CHARACTERIZATION OF *AEROMONAS HYDROPHILA* : A COMPARATIVE STUDY OF STRAINS ISOLATED FROM DIARRHEAL FECES AND THE ENVIRONMENT

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Abstract. Thirty-five strains of *Aeromonas hydrophila* isolated from feces of diarrheal patients and from the environments were collected from Thailand and Japan. The physiological, biochemical, and serological characteristics, antibiotic resistance patterns and cell surface-related properties were compared. The diarrheal and environmental isolates of *A. hydrophila* were found to be remarkably consistent in general culture and biochemical characteristics, with the exception of the reaction to D-arabinose in which the diarrheal strains were positive and environmental strains were negative. The plasmid patterns and cell surface-related properties of the environmental and diarrheal isolates were different. All strains produced Vero cell cytotoxin, hemolysin and lecithinase at 37°, 30° and 15°C. In contrast, 83% of the environmental strains produced these virulence factors even at 4°C. All strains indicated almost uniform susceptibility to the 16 antibiotics tested. Variations were found in the plasmid profile, toxin production in relation to the differences of temperature and cell surface-related properties of the strains. These variations between the clinical and environmental isolates could have potential as epidemiological markers for the sources of strains.

INTRODUCTION

Aeromonas spp is a member of the family Vibrionaceae. A ubiquitous microorganism, especially in the tropical countries, it can be isolated from surface water as well as clinical cases and is one of the causative agents of human gastroenteritis and other diseases such as septicemia and wound infection (Joseph *et al*, 1979; Ketover *et al*, 1973). This organism is also related to the infection of aquatic animals (Shotts *et al*, 1972). There are three phenospecies of mesophilic aeromonads - *A. hydrophila, A. sobria* and *A. caviae*. Among these, *A. hydrophila* is the commonest microbial pathogen. This organism has drawn special attention due to its pathogenesis in human intestinal and extraintestinal infections.

The aim of the present study was to compare strains of *A. hydrophila* isolated from diarrheal feces and the environment according to biochemical, serological and physiological characteristics, plasmid profiles and antibiotic susceptibility patterns. The cell surface-related properties of bacteria are associated with their pathogenesis. These properties were also investigated by hydrophobicity and binding of the dyes Congo red and Coomassie brilliant blue. Adhesion and invasion of bacteria into mucous membranes and epithelial cells are related to the pathogenecity of enteric infection. The adhesion and invasiveness of the strains to Vero and HEp-2 cells were also compared. Vero cell cytotoxin and hemolysin are recognized as important virulence factors produced by this organism (Asao *et al*, 1984; Vadivelu *et al*, 1995). Thus, we compared the production of cytotoxin and hemolysin by these strains at different temperatures.

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MATERIALS AND METHODS

Bacterial strains

Thirty-five strains of environmental and diarrheal isolates of *A. hydrophila* were studied. Among

them, twelve environmental strains were collected from river and waste water in Thailand. Human diarrheal strains were isolated from Thailand and Japan (12 and 11 isolates, respectively).

Biochemical and physiological tests

Frozen stock (-80°C) strains were grown on Trypticase Soy Agar (BBL, Maryland, USA) plates. Single colonies were then inoculated into Peptone Water Andrade (Oxoid, Hampshire, England) containing different carbohydrates. Reactions towards amino acids, proteins and lipids were investigated according to the method described elsewhere (Barrow *et al*, 1993).

Hemolytic activity was detected as a zone of hemolysis on 7% horse or sheep blood containing Blood Agar (Oxoid) plates after incubation for 2 ~ 4 days at 4°C, 15°C, 30°C and 37°C. Lecithinase production (Barrow *et al*, 1993) was investigated on 2.5%, 5% and 10% egg yolk-containing agar plates at 4°C, 15°C, 30°C and 37°C. The strains were inoculated in nutrient broth with 0%, 3%, 6% or 10% sodium chloride.

