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Distribution of six virulence factors in *Aeromonas* species isolated from US drinking water utilities: a PCR identification

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ABSTRACT

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Aims: To examine whether *Aeromonas* bacteria isolated from municipally treated water had virulence factor genes.

Methods and Results: A polymerase chain reaction-based genetic characterization determined the presence of six virulence factors genes, elastase (*ahyB*), lipase (*pla/lip/lipH3/alp-1*) flagella A and B (*flaA* and *flaB*), the enterotoxins, *act*, *alt* and *ast*, in these isolates. New primer sets were designed for all the target genes, except for *act*. The genes were present in 88% (*ahyB*), 88% (*lip*), 59% (*fla*), 43% (*alt*), 70% (*act*) and 30% (*ast*) of the strains, respectively. Of the 205 isolates tested only one isolate had all the virulence genes. There was a variety of combinations of virulence factors within different strains of the same species. However, a dominant strain having the same set of virulence factors, was usually isolated from any given tap in different rounds of sampling from a single tap.

Conclusions: These results show that *Aeromonas* bacteria found in drinking water possess a wide variety of virulence-related genes and suggest the importance of examining as many isolates as possible in order to better understand the health risk these bacteria may present.

Significance and Impact of the Study: This study presents a rapid method for characterizing the virulence factors of *Aeromonas* bacteria and suggests that municipally treated drinking water is a source of potentially pathogenic *Aeromonas* bacteria.

Keywords: *Aeromonas*, drinking water, polymerase chain reaction, virulence factors.

INTRODUCTION

Aeromonads, a class of Gram-negative organisms, are waterborne bacteria that are often found as environmental and food contaminants (Hanninen and Siitonen 1995). Having been implicated in clinical cases of diarrhoea, where they were isolated as the sole pathogen, these bacteria are considered as emerging pathogens (Janda and Abbott 1998; Isonhood and Drake 2002). The high prevalence of *Aeromonas* in the environment lends support to the hypothesis that infections are mainly acquired through the consumption of food and water, although no major outbreak has been documented (Chopra and Houston 1999). Besides diarrhoea, *Aeromonas* spp. have been known to cause other infections such as septicemia, wound infections, endocarditis, meningitis and pneumonia (Janda and Abbott 1998; Isonhood and Drake 2002).

Although most drinking water treatment strategies such as rapid/slow sand filtration, hyperchlorination/direct filtration and use of granular activated carbon, should reduce the number of aeromonads to low levels, the ability of *Aeromonas* to survive and grow in drinking water has been shown in several studies (Burke *et al.* 1984; Havelaar *et al.* 1990; Stelzer *et al.* 1992; Baloda *et al.* 1995; Huys *et al.* 1997; Kuhn *et al.* 1997; Gavriel *et al.* 1998; Janda and Abbott 1998; Brandi *et al.* 1999; Borchardt *et al.* 2003). This is because it has the potential to grow in water distribution systems especially in biofilms, where it is resistant to chlorination. In one study, Havelaar *et al.* (1990), reported

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the regrowth of *Aeromonas* in the distal parts of 16 of 20 Dutch distribution systems they examined, and especially drinking water that was derived from anaerobic ground waters containing methane. In this study they showed maximum recovery during the summer months when the temperatures were high; there was no correlation between total organic carbon or heterotropic plate counts. Other workers have demonstrated that water temperatures and free chlorine are the principal factors that affect the regrowth of *Aeromonas* (Burke *et al.* 1984; Holmes *et al.* 1996). Thus *Aer. hydrophila* could readily establish a biofilm together with other heterotrophic bacteria that could survive upto 0·6 mg l^{-1} of monochloramine, concentrations that were sufficient to remove *E. coli*-associated biofilms (Mackerness *et al.* 1991). Holmes *et al.* (1996) demonstrated that even after disinfection with 1 mg l^{-1} of chlorine, 10% of the pipe lengths still had aeromonads. Although free *Aeromonas* cells are susceptible to chlorine, chlorine dioxide and other chlorine-based disinfectants, it is thought that considerable time and elevated chlorine residual are needed to destroy the *Aeromonas* that are associated with biofilms.

