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Isolation and characterization of motile *Aeromonas* from human, food and environmental specimens

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SUMMARY

From July 1985 to March 1987, the occurrence of motile *Aeromonas* sp. in stool, food and environmental specimens was investigated to assess their pathogenic significance and to determine sources and routes of infection. A total of 9366 stool specimens were examined; *Aeromonas* was isolated from 11.1% of diarrhoeal stools and 2.2% of normal stools ($P < 0.001$). *Aeromonas* counts in food specimens, which included minced beef, pork and chicken, seafood and various vegetables and their products, were unexpectedly high suggesting that infection might be food-borne rather than water-borne. About 70% of the isolates from meat products were *A. hydrophila* and *A. sobria*, while *A. caviae* was the most common in sea-fish, vegetables and their products. Most *A. hydrophila* and *A. sobria* strains produced haemolysin, but haemagglutinin was found more frequently in *A. sobria*.

INTRODUCTION

Aeromonas organisms are considered to be autochthonous inhabitants of aquatic environments. Their roles in enteric infection in immunologically normal adults (Echeverria *et al.* 1984) and children (Burke *et al.* 1983) have been the subject of a number of studies. The illnesses reported range from mild to dysentery-like diarrhoea (Champsaur *et al.* 1982; Rahman & Willoughby, 1980) and include travellers' diarrhoea (Echeverria *et al.* 1981, 1984; Gracey *et al.* 1984). However the evidence associating *Aeromonas* with diarrhoea is largely circumstantial and epidemiological and lacks firm data. In some well-controlled studies, *Aeromonas* sp. were isolated significantly more frequently from diarrhoeal stools than from normal ones (Agger, McCormick & Gurwith, 1985; Burke *et al.* 1983; Cumberbatch *et al.* 1979), while in others, the isolation rates from the two groups were similar to each other (Figura *et al.* 1986; Millership, Curnow & Chattopadhyay, 1983; Pitarangsi *et al.* 1982).

Aeromonas strains produce a variety of biologically active extracellular substances including haemolysins (Asao *et al.* 1986; Stelma, Johnson & Spaulding, 1986) and enterotoxins (Chopra *et al.* 1986; Honda *et al.* 1985; Ljungh, Popoff & Wadström, 1977). Some strains are also invasive (Lawson, Burke & Chang, 1985; Watson *et al.* 1985) and produce haemagglutinins possibly associated with

diarrhoea (Atkinson & Trust, 1980; Burke *et al.* 1984*a*). There is no general consensus, however, in the roles of these factors in enteropathogenicity (Kindschuh *et al.* 1987; Turnbull *et al.* 1984). Oral administration of cytotoxic *A. hydrophila* (10^9 c.f.u.) failed to induce diarrhoea in rhesus monkeys (Pitarangsi *et al.* 1982) and in a study in human volunteers, the administration of various exotoxin-producing organisms failed to induce diarrhoea and they were not subsequently recovered from stools (Morgan *et al.* 1985).

Neither the sources nor the routes of infection are known though the organisms are most often isolated from man in summer when the bacillary population becomes the highest in aquatic habitats (Burke *et al.* 1983, 1984*b*). Some means of differentiation between the isolates pathogenic for man and those from the environment is clearly needed.

In this study, we examined stool, food, fish and environmental specimens to investigate the sources and routes of infection by *Aeromonas* sp. In addition, the isolates were examined for the haemolysin production and haemagglutination pattern in an attempt to correlate these properties with the species, pathogenicity and origin.

MATERIALS AND METHODS

Specimens. Stool specimens from healthy personnel including food handlers served as controls. Diarrhoeal stools were obtained from Osaka Municipal Momoyama Hospital and Juso Citizen's Hospital in Osaka.

Water samples were collected monthly at 25 sites on rivers in Osaka and assayed within 24 h: brackish water was sampled from 13 sites on the estuaries, and fresh water from 12 sites on the rivers. Tap water was collected every week for 6 months from April to the end of September 1986.

Fish were provided by the Laboratory of Food Hygiene, Osaka Municipal Wholesale Market. Sea-water fish examined were barracuda, cutlass fish, cuttlefish, flounder, halfbeak, horse mackerel, mackerel, niphonium, sardine, sea bream, sillago, sole and squid. Fresh-water fish (carp, eel and sweet fish) were also examined.

