ranged between 2 and 1,100 colonies per 100 ml in the intake underground well and between 3 and 43 colonies per 100 ml in samples from the distribution system. Samples positive for *Aeromonas* spp. from the network had a free chlorine level ranging between 0 and 0.4 mg l^{-1}. Multiple antibiotic-resistance was common among the isolated aeromonads; all were resistant to amoxycillin while 92% showed resistance to cephalexin. Haemolysis on blood agar was detected in 52% of the isolates recovered from the distribution network and 81% of isolates from the untreated underground source. The Biolog microbial identification system assigned identities to all of the isolated presumptive aeromonads (at least at the genus level), which was not the case with the API 20NE strips. Differences at the species level were observed when results from the Biolog system were compared with identification based on the MicroSeq 500 16S rDNA sequence analysis. The presence of *Aeromonas* spp. in drinking water can be an important threat to public health, thus greater awareness of *Aeromonas* strains as potential enteropathogens is warranted.

Key words | *Aeromonas*, antibiotic resistance, drinking water, 16S rDNA sequencing

Sima Tokajian
Fuad Hashwa (corresponding author)
Microbiology and Biotechnology,
Lebanese American University,
Byblos Campus,
PO Box 36 F-80,
Byblos,
Lebanon
Tel.: +961-9-547254 x2340
Fax: +961-9-547256
E-mail: *fhashwa@lau.edu.lb*

INTRODUCTION

Aeromonas are Gram-negative, oxidase-positive, facultative anaerobic, glucose fermenting, rod-shaped bacteria of the family Aeromonadaceae (Granum *et al*. 1998; Ghenghesh *et al*. 2001; Ormen & Ostensvik 2001). *Aeromonas* is widely distributed in aquatic environments and was frequently isolated from drinking water (Joseph & Carnahan 2000). A seasonal pattern, where higher numbers of *Aeromonas* were recovered during the summer, was previously reported (Kaper *et al*. 1981; Burke *et al*. 1984; *Gavriel et al*. 1998; Chauret *et al*. 2001), and a negative correlation was detected between *Aeromonas* isolation from drinking water and chlorination (Gavriel *et al*. 1998). Additionally, studying the survival ability of *A. hydrophila* in freshwaters and nutrient-poor waters revealed the ability of the organisms to survive under nutrient poor conditions in the absence of the autochthonous microbiota (Lowcock & Edwards 1994; Kersters *et al*. 1996).

Strains of *Aeromonas* species could be enteropathogens (Holmberg *et al*. 1986) and the main putative virulence factors are endotoxins (LPS), enetrotoxins, cytotoxins (aerolysins, phospholipases, haemolysins) and/or the ability to invade epithelial cells (Kirov 1993; Merino *et al*. 1995). The potential of *Aeromonas* to cause enteric and non-enteric diseases in humans and the susceptibility of patients with hepatobiliary disease to infection with *Aeromonas*, suggests that water for domestic use should be free of these organisms (Burke *et al*. 1984). The prevalence and distribution of *Aeromonas* in aquatic environments, its role as a contaminant of drinking water supplies and the potential for pathogenicity mediated by mesophilic *Aeromonas* are all of great public health concern

(Gavriel *et al*. 1998; Dumontet *et al*. 2000; Joseph & Carnahan 2000).

Many countries in the Middle East, including Lebanon, suffer from chronic water shortage. As a result supplies generally operate on an intermittent basis (Tokajian & Hashwa 2003), leaving the distribution system un-pressurized for long periods of time. Operating a distribution system in a non-continuous manner leads to conditions that favour the deterioration of water quality, due mainly to infiltration, regrowth within pipes and the detachment of the bacterial biofilm following variations in pressure and velocity. The aims of this study were to: determine the presence of *Aeromonas* spp. in chlorinated drinking water samples collected from an intermittent water distribution system; identify the isolated strains to species level using genotypic and phenotypic methods; investigate haemolytic activity; and test for multiple antibiotic resistance.

METHODS

Water samples

The study was conducted on a drinking water distribution network feeding a residential area in Lebanon. This residential area, fed by gravity, is supplied twice a week from underground water originating from an artesian well. Consumers receive water after it has been chlorinated and mixed in a 40 m^3-roofed reservoir. The distribution of water is intermittent. Water samples from the different sites on the distribution network and from the raw un-chlorinated underground source were collected in wide-mouthed polypropylene bottles that were sterilized at 121°C for 15 min, 24 h before the sampling day. Sodium thiosulfate was added to neutralize any residual chlorine in the drinking water samples (HMSO 1994).