Of the species isolated, *Aer. hydrophila*, *Aer. veronii* biovar *sobria* and *Aer. caviae* are the species most commonly implicated in human intestinal infections (Janda and Abbott 1998). Together these species account for >85% of the clinical isolates for this genus and thus they are considered major pathogens. *Aeromonas jandei*, *Aer. schubertii* and *Aer. veronii* biovar *veronii* are the other species that have been incriminated in infections, and are considered minor pathogens (Janda and Abbott 1998). *Aeromonas salmonicida* is an established fish pathogen, causing furunculosis that often results in the death of the fish (McGarey *et al.* 1991). *Aeromonas popoffii*, a species described recently (Huys *et al.* 1997; Demarta *et al.* 2000), was identified from freshwater and seawater and was shown to have most of the putative virulence genes (Soler *et al.* 2002). The mechanism of pathogenesis is complex and not well understood. *Aeromonas* virulence is considered to be multifactorial. Haemolysins, cytotoxins, enterotoxins, proteases (serine protease (AspA), elastase (AhpB)], lipases (Pla and Plc, Sat), DNAses and adhesins [type IV pili, polar flagella (FlaA and FlaB)] (Agarwal *et al.* 1998; Cascon *et al.* 2000; Rabaan *et al.* 2001) have all been identified as putative virulence factors in aeromonads. Several of these virulence factors have been identified in strains isolated from water (Handfield *et al.* 1996; Kuhn *et al.* 1997; Janda and Abbott 1998; Kingombe *et al.* 1999; Schubert 2000; Sechi *et al.* 2003). Of these, five factors have been shown by gene disruption techniques to be directly involved in the pathogenesis of the organism in animal models or cell lines. They are the enterotoxin, Act (Chopra *et al.* 1994, 1996; Xu *et al.* 1998; Sha *et al.* 2002) enterotoxin, Ast (Chakraborty *et al.* 1984; Sha *et al.* 2002), elastase (Cascon *et al.* 2000), and flagellin (Rabaan *et al.* 2001).

During 2000–2001, two small surveys were conducted by the USEPA to determine the frequency of occurrence of *Aeromonas* bacteria in drinking water. A total of 16 utilities in four states were sampled. Altogether 205 *Aeromonas* isolates were collected and identified. All the isolates were ampicillin and vancomycin resistant, and represented a wide variety of species (*Aer. hydrophila*, *Aer. salmonicida*, *Aer. bestiarum*, *Aer. veronii* biovar *sobria*, *Aer. caviae* and *Aer. enchelia*) (Birkenhauer, J. and Rodgers, M., US EPA, Cincinnati, OH, USA, in preparation). The goal of the present study was to determine how many of these isolates were potential pathogens, as measured by the presence of previously identified virulence-related genes. Using PCR, the presence of the following genes were determined: Act, *act* (Chopra *et al.* 1993), Alt, *alt* (Chopra *et al.* 1996), Ast, *ast* (Chopra *et al.* 1994), elastase, *ahyB* (Cascon *et al.* 2000), phospolipase, *pla/lipH3/apl-1/lip* (Anguita *et al.* 1993; Ingham and Pemberton 1995; Chuang *et al.* 1997; Merino *et al.* 1999) and flagellin, *fla* (Rabaan *et al.* 2001).

MATERIALS AND METHODS

Bacterial strains

The reference strains of *Aeromonas* used in this study (*Aer. hydrophila* ATCC 7966, *Aer. media*: ATCC 33907) were obtained from ATCC (Manassas, VA, USA). The *Aeromonas* water isolates were obtained from two surveys conducted by the US EPA during 2000 and 2000–2001. Stock cultures of the reference strains and the water isolates were frozen until the time of study.

Isolation of *Aeromonas* from drinking water

The *Aeromonas* strains were isolated by EPA method 1605. In this method *Aeromonas* in finished drinking water is isolated using ampicillin–dextrin agar with vancomycin (ADA-V) (Havelaar *et al.* 1987). Typically 500 ml of water was filtered through a sterile membrane filtration unit. This was performed within 30 h of sample collection. The filter was removed and placed on ADA-V plates. The plates were incubated at 35°C for 24 h. Yellow colonies were considered to be presumptive *Aeromonas*. Three additional biochemical tests, oxidase activity, trehalose fermentation and indole production, were used as confirmatory tests (Holt 1994). If a colony was positive for all three tests, then it was confirmed to be an aeromonad. The colony was archived in growth medium with 15% glycerol at −70°C, for further characterization, including species identification and virulence factors determination. The species identification of *Aeromonas* was performed by two methods: a suite of biochemical tests, including API-20E test system (bioMérieux, Marcy I'Etoile, France), as described by Abbott *et al.* (1992), and genetically by restriction fragment length polymorphism analysis of the 16S rRNA gene (RFLP) (Borrell *et al.* 1997) and will be described elsewhere (Birkenhauer, J. and Rodgers, M., US EPA, in preparation).

