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Short Communication

Effect of Culture Conditions on Production of Cytotoxic Enterotoxin of *Aeromonas sobria*

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Cytotoxic enterotoxin (Act) production of a cytotoxic enterotoxin (*act*) gene-positive isolate of *Aeromonas sobria* was studied in different bacteriological culture media, such as brain heart infusion (BHIB), trypticase soy broth with yeast extract (TSB), casamino acid yeast extract and Richardson's medium. Cytotoxin production was evaluated in Vero cell-line. Of the various media tested, higher titre of Act was produced in BHIB. Production of Act was enhanced when calcium (1 m*M*), Mg (10 m*M*), iron (200 μ*M*) and sodium chloride (0.5%) was supplemented in BHIB.

Keywords: *Aeromonas sobria*, cytotoxic enterotoxin (Act), Vero cell-assay

Aeromonas is one of the bacterial agents associated with enteric infections[1]. It is also associated with life-threatening diseases of humans such as septicaemia[2-3], meningitis[4], corneal ulcer[5], pulmonary infection[6] and haemolytic-uraemic syndrome[7-8]. *Aeromonas* species associated human wound infections have been reported due to exposure of divers in polluted water[9].

Production of extracellular heat-labile enterotoxin of *Aeromonas* was independently reported from India[10] and Sweden[11]. Other investigators subsequently confirmed the role of this enterotoxin in the pathogenesis of *Aeromonas*[12-13]. Haemolysin-free purified *Aeromonas* enterotoxin could induce change in morphological and biological properties of different cell lines[14-15]. *Aeromonas* enterotoxin has structural similarities with cholera toxin as demonstrated by immunological cross-reaction with cholera toxin[16-19]. This phenomenon is possibly due to close taxonomic relationship between *Aeromonas* and *Vibrio.*

Cytotoxic enterotoxin (*act*) gene has been cloned and sequenced from an isolate of *A. hydrophila* strain SSU[20]. The cytotoxic enterotoxin (Act) is a single polypeptide of 52 kDa[21]. Cytotoxicity is one of the biological activities of Act[22]. This toxin is secreted as an inactive precursor and subsequently converted into an active form by proteolytic activities[20]. This protein makes pores on the membranes of erythrocyte[23]. Haemolytic activity of Act is affected when pre-incubated with cholesterol. Some biological activities of this protein have been tested[21-22]. This study was undertaken to examine the effect of divalent cations (Ca^{2+}, Mg^{2+}), iron and different concentrations of NaCl on the production of Act of *A. sobria* isolated from the environment.

A. sobria (strain No. 71) used in this experiment was isolated from pond water. It possesses cytotoxic enterotoxin gene (*act)* and culture filtrate of this strain showed cytotoxicity in Vero cell-line[24]. The strain was stored at room temperature in T_1N_1 (1.0% trypticase, 1.0% NaCl, agar 7.0%, pH 7.2) soft agar. Various liquid culture media were evaluated in this study. These included brain heart infusion broth (BHIB; Difco, USA), trypticase soy broth with yeast extract (TSB; Difco, USA), casamino acid yeast extract (CYE) and Richardson's medium[25] (composition per litre: casamino acid 20.0 g, Na_2HPO_4 0.15 g, $NaCl_2$ 5 g, Tris-HCl l0.65 g, K_2HP0_4 3.67 g, yeast extract 6.0 g, trace salt 1.0 ml, pH 8.0).

BHIB medium was supplementation with different concentrations of divalent cations, iron and sodium chloride (NaCl). Stock solutions (500 m*M*) of calcium chloride ($CaCl_2$) and magnesium sulphate ($MgSO_4.7H_2O$) were prepared and they were sterilized by filtration (0.22 μm pore; Millipore Corporation, USA). The stock solutions of $CaCl_2$ and $MgSO_4.7H_2O$ were added to freshly prepared and sterilized BHIB to a final concentration of 1 m*M* and 10 m*M*. Stock solution (500 m*M*) of $FeCl_3$, sterilized by filtration, was added to freshly prepared and sterilized 10 ml BHIB to a final concentration of 200 μ*M* and 2,000 μ*M* in separate conical flasks. All supplemented media were pre-incubated overnight at 37°C to ensure sterility and the contamination free media were used. Freshly prepared BHIB medium was also supplemented with various concentration (0.5, 1.0, 1.5 and 2.0%) of NaCl prior to sterilization.

