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Virulence Characteristics of *Aeromonas* spp. in Relation to Source and Biotype

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The significance of *Aeromonas* spp. as potential water-borne enteric pathogens in Tasmania, Australia, an area with a mild climate and comparatively low year-round water temperatures, was investigated in view of the reported marked peak of *Aeromonas*-associated gastroenteritis in the summer and the apparent influence of temperature on levels of potentially pathogenic species in water supplies. Biochemical characteristics and virulence-associated properties—exotoxin production (hemolysin, enterotoxin), ability to grow at 43°C, and possession of pili—were determined for 105 Tasmanian isolates of *Aeromonas* spp.; 43 isolates were from clinical specimens (>75% diarrhea associated) and 62 were from water. Current classification schemes were evaluated for these isolates. *A. sobria* comprised 35% of the clinical isolates and 16% of the water isolates, *A. hydrophila* comprised 56 and 79%, and *A. caviae* comprised 9 and 5%. A total of 42% of the clinical isolates and 15% of the environmental isolates were enterotoxigenic (by the suckling mouse assay); these levels were significantly lower than those found in warmer environments. The majority (74%) of enterotoxigenic isolates were *A. sobria*. Enterotoxin-producing isolates possessed three or more of the following properties. They were Voges-Proskauer positive, did not hydrolyze arabinose, were positive for lysine decarboxylase, were able to grow at 43°C, and produced large amounts of hemolysin (titer, >128). Thus, the biochemical scheme proposed by Burke et al. (V. Burke, J. Robinson, H. M. Atkinson, and M. Gracey, J. Clin. Microbiol. 15:48–52, 1982) for identifying enterotoxigenic isolates appears to have widespread applicability. Environmental enterotoxigenic isolates possessed numerous pili, but these appeared to be lost once infection was established, as similar isolates from patients with diarrhea were poorly piliated.

In recent years *Aeromonas* spp. have become increasingly recognized as enteric pathogens, although their direct etiologic role remains to be documented definitively in animal models or studies on human volunteers. They appear to be an important cause of acute diarrhea in children (2, 24, 28, 54) and in adults, especially those older than 60 years; cause sporadic diarrhea or dysentery which can be severe, even life-threatening (12, 17, 23, 45); and they may be an important cause of travelers' diarrhea (18–20, 25). Many workers have reported *Aeromonas*-associated gastroenteritis to be distinctly seasonal, with a sharp summer peak, and it is suspected it may be water-borne (2, 11, 24).

Aeromonas spp. can be isolated from the feces of normal individuals at a frequency variously reported between 0.2 and 8% (13, 24, 39, 55, 56). However, exotoxin production, i.e., production of enterotoxins, cytotoxins, and hemolysins, is much more frequently found in isolates obtained from patients with gastrointestinal symptoms (24). A proportion of patients experience diarrheic-dysenteric symptoms or an *Aeromonas*-associated colitis (24, 36, 44). It has recently been reported that isolates from such patients are able to invade HEp-2 cell cultures (34, 57). Pathogenicity of *Aeromonas* spp. may therefore be somewhat akin to enteropathogenic, enterotoxigenic, and enteroinvasive strains of *Escherichia coli* (26).

Aeromonas-associated gastroenteritis is probably underdiagnosed due to lack of recognition of its significance, confusion over the taxonomy of the *Aeromonas* genus, and the difficulty for a laboratory to routinely identify isolates with virulence-associated properties, e.g., enterotoxin production and enteroinvasiveness. A classification scheme based on biotyping (the Voges-Proskauer [VP] test and arabinose fermentation) and a hemolysin assay was proposed by Burke el al. (7) from studies of *Aeromonas* isolates from Perth, Western Australia; Adelaide, South Australia (Fig. 1); India; and Bangladesh. They proposed that diagnostic laboratories that routinely use these tests might correctly identify 97% of enterotoxigenic isolates without the need for in vivo assays. Their classification closely followed an earlier taxonomic classification proposed by Schubert (47) which divided the motile, mesophilic aeromonads into two species, with various subtypes and biotypes (group A, type 1: *A. hydrophila* subsp. *hydrophila* or *anaerogenes*; group A, type 2: *A. hydrophila* subsp. *hydrophila* or *anaerogenes*; group B: *A. punctata* subsp. *caviae*; group C: *A. punctata* subsp. *punctata*). To date, however, there has been no independent confirmation of its validity or widespread applicability. The most recent classification, which is becoming increasingly accepted, divides the motile aeromonads into three species, *A. hydrophila, A. caviae,* and *A. sobria* (42), although in the literature to date there appears to be no useful separation with regard to enterotoxigenicity by using this method of species identification (54).