Congo red binding : Bacteria were streaked onto Congo-red agar, which consisted of Tryptic Soy Broth (Difco, Michigan, USA) with 0.6% yeast extract, 0.003% Congo red (Nacalai Tesque, Inc, Kyoto, Japan) and 1.5% agar (Qadri *et al*, 1988). The plates were incubated at 15°C, 30°C or 37°C for 1 ~ 3 days, and the development of pigmented colonies was observed.

Coomassie brilliant blue binding : Coomassie brilliant blue, 100 μg/ml was incorporated into brain heart infusion agar (Wilson and Horne, 1986). Colonies were inoculated and agar plates were incubated for 1 ~ 3 days at 15°C, 30°C and 37°C.

Cell-culture adherence test : Adherence of the bacteria was tested on Vero and HEp-2 cells. Confluent monolayers of Vero and HEp-2 cells were grown in Eagle's minimal essential medium (EMEM) on coverslips in an atmosphere of 5% CO_2 at 37°C. Bacteria were grown in Luria broth. Culture broth of the test strains of *A. hydrophila* containing 9×10^9 cfu was added into Vero and HEp-2 cells and then incubated at 37°C for 3 hours. A localized adherence-positive strain of *Escherichia coli* O127 (T41-F8/93) and a non-adherent strain of *E. coli* K-12 EC101 were used as positive and negative controls, respectively (Nakai *et al*, 1988; Pal and Hale, 1989). The non-adherent bacteria were washed out with phosphate buffered saline (PBS), pH 7.4. Cells were fixed with 70% methanol and stained with Giemsa. Adhesion of bacteria was observed under a microscope.

Cell-culture invasion test : Test strains of *A. hydrophila* were grown into Luria broth in a shaking incubator at 37°C for 4 hours. Approximately 1×10^6 cfu were added to the monolayers of Vero and HEp-2 cells grown in tissue culture plates. The plates were placed on an orbital shaker and rotated at 100 rpm for 2 minutes and then incubated in an atmosphere of 5% CO_2 and 95% O_2 at 37°C for 4 hours. The non-adherent (non-invasive) bacteria were washed out with PBS, pH 7.4, followed by incubation for 1 hour in EMEM containing gentamycin 100 μg/ml to kill the extracellular bacteria (Small and Falkaw, 1988). The confluent monolayers were washed and the internalized bacteria were released by lysis of the cells with Triton X-100. Bacteria were quantified by plate count. A strain of *Shigella flexneri* 2a (67001) was used as a positive control and *E. coli* K-12 EC101 was used as a negative control. Each strain was tested three times in duplicate and the values were averaged.

Salt agglutination test : Colonies were grown on Trypticase Soy Agar (BBL) at 37°C for 18 hours. Agglutination at different concentrations (0.5 ~ 3M ammonium sulfate in 0.02 M sodium phosphate buffer, pH 6.8) of ammonium sulfate on slides was then performed (Larsen *et al*, 1988; Rozgonyi *et al*, 1985). Autoagglutination of the colonies in physiological saline was routinely performed first.

Effect of temperature on production of cytotoxin

Bacteria were grown in Trypticase Soy Broth (BBL) at 4°C, 15°C, 30°C and 37°C and the culture filtrates were added to confluent monolayers of Vero cells. The cytotoxicities of the strains were assayed as described previously (Haque *et al*, 1995).

O-serogrouping

O-serogrouping of the strains of *A. hydrophila* was performed by agglutination according to the method described previously (Sakazaki and Shimada, 1984; Shimada *et al*, 1983, 1991).

Antibiotic sensitivity test

Antibiotic susceptibility of the strains were tested by the disk diffusion method (Bauer *et al*, 1966) with commercial disks (BBL). Antibiotic disks were placed on agar plates and then incubated at 37°C overnight. Bacterial growth inhibitory zones were measured and susceptibilities were determined according to the manufacturer's instructions.

