Comparison of Putative Virulence Factors in *Aeromonas hydrophila* Strains Isolated from the Marine Environment and Human Diarrheal Cases in Southern Italy

KAREL KROVACEK,1,* VINCENZO PASQUALE,2 SURAJ B. BALODA,1 VITTORIO SOPRANO,3 MARCO CONTE,3 AND STEFANO DUMONTET2

Section of Bacteriology and Epizootology, Department of Veterinary Microbiology, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Biomedical Centre, S-751 23 Uppsala, Sweden,1 and Faculty of Agricultural Sciences, University of Basilicata, Potenza,2 Experimental Institute for Animal Diseases, Portici,3 and Laboratory of Bacteriology, “D. Cotugno” Hospital, Naples,4 Italy

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*Aeromonas hydrophila* strains isolated from the same geographical region (southern Italy) but from different sources (sea sediments and human diarrhea cases) were characterized for the production of potential virulence determinants, such as production of cytotoxins, cytotoxic toxins, hemolysin, and dermonecrotic factors and their capacity to adhere to human intestinal 407 cells in vitro. The results showed that isolates from both the sources produced all or some of the virulence factors which may be involved in the pathogenesis of *Aeromonas*-associated infections. Our study indicates that further epidemiological studies are necessary to elucidate the public health significance of infections caused by *Aeromonas* spp.

*Aeromonas* spp. are commonly found in a wide range of aquatic systems and foods and have been isolated from coastal waters, lakes, rivers, drinking water, and a variety of foods (2, 3, 7, 14). These species have long been known to cause different infections in poikilothermic animals such as fish, reptiles, and amphibians (1, 26, 28). Only in recent years has the clinical importance of motile *Aeromonas* isolates been recognized (19). These pathogens have been associated with several categories of human infections, such as gastroenteritis, peritonitis, endocarditis, meningitis, septicemia, and urinary tract and wound infections (10).

Various putative virulence factors have been ascribed to *Aeromonas* spp. to explain the process of pathogenicity of these organisms. Such factors include the production of exotoxins (cytotoxin or enterotoxin) and α- and β-hemolysins and the ability to bind to and invade epithelial cells (13, 16, 19, 25, 29).

The occurrence and isolation of *Vibrio* spp., including *Aeromonas* spp., from marine water in the Gulf of Naples in Italy has recently been reported by Dumontet (7). Since these aquatic environments may represent a source of human infections, we studied the putative virulence markers of these pathogens, namely, the production of cytotoxin, enterotoxin, hemolysin, and dermonecrotic factors and adhesion to human intestinal cells. Furthermore, the purpose of this study was also to compare the virulence profiles of different *Aeromonas* strains isolated from an aquatic environment and from cases of human infection in southern Italy and to determine whether they had similar putative virulence factors which may be involved in the pathogenesis of *Aeromonas*-associated human disease.

Sea sediment samples were collected along the Gulf of Naples (Tyrrhenian Sea). The sediments (100 g) were sampled with an Eckman grab sampler, stored at 4°C, and transported to the laboratory, where microbiological analyses were initiated within 24 h of sample collection. Human strains were isolated at the clinical laboratory at the Domenico Cotugno Hospital of the Neapolitan Unita Sanitaria Locale. All strains were isolated from the feces of human patients with diarrhea (five male and three female patients, ranging in age from 8 to 64 years). In one case, *Aeromonas hydrophila* (strain 7H) was isolated together with another enteropathogen, *Salmonella* spp. group B, whereas *A. hydrophila* was the only pathogen isolated from the remaining patients.

The homogenized samples were inoculated into peptone-water (Merck, Darmstadt, Germany) and incubated at 37°C for 24 h. The materials from these enrichment samples were transferred with a loop to glutamate-starch-phenol red (GSP) agar and thiosulfate-citrate-bile-sucrose (TCBS) agar (E. Merck, Darmstadt, Germany) and incubated at 37°C for 24 h. Yellow colonies 2 to 3 mm in diameter surrounded by a yellow zone on GSP agar and yellow or blue colonies on TCBS agar which were gram-negative rods, motile, and oxidase positive and fermented glucose (O/F test) were further biotyped with the API 20 NE system according to the manufacturer’s instructions (API 20 NE 69280; Analytab Products, Marcy-l’Étoile, France). All of the human isolates showed identical biochemical patterns, while the environmental strains showed variable patterns of reaction for amygdalin only.