Vegetables and their products were sampled in our routine monitoring scheme. They were cabbage, carrot, cucumber, eggplant, lettuce, green peas, onion, pasta, potato, radish-sprouts, rice, spinach, tofu (soybean curd), tomato and vegetable salad.

Minced meat (raw) specimens were purchased at local retail markets.

Isolation. Stools were enriched in alkaline peptone water, pH 8·8 (APW), for 24 h at 30 °C, and subcultured for the first 7 months of the survey on Salmonella–Shigella agar and subsequently on bile salts brilliant green agar (BBG) (Millership & Chattopadhyay, 1984). Carbohydrates present in enteric agars have been reported to be inhibitory for *Aeromonas* sp., presumably because of by-products of carbohydrates metabolism (Millership & Chattopadhyay, 1984). Furthermore, acid production on carbohydrates containing media may result in a false negative oxidase result. Hence, we introduced BBG instead of Salmonella–Shigella agar in the middle of the survey. After incubation for 24 h at 30 °C, the plates were sprayed with oxidase reagent and oxidase-positive colonies were tested in Kaper's multiple test medium (Kaper *et al.* 1979).

For examination of river water, 0·1 ml of a tenfold dilution of the sample was spread on bile salts brilliant green starch agar (BBGS), which was incubated at 30 °C for 24–48 h (Nishikawa & Kishi, 1987). The plates were flooded with 0·5–1·0 ml of Lugol solution and amylase-positive colonies with surrounding clear zones were selected as presumptive *Aeromonas*. Tap water was counted for *Aeromonas* by the three-tube most probable number (MPN) method (American Public Health Association, 1971). Triplicate samples (1000, 100, 10 ml) were added to double-strength APW containing sodium thiosulphate.

Approximately 10 g of food sample in a sterile bag containing 90 ml of saline were macerated with a Stomacher 400 for 3 min. *Aeromonas* was counted by the three-tube MPN method. In the investigation of fish, only the digestive tracts were removed and examined; *Aeromonas* counts were expressed as the number per 10 g of digestive organs.

Identification. Motile strains yielding an alkaline surface and an acid butt in Kaper's multiple test medium were tested for Gram-negativity by the KOH method (Gregersen, 1978), cytochrome oxidase, catalase and indole production, gelatin hydrolysis, the absence of growth on TCBS, and resistance to 0/129 (Furniss, Lee & Donovan, 1978). Species identification was according to Bergey's Manual (Popoff, 1984); Voges-Proskauer reaction, H_2S production from cysteine, gas production from glucose, aesculin hydrolysis and salicin fermentation.

Haemolysin. Haemolysin was detected on trypticase soy agar plates containing 5% (v/v) rabbit blood in 48 h at 37 °C. β-haemolytic zones 2 mm or more around the colonies were regarded as the sign of positive haemolysis.

Haemagglutinin. Human group-O blood was collected, immediately placed into Alserver's solution, and stored at 4 °C for no longer than 7 days before use. Blood cells were washed three times and resuspended in Dulbecco phosphate-buffered saline (PBS), at 3% (v/v). *Aeromonas* strains were grown for 18 h on trypticase soy agar slants and suspended in PBS. Slide haemagglutination was performed at room temperature by mixing 20 μl of the blood cell suspension with the same volume of a bacterial suspension (10^{11} c.f.u./ml). Haemagglutination was read in 5 min and scored as ++ (immediate and complete agglutination) and + (incomplete agglutination occurring within 5 min). Fucose, galactose, and mannose were used singly at 1% (w/v) in the suspension of blood cells. Inhibition was scored as + when the haemagglutination became negative.

RESULTS

Isolation of Aeromonas *from stools.*

From a total of 9366 specimens, *Aeromonas* organisms were isolated from 11·1% (29/262) of diarrhoeal stools and from 2·2% (202/9104) of those from controls (χ^2, $P < 0{\cdot}001$). Most strains were isolated in summer (Fig. 1). 47·2% (109/231) of the stool isolates were *A. caviae* (Table 1). From the 29 patients (22 male and 5 female adults and 2 children) 30 *Aeromonas* strains were isolated: *A. hydrophila* and *A. sobria* were simultaneously recovered from one diarrhoeal stool. A second pathogen was found in the stools of 6 of the 29 patients. These were *Campylobacter jejuni*, *Salmonella* sp. and *Vibrio parahaemolyticus* from one each and *Entamoeba histolytica* from the other three.