Plasmid analysis

The plasmids of the strains were extracted by the alkaline lysis method (Maniatis *et al*, 1982). DNA was separated by agarose gel electrophoresis, and gels were stained with ethidium bromide and photographed under UV illumination.

RESULTS

Biochemical reactions of the strains

Fermentation of carbohydrates : The results of the reactions towards carbohydrates are summarized in Table 1. All strains were positive for the following : glucose, fructose, galactose, mannitol, maltose and starch. All strains were negative for the following: xylose, dulcitol, rhamnose, cellobiose, inositol, lactose, melibiose, raffinose, and inulin. Variable results were found with regard to L-arabinose, glycerol, mannose, sorbitol, and sucrose. Environmental strains showed positive and diarrheal strains showed negative reactions for D-arabinose.

Activities towards amino acids, proteins and lipids : All strains were positive to arginine and indole but all isolates were negative to ornithine and lysine, liquefied gelatin and hydrolyzed casein, hydrolyzed lipase and produced lecithinase on egg yolk medium. The diarrheal isolates showed some variations for the hydrolysis of casein (Table 2).

Physiological properties

Growth : All strains grew in peptone water with 0%, or 3% NaCl. However, only environmental strains (83%) of *A. hydrophila* were able to grow in 6% NaCl (Table 3). None but 83% of the environmental strains grew on Trypticase Soy Agar plates at 4°C.

Table 1

Activities of *A. hydrophila* towards carbohydrates and glycosides (number of strains tested).

	Diarrheal isolates from		Environmental
	Japan (11)	Thailand (12)	isolates (12)
Trioses			
glycerol	+	+9	+
Pentoses			
D-arabinose	-	-	+
L-arabinose	+2	+	+
xylose	-	-	-
Hexoses			
glucose	+	+	+
glucose gas	-	-	-
dulcitol	-	-	-
fructose	+	+	+
galactose	+	+	+
mannitol	+	+	+
mannose	+	+9	+
rhamnose	-	-	-
sorbitol	+	+7	+
Disaccharides			
cellobiose	-	-	-
inositol	-	-	-
lactose	-	-	-
maltose	+	+	+
sucrose	+	+	+3
melibiose	-	-	-
Trisaccharides			
raffinose	-	-	-
Polysaccharides			
inulin	-	-	-
starch	+	+	+
Glycosides			
esculin	+	+	+
salicin	-	-	-
ONPG	+	+	+

+, All strains positive; -, all strains negative; numerical values indicate the number of positive/negative strains.

Congo red binding : Diarrheal strains of *A. hydrophila* showed the ability to bind to Congo red within 24 hours as they produced pigmented colonies on Congo red agar. In contrast, the environmental strains could not bind to the dye within 24 hours, but did bind by 48 hours.

Table 2

Activities of *A. hydrophila* towards amino acids, proteins and lipids (number of strains tested).

	Diarrheal isolates from		Environmental
	Japan (11)	Thailand (12)	isolates (12)
Amino acids			
arginine dihydrolase	+	+	+
lysine decarboxylase	-	-	-
ornithine decarboxylase	-	-	-
tryptophane deaminase (indole)	+	+	+
Proteins			
gelatin liquefaction	+	+	+
hydrolysis of casein	+4	+7	+
Lipids			
lipase (Tween 80)	+	+	+
lecithinase	+	+	+

+, All strains positive; -, all strains negative; numerical values indicate the number of positive/negative strains.

Coomassie brilliant blue binding : Colonies were scored as wholly positive, negative or mixed with regard to binding of Coomassie brilliant blue. All diarrheal isolates but none of the environmental strains showed the presence of wholly positive colonies; 2 of the later strains contained mixed and 10 contained negative colonies (Table 3).

Salt agglutination test : All strains were highly hydrophobic and agglutinated in 0.06 M ammonium sulfate. However, all produced smooth suspensions in physiological saline.

Cell culture adherence : All diarrheal isolates showed good adherence to Vero and HEp-2 cells. Three environmental strains showed adherence to HEp-2 cells and only 2 showed adherence to Vero cells (Table 3).