Preparation of DNA

Total chromosomal DNA from *Aeromonas* was prepared by using the Wizard Genomic DNA purification kit as specified by the manufacturer (Promega, Inc., Madison, WI, USA). DNA was extracted in a final volume of 100 μl of Tris-HCl/EDTA buffer, pH 8. The DNA was brought to a concentration of 20 ng μl by dilution with Tris-HCl buffer, and 5 μl was used in a 25-μl of PCR reaction.

Design of primers

Unique primers were designed for the amplification of the genes coding for the virulence factors lipase, elastase, flagella A and flagella B of *Aeromonas*. All the available partial and full-length gene sequences for a given virulence factor, in the GenBank database, were aligned by the ClustalW Multiple alignment program of the BioEdit package (BioEdit version 5. 0. 6; Tom Hall, North Carolina State University, NC, USA). Primers were designed from the conserved regions, having a length of 18–20 nucleotides. The primers were then searched for their uniqueness and specificity to the respective gene from different *Aeromonas* species only, but not to that of any other species, by using the basic local alignment search tool (BLAST). Care was taken to see that a primer set generated an amplicon of a size that could easily distinguish it from the amplicons generated by the other primer sets. For *alt* and *ast*, only one gene for each was available in the GenBank data base. For *act*, Kingombe *et al.*'s (1999) primer set AHCF1 and AHCR1 was used. All primers were made by Sigma Genosys (The Woodlands, TX, USA).

PCR analysis

Reactions were performed in 25-μl volumes in 0·2 ml optical-grade PCR tubes (PE Applied Biosystems, Foster City, CA, USA) or in 24- or 48-well Pure-Elite Thermal Cycler plates (Lab Source, Chicago, IL, USA). Each 25 μl of reaction mix contained 1 μM of each primer, 12·5 μl of AmpliTaq^{TM} Gold PCR Master mix (2X) containing, $MgCl_2$, AmpliTaq^{TM} Gold DNA polymerase, and dNTPs (Applied Biosystems). The amount of template used was 80 ng in 5 μl volume. Cycling conditions consisted of an initial single cycle at 95°C for 5 min, followed by 25 cycles of melting for 25 s at 95°C, annealing for 30 s at 55°C, elongation for 1 min at 72°C and a final single cycle at 70°C for 5 min. PCR was performed in Eppendorf Master Cycler gradient thermocycler (Eppendorf AG, Hamburg, Germany). Each DNA extract was first evaluated with a single primer set. The assay was then made multiplex by including the second primer set. Before performing PCR on the water isolates, each primer set was tested with ATCC control strains to confirm the production of an amplicon of predicted size. The following primer sets were used together: Act and Ast; Lipase and Elastase; and Alt and Fla.

Post-PCR analysis

The PCR products were detected by subjecting a sample from each reaction tube to 2% agarose gel electrophoresis stained with ethidium bromide. Representative PCR amplicons from each primer set were purified by QIAquick PCR purification kit (Qiagen Corp., Santa Clarita, CA, USA) and sequenced by the DNA Core facility of Children's Hospital Medical Center (Cincinnati, OH, USA). The sequences of the amplicons were determined in order to confirm the successful amplification of the target genes.