It has been shown for enterotoxigenic organisms, such as enterotoxigenic *E. coli* and *Vibrio cholerae*, that the ability to adhere to the intestinal mucosa, as well as enterotoxin production, is necessary to produce diarrhea (22). For *E. coli*, pili mediate this attachment in some instances (29, 51). The importance of both virulence factors has been confirmed by experiments in human volunteers (21, 46) and by the success of antipilin vaccines in preventing enterotoxigenic *E. coli* diarrhea in animals (1, 41).

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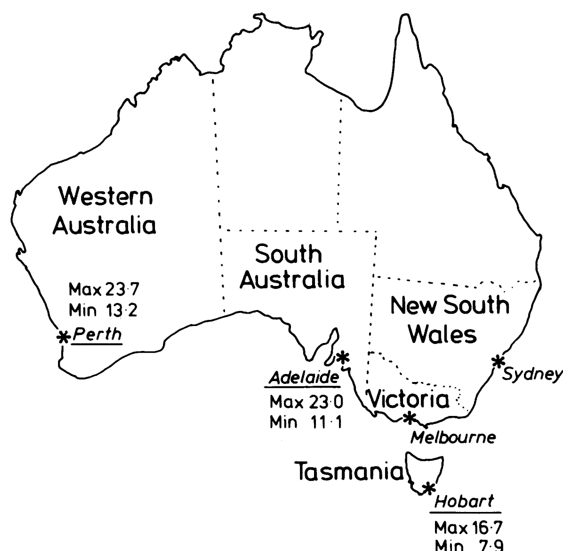

FIG. 1. Geographical centers in Australia from which the *Aeromonas* isolates described in this study were obtained. The values shown represent the mean temperatures for the hottest (max) and coldest (min) months of the year in degrees Celsius.

At present adhesins that may be important virulence factors for *Aeromonas* spp. are not defined. Atkinson and Trust (3) described patterns of hemagglutination which correlated well with the ability to adhere to human erythrocytes and buccal epithelial cells. Hemagglutination patterns have also been correlated recently with biotype and source of *Aeromonas* isolates (5). More than one mechanism appears to be involved in this hemagglutination, one of which is pili mediated (3, 16). However, studies of pili production by *Aeromonas* spp. in relation to source, biotype, and virulence have been few and the results have been conflicting (3, 16, 35).

The public health significance of *Aeromonas* spp. in water supplies has been given little attention. The few studies done prior to 1984 examined only small numbers of isolates and suffer from the prevailing taxonomic confusion, but the results suggest that a high proportion (60 to 80%) of isolates from water produce cytotoxins (26, 32, 35). More recent studies by Burke et al. (9–11) done in Perth, Western Australia, have reported, similarly, that high numbers of isolates from water produce enterotoxin (as determined by the suckling mouse assay) and that many of these enterotoxigenic isolates are indistinguishable from clinical isolates. It appears that the presence of such strains in the water may pose a significant public health risk. They are found in increased numbers in water supplies during the summer months, paralleling the incidence of *Aeromonas*-associated gastroenteritis. Perth, a city with 969,000 people situated on the southwest coast of Australia, has a typical Mediterranean climate, with long, hot, and dry summers and short, mild, and wet winters. By contrast, Tasmania, the island state of Australia (population, 437,000), has a much milder climate with significantly lower temperatures all year. The relative geography and mean temperatures for the hottest and coldest months of these Australian centers are shown in Fig. 1.

A preliminary survey of the causative agents of acute childhood (<5 years) diarrhea in Tasmania established that *Aeromonas* spp. were readily isolated as the sole potential pathogen, even with the milder climate conditions and during the winter months. They were more frequently isolated (4.7%; 5 of 107), than species of *Salmonella* (3.7%; 4 of 107), *Shigella* (0.9%; 1 of 107), or *Campylobacter* (0.9%; 1 of 107) (33).

In this study we examined clinical and environmental (water) isolates of *Aeromonas* from several sites in Tasmania. Detailed biochemical profiles for each isolate were determined, and each isolate was tested for virulence-associated properties—exotoxin (hemolysin and enterotoxin) production, possession of pili, and the ability to grow at elevated temperatures—to evaluate the significance of *Aeromonas* spp. as potential water-borne pathogens in such regions with much milder climates and to assess the usefulness of the biotyping scheme proposed by Burke et al. (7) for identifying toxigenic isolates likely to be of significance.

