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Detection of the Aerolysin Gene in *Aeromonas hydrophila* by the Polymerase Chain Reaction

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Synthetic oligonucleotide primers were used in a polymerase chain reaction (PCR) technique to detect the gene for aerolysin in strains of *Aeromonas hydrophila* and to screen for identical genes in *A. caviae*, *A. sobria*, and *A. veronii* isolated from patients with diarrheal disease. Primers targeted a 209-bp fragment of the *aer* gene coding for the beta-hemolysin and detected template DNA only in the PCR using nucleic acid (NA) from hemolytic strains of *A. hydrophila* which were also cytotoxic to Vero and CHO cells and enterotoxic in suckling-mouse assays. PCR amplification of NA from hemolytic *A. sobria* or nonhemolytic *A. hydrophila* and *A. caviae* strains was consistently negative. Primer specificity was determined in the PCR by using NA extracted from 56 strains of bacteria, including hemolytic *Escherichia coli* and *Listeria monocytogenes* as well as several recognized enteric pathogens defined in terms of their toxigenicity. The detection limit for the aerolysin gene by PCR amplification was 1 ng of total NA. The PCR clearly identified aerolysin-producing strains of *A. hydrophila* and may have application as a species-specific virulence test because other hemolytic *Aeromonas* species tested were negative.

Aeromonas hydrophila and *A. sobria* are associated with both diarrheal and extraintestinal infections in human disease (2, 15). Immunocompromised hosts can develop *Aeromonas* sepsis or meningitis, and both immunocompetent and immunocompromised hosts can suffer *Aeromonas* wound infections. The majority of human isolates of *Aeromonas* spp. are hemolytic and soft tissue necrosis is characteristic of extraintestinal *Aeromonas* infections, thereby suggesting that hemolysin may be a virulence factor. Diarrhea has been associated with some strains of *Aeromonas* spp., and both antibiotic therapy and drinking of untreated water are significant risk factors for susceptible hosts (20). Several extracellular products of *A. hydrophila* which have been suggested as possible contributory factors in the pathogenesis of these diseases include a beta-hemolysin also known as Asao toxin (3), cytotoxic enterotoxin (6, 16), and a cholera toxin-cross-reactive cytolytic and hemolytic enterotoxin from supernatants of a human diarrheal isolate (SSU) (26). An alpha-hemolysin which causes incomplete lysis of erythrocytes is probably of minor, if any, importance in the pathogenesis of *Aeromonas* infections (2). The cholera toxin-reactive properties were established by enzyme-linked immunosorbent assays and Western immunoblot analyses, although cholera antitoxin failed to neutralize the hemolytic and enterotoxic activities. It is theorized that all of the molecules described above are similar to the aerolysin first described by Bernheimer and Avigad (4) and that *A. hydrophila* cytolytic enterotoxin is one protein molecule with tissue-specific activities which are enterotoxic or cytotoxic (26). Rose et al. (26) suggested that the cholera toxin-cross-reactive antigenic determinants are located in different regions of the molecule from the biologically active sites.

Aerolysin, produced by some strains of *A. hydrophila*, is an extracellular, soluble, hydrophilic protein exhibiting both hemolytic and cytolytic properties. Aerolysin binds to specific glycoprotein receptors on the surface of eucaryotic cells before inserting into the lipid bilayer and forming holes. The hole-forming aerolysin toxin crosses the inner bacterial membrane as a preprotoxin containing a signal peptide which is removed cotranslationally (11). The protoxin thus released is an inactive precursor protoxin (proaerolysin) which is subsequently activated by proteolytic removal of about 25 amino acids from the carboxy terminus (12). The preproaerolysin gene was cloned into *Escherichia coli*, and its nucleotide sequence was determined (13). The synthetic oligonucleotide primers used in the polymerase chain reaction (PCR) described herein targeted a 209-bp fragment of the longest open reading frame of the published *aer* gene sequence. Downstream and upstream genes referred to as *aerB* and *aerC* reportedly modulate the expression and activity of the aerolysin *aerA* gene (7) and were not considered as suitable targets for the PCR probes.