RESULTS

Cytotoxic enterotoxin (*act*), haemolysin *(hlyA)*/aerolysin *(aerA)*

The cytotoxic enterotoxin encoded by the *act* gene of *Aer. hydrophila*, has multifunctional activities: it has cytotoxic and haemolytic activities, in addition to having enterotoxic activity (Xu *et al.* 1998; Chopra and Houston 1999). Other *Aeromonas* spp. possess haemolytic activity by virtue of other genes, namely *hlyA* and *aerA*, and it is possible for strains to have more than one of these genes (Howard *et al.* 1987; Kozaki *et al.* 1989; Hirono and Aoki 1991; Heuzenroeder *et al.* 1999). In the last decade the contribution of haemolysins to the virulence of aeromonads has been the subject of several studies. However, these studies have been limited because only one or the other virulence gene was targeted (Pollard *et al.* 1990; Baloda *et al.* 1995; Shibata *et al.* 1996; Gonzalez-Serrano *et al.* 2002). Kingombe *et al.* (1999) aligned the *act* gene from *Aer. hydrophila* with haemolysin genes from other *Aeromonas* spp. and found conserved regions. From these conserved regions they designed primers (AHCF1/AHCR1) that directed the amplification of a 232-bp fragment from most of these genes. This well-characterized primer set was selected for detecting the *act*/*aerA*/*hlyA* gene in the drinking water isolates (Table 1). In the present study, 70% of the drinking water *Aeromonas* isolates yielded the 232-bp fragment (Fig. 1), while in their report, Kingombe *et al.* 1999 showed that 65% of *Aeromonas* isolates tested had these virulence genes.

Table 1 Sequence of the primers used for amplification of the different virulence factor genes

Name of gene	Primer sequence	Accession no.*	Reference	Size of product	Control strain
Act					
F	5′-AGAAGGTGACCACCAAGAACA-3′	M84709†	Kingombe *et al.* (1999)	232 bp	ATCC 7966
R	5′-AACTGACATCGGCCTTGAACTC-3′				
Ast					
F	5′-TCTCCATGCTTCCCTTCCACT-3′	AF419157	This study	331 bp	ATCC 7966
R	5′-GTGTAGGGATTGAAGAAGCCG-3′				
Fla					
F	5′-TCCAACCGTYTGACCTC-3′	AF198617‡	This study	608 bp	ATCC 33907
R	5′GMYTGGTTGCGRATGGT-3′	AF002709‡			
Alt					
F	5′-TGACCCAGTCCTGGCACGGC-3′	L77573	This study	442 bp	ATCC 33907
R	5′GGTGATCGATCACCACCAGC-3′				
Lipase (Lip)					
F	5′-ATCTTCTCCGACTGGTTCGG 3′	AF092033	This tudy	382 bp	ATCC 7966
R	5′-CCGTGCCAGGACTGGGTCTT-3′	S65123			
		U63543			
		U14011			
Elastase (Ela)					
F	5′-ACACGGTCAAGGAGATCAAC-3′	AB022174	This study	513 bp	ATCC 7966
R	5-′CGCTGGTGTTGGCCAGCAGG-3′	AF193422			
		AB024302			

*Accession no. of the gene/s that were used for aligning and developing the primers are given in this column.
†This primer set was developed by Kingombe *et al.* 1999 and details of homology to other aerolysin/haemolysin genes are described in the reference.
‡For developing Fla -forward (F) and reverse primers(R), nucleotides 1969–2889 (*flaA* gene region) and nucleotides 3515–4432 (*flaB* gene region) from *Aer. caviae*, gene AF 198617, were aligned with nucleotides 536–1453 (*flaA* gene region) and nucleotides 2035–2946 (*flaB* gene region) from *Aer. salmonicida*, gene AF002709.

Cytotonic enterotoxins (ast, alt). The cytotonic enterotoxins do not cause degeneration of crypts and villi of the small intestine-like cytotoxic enterotoxin (Chopra and Houston 1999). Knockout mutations in either the *alt* or *ast* gene of *Aer. hydrophila*, and subsequent challenge of mice with these mutant strains, showed significantly reduced accumulation of fluid in the ligated ileal loop of the animal model, compared with that of wild type, indicating a distinct role of these factors in diarrhoea (Sha *et al.* 2002). The *alt* gene, which is 1371 nucleotides long, has 88% sequence similarity with 1371 nucleotides present at the c-terminus end of *pla* lipase gene, which is 2602 bp long (cds 176–2593 bases). The *alt* gene however does not manifest any lipase activity (Chopra *et al.* 1996). Primers had to be designed carefully so that only *alt* was amplified and not *pla/lip/lipH3/alp-1*. The primer pairs AltF and AltR were chosen after alignment of all the lipase gene sequences available in the gene bank data bases and *alt*. This was confirmed by sequencing the PCR product generated by the AltF and AltR primers (Table 1). A subsequent search revealed that the 442-bp band had 95% homology to *alt* and 88% to *pla*. ATCC 33907 had the *alt* gene but not *lipase* and was used as a control. The *alt* gene was found in 46% of the strains.