MATERIALS AND METHODS

Sources of isolates. A total of 105 isolates from the island state of Tasmania, comprising 43 isolates from clinical sources and 62 isolates from environmental sources, were included in this study. The majority (85%) of the clinical isolates were from feces, and 75% were from patients with gastroenteritis. The remaining isolates from humans (15%) were from miscellaneous sites, including sputum, ear, blood, and wounds. Environmental isolates were obtained from water reservoirs, rivers, and chlorinated tap water around Tasmania. The method of isolation of *Aeromonas* spp. from water samples was that used by the Public Health Laboratory, Royal Hobart Hospital, Hobart, Tasmania. Briefly, a 100-ml quantity of the water sample was filtered through a 0.45-μm-pore-size membrane filter (Schleicher & Schüll, Inc., Dassel, Federal Republic of Germany). The filter was then transferred to a lauryl sulfate agar plate and incubated overnight at 37°C. Isolated colonies were tested for oxidase positivity, and positive colonies were subcultured onto blood agar prior to further identification. If single colonies were not apparent on the preliminary isolation media, the filter was rinsed in 10 ml of sterile normal saline (0.85%), and a portion of the liquid was inoculated onto blood agar with 10 mg of ampicillin per liter. An additional 26 isolates obtained from the centers in Australia (Perth and Adelaide; Fig. 1) were also examined.

Following the initial isolation on blood agar containing ampicillin (10 mg/liter) (24), oxidase-positive colonies were confirmed as *Aeromonas* spp. in Kaper medium (31) and with the API 20E System (Analytab Products, La Balme-Les Grottes, Montalieu-Vercieu, France). Isolates were stored in maintenance medium at room temperature (9).

Biochemical characteristics. In addition to the reactions recorded with API 20E test kits, the following biochemical tests were performed on each isolate: gas from glucose, gas from glycerol, and gluconate oxidation (15); esculin hydrolysis, fermentation of salicin, and growth in KCN medium, as described by Cowan (14); ability to use L-histidine or L-arginine as the sole carbon source (52); and the ability to produce H_2S from cysteine (53).

API 20E test kits were read after 18 to 24 h at 37°C and reexamined at 48 h if definitive results were not obtained. The additional tests were incubated at 30°C and observed daily for 4 days; esculin hydrolysis was observed daily for 7 days. Key tests, VP (MRVP medium; Oxoid Ltd., Basingstoke, England) and arabinose fermentation (15), were also repeated separately at 30°C and were read daily for 3 and 4 days, respectively.

Isolates were classified into species *A. sobria, A. hydrophila*, and *A. caviae* by the criteria of Popoff (42) and also into groups based on the earlier classification of Schubert (47), as outlined by Burke et al. (7). The ability of each isolate to grow on blood agar at 43°C was estimated at 24 h. Growth was scored from 0 (no growth) to 4+ (heavy growth).

Exotoxin assays. For exotoxin assays several colonies from 18- to 24-h-old cultures on blood agar were inoculated into 5 ml of tryptone soya broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.6% (wt/vol) yeast extract (Oxoid) in 25-ml Erlenmeyer flasks. These were incubated (37°C, 20 to 24 h) with agitation (200 to 300 rpm; Orbital shaker; Paton Industries, Adelaide, Australia). Cell-free preparations were then prepared by centrifugation (10,000 × *g*, 30 min) and filtration (0.2-μm-pore-size disposable filters, Schleicher & Schüll). Supernatant preparations were tested on the day of preparation.

(i) Suckling mouse assay. The heat-labile enterotoxin of *Aeromonas* spp. (6, 8) was detected by the method described in detail by Berry et al. (4). The test, in brief, involved intragastric inoculation of the cell-free supernatant test solutions (100 μl) to which had been added 0.02 ml of 2.5% pontamine sky-blue dye (Gurr, Hopkin and Williams, Chadwell Heath, Essex, England). The mice were subsequently kept for 3 h at 28°C in a compartmentalized metal tray with a blotting paper base, before they were killed by cervical dislocation. The intestines were removed, and the ratio of the intestinal weight to the remaining body weight was determined. The presence of diarrhea (blue staining on the blotting paper) was also recorded. A scoring system incorporating both these parameters allowed identification of enterotoxigenic strains (4).

(ii) Hemolysin assay. Doubling dilutions of the cell-free supernatant test solutions in phosphate-buffered saline (pH 7.4) were made in microtiter trays (Linbro, Hamden, Conn.), and an equal volume (100 μl) of a 1% suspension of fresh, washed (5 times) rabbit erythrocytes was added. Phosphate-buffered saline and broth blanks were included in each tray, and each isolate was assayed in duplicate from replicate broth supernatants. Trays were sealed and incubated for 1 h at 37°C and then for 1 h at 4°C. The hemolysin titer was recorded as the last dilution showing 50% hemolysis of the erythrocytes.

