Iranian Biomedical Journal 8 (4): 199-203 (October 2004)

Characterization and Distribution of Virulence Factors in *Aeromonas hydrophila* Strains Isolated from Fecal Samples of Diarrheal and Asymptomatic Healthy Persons, in Ilam, Iran

Mohammad Mehdi Aslani*[1] and Hassan Seyyed Hamzeh[2]

[1]*Dept. of Microbiology, the Pasteur Institute of Iran, Tehran;* [2]*Dept. of Biology, Azad University, Lahijan, Iran*

Received; revised ; accepted

ABSTRACT

Aeromonas hydrophila **secretes several extracellular proteins including enterotoxin, hemolysin and aerolysin that are associated with the bacterial virulence. Previous studies have shown that two hemolytic toxins, hemolysin A and aerolysin A contribute to the virulence of *Aeromonas hydrophila*. In the current study, a total of 50 strains of *Aeromonas hydrophila*, including 28 (56%) strains isolated from diarrheal cases and 22 (44%) strains isolated from healthy asymptomatic controls, were used. These strains were tested for cytochrome oxidase activity based on Kovac's method and confirmed as *A. hydrophila* with API 20E multi-test systems. To determine hemolysin, cytotoxin and enterotoxin activities, horse blood agar, Vero cells and the suckling mouse model were used, respectively. The presence of two hemolytic toxin genes: *hlyA* and *aerA* was determined by PCR assay. About 89% of diarrheal strains tested were positive for hemolysins, 68% for cytotoxin and 89% for enterotoxin activity. These figures for healthy asymptomatic isolates were 72%, 23% and 14%, respectively. A significant association was found between cytotoxin and enterotoxin activity in diarrheal disease, whereas such association was not found in case of hemolysin. Almost 93% of diarrheal isolates and 73% of healthy person strains were PCR positive for *hlyA* gene. The corresponding figures for *aerA* gene were 86% and 45.5% respectively. The *aerA*$^+$ *hlyA*$^+$ genotype and in some cases *aerA*$^+$ genotype could be considered as reasonable predictors of human diarrheal disease. However, the role of other unknown hemolytic and cytolytic factors cannot be discounted.** *Iran. Biomed. J. 8 (4): 199-203, 2004*

Keywords: *Aeromonas*; Suckling mouse; Aerolysin; Hemolysin; Cytotoxin

INTRODUCTION

Aeromonads are ubiquitous organisms found in aquatic environments, food items, including meat, fish and vegetables and the intestine of apparently healthy humans with diarrhea [1]. Among various Aeromonas species, *A.hydrophila* is the most commonly involved in human infection such as septicemia and gastroenteritis [2]. The pathogenesis of *A. hydrophila* infection is complex and multi-factorial with the involvement of a number of virulence factors [3].

A. hydrophila may cause diarrhea by producing cytotoxic enterotoxin [4]. To date, two hemolytic toxins have been described: the *A. hydrophila* hemolysin (*hlyA*) [5] and aerolysin (*aerA*) [6]. Most *Aeromonas* hemolysins described are related to one of these toxins. A virulent strain of *A. hydrophila* (A6) [7] produces both hemolysins. The combined effect of these toxins causes hemolysis and cytotoxicity and contributes to the virulence in the suckling mouse.

It is estimated that aeromonads may cause up to 13% of the reported gastroenteritis cases in the United States [8]. Studies carried out in Thailand indicated a higher isolation rate in patients with diarrhea, while there was no difference in isolation rates among native Thais living in the same area [9]. Studies in Australia showed a 10% isolation rate in diarrhea patients compared with no isolation in healthy individuals [10].

The current study aimed to determine different virulence factors such as hemolysin, cytotoxin and enterotoxins phenotypically and genetically.

*Corresponding Author; Tel. (+98-21) 640 5535; Fax: (+98-21) 646 5132; E.mail: mmaslani@ institute.pasteur.ac.ir

[DOR: 20.1001.1.1028852.2004.8.4.6.7]

MATERIALS AND METHODS

Bacterial strains. A total of 50 strains of *A. hydrophila* isolated from fecal samples of diarrheal and asymptomatic healthy persons in Ilam (Iran) were used. All strains were tested for cytochrome oxidase activity based on Kovac's method [11] and confirmed as *A. hydrophila* with API 20E (Analytab Products, Inc., plain view NY) multi-test systems [12].

Culture conditions. Bacterial strains were grown on blood agar (oxoid blood agar base with 5% added defibrinated horse blood) at 37ºC for 18 h.