Culture media

Aeromonas agar base (Oxoid) was used for the isolation of presumptive *Aeromonas* spp. The medium was autoclaved for 15 min at 121°C, cooled to 50°C and freshly prepared ampicillin (10 mg l^{-1}) was added. Water samples (100 ml) were assayed with the membrane filter technique using 0.45 μm filters (Millipore). Aeromonas agar plates were incubated at 30°C for 24 h, and all presumptive *Aeromonas* colonies were selected for further identification.

Phenotypic identification

Gram-negative and oxidase positive strains were further identified with API 20NE (bioMerieux, Marcy-L'Etoile, France) and Biolog (Biolog, Inc., Hayward, California) microbial identification systems. The metabolic profile of each organism using the Biolog microtiter plates was compared automatically, by using the MicroLog software, with the MicroLog GN database (release 4.01A). Biolog identifications were reported if the similarity index of the genus or species was 0.5 or greater (similarity index 88% or greater) after 24 h of incubation.

Antibiotic susceptibility testing

Antibiotic susceptibility was determined by the agar diffusion method, using Mueller-Hinton agar (Oxoid). Results were interpreted according to the current NCCLS guidelines (NCCLS 2000). Eight antibiotics corresponding to the drugs most commonly used in the treatment of human infections caused by Gram-negative bacteria were used. The antibiotics (Oxoid) tested and their sensidisk concentrations were: amikacin (AK), 30 μg; sulfamethoxazole/trimethoprim (SXT), 25 μg; tetracycline (TE), 30 μg; cephalexin (CL), 30 μg; kanamycin (K), 30 μg; chloramphenicol (C), 10 μg; gentamicin (GN), 10 μg; amoxycillin (AML), 25 μg.

Haemolysis assay

Haemolysis was assayed on tryptone soy agar (Oxoid) plates with 5% whole sheep blood (Lye & Dufour 1991). Plates were incubated at 30°C and were checked for the type (α or β) of haemolytic activity after 24 h.

DNA extraction

Based on morphological characteristics a number of colonies representing all recovered aeromonads in this study

were chosen for identification based on 16S rDNA analysis and were designated as HST-109 through HST-122. DNA extractions were made from pure cultures using the Prepman protocol for Gram-negative bacteria as described by the manufacturer (Applied Biosystems, USA). DNA extraction was first evaluated by the amplification of the 16S rRNA gene using the gene sequence specific primers: forward primer 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) (Thermo Hybaid, Germany) (Stackebrandt *et al*. 1988; DeLong 1992).

The amplification was performed on 1.5 μl DNA extract in 20 μl using Expand High Fidelity PCR System (Roche, Germany). PCR products were visualized by ethidium bromide staining on agarose gel electrophoresis.

Sequencing of 16S rDNA

A 500-bp 16S rDNA fragment was amplified using the MicroSeq 500 kit (Applied Biosystems, USA) in a reaction volume of 20 μl (10 μl of MicroSeq PCR master mix, 8.5 μl of molecular-grade water, 0.05 units of AmpliTaq Gold (Applied Biosystems, USA) and 1.5 μl DNA extract). Prior to sequencing the amplified products were purified (0.2 units of shrimp alkaline phosphatase and exonuclease 1 (USB, Cleveland, Ohio) were mixed with 10 μl of the amplified product and incubated at 37°C for 30 min, and then at 80°C for 15 min). Forward and reverse sequencing reactions were performed for each amplified product followed by purification using ethanol precipitation.

Sequence determination and analysis

Sequences were analysed on the ABI PRISM 310 (Applied Biosystems, USA) automated DNA sequencer. Using MicroSeq 500 microbial identification and analysis software, fragmentary sequence information were aligned and assembled, and the final consensus sequence was compared with over 1,200 validated 16S ribosomal DNA sequences in the database (Suzuki & Giovannoni 1996; Turenne *et al*. 2001). Sequences were compared with published 16S rDNA sequences from the EMBL (FASTA version 3.3t09) (Pearson & Lipman 1988). The 16S rDNA sequences of *Aeromonas* spp. and their accession numbers were derived from the GenBank. A phylogenetic tree was constructed using the TREECON software. Bootstrap analysis was also used to place confidence intervals on phylogenies (Van de Peer & Watcher 1994).