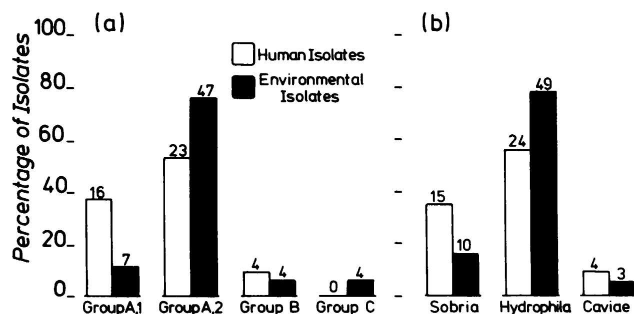

FIG. 2. Biochemical classification of Tasmanian *Aeromonas* isolates. (a) Groups classified by the method of Burke et al. (7) based on the earlier classification of Schubert (47) (group A, type 1: VP positive, arabinose negative; group A, type 2: VP positive, arabinose positive; group B: gluconate oxidation negative, gas from glucose negative; group C: gluconate oxidation, gas from glucose (one or both) positive, gas from glycerol negative). (b) Groups classified to the species level by the criteria of Popoff (42).

TABLE 1. Differentiation of *A. hydrophila* and *A. sobria*

Test	No. (%) of positive strains of:			
	A. hydrophila		*A. sobria*	
	Human	Environmental	Human	Environmental
Esculin hydrolysis	24 (100)	49 (100)	1 (7)	0 (0)
Salicin fermentation	24 (100)	42 (86)	0 (0)	0 (0)
Arabinose fermentation	21 (88)	49 (100)	1 (7)	1 (10)
Growth in KCN	24 (100)	49 (100)	10 (67)	4 (40)[a]
Histidine and arginine utilization	24 (100)	49 (100)	1 (7)	3 (30)[b]

[a] Positive growth in KCN for *A. sobria* was ± to 1+ compared with 2+ to 3+ growth with *A. hydrophila*.

[b] Histidine and arginine utilization was ± to 2+ in 48 to 72 h for *A. sobria* versus 2+ to 3+ at 48 h for *A. hydrophila*.

Electron microscopy studies. (i) Technique of negative staining. The tops of 18- to 24-h-old colonies on blood agar were scraped with a nichrome loop, and the organisms were dispersed into 3 ml of RPMI 1640 medium (Flow Laboratories, Inc., North Ryde, New South Wales, Australia) containing 2 g of $NaHCO_3$ per liter to obtain a slightly turbid solution. A drop of organism suspension was placed on a pioloform-coated (Agar Aids Stansted, Essex, United Kingdom) 100-mesh copper grid for 1.5 min. It was blotted from the edge with filter paper and floated for 2 min in a drop of 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The grid was again blotted from the edge with filter paper. A drop of distilled water was placed on it and immediately blotted. This step was repeated followed by placing a drop of 1% sodium phosphotungstate (pH 6.0) for 15 s on the grid. The grid was then blotted thoroughly around the edge and allowed to air dry. Preparations were examined with a Philips 410 electron microscope at 80 kV.

(ii) Examination technique. Each isolate was coded and randomly examined, without indication of its source or biotype, and on a minimum of two separate occasions. Isolates were obtained from different cultures.

Percentage of microorganisms with pili. Areas of the grid with sufficient dispersion of microorganisms, so that they could be examined individually, were selected, and the grid was moved in one direction and examined at ×32,000 magnification. The first 20 microorganisms encountered were studied to determine the percentage with one or more pili.

Number of pili per microorganism. The number of individual pili emanating from the surface of the next three single microorganisms was counted and scored as follows: 1, <10 pili; 2, 10 to 100 pili; 3, >100 pili.

RESULTS

Biochemical test characteristics. (i) Classification of isolates. Classification of isolates by the scheme of Burke et al. (7) and the criteria of Popoff and Véron (42, 43) is shown in Fig. 2. In the former scheme, *Aeromonas* isolates were subdivided into VP-positive and VP-negative groups. VP-positive isolates (group A) were further subdivided on the basis of arabinose fermentation into arabinose-negative (group A, type 1) and arabinose-positive (group A, type 2) groups. VP-negative isolates were classified into those which did not oxidize gluconate or produce gas from glucose (group B) and those which had one or both of these properties and did not produce gas from glycerol (group C).

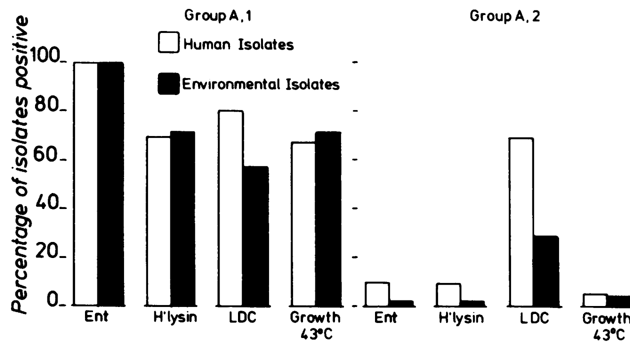

FIG. 3. Comparison of exotoxin production—enterotoxin (Ent) and hemolysin titer >128 (H'lysin)—LDC positivity, and ability to produce 2+ growth at 43°C in 24 h (growth at 43°C) for the two major biogroups of *Aeromonas* spp. Group A, type 1 (VP positive, arabinose negative), comprised 16 human and 7 environmental isolates; group A, type 2 (VP positive, arabinose positive), comprised 23 human and 47 environmental isolates.