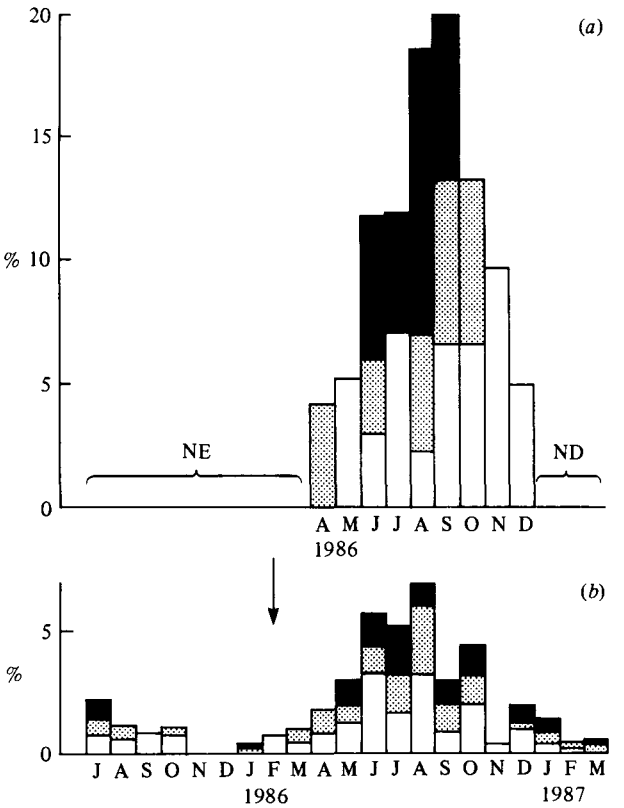

Fig. 1. Isolation (%) of *Aeromonas* from faecal specimens by month. (*a*) Isolation from diarrhoeal stools. (*b*) Isolation from normal stools. The arrow indicates introduction of BBG agar. Salmonella–Shigella agar had been used before that. NE, not examined; ND, not detected. ■, *A. hydrophila*; ▨, *A. Sobria*; □, *A. caviae*.

Table 1. *Species distribution of* Aeromonas *isolates by source*

Source	*Aeromonas* sp.	*A. hydrophila*	*A. sobria*	*A. caviae*
Normal stool	202/9104*	†24·3(49)†	28·6(58)	47·0(95)
Diarrhoeal stool	29/262	33·3(10)	20·0(6)	46·7(14)
River water	224/275	52·9(194)	9·6(35)	37·5(137)
Sea fish (summer)	22/33	30·6(11)	16·6(6)	52·8(19)
Sea fish (winter)	1/13	100·0(1)	0·0(0)	0·0(0)
Fresh-water fish (summer)	6/6	50·0(6)	33·3(4)	16·7(2)
Vegetables	22/43	37·0(10)	0·0(0)	63·0(17)
Meat products	37/48	45·5(30)	24·2(16)	30·3(20)

* Number of positives/samples examined.

† Relative percentages of isolates of each species. Figures in parentheses are number of strains isolated.

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Table 2. *Species distribution of* Aeromonas *in meat products*

Item	*Aeromonas* sp.	*A. hydrophila*	*A. sobria*	*A. caviae*
Cooked beef	* 4/8(0·3 to > 140)†	3/8	ND	2/8
Cow liver	1/1(24)	1/1	ND	1/1
Ground beef	10/10(91 to > 14000)	9/10	6/10	4/10
Ground Pork	10/10(150–46000)	9/10	6/10	5/10
Ground chicken	5/5(530 to > 140000)	5/5	3/5	4/5
Roast beef	2/7(0·36–0·73)	1/7	ND	1/7
Sausage & Ham	5/7(0·73 to > 140)	2/7	1/7	3/7

* Number of positives/samples examined.
† Figures in parentheses are the range of MPN/g of the product.