RESULTS

Aeromonas spp. in the underground well and the distribution network

An overview of the types, different properties and sources of *Aeromonas* isolated during this study is shown in Tables 1 and 2. *Aeromonas* spp. were detected in samples collected from both the untreated underground water (Table 1) and the network (Table 2). *Aeromonas* spp. counts ranged between 2 and 1,100 colonies per 100 ml in the underground well and between 3 and 43 colonies per 100 ml in samples from the distribution system. Samples positive for *Aeromonas* spp. from the network had a free residual chlorine level ranging between 0 and 0.4 mg l^{-1} (Table 2). *A. veronii*, *A. hydrophila*, *A. caviae* and *A. jandaei* were recovered from the underground well and the network, while *A. allosaccharophila* was detected only in the raw water source (Table 1).

All isolates were resistant to amoxycillin and 92% showed additional resistance to cephalexin. Resistance to tetracycline was detected only with *Aeromonas* DNA Group 5A isolated from the distribution system (Table 2). Multiple antibiotic resistance was detected in 94% of the isolates. All of the isolates were sensitive to amikacin, kanamycin, chloramphenicol and gentamicin. Haemolysis on blood agar was detected in 52% of the isolates recovered from the distribution network and in 81% of isolates from the untreated groundwater source.

The Biolog microbial identification system assigned identities to all of the isolated presumptive aeromonads, which was not the case with the API 20NE strips.

Identification of *Aeromonas* spp. based on 16S rDNA sequence analysis

The 16S rDNA sequence analysis of aeromonads recovered from untreated and treated water samples showed

Table 1 | Types (based on Biolog identification), antibiotic resistance and haemolytic activity of *Aeromonas* spp. in samples from the underground well

Types isolated	Number 100 ml^{-1}	Antibiotic resistance*	Haemolytic activity
Aeromonas veronii DNA Group 10	25	AML25 + CL30	+
Aeromonas MEDIA-Like DNA Group 5A	18	AML25 + CL30	+
Aeromonas caviae DNA Group 4	4	AML25 + CL30 SXT25 + C10	–
Aeromonas caviae DNA Group 4	25	AML25	+
Aeromonas hydrophila DNA Group 1	7	AML25 + CL30 SXT25	+
Aeromonas DNA Group 5B	8	AML25 + CL30	+
Aeromonas DNA Group 5A	60	AML25 + CL30	+
Aeromonas jandaei DNA Group 9	1,100	AML25 + CL30	–
Aeromonas allosaccharophila	66	AML25 + CL30	+
Aeromonas hydrophila DNA Group 5A	2	AML25	+
Aeromonas DNA Group 5B	4	AML25	+

*Sulfamethoxazole/trimethoprim (SXT); cephalexin (CL); amoxycillin (AML); chloramphenicol (C).

98–99% similarities with their closest match in the GenBank (Table 3). The phylogenetic tree reflects the phylogenetic relations of the isolates to their closest relatives (Figure 1). *Aeromonas hydrophila* strain 45/90 had 99% similarity to HST-110, HST-116 and HST-117 (Table 3 and Figure 1). HST-120 and HST-121 had 96% and 98% similarity to *Aeromonas veronii*, respectively. *Aeromonas media* and *Aeromonas caviae* had 98% similarity to HST-112 and HST-122, respectively.

Differences only at the species level were detected when the identification of *Aeromonas* using the Biolog system was compared with the outcome of identification based on phylogenetic analysis.

DISCUSSION

In the present study *Aeromonas* spp. were recovered in samples from the network with residual free chlorine ranging between 0 and 0.4 mg l^{-1} and in samples from the untreated underground water source. *Aeromonas hydrophila* and *A. caviae* were isolated from the source and the distribution network. *A. veronii*, the most invasive species known to cause severe *Aeromonas* gastroenteritis (Granum *et al.* 1998; Ormen & Ostensvik 2001), was also isolated from the chlorinated distribution network. Decreasing levels of chlorination increased the probability of detecting *Aeromonas* in drinking water reservoirs (Gavriel *et al.* 1998). Enteropathogenic *Aeromonas* species were found to be commonly present in untreated drinking water obtained from wells in Libya (Ghenghesh *et al.* 2001), and it has been shown that enteropathogenic strains can be acquired by drinking untreated water (Holmberg *et al.* 1986).