The majority of the Tasmanian human and environmental isolates (90 and 87%, respectively) were VP positive and thus were placed in group A, types 1 and 2. However, a greater proportion (37% [16 of 43] versus 11% [7 of 62]) of the human isolates belonged to group A, type 1, the group found to contain the majority of enterotoxin positive isolates in the studies done in Perth (7). No human isolates were obtained that belonged to group C, which was also postulated by Burke et al. (7) to be an enterotoxin-positive biogroup. However, four environmental isolates were identified from this group. Small numbers only (9 and 6%, respectively) of human and environmental isolates fell into group B, corresponding to the nonenterotoxigenic group (*A. caviae*).

When the isolates were divided into species according to the criteria of Popoff and Véron (42, 43), *A. hydrophila* was the predominant isolate, comprising 56% (24 of 43) of the human isolates and 79% (49 of 62) of the environmental isolates. *A. sobria* was more common in the human (35%; 15 of 43) than in the environmental (16%; 10 of 62) isolates. Of interest, 13 of the 16 human isolates and all 7 environmental isolates in group A, type 1, were *A. sobria*, while the putative nonenterotoxigenic isolates in group A, type 2, were predominantly *A. hydrophila* (21 of 23 human isolates and 46 of 47 environmental isolates) (Fig. 2). Thus, if the correlations of Burke et al. (7) of these biogroups and enterotoxigenicity held true for the Tasmanian isolates, there would be clear value in carrying out identification to the species level by the method of Popoff and Véron (43).

Of the 26 isolates obtained from Adelaide and Perth, those from feces were predominantly *A. sobria* (8 of 9; group A, type 1), while group A, type 2, isolates were *A. hydrophila* (5 of 5) and 1 isolate was *A. caviae* (group B). Environmental isolates proved more difficult to classify. Of 10 group A, type 1, isolates, only 4 were readily identified as *A. sobria*, while the remaining group A, type 2, nontoxigenic isolate was *A. hydrophila*.

(ii) Evaluation of biochemical test criteria. Comparison of results of key tests (VP and arabinose fermentation) performed with the API 20E system and individually at 30°C showed that although there was reasonable agreement by both methods (88 and 80%, respectively) for the human isolates, the API 20E system was less accurate (74 and 56% agreement, respectively) for the environmental isolates. Individual testing at 30°C yielded a greater proportion of easily read positive reactions compared with negative or doubtful positive reactions recorded with the API 20E kits (37°C), particularly for the environmental isolates.

Analysis of the tests used in the differentiation of *A. hydrophila* from *A. sobria* is shown in Table 1.

Esculin hydrolysis and salicin and arabinose fermentation were the most useful as discriminatory tests. Other proposed discriminatory reactions, such as the ability to grow in KCN medium and the use of histidine and arginine as sole carbon sources, were less useful, as they required experience gained from testing large numbers of isolates, with positive results often being a question of degree or timing of reaction (Table 1).

Exotoxin production in relation to biotype. In Fig. 3 are summarized results of exotoxin assays (enterotoxin and hemolysin) for the majority of isolates (group A, type 1: VP positive, arabinose negative; group A, type 2: VP positive, arabinose positive).

(i) Enterotoxin. The relationship between biotype and enterotoxin, as described by Burke et al. (7), was confirmed for the Tasmanian isolates. All group A, type 1, isolates (16 human and 7 environmental) were enterotoxigenic (20 of these 23 were *A. sobria*), as was one of four group C isolates. By contrast, few isolates (2 of 23 human and 1 of 47 environmental) in group A, type 2, produced enterotoxin, and as expected none of the isolates (human or environmental) in group B (*A. caviae*) were enterotoxigenic. The proportion of environmental enterotoxigenic isolates in Tasmania (15%; 9 of 62) appears to be very much less than that described for Perth and its environs (≈70%).

(ii) Hemolysin. Enterotoxigenic human and environmental isolates in group A, type 1, tended to have high hemolysin titers (128 to 2,048), as did the few toxigenic isolates in group A, type 2 (Fig. 3), and the toxigenic environmental group C isolate. Nonenterotoxigenic isolates in group A, type 2, had no hemolysin titers (25%) or low hemolysin titers of between 4 and 32 (75%). None of the group B isolates produced hemolysin.