Hemolysin detection. The production of hemolysin was assayed by recording the lysis of horse erythrocytes in agar plate (5% horse blood in brain heart infusion agar) by the sterile cell-free culture filtrates [7].

Cytotoxin assay. Cytotoxin assay was performed according to the modified method [7]. Briefly, the harvested culture from blood agar plates was inoculated into two containers with 15 ml of tryptic soy broth (Difco laboratories), and incubated at 37ºC for 24 h on a shaker (60 rpm). The cultures were then harvested by centrifugation (13000 ×g) at 4ºC for 30 minutes. The supernatants were filter sterilized by 0.22 µm membrane and tested for cytotoxic activity on Vero cells (African green monkey kidney cells, National Cell Bank, the Pasture Institute of Iran, Tehran) by exposing a monolayer of the cell to serial dilutions of the filtrates. After incubations at 37ºC for 24 h in 5% Co_2, destruction of at least 50% of the Vero cells was recorded as a positive result. Two strains of *A. hydrophila:* ATCC 7966 and ATCC 15467 were used as positive and negative controls, respectively.

Suckling mouse model. Preparation of feeding inocula, infection of suckling mice and the determination of fluid accumulation (FA) in the gut were studied according to the method of Wong *et al.* [7]. The FA ratio was expressed as the weight of the gut (mg) over the weight of the remaining carcass (mg). The arbitrary cut-off value for positive FA ratio is 80 mgg^{-1}. A FA ratio of 49 ± 4 mgg^{-1} was considered as a negative factor for FA in the intestine. *A. hydrophila* ATCC 7966 for positive and ATCC 15467 for negative controls were used.

DNA extraction. A bacterial colony was inoculated in tryptocasein soy broth and incubated at 35ºC overnight with shaking at 100 rpm. Culture medium (1.5 ml) was centrifuged at 2500 g for 10 min. The supernatant was discarded and the sediment was used for DNA extraction by a phenol-free salting-out method [13].

PCR amplification. PCR was performed using a pair of primers: H1 (5'-GGC CGG TGG CCC GAA GAT GCA GG-3') and H2 (5'-GGC GGC GCC GGA CGA GAC GGG-3') to amplify a 597-bp *hlyA* gene and a pair of primes: A1(5'-GCC TGA GCG AGA AGG T –3') and A2 (5'-CAG TCC CAC CCA CTT C-3') to amplify a 416-bp *aerA* gene based on Heuzenroeder's method [14]. *A. hydrophila* ATCC 7966 and ATCC 15467 were used as positive and negative controls, respectively. PCR assay was performed using a PCR mixture containing 5 µl of template DNA and 8 µl of a mixture containing each deoxynucleoside triphosphate at 0.2 mM (Roche Diagnostics GmbH, Germany), 5 µl of a 25 mM $MgCl_2$ solution (3.0 mM final concentration), 0.25 µl of a 200 mM solution of each primer (1 µM final concentration), 0.8 µl of *Taq* polymerase (5 u/µl, Roche Diagnostics, GmbH, Germany), 5 µl of 10 × PCR buffer (Roche Diagnostic, GmbH, Germany) and 25.70 µl of sterile double distilled water, to make the final volume of 50 µl. The amplification programme consisted of 35 cycles of 30 seconds at 94ºC, annealing for 30 seconds at 62ºC for (*hlyA*) or 52ºC for (*aerA*) and a 2-minute extension at 72ºC with a final extension at 72ºC in an Eppendrof Thermal Cycler (Master Cycler personal, Germany). The PCR products were separated in a 1.7% agarose gel. Electrophoresis was performed in 0.5 X Tris-borate-EDTA buffer for 1 h at 100 V. Amplicons were visualized with UV light after the agarose gel had been soaked in ethidium bromide solution (0.5 µg/ml) for 15 min.

RESULTS

Out of 50 *A. hydrophila* isolates, 28 strains (56%) were isolated from diarrheal and 22 strains (44%) from healthy persons.

Hemolytic assay. Out of 50 isolated strains, 41 strains (82%) were β-hemolytic of which 25 strains (61%) had been isolated from diarrheal cases (Table 1).

Cytotoxic assay. The characteristic cytopathic effect of *A. hydrophila* cytotoxin is shown in Figure 1. The cytotoxin effect was characterized

[DOR: 20.1001.1.1028852.2004.8.4.6.7]

Table 1. Phenotypic characterization of *A. hydrophila* strains used in this study.