Table 3. *Number* (%) *of β-haemolytic* Aeromonas *strains isolated from various sources*

Source	*A. hydrophila*	*A. sobria*	*A. caviae*
Normal stool	22(91·7)	25(89·3)	1(2·2)
Diarrhoeal stool	9(90·0)	6(100·0)	0(0·0)
River water	74(97·4)	16(72·7)	1(2·1)
Sea fish	8(100·0)	7(100·0)	0(0·0)
Vegetables	11(91·7)	ND	0(0·0)
Meat products	28(96·6)	3(100·0)	1(11·1)

Recovery of Aeromonas *from aquatic environments.*

All samples yielded *Aeromonas* organisms, from river water *A. hydrophila* being the most common. No seasonal variation was noted. The density was lower in brackish water (mean concentration $10^{2·41}$/ml) than fresh water (mean concentration $10^{3·30}$/ml). *Aeromonas* was isolated only once from tap water (MPN = 2·3/1).

Recovery of Aeromonas *from foods*

Fish. Aeromonas was isolated from seawater fish more frequently in summer than in winter (Table 1). The number of organisms in seawater fish were small (3-15/fish) but were tenfold higher in fresh water fish (53 to >140/fish).

Meat. The findings in fresh and cooked or processed meats are presented in Table 2. The counts in minced beef, pork and chicken though variable from sample to sample, indicated a substantial level of contamination.

Vegetables. A. caviae was found in 11 of the 15 kinds of vegetables tested and *A. hydrophila* in 5. *A. sobria* was not present in any. Organisms were not detected in lettuce, pasta, potato or tomato and were present in the other vegetables in low numbers (0·3 to >140/g).

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Table 4. *Haemagglutination patterns* by species of* Aeromonas

Species	Number of strains								
	$F^-G^-M^-$	$F^-G^-M^+$	$F^-G^+M^-$	$F^-G^+M^+$	$F^+G^-M^-$	$F^+G^+M^-$	$F^+G^+M^+$	$F^+G^+M^-$	NHA**
A. hydrophila ($n = 205$)	1	2	0	3	0	0	3	33	163
A. sobria ($n = 100$)	3	11	0	10	4	0	2	13	57
A. caviae ($n = 193$)	3	0	0	0	0	0	0	13	177

* Abbreviations: F, fucose; G, glactose; M, mannose. + indicates inhibition of haemagglutination; − indicates no inhibition.
† Strains with no haemagglutinin.

Table 5. *Haemagglutination patterns of* Aeromonas *isolated from stools and non-faecal specimens*

Agglutination* pattern	Strains (%) from faeces		Strains (%) from non-faecal specimens	
	A. hydrophila	*A. sobria*	*A. hydrophila*	*A. sobria*
$F^-G^-M^-$	1(2·9)	0(0)	0(0)	3(4·1)
$F^-G^-M^+$	0(0)	6(17·6)	2(1·1)	6(8·2)
$F^-G^+M^+$	0(0)	4(11·8)	3(1·7)	7(9·6)
$F^+G^-M^-$	0(0)	4(11·8)	0(0)	0(0)
$F^+G^+M^+$	0(0)	0(0)	3(1·7)	2(2·7)
$F^+G^-M^+$	8(23·5)	5(14·7)	25(14·0)	8(11·0)
NHA	25(73·5)	15(44·1)	145(81·5)	47(64·4)

* Abbreviations and symbols are the same as those in Table 6.

Table 6. *Number* (%) *of haemagglutinin-positive* Aeromonas *strains isolated from various sources*

Source	*A. hydrophila*	*A. sobria*	*A. caviae*
Normal stool	6(25·0)	18(64·3)	5(10·9)
Diarrhoeal stool	3(30·0)	1(16·7)*	2(13·3)
River water	12(15·8)	8(36·4)	4(8·5)
Fish	0(0·0)	2(28·6)	0(0·0)
Vegetables	3(12·5)	ND	1(4·0)
Meat products	10(34·5)	2(66·7)	1(11·1)

* Significantly different from the value for normal stool at $P < 0{\cdot}001$.

Haemolysin production

One hundred and fifty-two of 159 (95·6%) of strains of *A. hydrophila*, 57 of 66 (86·4%) strains of *A. sobria* and 3 of 157 (1·9%) strains of *A. caviae* were haemolytic. No relationship between haemolysin production and the source of the organism could be demonstrated (Table 3).