Other properties related to exotoxin production. (i) Lysine decarboxylase activity. Lysine decarboxylase (LDC) activity has been reported to correlate with cytotoxin production (2, 16) and, by implication, enterotoxigenicity. Nonenterotoxigenic, hemolysin-negative group B isolates were all LDC negative, and LDC positivity was most frequently found (80%) in toxigenic human isolates. However, a high proportion (68%) of nonenterotoxigenic group A, type 2, human

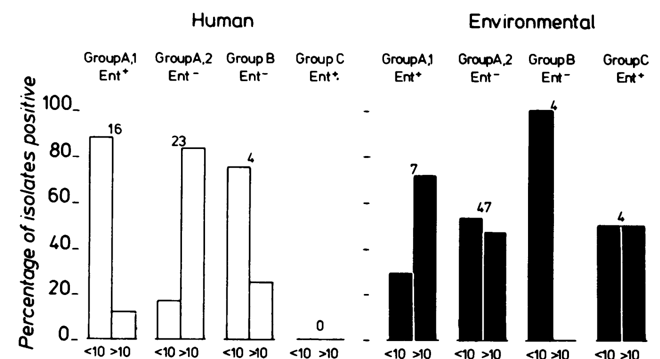

FIG. 4. Piliation of human and environmental *Aeromonas* isolates in relation to biotype (Ent, enterotoxigenic). The percentages of isolates in each group for which the majority of organisms possessed no or few pili (<10) or 10 or more pili (>10) are shown.