Although the number of *Aeromonas* isolates recovered from the network did not exceed 43 colonies per 100 ml, Legnani *et al.* (1998) demonstrated that under

Table 2 | Types (based on Biolog identification), antibiotic resistance and haemolytic activity of *Aeromonas* spp. in samples from the distribution network

Types isolated	Number 100 ml^{-1}	Antibiotic resistance*	Free chlorine mg l^{-1}	Haemolytic activity
Aeromonas caviae DNA Group 4	5	AML25 + CL30	0.04	+
Aeromonas DNA Group 5B	43	AML25	0	+
Aeromonas DNA Group 5A	21	TE30	0.1	–
Aeromonas DNA Group 5B	16	AML25 + CL30 SXT25	0.1	–
Aeromonas caviae DNA Group 4	18	AML25	0.1	+
Aeromonas veronii DNA Group 10	11	AML25 + CL30 C10	0	+
Aeromonas DNA Group 5B	13	AML25 + CL30	0	+
Aeromonas DNA Group 5A	9	AML25 + CL30	0	+
Aeromonas jandaei DNA Group 9	37	AML25 + CL30	0	–
Aeromonas hydrophila DNA Group 2	3	AML25 + CL30	0.4	+

*Sulfamethoxazole/trimethoprim (SXT); cephalexin (CL); amoxycillin (AML); chloramphenicol (C); tetracycline (TE).

particular conditions these bacteria might reach higher concentrations (regrowth) and thus pose a risk particularly to susceptible subjects in the population. Assanta *et al*. (1998) demonstrated that *Aeromonas* strains could easily attach to different water distribution pipe surfaces after exposures as short as 1–4 h, and were occasionally recovered from drinking water reservoirs exposed to sustained chlorine disinfection. On the other hand, secondary enrichment, as is the case with food production, can lead to human diseases even in the presence of low microbial counts of pathogenic *Aeromonas* strains in drinking water (Schubert 2000).

In the present study haemolysis on blood agar was detected in 52% and 81% of the isolates recovered from the distribution network and the untreated underground source, respectively. Bondi *et al.* (2000) showed that 87% of 30 *A. hydrophila* strains isolated from superficial swimming pool water were haemolytic and that haemolytic and cytotoxic activities were frequently associated.

The antibiotic susceptibilities in this study were in broad agreement with those of other workers (LeChevallier *et al*. 1982; Krovacek *et al.* 1989; Miranda & Castillo 1998; Goni-Urriza *et al.* 2000; Ghenghesh *et al.* 2001). The isolates were resistant to amoxycillin and 92% showed resistance to cephalexin, while all were sensitive to amikacin, kanamycin, chloramphenicol and gentamicin.

This is the first study reporting the isolation and identification of *Aeromonas* spp. from an intermittent drinking water distribution network in Lebanon. Identification of presumptive aeromonads to the genus level could be successfully achieved using the Biolog microbial identification system and the 16S rDNA gene sequence analysis. The present study, similar to others reported in the literature (Lane *et al*. 1985; Woese 1987; Böttger 1989; Braun-Howland *et al*. 1993; Suzuki & Giovannoni 1996; Abraham *et al*. 1999; Boye *et al*. 1999; Kolbert & Persing 1999; Drancourt *et al*. 2000; Patel *et al*. 2000; Turenne