In the present study, we report the development of a PCR procedure (27) which will rapidly and specifically detect aerolysin genes in strains of hemolytic *A. hydrophila* associated with human disease. The application of PCR technology to the detection of toxin genes in virulent enteric pathogens has been recently reported for the verotoxins of *E. coli* (24). The oligonucleotide primers used in the aerolysin study were tested in the PCR with template nucleic acid (NA) extracted from a collection of reference strains and *Aeromonas* spp. isolated from human sources. PCR results were compared with biological assays for hemolytic, cytotoxic, and enterotoxic activity.

MATERIALS AND METHODS

Bacterial strains and culture media. A complete list of bacterial strains used in this study appears in Table 1. Clinical isolates and reference strains were stored on maintenance media and held in the culture collection of the National Laboratory for Enteric Pathogens, Laboratory Centre for Disease Control. Reference strains were defined in terms of established toxigenicity. *Aeromonas* and *Plesiomonas shigelloides* strains were fecal isolates from patients with diarrhea and were identified to the species level by

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TABLE 1. Summary of organisms, hemolysin production, toxin profiles, and PCR probe results

Organism	Serotype	No. of strains	Toxin(s)[a]	Hemolysin	PCR amplification product (209 bp)
Aeromonas and *Plesiomonas* strains					
Aeromonas hydrophila		10	Cytotoxin,[b] enterotoxin	+	+
A. hydrophila		4	Cytotoxin, enterotoxin	−	−
A. hydrophila		1	Cytotoxin	−	−
A. hydrophila		5		−	−
A. sobria		6	Cytotoxin, enterotoxin	+	−
A. sobria		1	Cytotoxin	−	−
A. caviae		5		−	−
A. veronii		1		−	−
Plesiomonas shigelloides		3		−	−
Other toxigenic pathogens					
Listeria monocytogenes		1	Listeriolysin	+	−
Vibrio cholerae	O1	1	Cholera toxin	−	−
Shigella dysenteriae	1	1	Shiga toxin	−	−
S. dysenteriae	2	1	CLDT	−	−
Escherichia coli	O22:H43	1	Hemolysin	+	−
E. coli	O55:H4	1	CLDT	−	−
E. coli	O91:H21	1	VT2, CLDT	−	−
Campylobacter jejuni		1	CLDT	−	−
C. coli		1	CLDT	−	−
C. fetus subsp. *fetus*		1	CLDT	−	−
C. upsaliensis		1	CLDT	−	−
Reference strains					
E. coli H19	O26:H11	1	VT1	−	−
E. coli E32511	O157:H−	1	VT2	−	−
E. coli 412	O139:H1	1	VTe[c]	−	−
E. coli TD427c2	O25:H−	1	LT	−	−
E. coli TD213c2	O128	1	ST	−	−
E. coli H10407	O78:H11	1	LT, ST	−	−
E. coli HB101		1		−	−
E. coli C600		1		−	−
E. coli ATCC 25992	O6:H1	1		−	−

[a] VT, Verotoxin; LT, heat-labile enterotoxin; ST, heat-stable enterotoxin; CLDT, cytolethal distending toxin.
[b] Cytotoxic to Vero and CHO cells.
[c] VTe or SLT-IIv associated with edema disease in pigs; noncytotoxic to HeLa cells (19).

using the characteristics defined by Popoff (25). Strains of *Aeromonas* spp., *P. shigelloides*, *Shigella* spp., *E. coli*, and *Campylobacter* spp. were grown as previously described (24). *Vibrio cholerae* O1 was grown for 18 h in syncase broth at 37°C with agitation, and all *Streptococcus* spp. and *Listeria monocytogenes* were grown overnight on Columbia blood agar containing 5% sheep erythrocytes.

Determination of hemolytic, cytotoxic, and enterotoxic activity. Strains of *Aeromonas* spp. and *P. shigelloides* were scored for beta-hemolysin activity after 24 h of growth at 30°C on Mueller-Hinton agar (Oxoid Ltd.) containing either 5% rabbit erythrocytes or 5% sheep erythrocytes. Strains were considered hemolysin positive only if beta-hemolysis was observed on both types of blood agar plates. All other bacterial strains tested were scored for hemolysin by using Mueller-Hinton agar medium supplemented with 5% sheep erythrocytes.