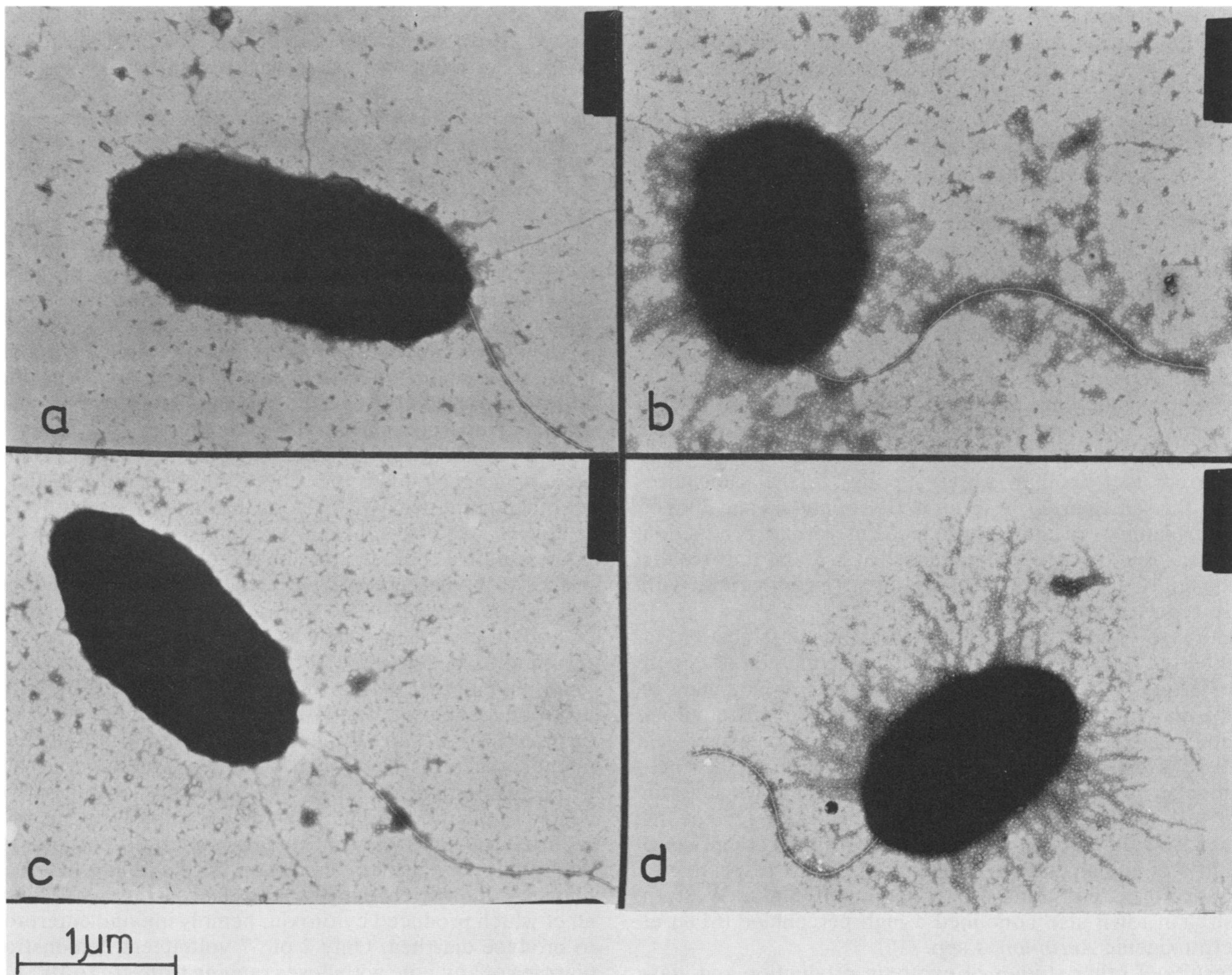

FIG. 5. Electron micrographs (negative stain) of representative *Aeromonas* isolates. (a) Nonpiliated, *A. caviae* (group B). (b) Heavily piliated, enterotoxigenic environmental *A. sobria* (group A, type 1). (c) Poorly piliated, enterotoxigenic human *A. sobria* (group A, type 1). (d) Heavily piliated, nonenterotoxigenic human *A. hydrophila* (group A, type 2).

isolates possessed the LDC marker. LDC positivity was thus more frequently associated with human rather than environmental isolates (Fig. 3).

(ii) Growth at 43°C. The ability to grow at 43°C also was proposed as a marker for virulence. All the enterotoxigenic isolates grew at 43°C, and the majority showed >2+ growth at 24 h, whereas the majority of nonenterotoxigenic human isolates (70%; 13 of 20) showed no or <1+ growth. Almost all (45 of 47) nonenterotoxigenic, environmental isolates and all group B isolates were unable to grow at 43°C.

Evaluation of the extent of piliation. A clear and unexpected pattern emerged when the degree of piliation of *Aeromonas* isolates was correlated with source and biotype (Fig. 4).

While the environmental isolates followed the pattern which may have been expected if the presence of pili is considered a virulence-associated property, with enterotoxigenic group A, type 1, isolates being heavily piliated (>>100 pili on 80% or more of the microorganisms) (Fig. 5b) and nonenterotoxigenic isolates from group A, type 2, and group B having much fewer or no pili, the human isolates showed the reverse pattern. Surprisingly, the majority of human enterotoxigenic isolates (group A, type 1) were poorly piliated (Fig. 5c), while the nonenterotoxigenic (group A, type 2) isolates possessed pili (Fig. 5d).

DISCUSSION

Review of the current literature on *Aeromonas* spp. suggests that several subsets (enterotoxigenic and enteroinvasive) are important water-borne, human enteric pathogens. The increased frequency of isolation of enterotoxigenic *Aeromonas* spp. from feces is associated with a peak of gastroenteritis over the summer months, presumably when these organisms, which appear to survive chlorination, are present in high numbers in the water supply (2, 11, 24). In view of this sharp, apparently temperature-related incidence of *Aeromonas* gastroenteritis, it was of interest to see whether in an area such as Tasmania, which has a cooler climate and low water temperatures year-round, *Aeromonas* spp. are similarly prevalent.

Enterotoxigenic *Aeromonas* spp. were isolated from patients with diarrhea in Tasmania, often as the sole potential enteric pathogen, and similar isolates were found in tap

water and freshwater supplies. They comprised 42% (18 of 43) of the clinical isolates and 15% (9 of 62) of the water isolates. The majority (74%) of these toxigenic isolates were *A. sobria*.

There have been few comparative studies of *Aeromonas* spp. from clinical and environmental sources. Daily et al. (16) investigated only small numbers of isolates (15 clinical and 9 water isolates), and these were not from the same geographical source. Nevertheless, they concluded that the majority of clinical isolates (in Indonesia and the United States) were *A. sobria*, while *A. hydrophila* was the predominant species in the environment (in the United States). Based on 50% lethal dose studies in mice and exoenzyme production, they postulated that *A. sobria* is the more virulent species in humans.

A more comprehensive study by Burke et al. (9) on *Aeromonas* spp. from Perth, Western Australia, compared 147 diarrhea-associated isolates from humans with 94 isolates from metropolitan water supplies in the same area during the same period. A total of 91% of human and 70% of water isolates were enterotoxigenic. Enterotoxin production was measured by the suckling mouse assay, and thus results of the study done in Perth (9) are directly comparable with those reported here.

Although Burke et al. (9) prefer the classification of Schubert (47), species classification by the method of Popoff and Véron (43) revealed that *A. sobria* was predominant in their isolates, comprising 87 and 45% of human and environmental isolates, respectively. The latter percentage may well have been higher, as a large proportion (45%) of the environmental isolates were not readily classified into species in their test system. Classification into species of 12% of the human isolates was also in doubt. *A. hydrophila* comprised 3 and 14% of human and environmental isolates, respectively. An unchlorinated, domestic water supply in a Western Australian town also contained a high percentage (61%) of enterotoxigenic *Aeromonas* spp. (10).