Invasion : The quantitative cell culture invasion assay showed that the intracellular survival of bacteria of the clinical isolates in Vero cells were $2.8 \times 10^3 \sim 3.2 \times 10^3$ and in HEp-2 cells were $3.8 \times 10^3 \sim 4.1 \times 10^3$. The environmental strains were non-invasive (Table 3).

Production of toxins at different temperatures

Cytotoxin : Culture filtrates of all strains produced cytotoxins at 15°C, 30°C and 37°C which was demonstrated by the rounding and shrinkage of Vero cells. The diarrheal isolates could not produce cytotoxin at 4°C due to non-growth. Four environmental strains produced cytotoxins at 4°C.

Hemolysin : All Japanese and Thai diarrheal and environmental strains produced hemolysin at 15°C, 30°C and 37°C. Eighty-three percent of environmental strains produced hemolysin at 4°C. No clinical isolate of *A. hydrophila* produced hemolysin at 4°C (Table 4). The optimum temperature for the production of hemolysin was 37°C.

Lecithinase

The Japanese diarrheal isolates produced lecithinase at 30°C and 37°C but failed to do so at 4°C (Due to non-growth) or 15°C. The Thai clinical isolates produced lecithinase at 15°C, 30°C and 37°C. However, 83% of environmental strains could produce lecithinase even at 4°C (Table 4).

O-serogrouping

Japanese diarrheal isolates were mostly serogroup O5. Thai diarrheal isolates belonged to O5, O16 and O38. Thai environmental strains were mostly O16. Twenty-eight percent of all strains were rough.

Table 3

Characterization of dye binding, adhesion and invasion to cell lines and growth in different concentrations of NaCl (number of strains tested).

	Diarrheal isolates from		Environmental
	Japan (11)	Thailand (12)	isolates (12)
Dye binding			
Congo red	+	+	$+^{d}$
Coomassie blue	+	+	-10
Adhesion to			
Vero cells	+	+	+2
HEp-2 cells	+	+	+3
Invasion to			
Vero-cells	+	+	-
HEp-2 cells	+	+	-
Growth in NaCl			
0%	+	+	+
3%	+	+	+
6%	-	-	+10
10%	-	-	-

+, All strains positive; -, all strains negative; numerical values indicate the number of positive/negative strains; $+^{d}$ delayed binding.

Plasmid profile

No plasmids were found in the environmental isolates. Among the 23 diarrheal isolates of *A. hydrophila* only 16 strains harbored plasmid DNA of 2.14 ~ 23.5 kb.

Antibiotic susceptibility

All isolates of *A. hydrophila* were uniformly sensitive to tetracycline, chloramphenicol, kanamycin, gentamycin, streptomycin, polymyxin B, neomycin and nalidixic acid but resistant to ampicillin, cephalothin, penicillin, aminobenzyl penicillin, colistin, trimethoprim, sulfamonomethoxine and sulfisoxozol.

DISCUSSION

In this study, *A. hydrophila* was found in stool specimens from patients with diarrhea and water specimens collected from river and waste water. The biochemical reactions of 35 strains of *A. hydrophila* from diarrheal feces and the environment were almost homogenous and no basis was found for setting up different isolates regarding the source. The diarrheal and environmental strains differed with regard to reaction for D-arabinose; the diarrheal strains showed negative results and environmental strains positive results. Some divergent results of biochemical and physiological properties of *A. hydrophila* may be related to diarrheal or environmental origin as well as the geographical source.

The protein-specific dyes Congo red and Coomassie brilliant blue have frequently been used to distinguish polymorphic forms of bacterial species in solid media (Qadri *et al*, 1988; Wilson *et al*, 1986). Congo red binding is an indicator of pathogenesis in some bacteria. It interacts with the outer

Table 4

Production of hemolysin in 7% blood-agar and lecithinase in 5% egg yolk plates by *A. hydrophila* in relation to temperature (°C) variation.