These *A. hydrophila* isolates were streaked onto blood agar (5% horse erythrocytes in brain heart infusion [BHI] agar [Difco Laboratories]) and incubated at 37°C for 20 h. The bacteria were further inoculated into BHI broth and incubated at 37°C for 20 h. Cultures were harvested by centrifugation at 16,000 × g and 4°C for 30 min, and the supernatants were membrane filtered (Millipore, 0.22-μm pore size). The sterile cell-free culture filtrates were tested for the presence of hemolysins, cytotoxins, cytotoxic toxins, enterotoxins, and dermonecrotic factors.

The cytotoxic/cytopathic activity of the culture filtrates of *A. hydrophila* strains was detected by using Chinese hamster ovary (CHO)-K1 cells as described previously (7). Briefly, these activities were determined by microscopic examination of the cell monolayers after 18 to 24 h of incubation at 37°C of CHO-K1 cells with the sterile culture filtrates. The highest

**Author affiliations** are listed at the end of this issue. **Corresponding author.** Mailing address: Section of Bacteriology and Epizootology, Biomedical Center, Box 583, S-751 23 Uppsala, Sweden. Phone: 46-18-17 45 92, Fax: 46-18-50 63 38.
dilution of the culture filtrate producing cytopathic effects, identified as rounding of the cells, was designated the cytotoxic titer, whereas the elongation of CHO-K₁ cells was attributed to cytotonic toxin activity.

The enterotoxic activity of the culture filtrates was tested in the rabbit ligated intestinal loop assay, which was performed in rabbits weighing 2.5 kg (27). The production of dermonecrotic factors was investigated by the rabbit skin test as described by Craig (6). Culture filtrates from A. hydrophila strains were assayed for hemolytic activity against bovine erythrocytes (1%, vol/vol) as described previously (17).

A. hydrophila strains were cultured at 37°C for 24 h with and without shaking (60 rpm), and their ability to adhere to human intestinal cells (intestine 407, ATCC CCL6; Flow Laboratories, Irvine, Scotland) was studied by methods described earlier (12).

A total of 14 isolates from sea sediment and 8 isolates from human diarrhea cases were identified and confirmed as A. hydrophila, and each of these isolates produced a variety of virulence factors to different extents (Table 1). None of the sea sediment isolates produced cytotonic toxins, and only three isolates showed the production of dermonecrotic factors (Table 1). Although a majority of these isolates produced demonstrable amounts of cytotropic factors in CHO-K₁ cells, the activities of only three isolates could be observed at a reciprocal titer of 1,000. The human isolates, on the other hand, produced relatively greater amounts of cytotropic activities in CHO-K₁ cells. These isolates produced weak cytotonic toxin activity demonstrable only in undiluted cell-free culture supernatants. Although all 22 isolates of A. hydrophila from sea sediment and clinical sources produced beta-hemolytic activity against bovine erythrocytes, it was stronger in the clinical isolates (Table 1). Furthermore, four clinical isolates and three sea sediment isolates produced dermonecrotic factors.

As shown in Table 1, of the 16 isolates (10 from sea sediment and 6 clinical isolates) selected for assay of the production of enterotoxic activity in rabbit loops, only 6 isolates (3 from each group) gave positive results. All 22 A. hydrophila isolates from the marine environment and clinical sources bound to human intestinal 407 cells to various degrees (Table 1). The clinical isolates, however, showed less adherence to human intestinal 407 cells, since the majority of these isolates showed fewer than 5 bacteria bound per intestinal cell. Interestingly, the majority of the clinical isolates showed localized adhesion to intestinal 407 cells in this in vitro assay, whereas the environmental isolates showed diffuse adhesion (Fig. 1).