Source	β-hemolysis[a]		Cytotoxicity[b]		Enterotoxicity[c]	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Diarrheal	25 (89.2)	3 (10.8)	19 (67.9)	9 (32.1)	25 (89.3)	3 (10.7)
Health/asymptomatic	16 (72.7)	6 (27.3)	5 (22.7)	17 (77.3)	3 (13.6)	19 (86.4)
Total	41 (82.0)	9 (18.0)	24 (48.0)	26 (52.0)	28 (56.0)	22 (44.0)
P value	NS		*P*<0.05		*P*<0.05	

[a]β-hemolysis on horse blood agar; [b] Cytopathic effect on Vero cells; c On suckling mouse model; NS; not significant.

by rounding-up of the Vero cells and finally extensive destruction of the monolayer with cell detachment. Cytotoxic assay showed that only 24 strains (48%) had cytopathic effect of which 19 strains (79.2%) were isolated from diarrheal cases and the remainder 5 strains (20.8%) were isolated from healthy/asymptomatic persons (Table 1).

Enterotoxic assay. Enterotoxic assay showed that overall 28 strains were positive with suckling mouse models, of which 25 (89.3%) strains were isolated from diarrheal cases and the reminder 3 strains (10.7%) were isolated from healthy/ asymptomatic persons.

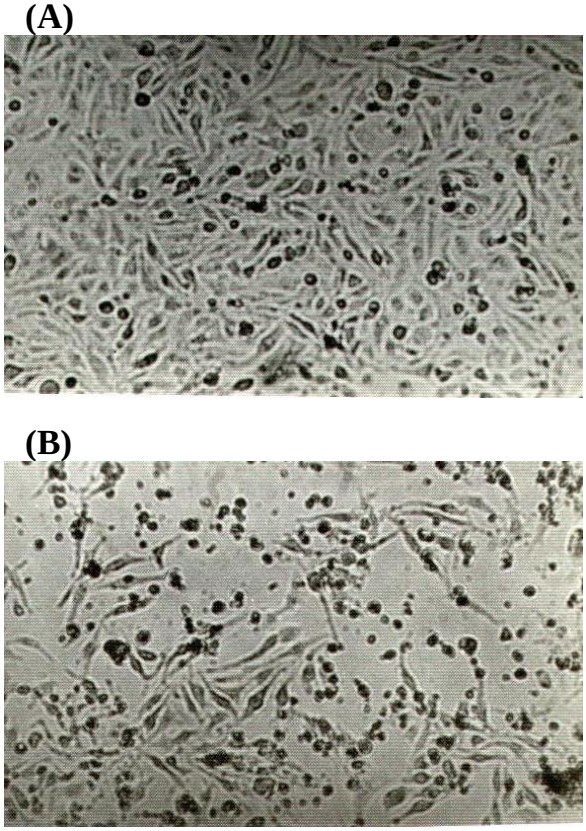

Fig. 1. Characteristic cytopathic effect of *A. hydrophila* cytotoxin in Vero cells. Normal Vero cell monolayer on the top; *A .hydrophila* cytopathic effect on the bottom.

Distribution of hlyA and aerA genes. PCR assay, using primer H1 and H2, showed that 43 (86%) of the isolated strains were positive for hemolysis genes, of which 26 (60.5%) were isolated from diarrheal cases and 17 (39.5%) from healthy/ asymptomatic persons. All of the hemolytic strains on horse blood agar were PCR positive for *hlyA* gene. PCR products of *aerA* genes with A1 and A2 primers were found in 28 strains, of which 24 strains (85.7%) were from diarrheal cases and 4 (14.3%) from healthy/asymptomatic persons. Three genotypes in *A. hydrophila* isolates were found. The $aerA^+$ $hlyA^+$ genotype was the most common genotype among diarrheal isolates which was found in 22 (78.6%) of diarrheal isolates. The summary of the presence of aerolysin and hemolysin genes in 50 *A. hydrophila* strains is shown in Table 2.

Table 2. Summary of the presence of Aerolysin (*aerA*) and hemolysin (*hlyA*) genes in 50 *A. hydrophila* strain by PCR .

Genotype	Diarrhea No (%)	healthy/asymptomatic No (%)
$aerA^+$ $hlyA^+$	22 (44)	4 (8)
$aerA^+$ $hlyA^-$	2 (4)	6 (12)
$aerA^-$ $hlyA^+$	4 (8)	12 (24)
Total	28 (56)	22 (44)

DISCUSSION

A. hydrophila is a causative agent of a number of human infections [2-4]. The role of these organisms in acute diarrhea has epidemiologically approved in some controlled studies [15], but not in others [9, 16]. *Aeromonas* spp. in the oral-challenge study failed to cause diarrhea in adult volunteers [17]. On the other hand, there are several case reports that support an etiological role of aeromonds in diarrheal disease [4, 10].