Table 3 | 16S rDNA-based identification of *Aeromonas* from drinking water

Isolate	16S rDNA-based identification	GenBank accession no.
Isolates exhibiting 99% 16S rDNA sequence homology with a deposited sequence		
HST-110	*Aeromonas hydrophila* strain 45/90	AF468055
HST-114	*Aeromonas caviae* ATCC 15468T	X74674
HST-116	*Aeromonas hydrophila* strain 45/90	AF468055
HST-117	*Aeromonas hydrophila* strain 45/90	AF468055
Isolates exhibiting 98% 16S rDNA sequence homology with a deposited sequence		
HST-109	*Aeromonas hydrophila*	X97271
HST-112	*Aeromonas media* ATCC 33907T	AY190157
HST-115	*Aeromonas hydrophila* strain 45/90	AF468055
HST-121	*Aeromonas veronii* clone 4	AF418212
HST-122	*Aeromonas caviae* ATCC 15468T	X74674
Isolates exhibiting 96–97% 16S rDNA sequence homology with a deposited sequence		
HST-118	*Aeromonas* sp. PAR3	AF108136
HST-119	*Aeromonas hydrophila* ATCC7966T	X74674
HST-120	*Aeromonas veronii* ATCC 35624T	AB033950

et al. 2001), revealed that the 16S rRNA gene sequences frequently provide phylogenetically useful information. However, identification based on phylogenetic analysis could be simplified by the determination of the first 500-bp sequence of the 16S rRNA gene.

CONCLUSION

In recent years there has been an increasing number of reports on *Aeromonas* spp. associated with health problems; drinking water may represent an important source of pathogenic *Aeromonas* spp. (Handfield *et al*. 1996). The isolation of *Aeromonas* spp. from samples collected from the distribution network in this study could be attributed to the ineffective treatment strategy employed in the system, which may enhance the survival of the organism as suspended or as part of the biofilm community. This preliminary study further emphasizes the need to survey the incidence of *Aeromonas* spp. in chlorinated drinking water in relation to *Aeromonas*-associated enteritis in Lebanon. Moreover, studies on *Aeromonas* in drinking water should include not only enumeration but also assessment of the virulence factors of the predominant strains.

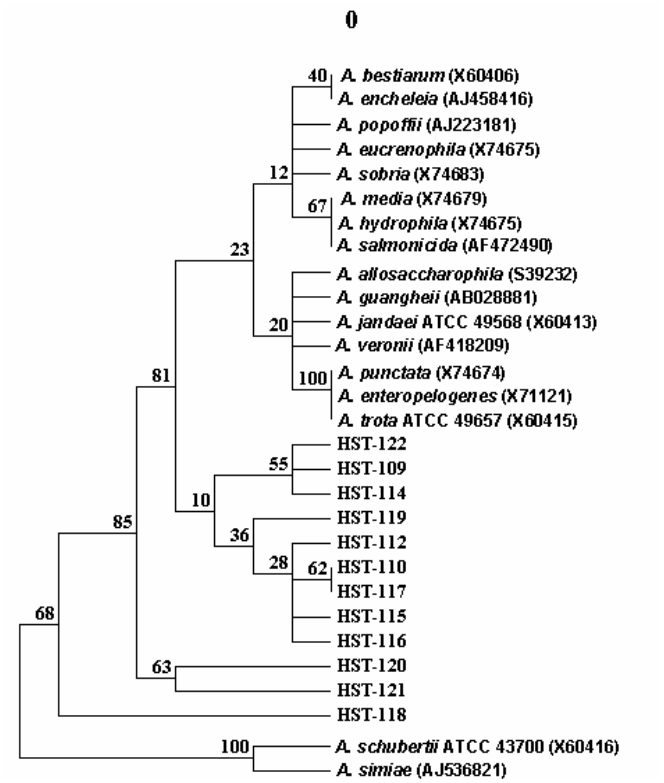

Figure 1 | Phylogenetic tree relating sequence-based identification of *Aeromonas* spp. GenBank accession numbers for the different aeromonads are presented between brackets. The algorithm used to construct the tree is the unweighted pair group method using averages (UPGMA). Bootstrap values are displayed as percentages of 100 replicons.

REFERENCES

Abraham, W., Strompl, C., Meyer, H., Lindholst, S., Moore, E., Christ, M., Vancanneyt, B., Tindall, B., Bennasa, J., Smit, J. & Tesar, M. 1999 Phylogeny and polyphasic taxonomy of *Caulobacter* species. Proposal of *Maricaulis* gen. nov. with *Maricaulis maris* (Poindexter) comb. Nov. as the type species, and emended description of the genera *Brevundimonas* and *Caulobacter*. *Int. J. Syst. Bacteriol.* **49**, 1053–1073.

Assanta, M., Roy, D. & Montpetit, D. 1998 Adhesion of *Aeromonas hydrophila* to water distribution system pipes after different contact times. *J. Fd Prot.* **61**, 1321–1329.