Results of other studies of exotoxin production and classification by species of *Aeromonas* spp. isolated from water are conflicting. Kaper et al. (32) examined 118 isolates from the Chesapeake Bay in the United States, and all were classified as *A. hydrophila*. A total of 71% (83 of 116) produced cytotoxins and 73% (8 of 11) gave a positive reaction for enterotoxin production in the rabbit ileal loop test. Le Chevallier et al. (35) investigated species isolated from chlorinated drinking water and classified all 20 isolates as *A. sobria*. Eighty percent (16 of 20) produced cytotoxins but were negative in the rabbit ileal loop assay.

Clearly, there are marked regional differences in the prevalence of different species of *Aeromonas* and the proportion of enterotoxigenic strains. Water temperature, residual chlorine levels, and interaction between these variables have been shown to influence the growth of *Aeromonas* spp. (11) and probably contribute to such differences.

The Tasmanian isolates were readily identifiable (>95%) to the species level by using the criteria of Popoff and Véron (43). Similarly, Janda et al. (30) were able to classify >93% of a group of 147 *Aeromonas* spp. isolated from diverse clinical (131 isolates), environmental-animal (13 isolates), and unknown (3 isolates) sources. These consisted of 48% *A. hydrophila*, 25% *A. sobria*, and 27% *A. caviae*. The temperature of incubation (30 versus 37°C) and the method of performing key tests (separately versus the API 20E test system) influences biotyping results, particularly for the environmental isolates; nevertheless, it may well be that in warmer climates, environmental isolates of *Aeromonas* are more phenotypically diverse. Our limited examination of isolates from such areas parallel the findings of the study done in Perth (9) which suggest this result.

The validity of the biochemical classification scheme proposed by Burke et al. (7) for identification of toxigenic isolates was essentially confirmed for the Tasmanian isolates. Toxigenic isolates (*A. sobria* and *A. hydrophila*) invariably possessed three or more of the following properties: positive VP reaction, negative arabinose fermentation, positive LDC activity, growth at 43°C, and high hemolysin titer (>128). The titer of hemolysin was important, as 75% of nonenterotoxigenic isolates produced low amounts of hemolysin (titers of 4 to 32).

The marked absence of pili on the enterotoxigenic human isolates was an unexpected finding. It is unlikely to be due to culture conditions or repeated subculturing, as fresh isolates (two subcultures) followed the same pattern. Moreover, the heavily piliated, environmental, enterotoxigenic isolates were maintained under the same culture conditions, and pili on other gram-negative bacteria have been reported to be relatively stable to repeated subculturing (38, 50). We hypothesize that pili are an important virulence-associated property of environmental isolates but that once infection is established, there is a shift toward nonpiliated forms because of growth conditions such as iron deficiency or selection resulting from decreased survival of piliated organisms beyond the mucosal barrier due to their enhanced susceptibility to phagocytosis. The latter phenomenon has been demonstrated for *E. coli* (48), and similar shifts from piliated to nonpiliated forms have been observed with several pathogenic microorganisms (27, 37, 49). The relationship between degree of piliation and biotype may account for some of the reported correlations of hemagglutination patterns and biotype. It may also be one reason for the failure in a recent human volunteer challenge trial of five *Aeromonas* isolates, all of which produced cytotoxin, hemolysin, and enterotoxin to produce diarrhea. Only 2 of 57 volunteers demonstrated diarrhea of any note with doses ranging from 10^4 to 10^{10} CFU (40). It would be of interest to test environmental, enterotoxigenic isolates for enteropathogenicity in a similar trial.

It is generally agreed that *A. caviae* is not an enteric pathogen (24, 30), and all the Tasmanian isolates of this species were negative for enterotoxin and hemolysin production. They were poorly piliated and possessed none of the other virulence-associated markers.

It is concluded from the results of this study that, although potentially pathogenic *Aeromonas* spp. may be more prevalent in warm environments, nevertheless they are found in water supplies in mild-cold regions where they may also pose a health risk. They are readily isolated from patients with diarrhea, particularly children during the summer months, often as the sole potential enteric pathogen (33). The majority (>97%) of such isolates are able to be recognized by the biotyping scheme, in conjunction with hemolysin titer, first proposed by Burke et al. (7). Classification to the species level by the method of Popoff and Véron (43) is also of value in such areas, with the majority (74%) of enterotoxigenic isolates being *A. sobria* and the majority (83%) of *A. sobria* being enterotoxigenic.

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