Cytotoxic and enterotoxic activities were determined as previously described (24) with culture supernatants which were passed through 0.22-μm-pore-size cellulose acetate membrane filters (Nalge Co., Rochester, N.Y.). Both *Aeromonas* enterotoxin (6) and *E. coli* heat-stable enterotoxin (8) were assayed with 3-day-old suckling mice. Ratios of intestinal weight to remaining body weight higher than 0.085 were considered enterotoxin positive.

NA isolation and PCR. Procedures for NA isolation and PCR were previously described (24). A list of bacterial strains tested appears in Table 1, and the aerolysin-specific oligonucleotide primers (Oligonucleotide Synthesis Laboratory, Queen's University, Kingston, Ontario, Canada) are described in Table 2. PCR was performed with 10 ng of NA with the following amplification cycles: denaturation for 2 min at 94°C, annealing of primers for 2 min at 55°C, and primer extension for 1 min at 72°C with autoextension. To test the sensitivity of the PCR procedure in detecting the aerolysin gene, NA from a strain of *A. hydrophila* carrying the aerolysin gene was adjusted to a concentration of 200 μg/ml and serial 10-fold dilutions were made in TE buffer (10 mM Tris chloride, 1 mM EDTA [pH 8.0]) before the NA was used as a template in the PCR.

Restriction endonuclease digestions. Ten-microliter sam-

TABLE 2. Base sequences, locations, and predicted sizes of amplified products for the aerolysin-specific oligonucleotide primers

Primer	Oligonucleotide sequence (5′–3′)[a]	Location within gene[b]	Size of amplified product (bp)
Aero1a	ccaaggggtctgtggcgaca	645–664	
Aero1b	tttcaccggtaacaggattg	834–853	209

[a] From the published *aer* gene for aerolysin (13).
[b] In nucleotides.

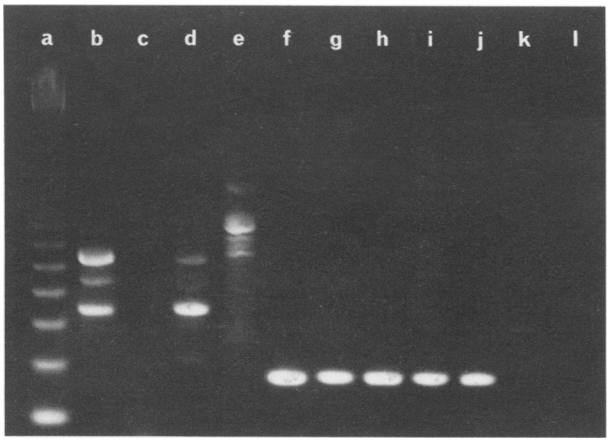

FIG. 1. Occurrence and distribution of a 209-bp amplification fragment in the PCR. Lanes: a, 123-bp ladder (Bethesda Research Laboratories, Inc., Gaithersburg, Md.); b, *P. shigelloides* (hemolysin negative); c, *A. veronii* (hemolysin negative); d, *A. caviae* (hemolysin negative); e, *A. hydrophila* (hemolysin negative); f through j, *A. hydrophila* (aerolysin positive); k, *A. sobria* (hemolysin positive); l, *E. coli* O22:H43 (hemolysin positive).

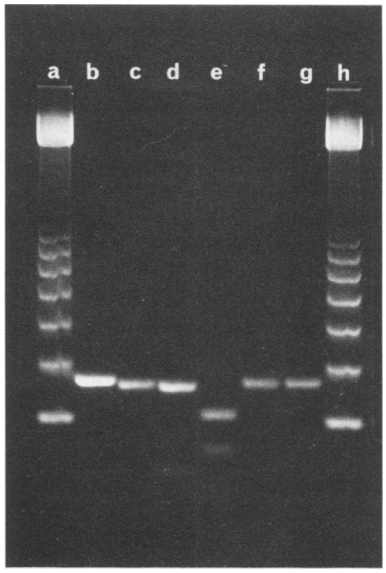

FIG. 2. Restriction endonuclease digestion with *Nci*I. Undigested PCR amplification fragments appear in lanes b through d, and those digested with *Nci*I appear in lanes e through g. Lanes: a and h, 123-bp ladder; b and e, *A. hydrophila* (aerolysin positive); c and f, *S. pyogenes* (SLO positive); d and g, *S. pyogenes* (erythrogenic toxin positive).