The members of the family Vibrionaceae, including Aeromonas spp., are widely distributed in the environment, particularly in aquatic systems. It is thus possible that the environment, animals, food, seafood, and drinking water are important reservoirs of Aeromonas spp. that are capable of causing human infections. Our study shows that the Aeromonas strains isolated from marine water sediment and clinical human sources and from the same geographical region possessed a variety of common putative virulence markers. They produced exotoxins, hemolysins, and dermonecrotic factors and were able to bind to human intestinal cells in vitro. The virulence characteristics of Aeromonas strains in relation to the source have been reported previously, especially from Australia and the United States (4, 11, 21). Recently, Gray et al. (9) reported that many Aeromonas isolates from water and animals (cows and pigs) in Great Britain possessed a full battery of virulence factors, such as cytoxins, cytotoxic toxins, hemagglutinins, and invasins. In addition, Mateos et al. (20) showed that A. hydrophila isolates from the environment were avirulent for mice, whereas human isolates caused lesions and death in these laboratory animals. In two comprehensive studies from Australia, Burke et al. (4) and Kirov et al. (11) compared

<table>
<thead>
<tr>
<th>Source</th>
<th>Strain</th>
<th>Cytotoxin (reciprocal titer)*</th>
<th>Cytotonic toxin (reciprocal titer)*</th>
<th>Hemolysin (reciprocal titer)*</th>
<th>Dermonecrotic factors*</th>
<th>Enteroxin production*</th>
<th>Adhesion to 407 cells*</th>
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<td></td>
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<tr>
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<tr>
<td></td>
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<td>10</td>
<td>1</td>
<td>256</td>
<td>+</td>
<td>Neg.</td>
<td>&lt;5</td>
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</table>

* Dilutions of supernatant showing a cytotoxic or cytotropic reaction as lysis or elongation of cells, respectively. ––, no reaction.
* Cytotoxicity reaction in the rabbit skin test (>10 mm diameter) after injection of 0.1 ml of sterile culture filtrate.
* Values represent the amount of fluid (in milliliters) accumulated per centimeter of rabbit ileal loop after injection of 1 ml of sterile culture filtrate. Neg., no fluid accumulation; NT, not tested.
* Number of adherent bacteria per human intestinal 407 cell.
Aeromonas strains isolated from drinking water, rivers, water reservoirs, and humans. These investigators found that both clinical and environmental strains were capable of producing enterotoxins.

In our study, too, we found that production of enterotoxins, cytotoxins, hemolysins, and dermonecrotic toxins was prevalent in Aeromonas strains isolated from the marine environment and clinical sources. However, Figura et al. (8) reported that there was no significant difference in the occurrence of Aeromonas enterotoxins between strains isolated from human patients and controls. Moreover, our observations are in agreement with those of Okitsu et al. (22), who found poor correlation between production of hemolysin and enterotoxigenicity in Aeromonas spp., although Burke et al. (4) found a 99% correlation between hemolysin production and enterotoxigenicity. Our findings on the production of cytotoxin, which was observed in both clinical and environmental Aeromonas isolates, correlated with previous findings indicating that cytotoxin production and enterotoxin production were independent properties. Although the skin test has been used previously for the detection of cholera and Escherichia coli, Aeromonas, and Salmonella enterotoxins, the dermonecrotic effect of fish pathogens such as A. hydrophila, Vibrio anguillarum, and Yersinia ruckeri has also recently been reported (15, 23, 24). In our investigation, both environmental and clinical isolates were capable of producing dermonecrotic factors to various degrees. However, there was no correlation between the skin reaction and the production of enterotoxins or hemolysin.

For many bacterial enteropathogens, the ability to adhere to intestinal mucosa is a first step in the colonization and development of disease. It has been reported that Aeromonas strains adhere to and invade epithelial cells grown in culture (5, 13, 16). In our comparative study, all of the strains isolated from marine water sediments and cases of human diarrhea that were investigated had the capacity to adhere to intestinal cells in vitro, and the majority of the isolates were classified as low adherers, with <10 bacteria per intestinal cell. On the other hand, Carrello et al. (5) reported that human diarrheal Aeromonas isolates bound to larynx carcinoma (HEp-2) cells in higher numbers than the environmental isolates, none of which bound at >20 bacteria per cell.

This study shows that a vast majority of A. hydrophila strains isolated from the environment and from clinical samples from the same geographical area in southern Italy were capable of producing a full range of different potential virulence factors, although we did not observe any close correlation between different virulence factors, as reported by other investigators. The increasing frequency of reports on aquatic environment-associated human infections thus makes it important to know whether the environmental isolates possess virulence-associated factors and therefore represent an increased health risk for humans.

The results of the present investigation indicate that further epidemiological studies are necessary to elucidate the public health significance of aeromonads in the aquatic environment. Further studies at our laboratory with the PhenePlate biochemical fingerprinting system (18), based on the evaluation of the kinetics of biochemical reactions, to investigate this phenomenon are in progress.

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