A. hydrophila	Production of hemolysin at				Production of lecithinase at			
(number of strains)	4°C	15°C	30°C	37°C	4°C	15°C	30°C	37°C
Diarrheal								
Japan (11)	ng	+	+	+	ng	+	+	+
Thailand (12)	ng	+	+	+	ng	+8	+8	+8
Environmental								
Thailand (12)	+10	+	+	+	+10	+	+	+

+, All strains positive; -, all strains negative; numerical values indicate the number of positive/negative strains; ng, non-growth.

membrane and outer membrane proteins (Ishiguro *et al*, 1985; Qadri *et al*, 1988). Rough strains are deficient in lipopolysaccharides, so their binding with Congo red may be misinterpreted with regard to virulence. The diarrheal isolates showed higher binding affinities with these dyes.

The salt agglutination test (SAT) has been used successfully to determine the degree of bacterial cell-surface hydrophobicity (Rozgonyi *et al*, 1985). All of the strains of *A. hydrophila* examined here were highly hydrophobic, regardless of their source.

The environmental isolates were viable at higher salinity (NaCl 6%) than the clinical isolates. The identification of *Aeromonas* spp is often confused with *V. fluvialis*. The strains examined here were differentiated from *V. fluvialis* as they were resistant to 150 µg of O/129, a vibriostatic agent.

The adherence of pathogenic bacteria to the mucosal surface is recognized as an important first step in colonization and infection (Law, 1994). *In vitro* adherence of this organism to Vero and HEp-2 cells may give some insight to its pathogenecity. The higher ability of invasion and adhesion of the diarrheal isolates of *A. hydrophila* to Vero and HEp-2 cells may represent some mechanism of pathogenesis and/or infection.

The plasmid profiles of the strains were different; while no plasmids could be found in the environmental isolates, plasmids were frequently isolated from the diarrheal strains. Origin of these plasmids in diarrheal isolates of *A. hydrophila* remains to be determined.

The ability of bacteria to enter and survive within host epithelial cells indicates that several plasmid-mediated determinants appear to be necessary for invasiveness in some species (Small *et al*, 1988). The diarrheal isolates showed better adhesion to both Vero and HEp-2 cells than the environmental isolates. The diarrheal strains also showed better invasion of Vero and HEp-2 cells than the environmental strains.

Although we could not determine the relationship between the presence of plasmids and invasiveness of these organisms, among the diarrheal isolates the plasmid-harboring strains showed greater adhesiveness and invasiveness. Thus, the presence of plasmids, in the diarrheal isolates may have influenced the adhesion or invasion of bacteria to Vero and HEp-2 cells.

O-serogrouping of *A. hydrophila* (Shimada *et al*, 1991) is an important classification system for this organism. In this study, we found no correlation between the biochemical properties of O-serogrouping of the tested strains of *A. hydrophila*.

Production of hemolysin by the environmental strains at low temperature is an important finding with regarding to the source of the strains. None of the diarrheal isolates produced hemolysin at 4°C due to non-growth, but 83% of the environmental isolates were able to produce hemolysin at 4°C. The incidence of production of lecithinase by the environmental isolates at lower temperature was also higher in comparison to the diarrheal isolates.

Singh *et al* (1992) described the production of stronger enterotoxic effects of environmental than diarrheal isolates of *A. hydrophila*. In this study, we found stronger cytotoxic effects of the environmental strains on Vero cells. Moreover, the environmental strains could produce cytotoxin at refrigeration temperature, and also produced hemolysin at lower temperatures than the diarrheal isolates. Though the number of the strains tested in this study was limited, we found some divergent characteristics between the diarrheal and environmental strains.

Thus, reaction for D-arabinose, plasmid profile, growth in NaCl, surface-related properties and production of virulence factors in relation to temperature may be the distinctive markers between diarrheal and environmental isolates of *A. hydrophila*.

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