Bondi, M., Messi, P., Guerrieri, E. & Bitonte, F. 2000 Virulence profiles and other biological characters in water isolated *Aeromonas hydrophila*. *Microbiologica* **23**, 347–356.

Böttger, E. 1989 Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. *FEMS Microbiol. Lett.* **65**, 171–176.

Boye, K., Hogdall, E. & Borre, M. 1999 Identification of bacteria using two degenerate 16S rDNA sequencing primers. *Microbiol. Res.* **154**, 23–26.

Braun-Howland, E., Vescio, P. & Nierzwicki-Bauer, S. 1993 Use of simplified cell blot technique and 16S rRNA-directed probes for the identification of common environmental isolates. *Appl. Environ. Mirobiol.* **59**, 3219–3224.

Burke, V., Robinson, J., Gracey, M., Peterson, D., Norman, M. & Haley, V. 1984 Isolation of *Aeromonas hydrophila* from the metropolitan water supply: seasonal correlation with clinical isolates. *Appl. Environ. Microbiol.* **48**, 367–370.

Chauret, C., Volk, C., Creason, R., Jarosh, J., Robinson, J. & Warnes, C. 2001 Detection of *Aeromonas hydrophila* in drinking-water distribution system: a field and pilot study. *Can. J. Microbiol.* **47**, 782–786.

DeLong, E. 1992 Archaea in coastal marine environments. *Proc. Natn Acad. Sci.* **89**, 5685–5689.

Drancourt, M., Bollet, C., Carlioz, A., Martellin, R., Gayral, J. & Raoult, D. 2000 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *J. Clin. Microbiol.* **38**, 3623–3630.

Dumontet, S., Krovacek, K., Svenson, S., Pasquale, V., Baloda, S. & Figiuolo, G. 2000 Prevalence and diversity of *Aeromonas* and *Vibrio* spp. in coastal water of Southern Italy. *Comp. Immunol. Microb.* **23**, 53–72.

Gavriel, A., Landre, J. & Lamb, A. 1998 Incidence of mesophilic *Aeromonas* within public drinking water supply in north-east Scotland. *J. Appl. Microbiol.* **84**, 383–392.

Ghenghesh, K., El-Ghodban, A., Dkakni, R., Abeid, S., Altomi, A., Tarhuni, A. & Marialigeti, K. 2001 Prevalence, species differentiation, hemolytic activity, and antibiotic susceptibility of Aeromonads in untreated well water. *Mem. Inst. Oswaldo Cruz* **96**, 169–173.

Goni-Urrizza, M., Pineau, L., Capdepuy, M., Roques, C., Caumette, P. & Quentin, C. 2000 Antimicrobial resistance of mesophilic *Aeromonas* spp. isolated from European rivers. *J. Antimicrob. Chemother.* **46**, 297–301.

Granum, P., O'Sullivan, K., Tomas, J. & Ormen, O. 1998 Possible virulence factors of *Aeromonas* spp. from food and water. *FEMS Immunol. Med. Microbiol.* **21**, 131–137.

Handfield, M., Simard, P. & Letarte, R. 1996 Differential media for quantitative recovery of waterborne *Aeromonas hydrophila*. *Appl. Environ. Microbiol.* **62**, 3544–3547.

HMSO 1994 The microbiological examination of water. Reports on Public Health and Medical Subjects Memorandum No. 71, Department of Health and Social Security, London.

Holmberg, S., Schell, W., Fanning, G., Wachsmuth, K., Hickman-Brenner, F., Blake, P., Brenner, D. & Framer, J. 1986 *Aeromonas* intestinal infections in the United States. *Ann. Intern. Med.* **105**, 683–689.

Joseph, S. & Carnahan, A. 2000 Update on the genus *Aeromonas*. *ASM News* **66**, 218–223.

Kaper, J., Lockman, H. & Colwell, R. 1981 *Aeromonas hydrophila*: ecology and toxigenicity of isolates from an estuary. *J. Appl. Bacteriol.* **50**, 359–377.

Kersters, I., Huys, G., Duffel, H., Vancanneyt, M., Kersters, K. & Verstraete, W. 1996 Survival potential of *Aeromonas hydrophila* in freshwaters and nutrient-poor waters in comparison with other bacteria. *J. Appl. Bacteriol.* **80**, 266–276.