The *ast* gene was found in 30% of the strains (Fig. 1). The control used for this assay was ATCC 7966, which exhibited both the *act/hlyA/aerA* and *ast* genes.

Elastase (ahpB). The disruption of the *ahpB* gene in *Aer. hydrophila* results in a 100-fold increase in the 50% lethal dose (LD_{50}) of *Aer. hydrophila* in fish, suggesting that elastase, a zinc metalloprotease, is an important virulence factor in the pathogenesis of the organism (Cascon *et al.* 2000). A search in the Genbank data bases revealed three elastase genes, two from *Aer. caviae* species (accession nos AB022174 and ABO24302) and one from *Aer. hydrophila* (accession no. AF193422). The primers ElasF and ElasR were designed to amplify a 513-bp fragment of the conserved regions between the three genes (Fig. 1). The control strain used for this assay was ATCC 7966. The *ahpB* gene was found in 88% of the strains (Fig. 1). An additional band of about 200 bp was also generated when the two primer sets were used together, in most of the cases (Fig. 1, lanes 2–11). This band was not related to lipase or elastase, because when the primer sets were used individually, this band did not appear.

Flagella. The majority of *Aeromonas* species and all of the species recognized as human pathogens, are motile by polar

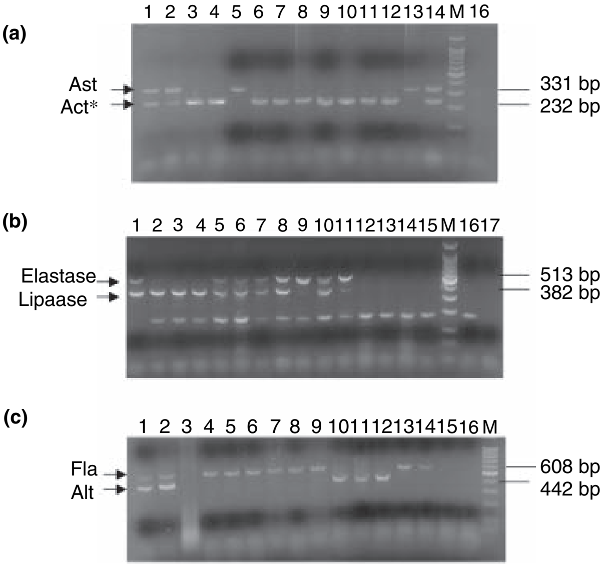

Fig. 1 Agarose gel electrophoresis of PCR products from representative *Aeromonas* isolates. Chromosomal DNA (80 ng) from pure cultures of *Aeromonas* isolates were used as templates for PCR with primer sets for the genes for Ast and Act (a), for the genes for elastase and lipase in (b) and for the genes for Fla and Alt in (c). (a) Lane 1, corresponds to PCR product from the control strain ATCC 7966, expressing both Act and Ast, Lane 2-16 contain products from isolates 321-06-B1, 492-20-D9, 492-20-D10, 407-08- C10, 492-24-D8, 492-24-D10, 492-31-D5, 492-31-D6, 492-31-D8, 492-32-D5, 492-32- D9, 407-07-D3, 407-07-D4, no template, respectively. (b) Lane 1 corresponds to PCR product from strain, AMC 12723W, expressing both Elastase and Lipase, Lanes 2-17 contain products from isolates ATCC 33658, ATCC 14174, 115-07-A3, 115-07-A4, 115-07-A1, 703-03-A1, ATCC 7966, ATCC 33907, 407-07-C18, 407-07-C20, 492-24- D5, 492-32-D8, ATCC 35993, ATCC 9071, ATCC 51208, no template, respectively. (c) Lane 1 corresponds to PCR product from control strain ATCC 33907 expressing Alt and Fla. 492-21-D6, 492-21-D7, 492-32-D5, 492-32-D6, 492-32-D7, 492-32-D8, 492-32-D9, 492-32-D10, 492-06-D8, 492-06-D9, 492-06-D10, 492-07- B6, 492-07- B7, 492-07- B8, No template, respectively. **M** in each lane stands for the molecular weight standard: 100 bp ladder (Promega). Act* represents *act/hlyA/aerA* genes