Haemagglutinin

Haemagglutinin was more common in *A. sobria* (43%) than in *A. hydrophila* (20·5%) or *A. caviae* (8·3%). Fucose-resistant haemagglutinin was common in *A. sobria*. Haemagglutinin sensitive to fucose and mannose but resistant to galactose was common in *A. hydrophila* and *A. caviae* (Table 4). No haemagglutinin pattern specific for faecal isolates were recognized (Table 5). The proportion of haemagglutinin positives among *A. sobria* isolated from normal stools was about the same for those isolated from meat products. Among *A. sobria* strains isolated from diarrhoeal stools, only one was haemagglutinin positive. The proportion of haemagglutinin-positives among *A. hydrophila* strains from stools were identical to that from meat products (Table 6).

DISCUSSION

Aeromonas was isolated from normal and diarrhoeal stools at an increased frequency in summer as others have found (Burke *et al.* 1983; Janda, Bottone &

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Reitano, 1983), and more often from diarrhoeal than from normal stools (Agger, McCormick & Gurwith, 1985; Burke *et al.* 1983; Cumberbatch *et al.* 1979). *A. caviae* was the most common accounting for 47% of the stool isolates (Altwegg, 1985; Gosling, 1986).

Aeromonas infection is generally regarded as water-borne (Burke *et al.* 1984*b*; Le-Chevallier *et al.* 1982). However, no marked increase in the numbers of organisms was found in river water or in tap water in summer at the time when *Aeromonas* was detected more frequently in human stools. Fish might be considered an important source of infection, but the numbers of organisms recovered from seafish were very few making them unlikely to be a major source of infection. *Aeromonas* seems to prefer fresh water to brackish water, and freshwater fish showed heavier contamination. Freshwater fish, however, are not so commonly consumed in Japan. The present findings suggest that meat products may be a more important source of infection than fish. The organisms were present in most meat samples and 75% of those from minced meat were *A. hydrophila* and *A. sobria*, which are considered to be enteric pathogens. That the rate of haemagglutinin positive isolates from meat was the same as that among those from stools might be interpreted as an indication of the importance of meat as a source of infection.

A. caviae was the major species isolated from vegetables and their products which are often eaten raw. The high prevalence of *A. caviae* among the isolates from stools may be associated with the consumption of these foods.

Aeromonas enterotoxin has been a subject of controversy. Although some workers have ascribed the enteropathogenicity to a cytotonic enterotoxin, Stelma, Johnson & Spaulding (1986) consider β-haemolysin to be a more likely pathogenic factor. However, failure of haemolytic strains to cause diarrhoea in human volunteers suggests that haemolysin *per se* is not the sole determinant of virulence (Morgan *et al.* 1985). Indeed the significance of the finding that *Aeromonas* can be detected more frequently from diarrhoeal stools has still to be clarified.

Escherichia coli and *Vibrio cholerae* induce diarrhoea by elaboration of enterotoxins after adhesion to the intestinal mucosa (Freter, 1978). Both adhesins and enterotoxins were necessary to produce diarrhoea in volunteers fed orally with *E. coli* (Evans *et al.* 1978; Satterwhite *et al.* 1978). Although most *A. hydrophila* and *A. sobria* strains produce β-haemolysin, few strains possess adhesin and only a small proportion of human beings have intestinal epithelial cells sensitive to the adhesin, which may account for the rarity of outbreaks.

The ability of an enteric bacterium to adhere to human intestinal epithelium is often ascribed to haemagglutinin (Evans, Evans & DuPont, 1979). Atkinson & Trust (1980) stated that enterotoxigenic strains of *A. hydrophila* strongly agglutinate human blood cells, and Burke *et al.* (1984*a*) correlated the haemagglutination pattern to the source of *Aeromonas*. Although we identified the haemagglutination pattern by Burke's method, our patterns did not correlate with the source although they did with the species. Substantially more isolates of *A. sobria* from normal stools were haemagglutinin positive than were those from *A. hydrophila* or *A. caviae*, which may account for the finding that comparable numbers of *A. sobria* and *A. hydrophila* strains were isolated from stools, although the former is far less often found in the environment. Daily *et al.* (1981) also

concluded that the majority of clinical isolates were *A. sobria*, while *A. hydrophila* was predominant in the environment.

The present study shows that *Aeromonas* is ubiquitous not only in aquatic environments but also in various foods. Haemolysin production serves only as a marker for species differentiation but does not distinguish between the pathogenic and nonpathogenic strains. The involvement of other factors such as adhesin clearly require investigation to advance the understanding of the pathogenicity of *Aeromonas*.

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