ples of amplification fragments recovered after 60 cycles in the PCR by using 1 μg of NA from either aerolysin-positive *A. hydrophila* or *Streptococcus pyogenes* were subjected to restriction endonuclease digestion with *Nci*I (GIBCO-Bethesda Research Laboratories) as recommended by the manufacturer. The digested samples were analyzed by standard submarine gel electrophoresis.

RESULTS

Aerolysin oligonucleotide probes. One pair of synthetic aerolysin-specific oligonucleotide primers targeting a 209-bp fragment of the *aer* gene coding for the hole-forming aerolysin toxin was used in the PCR. The primers were designed by computer analysis (9) by using published sequences of the *aer* gene (13). Figure 1 shows the presence and occurrence of the amplified product when NA from representative *Aeromonas*, *Plesiomonas*, and hemolytic *E. coli* strains were used as templates. Results of the PCR are summarized in Table 1. An amplification product of the expected size (Table 2) was observed only in the PCR in which NA from beta-hemolytic strains of *A. hydrophila* was used (Fig. 1, lanes f to j). No similar fragments were observed in the PCR when template NA from nonhemolytic *A. hydrophila* (Fig. 1, lane e), hemolytic *E. coli* (Fig. 1, lane l), hemolytic *A. sobria* (Fig. 1, lane k), nonhemolytic *A. caviae* (Fig. 1, lane d), nonhemolytic *P. shigelloides* (Fig. 1, lane b), and nonhemolytic *A. veronii* (Fig. 1, lane c) was used. The sensitivity limit of the PCR protocol in detecting *aer* genes in beta-hemolytic strains of *A. hydrophila* was 1 ng of total NA (data not shown).

Specificity of oligonucleotide probes for detection of aerolysin gene sequences. A summary of strains, hemolytic activity, cytotoxicity to Vero and CHO cells, toxigenicity, and the PCR results appears in Table 1. All strains of beta-hemolytic *A. hydrophila* produced typical lysis on both rabbit and sheep blood agar plates. Primer specificity was determined in the PCR by using NA extracted from 56 strains of common enteric pathogens, including representative and reference strains of *V. cholerae*, *Shigella dysenteriae*, *E. coli*, and *Campylobacter* spp. defined in terms of their toxigenicity. The PCR clearly identified all 10 aerolysin-positive strains of *A. hydrophila* which were also cytotoxic to Vero and CHO cells and enterotoxic in suckling mice. Ten nonhemolytic isolates of *A. hydrophila* were selected for this study to establish primer specificity. No specific amplification fragments were observed in the PCR when NA from any of the other *Aeromonas* or *Plesiomonas* strains, including several cytotoxic and enterotoxic isolates, was used. The results of PCR using NA from strains of hemolytic *E. coli* and listeriolysin-positive *L. monocytogenes* were also found to be negative.

Nonspecific amplification in some *Streptococcus* spp. NA extracted from clinical strains of *S. pyogenes* and *S. agalactiae* were tested in the PCR. Although the three strains of *S. agalactiae* tested were completely negative, strains of *S. pyogenes* producing erythrogenic toxin and streptolysin O were typified by a common amplification fragment approximately 200 bp in size. Although the 200- and 209-bp fragments could be differentiated in 2% agarose gels, increasing the agarose concentration to 4% allowed better resolution of the two products (data not shown). Both the 200-bp streptococcal and 209-bp aerolysin amplification fragments were subjected to restriction endonuclease digestion with *Nci*I. The aerolysin-specific 209-bp fragment was cleaved into two smaller segments of 132 and 77 bp, whereas the 200-bp streptococcal fragments remained undigested (Fig. 2). Although 30 cycles of amplification using 10 ng of NA was adequate in the PCR in which aerolysin-positive *A. hydrophila* was used, it was found that 60 cycles with 1 μg of NA was required for consistent amplification of the streptococcal fragments.