Our data show that 82% of *A. hydrophila* strains are β-hemolytic on horse blood agar, but 84% PCR positive for *hlyA* gene with no correlation with diarrhea. Therefore, β-hemolysis may not be a predictor of virulence.

[DOR: 20.1001.1.1028852.2004.8.4.6.7]

Burk *et al.* [18] reported intragastric administration of Aeromonas culture supernatants to suckling mice. Two parameters of virulence were reported: FA and diarrhea. The FA in the intestine of the infected mice was consistent with findings of other workers [7, 19]. These observations suggest that a secreted product may contribute to virulence of aeromonds. In the present study, 89.3% of diarrheal isolates were enterotoxic on suckling mice model and there was a significant correlation (P<0.05) between this virulence factor and diarrhea.

Vero cells are reported to be the most sensitive cell line for detection of Aeromonas cytotoxin [20]. In this study, 67.9% of diarrheal strains showed vero cytotoxic activity that had a significant association with diarrhea. In a study conducted in Australia [7], vero cytotoxicity was detected in all virulent Aeromonas isolates tested and a low level, in one of the six avirulent strains. Also, a significant level of correlation was observed between cytotoxicity and virulence in suckling mice. In other studies, the production of cytotoxin has been also correlated with gastroenteritis caused by Aeromonas infection [21]. In our study, all of cytotoxic strains were also virulent in suckling mouse model.

Previous studies have shown that two hemolytic toxins: HlyA and AerA contribute to the virulence of *A. hydrophila* [22, 23]. All of the hemolytic strains on horse blood agar yielded PCR products for *hlyA* gene. There was no statistically significant correlation between the presence of this trait and diarrhea. However, the corresponding association was found significant (P<0.05) in relation to a*erA* gene. Among the different genotypes (Table 2), the *hlyA*$^+$*aerA*$^+$ was the most common genotype in diarrheal isolated *A. hydrophila* strains. There was a significant correlation between this genotype and diarrhea (P<0.05). Wong *et al.* [22] reported that the inactivation of either *aerA* or *hlyA* alone reduced but did not totally cancel the hemolytic and cytotoxic activities of virulent strains of *A. hydrophila* (strain A6). These activities were removed only when both the *hlyA* and *aerA* genes were inactivated. It is likely that both toxins act by pore-formation and the effects are likely to be synergistic.

In conclusion, these observations suggest that an evaluation of *Aeromonas* virulence requires the assessment of virulence phenotypes and complete virulence gene set. Screening of specific cytotoxin and hemolysin genes appear to be the most effective way of detecting and characterizing *Aeromonas* virulence factors. Since a significant correlation was found between the *aerA*$^+$ *hlyA*$^+$ genotype and diarrhea and the frequency of these genotypes was the most common genotype in the diarrheal isolates, the *aerA*$^+$ *hlyA*$^+$ genotype could be good predictors of the human diarrheal disease.

ACKNOWLEDGMENTS

The authors are grateful to Dr. S. Bouzari for critical discussion and Mr. E. Shafie, Mr. N. Islami, Mr. Raisi, Miss F. Shoraj and Miss Khamseh for their technical help.

REFERENCES

1. Hanninen, M.L. and Siitonen, A. (1995) Distribution of *Aeromonas* phenospecies and genospecies among strains from water, food or from clinical samples. *Epidemiol. Infect. 115: 39-50*.
2. Chopra, A.K. and Houston, C.W. (1999) Enterotoxins in *Aeromonas*-associated gastroenteritis. *Microbes. Infect. 1: 1129-1137*.
3. Albert, M.J., Ansaruzzaman, M., Talukder, K.A., Chopra, A.K., Kuhn, I., Rahman, M. Faruque, A.S.G, Sirajul Islam, M., Bradleysack R.O. and Mollby, R. (2000) Prevalence of Enterotoxin genes in *Aeromonas spp*. isolated from children with diarrhea, healthy controls and the environment. *J. Clin. Microbiol. 38: 3785-3700*.
4. Sha, J., Kozlova, E.V. and Chopra, A.K. (2002) Role of various enterotoxins in *Aeromonas hydrophila* induced gastro enteritis: Generation of entertoxin gene-deficient mutants and evaluation of their enterotoxic activity. *Infect. Immun. 70: 1924-1935*.
5. Hirono, I. and Aoki, T. (1991) Nucleotide sequence and expression of an extra cellular hemolysin gene of *Aeromonas hydrophila*. *Microb. Pathog. 11: 189-197*.
6. Howard, S.P., Garland, W.J., Green, M.J. and Buckley, J.T. (1987) Nucleotide sequence of the gene for the hole forming toxin aerolysin of *Aeromonas hydrophila*. *J. Bacteriol. 169: 2869-2871*.
7. Wong, C.Y.F., Mayrhofer, G., Heuzenroeder, M.W., Atkinson, H.M., Quinn, D.M. and Flower, R.L.P. (1996) Measurement of virulence of aeromonads using a suckling mouse model of infection. *FEMS Immunol. Med. Microbiol. 15: 233-241*.
8. Buchanan, R.L. (1984) The new pathogens. An update of selected examples. *Assoc. Food Drug off. Q. Bull. 48:142-155*.
9. Pitarangsi, C., Echeverria, P., Whitmire, R., Tirapat, C., Formal, S., Dammin, G.J. and Tingtalapong, M. (1982) Enteropathogenicity of *Aeromonas hydrophila* and *Plesiomonas shigelloides*:

[DOR: 20.1001.1.1028852.2004.8.4.6.7]

prevalence among individuals with and without diarrhea in Thailand. *Infect. Immun. 35: 666-673*.

10. Khardori, N., Fainstein, V. (1988) *Aeromonas* and *Plesiomonas* as etiological agents. *Annu. Rev. Microbial. 42: 395-412*.
11. Cowan, S.T. and Steel, K.J. (1973) Manual for the identification of medical bacteria. Cambridge University press. New York.
12. Mugg, P. and Hill, A. (1981) Comparison of the Microbact-12E and 24 E systems and the API-20E system for the identification of *Enterobacteriaceae*. *J. Hyg. 87: 287-297*.
13. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: A laboratory manual. 2nd ed. Cold spring Harbor laboratory press. Cold Spring Harbor. N.Y.
14. Heuzenroeder, M.W., Wong, C.Y.F. and Flower, R.L.P (1999) Distribution of two hemolytic toxins genes in clinical and environmental isolates of *Aeromonas* spp. correlation with virulence in a suckling mouse model. *FEMS Microbiol. Lett. (174): 131-136*.
15. Albert, M.J., Faruque, A.S.G., Faruque, S.M., Sach K.B. and Mahalanabis, D. (1999) Case control study of enteropathogenes associated with childhood diarrhea in Dhaka, Bangladesh. *J. Clin. Microbiol. 37: 3458-3464*.
16. Figura, N., Marri, L., Verdiani, S., Ceccherini, C. and Barberi, A. (1981) Prevalence, species differentiation and toxicity of *Aeromonas* strains in cases of childhood gastroenteritis and in controls. *J. Clin. Microbiol. 23: 595-599*.
17. Morgan, D.R., Johnson, P.C., Dupont, H.L., Satterwhite, T.K. and Wood, L.V. (1985) Lack of correlation between known virulence properties of *Aeromonas hydrophila* and enteroathogencity of humans. *Infect. Immun. 50: 62-65*.
18. Burke, V., Robinson, J., Berry, R.J. and Gracey, M. (1981) Detection of enterotoxins of *Aeromonas hydrophila* by a suckling mouse test. *J. Med. Microbiol. 14: 401-408*.
19. Daily, O.P., Joseph, S.W., Coolbaulgh, J.C., Walker, R.I., Merrell, B.R., Rollins, D.M., Colwell, R.R. and Lissner, C.R. (1981) Association of *Aeromonas sobria* with human infection. *J. Clin. Microbiol. 13: 769-777*.
20. Barer, M.R., Millership, S.E. and Tabaqchali, S., (1986) Relationship of toxin production to species in genus Aeromonas. *J. Med. Microbiol. 22: 303-309*.
21. Millership, S.E., Barer, M.R. and Tabaquchail, S. (1986) Toxin production by *Aeromonas spp.* from different sources. *J. Med. Microbiol. 22: 311-314*.
22. Wong, C.Y.F, Heuzenroeder, M.W. and Flower, R.L.P. (1998) Inactivation of two hemolytic toxin genes in *Aeromonas hydrophila* attenuates virulence in a suckling mouse model. *Microbiology 144: 291-298*.
23. Wong, G., Clark, C.G., Liu, C., Pucknell, G., Munro, C.K., Kurk, T.M.A.C., Calderia, R., Woodward, D.L. and Rodgers, F. (2003) Detection and characterization of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR. *J. Clin. Microbiol. 41: 1048-1054*.

[DOR: 20.1001.1.1028852.2004.8.4.6.7]