flagella. Each polar flagellum consists of two flagellin subunits Fla A and Fla B and the *flaA* and *flaB* genes have been cloned from *Aer. salmonicida* and sequenced (Umelo and Trust 1997; Rabaan *et al.* 2001). Motility is considered a virulence factor for aeromonads because mutations in both of these two genes result in complete loss of motility and adherence to human HEp-2 cells (Rabaan *et al.* 2001). In *Aer. caviae* there is 84% identity at the nucleotide level between FlaA and FlaB, 92% identity at the amino acid level (Rabaan *et al.* 2001) and 79% identity at the nucleotide level in *Aer. salmonicida* (Umelo and Trust 1997). These two *flaA* genes and two *flaB* genes were aligned and one set of primers were designed to amplify a portion of the conserved regions from the four genes. This was performed to limit the number of primers used. FlaF and FlaR recognized both *flaA* and *flaB* genes and generated a band of 608 bp (Fig. 1). The control strain used for this assay was ATCC 33907. The *fla* genes were found in 59% of the strains.

Lipase. The phospholipase gene has been cloned from several strains of *Aeromonas* and has been alternately called *pla* (accession no. AF092033 (Merino *et al.* 1999), *lipH3* (accession no. S65123 (Anguita *et al.* 1993) and *lip* (accession no. U63543 (Chuang *et al.* 1997). The *pla* gene also shows homology to the phospholipase C gene *apl-1* (accession no. U14011 (Ingham and Pemberton 1995). The LipF and LipR primers used in the present study were designed by aligning all of the *Aeromonas* lipase gene sequences found in GenBank, including *apl-1*, and targeting conserved regions within these sequences. A 382-bp product was amplified with the lipase F and lipase R, primer set (Table 1). The control strain used for this gene was ATCC 7966. The *pla/lip/lipH3/apl-1* gene was present in 88% of the strains.

Combination of virulence factors

In general there was a wide variety in the combinations of virulence factors in the *Aeromonas* isolates from drinking water (Table 2.) Only one isolate (*Aer. hydrophila*) had all six genes; all other isolates had at least one gene. Sixty-seven per cent of the isolates had at least four virulence genes. The *act* gene was the most frequently found entertoxin gene among all isolates (70%), although it was relatively uncommon to find this gene in the *Aer. hydrophila* isolates. The *ast* and *alt* genes were found in 30 and 43% of the isolates, respectively. The *Aer. hydrophila* isolates accounted for all but one of the positive results for the a *st* gene. All species, except for the *Aer. caviae* isolates, had at least one entertoxin gene. There was no obvious association among the isolates with regard to the enterotoxin genes, although no isolates were recovered having only the *alt* gene. Several of the utilities (407, 492 and 649) yielded isolates from more than one species and these isolates often differed in the combination of virulence factors present. For example, from utility 407, tap D, 4 *Aer. hydrophila* isolates were recovered, displaying three different combinations of virulence factors and from utility 115, tap A, three *Aer. salmonicida* strains were recovered with two different combinations. In many cases, however, from a given tap, a dominant species with a specific combination of virulence factors was isolated. Thus in utility 407, tap C, one dominant (18/36 or 50%) combination of virulence factors in the *Aer. hydrophila* isolates was seen (Alt, Ast, elastase, lipase and flagella). From tap E two different species, a dominant species of *Aer.*