Stock culture of *A. sobria* from T_1N_1 soft agar was aseptically sub-cultured onto gelatine agar plate and incubated overnight at 37°C. Following incubation, one well-isolated colony was inoculated into 10 ml BHIB and incubated overnight at 37°C in a shaking (120 oscillation/min) water bath (Water bath shaker, MM-10, Ogawa Seiki Co Ltd, Japan). The overnight broth culture of *A. sobria* (0.5 ml) was added to 10 ml pre-sterilized BHIB with and without supplement(s) in

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BHIB and incubated overnight at 37°C in a shaking water bath (120 oscillation/min). Following incubation, bacterial concentrations in incubated cultures (BHIB with and without supplements) were measured on a nutrient agar plate (Difco, USA) following drop plate technique[26]. To prepare the supernatant, overnight culture BHIB (5.0 ml) was centrifuged at 10,000 rpm using Sorvall centrifuge (RC5C, Sorvall Instruments, USA) at 4°C. The supernatant was collected in pre-sterilized glass vial and further sterilized by filtration (0.22 μm pore). Each of the experiments with BHIB having different supplements was repeated twice for the production Act and the cytotoxin titre was measured in Vero cell-assay.

Production of cytotoxic enterotoxin (Act) in BHIB with different supplements was tested in Vero cell-line (African green monkey kidney cells)[24] with slight modification as adopted in our laboratory. In short, Vero cell-line was grown in tissue culture medium 199 supplemented with Eagles salts and L-glutamate (2.3 m*M*), heat-inactivated foetal bovine serum (10%, Gibco, USA), penicillin (100,000 U/l), streptomycin (100 mg/l) and gentamicin (50 mg/l). Vero cell suspension (200 μl) in tissue culture medium 199 (2×10^3/200 μl) was added in each well of microtitre plate (96 well, flat bottom, Falcon, USA) and incubated at 37°C in a humidified CO_2 incubator for 3 h to allow adhesion. Following incubation, 200 μl diluted culture filtrate (serial two-fold dilution of crude toxin in PBS) was added into each well of the microtitre plate. Plate containing Vero cell and toxin were incubated at 37°C in humidified CO_2 incubator. Change of the cell morphology (cell death or rounding) was studied using an inverted phase contrast microscope after 18-24 h of incubation. Wells containing 50% affected cells were considered positive.

Of the four liquid media tested, the highest titre of Act was produced in BHIB. Therefore, subsequent experiments were conducted using BHIB medium. The effect of calcium on the production of Act was studied in BHIB supplemented with $CaCl_2$. Compared to BHIB without supplemented, 2-fold rise of Act titre was observed when BHIB was supplemented with 1 m*M* $CaCl_2$. At this concentration of $CaCl_2$, density of *Aeromonas* decreased by one log compared to BHIB without supplement. When BHIB was supplemented with 10 m*M* $CaCl_2$, production of Act decreased but the bacterial concentration was comparable with that of BHIB without supplement (Table 1).

Table 1. *Effect of different supplements of BHIB on production of cytotoxic enterotoxin (Act) and bacterial growth of an act gene-positive A. sobria isolate*

Treatment	Concentration	Cytotoxin titre	Bacterial cell concentration (No./ml)
Control	-	120	2.2×10^{10}
Calcium chloride	1	240	7.5×10^9
(m*M*)	10	120	2.2×10^{10}
Magnesium	1	120	3.39×10^{10}
sulphate (m*M*)	10	320	3.6×10^{10}
Ferric chloride	200	160	6.4×10^{10}
(μ*M*)	2000	160	1.7×10^{10}
Sodium chloride	0.50%	160	1.7×10^{10}
(%)	1.00%	120	2.1×10^{10}
	1.50%	120	6.1×10^{10}
	2.00%	80	4.5×10^9

The influence Mg^{2+} ion on the production of Act by the organism was studies using BHIB medium supplemented with $MgSO_4$. Supplementation of BHIB with 10 m*M* $MgSO_4$ induced production of 2.7-fold rise of Act titre compared to the BHIB medium without supplement (Table 1). However, addition of 1 m*M* $MgSO_4$ did not increase production of Act compared to non-supplemented BHIB. Bacterial concentrations in BHIB supplemented with the two concentrations of $MgSO_4$ were comparable with that of BHIB without supplement. These results suggest that divalent cations, such as Ca^{2+} and Mg^{2+} might be required to supplement BHIB for the production of higher titre of Act.