Kirov, S. 1993 The public health significance of *Aeromonas* spp. in foods. *Int. J. Fd Mirobiol.* **20**, 178–198.

Kolbert, C. & Persing, D. 1999 Ribosomal DNA sequencing as a tool for the identification of bacterial pathogens. *Curr. Opin. Mirobiol.* **2**, 200–305.

Krovacek, K., Peterz, M., Ahmed, F. & Mansson, I. 1989 Enterotoxigenicity and drug sensitivity of *Aeromonas hydrophila* isolated from well water in Sweden: a case study. *Int. J. Fd Microbiol.* **8**, 149–154.

Lane, D., Pace, B., Olsen, G., Stahl, D., Sogin, M. & Pace, N. 1985 Rapid determination of 16S ribosomal RNA sequence for phylogenetic analyses. *Proc. Natn. Acad. Sci.* **82**, 6955–6959.

LeChevallier, M., Evans, T., Seidler, R., Daily, O., Merrell, B., Rollins, D. & Joseph, S. 1982 *Aeromonas sobria* in chlorinated drinking water supplies. *Microbial Ecol.* **8**, 325–333.

Legnani, P., Leoni, E., Soppelsa, F. & Burigo, R. 1998 The occurrence of *Aeromonas* species in drinking water supplies of an area of Dolomite Mountains, Italy. *J. Appl. Microbiol.* **85**, 271–276.

Lowcock, D. & Edwards, C. 1994 Survival of genetically-marked *Aeromonas hydrophila* in water. *Lett. Appl. Microbiol.* **19**, 121–123.

Lye, D. & Dufour, A. 1991 A membrane filter procedure for assaying cytotoxic activity in heterotrophic bacteria isolated from drinking water. *J. Appl. Bacteriol.* **70**, 89–94.

Merino, S., Ribiers, X., Knochel, S. & Tomas, J. 1995 Emerging pathogens: *Aeromonas* spp. *Int. J. Fd Microbiol.* **28**, 157–168.

Miranda, C. & Castillo, G. 1998 Resistance to antibiotic and heavy metals of motile aeromonads from Chilean freshwater. *Sci. Total Environ.* **224**, 167–176.

NCCLS (National Committee for Clinical Laboratory Standards) 2000 Performance standards for disk susceptability. Approved standards NCCLS document M2-A7, 7th edn. Villanova, Pennsylvania.

Ormen, O. & Ostensvik, O. 2001 The occurrence of aerolysin-positive *Aeromonas* spp. and their cytotoxicity in Norwegian water sources. *J. Appl. Microbiol.* **90**, 797–802.

Patel, J., Leonard, D., Pan, X., Musser, J., Berman, R. & Nachamkin, I. 2000 Sequence-based identification of *Mycobacterium* species using the MicroSeq 500 16S rDNA bacterial identification system. *J. Clin. Microbiol.* **38**, 246–251.

Pearson, W. & Lipman, D. 1988 Improved tools for biological sequence comparison. *P. Natn. Acad. Sci.* **85**, 2444–2448.

Schubert, R. 2000 Intestinal cell adhesion and maximum growth temperature of psychrotrophic aeromonads from surface water. *Int. J. Hyg. Environ. Health* **203**, 83–85.

Stackebrandt, E., Murray, R. & Truper, H. 1988 *Proteobacteria* classis nov., a name for the phylogenetic taxon includes the 'purple bacteria and their relatives'. *Int. J. Syst. Bacteriol.* **38**, 321–325.

Suzuki, M. & Giovannoni, S. 1996 Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl. Environ. Microbiol.* **62**, 625–630.

Tokajian, S. & Hashwa, F. 2003 Water quality problems associated with intermittent water supply. *Wat. Sci. Technol.* **47**, 229–234.

Turenne, C., Tschetter, L., Wolfe, J. & Kabani, A. 2001 Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous *Mycobacterium* species. *J. Clin. Microbiol.* **39**, 3637–3648.

Van de Peer, Y. & De Watcher, R. 1994 TREECON for windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput. Appl. Biosci.* **10**, 569–570.

Woese, C. 1987 Bacterial evolution. *Microbiol. Rev.* **51**, 221–271.