Table 2 Number of isolates having a given combination of virulence factor genes

Strain	Number of isolates	Act	Alt	Ast	Elastase	Fla	Lipase
Aer. hydrophila (61), *321-B, 407-C, 407-D, 492-E, 649-E	24	−	+	+	+	+	+
	20	−	−	+	+	+	+
	9	+	−	+	+	+	+
	7	−	+	+	+	−	+
	1	+	+	+	+	+	+
Aer. bestiarum (17) *407-E, 492-D	17	+	−	−	+	+	+
Aer. salmonicida (94) *492-B, 492-D, 492-Y, 115-A	25	+	+	−	+	−	+
	34	+	−	−	+	−	+
	29	+	+	−	+	+	+
	2	−	−	−	+	+	+
	2	+	−	−	+	+	+
	2	−	+	−	+	+	+
Aer. caviae (6) *703-E, 649-E	4	−	−	−	+	−	+
	2	−	−	−	+	+	+
Aer. veronii bv *sobria* (25) *492-D, 492-A, 492-B	13	+	−	−	−	−	−
	12	+	−	−	−	+	−
Aer. enchelia (2) *702-A, 407-E	1	+	−	−	+	−	+
	1	+	−	+	+	+	+

The number in parenthesis in the first column represents the total number of isolates that were isolated from this species. Thus there were 205 isolates altogether. Numbers 115, 321, 702, 703, 407, 492 or 649 are different water utilities that were tested for *Aeromonas* contamination. The letters A, B, C, D or E represent the tap of a utility.

*Indicates the utilities and the corresponding taps that tested positive for that species.

+, Indicates the presence of a gene; −, indicates the absence of a gene.

bestiarum (eight of nine or 89%) with the virulence factors combination of Act, elastase, flagella and lipase, and one *Aer. enchelia* species (one of nine or 10%), were isolated. The elastase, flagella and lipase genes were commonly found among the isolates, present in 88, 59 and 88% of the isolates, respectively.

DISCUSSION

There is a need for a practical method of screening large number of *Aeromonas* isolates for potential virulence. One rational approach to determine whether a given microorganism has the potential to be virulent is to identify whether virulence factor genes are present. As virulence in *Aeromonas* is certain to be multifactorial, the PCR approach developed in this study has value in characterizing *Aeromonas* isolates from water. A similar approach has been used by other workers to detect one or more virulence genes in *Aeromonas* (Gustafson *et al.* 1992) (Shibata *et al.* 1996; Wang *et al.* 1996) (Khan *et al.* 1999) (Kingombe *et al.* 1999) (Heuzenroeder *et al.* 1999; Biscardi *et al.* 2002; Gonzalez-Serrano *et al.* 2002; Soler *et al.* 2002; Sechi *et al.* 2003). Thus, three duplex PCR assays were used to analyse for six potential *Aeromonas* virulence factor genes. The three enterotoxin genes were selected as targets because the cytotoxic enterotoxin, *act/hlyA/aerA* and the cytotonic enterotoxins, *alt* and *ast*, have all been implicated as important virulence factors in diarrhoeal disease (Albert *et al.* 2000; Sha *et al.* 2002). In the present study, of the 205 strains tested, one or more of these enterotoxin genes were found in 97% of the isolates, with the major enterotoxin *act/hlyA/aerA*, being present in 70% of the isolates. This result agrees with those reported by Kingombe *et al.* (1999), who, in their study of 350 clinical and environmental isolates, found 65% of the *Aeromonas* strains positive for *act/hlyA/aerA*. Albert *et al.* (2000), reported a significant correlation between *Aeromonas* isolates having both the *alt* and *ast* genes and diarrhoea in children harboring such strains. In their study 54% of the *Aer. hydrophila* strains isolated from diarrhoeal children had both genes, while only 15% of the strains recovered from environmental samples were found to have both genes. In the current study, 52% of the *Aer. hydrophila* isolates had both the *alt* and *ast* genes.