DISCUSSION

We have designed and described a PCR protocol to detect aerolysin genes in NA extracted from strains of *A. hydrophila* isolated from humans. Although it is possible to detect

the beta-hemolysin biologically, these tests do not distinguish beta-hemolysins from *A. hydrophila* and *A. sobria*. The primers used in the PCR targeted the *aer* gene and clearly detected the specific nucleotide sequence in template NA extracted from representative *A. hydrophila* isolated from human sources. The PCR was species specific in that amplification was observed only with NA template from hemolytic *A. hydrophila* and not with template from hemolytic *A. sobria*. PCR using NA from bacterial strains known to harbor virulence-associated toxins such as cholera toxin, listeriolysin, verotoxins (VT1, VT2, and VTe) (17, 19), Shiga toxin, and the classic heat-labile and heat-stable enterotoxins were consistently negative. *A. sobria* and nonhemolytic *A. hydrophila* that produced cytotoxic and enterotoxic factors were negative in the PCR, indicating that the presence or absence of these toxins did not affect the detection of the aerolysin gene. Direct detection of toxin genes in clinical isolates greatly simplifies the identification when compared with similar determinations in stool specimens. Although the concordance between phenotypic and genotypic analysis was 100% in this study, examination of larger numbers of strains may reveal discrepancies and identify strains which carry the aerolysin gene but have altered gene expression.

The nucleotide sequence used to design the oligonucleotide primers used in the PCR was based on the published *aer* sequence for an *A. hydrophila* isolate pathogenic for fish (13). The gene sequence subsequently published for the aerolysin of *A. sobria* (14) was compared with that of *A. hydrophila* and showed a 77% homology at both the nucleotide and amino acid level in the aerolysin-coding region. The PCR primers were designed to avoid regions of homology in the structural genes for *A. sobria* aerolysin (14), *E. coli* hemolysin A (10), or *Staphylococcus aureus* alpha-toxin, as previously documented (13). A computer-assisted nucleotide sequence comparison of the aerolysin primers with the published gene sequences for *speA* (streptococcal erythrogenic toxin [29]) and *slo* (streptolysin O [18]) genes failed to identify potential binding sites for the primers. The presence of an *Nci*I site in the 209-bp aerolysin-specific PCR amplification fragment and the absence of a similar site in the 200-bp fragments from the PCR in which NA containing *speA* or *slo* genes was used suggests that the observed streptococcal amplification is nonspecific.

It has been established that aerolysin is a virulence factor contributing to the pathogenesis of *A. hydrophila* infection (28), and a very recent report suggests that hemolytic, enterotoxic, and cytotoxic activities can be associated with a single polypeptide (26). A study characterizing strains of *A. hydrophila* and their association with clinical symptoms (5) revealed a statistically significant correlation between the production of cytotoxic hemolysin and the presence of diarrhea and no correlation between the production of enterotoxin and diarrhea. Some strains of nonhemolytic, cytotoxic, and enterotoxic *A. hydrophila* identified in the current study appear to be associated with diarrheal disease. Recent studies on the characterization of *A. hydrophila* isolated from retail foods of animal origin (23) indicated that most food and clinical isolates were cytotoxic and beta-hemolytic in human blood. Although the enteropathogenic significance of *A. caviae* in human diarrheal disease has not yet been well established (1), recent reports present microbiologic and clinical evidence that *A. caviae* may be a toxigenic pediatric enteric pathogen (21, 22). None of the *A. caviae* isolates tested in our study were hemolytic, cytotoxic, enterotoxic, or positive in the PCR. All of the *A. sobria* isolates were cytotoxic, although only the enterotoxic strains were beta-hemolytic, showing a close relationship between the hemolytic and enterotoxic factors in this species. The PCR protocol clearly identified aerolysin-producing strains of *A. hydrophila* and may have application as a rapid, species-specific virulence test, given that other hemolytic and cytotoxic species of *Aeromonas* and other enteric pathogens were negative.

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