Although enterotoxins are considered to be important factors in *Aeromonas*-induced gastroenteritis, the mere presence of these toxins may not be sufficient for virulence. Indeed these factors have been found in strains isolated from healthy humans (Pin *et al.* 1995; Schiavano *et al.* 1998). As the ability of a bacterium to adhere and invade the intestinal mucosa are also essential components of enteropathogen pathogenesis, the presence of the elastase, lipase and flagella genes were evaluated. In this study the elastase gene was shown to be present in 88% of the strains tested. Phospolipases have also been shown to be important virulence factors in several bacterial pathogens, including *Aeromonas* spp. (Straus *et al.* 1992; Konig *et al.* 1996) (Merino *et al.* 1999). In this study the primers Lip F/R amplified the *pla/lip/lipH3/alp-1* gene from 88% of the strains. There is a controversy about the phospolipase C gene in the literature and two sequences have been published, *alp1* and *plc* (Ingham and Pemberton 1995; Merino *et al.* 1999). The lipase primer set Lip F and Lip R designed in the current study, recognized *alp1* as expected. However, two other primer sets designed specifically against different regions of *plc* gene (accession no. AF092034) failed to give a product against a collection of 30 ATCC strains, including *Aer. salmonicida* ATCC 14174. Earlier it was shown that there were only four nucleotides difference between haemolysin ASH1 gene of ATCC 14174 and the *plc* gene of *Aer. hydrophila* AH-3 and therefore the primer sets should have recognized the ASH1 gene (Merino *et al.* 1999). Therefore, this gene could not be tested. Genes for other lipases and proteases such as glycerophospolipid:cholestrol acetyl transferase, GCAT, and serine proteases AspA and Ahp A have been identified in *Aeromonas*. However, these genes have been shown not to play a role in the pathogenesis of fish in gene disruption studies (Rivero *et al.* 1990; Vipond *et al.* 1998; Cascon *et al.* 2000) and hence they were not tested.

In the study by Kuhn *et al.* (1997), it was reported that 89% of the strains that produced haemolysins and enterotoxins also showed an increased ability to adhere to human intestinal Henley 407 cells. Flagella are important in the adherence process and in *Aeromonas* it has been shown that mutations in the polar flagellum *flaA* and *flaB* genes result in complete loss of motility and adherence to human epithelial HEp-2 cells. In this study, the genes coding for the polar flagellum protein was shown to be present in 59% of the strains. Type IV pilus adhesins have also been shown to be present in virulent strains of *Aeromonas* (Hokama and Iwanaga 1992; Iwanaga and Hokama 1992; Kirov *et al.* 1998, 1998). However, this gene cluster was not tested as it was recently shown by gene disruption studies that one type IV pilus, encoded by the gene cluster, *tap ABCD*, was not necessary for human intestinal adhesion and infection (Kirov *et al.* 2000).

Of the 205 isolates analysed, 18 different combinations of virulence factors were found among the different isolates. Only one strain, belonging to HG 1, *Aer. hydrophila*, had all six virulence factor genes. Although there was a wide range of species isolated from the different utilities, with different combinations of virulence factor genes, a single strain appeared to predominate in the *Aeromonas* populations from different rounds of sampling from a given water tap. This observation is in agreement with the Kuhn *et al.* (1997) study where they found multiple *Aeromonas* strains in a single water source although one strain was usually dominant (Kuhn *et al.* 1997).

The significance of these findings need to be evaluated carefully with regards to the public health significance of *Aeromonas* in drinking water. The exact relationship between the presence of virulence factor genes and the ability of a given strain to cause human disease has not been firmly established. Differences in susceptibility among people may also be a key factor. In an epidemiological study by Demarta *et al.* 2000, ribotyping was used to demonstrate that *Aeromonas* strains isolated from stool cultures of some symptomatic children, had the same riboprofile as strains found in asymptomatic family members, suggesting a relationship between the predisposition of the host and susceptibility to *Aeromonas* infections. The same study also showed that in two cases, strains isolated from a patient had the same ribotype as that of strains found in the drinking water supply in the same geographical region, although not in their immediate tap water or water pipes. Other published studies using ribotyping, multilocus enzyme analysis and pulse-field gel electrophoresis, have found no relationship between clinical and environmental *Aeromonas* isolates (Tonolla *et al.* 1991; Moyer *et al.* 1992; Hanninen and Siitonen 1995; Borchardt *et al.* 2003). It is perhaps important to note that the environmental samples tested in these other studies were not as geographically close to the actual patient's house compared with the Demarta *et al.* (2000) studys.

In conclusion, many *Aeromonas* strains isolated from drinking water have multiple virulence factors and thus have the potential to be pathogenic. This novel study also demonstrates the interesting potential for a PCR technique to rapidly (<6 h) identify the presence of six *Aeromonas* virulence factors. Additional evaluation to completely determine the applicability of this approach to virulence factor determination and that role in assessing *Aeromonas* influences on adverse public health issues is warranted. Future studies will be directed towards characterizing the virulence of the strains in animal models.

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