Effect of iron ($FeCl_3$) supplementation on production of Act was studied using BHIB medium. Supplementation of BHIB with both 200 and 2,000 μ*M* $FeCl_3$ exhibited a 1.3-fold rise of Act production compared to BHIB without supplement (Table 1). Bacterial growth in BHIB supplemented with the above two concentrations of $FeCl_3$ was comparable to that of BHIB without supplement. BHIB supplemented with different concentrations of NaCl showed little positive influence on Act production by the organism. Compared to non-supplemented BHIB, Act titre was slightly increased due to supplementation of BHIB with 0.5% NaCl. Supplementation of BHIB with 1.0 and 1.5% NaCl induced Act production but the titre was comparable with that of BHIB without supplement. Bacterial growth due to supplementation of BHIB with 0.5, 1.0 and 1.5% NaCl was comparable with that of non-supplemented BHIB. Both bacterial growth and Act production were inhibited when BHIB was supplemented with 2.0% NaCl (Table 1).

In this study, the highest titre of cytotoxin was produced in BHIB. This result is consistent with the finding of Tsai *et al.*[27]. It is possible that some components of these media enhance production and subsequent transportation of Act outside the cell wall. On the other hand, inability to produce significant quantities of Act in other media might be due to inhibition of toxin synthesis, or failure to transport significant quantities of toxin outside the cell wall. Metallic ions are used as co-factors of different enzymes. Supplementation of culture medium with some of these ions stimulates bacterial growth and production of pathogenic, such as β-haemolysin of *A. caviae*[28]. In the present investigation, enhanced production of Act due to the supplementation of BHIB with Ca^{2+} and Mg^{2+} indicates a similar mechanism of toxin production in *A. sobria* having cytotoxic enterotoxin gene (*act*). This observation is also supported by experiment relating to regulation of *act* in the presence of Ca^{2+} ion[29].

Bacterial growth requires soluble iron as one of the essential nutrients required for many biological processes of the organism including electron transport chain and also as co-factor of certain enzymes[30]. At iron limiting condition, *Aeromonas* species produces iron-chelating compound known as siderophores. It helps *Aeromonas* to solubilize iron and makes it bioactive. In this study, supplementation of BHIB with iron slightly increased production of Act. This observation is inconsistent to β-haemolysin production by *A. caviae*[28].

Sodium chloride is one of the important components of a bacteriological culture medium. Addition of sodium chloride to the medium enhances the production of virulence factors, such as enterotoxin[31] and haemolysin[32-33] for some members of

vibrionaceae. Excess amounts of sodium chloride may adversely affect bacterial growth. In the present study, Act production increased with addition of 0.5% NaCl in BHIB. With gradual increase in the concentration of sodium chloride in the medium, production of cytotoxin gradually decreased. This trend is consistent with the production of haemolysin by strains of *V. fluvialis*[33] and production of cytotoxin and haemolysin of *A. hydrophila*[27] in BHIB.

This study demonstrated that BHIB induced production of higher titre of Act compared to other media tested. The production of Act was enhanced when BHIB was supplemented with optimum concentrations of iron, calcium, magnesium and sodium chloride. Further study is needed to understand the mechanism of action of these chemicals in the pathogenesis of *A. sobria in vitro* and *in vivo*.

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References

1. Teka T, Faruque AS, Hossain MI & Fuchs GJ. 1999. *Aeromonas*-associated diarrhoea in Bangladeshi children: Clinical and epidemiological characteristics. *Ann Trop Pediatr*. **19**: 15-20.
2. Tabata A, Hatayama M & Shimizu Y. 1999. Three cases of *Aeromonas hydrophila* septicemia complicated with hepatic cirrhosis. *Nippon Shokakibyo Gakkai Zasshi*. **96**: 1181-1185.
3. Ko WC, Lee HC, Chuang YC, Liu CC & Wu JJ. 2000. Clinical features and therapeutic implications of 104 episodes of monomicrobial *Aeromonas* bacteraemia. *J Infect*. **40**: 267-273.
4. Lin CS & Cheng SH. 1998. *Aeromonas hydrophila* sepsis presenting as meningitis and necrotizing fasaciitis in a man with alcoholic liver cirrhosis. *J Formos Med Assoc*. **97**: 498-502.
5. Carta F, Pinna A, Zanetti S, Carta A, Sotgiu M, Fadda G. 1994. Corneal ulcer caused by *Aerommonas* species. *Am J Ophthalmol*. **118**: 530-531.
6. Lecler O, Pourriat JL, Hoang P, Fournier JL & Cupa M. 1990. Pulmonary infection by *Aeromonas hydrophila* following drowning in a swimming pool. *Cah Anesthesiol*. **38**: 435-436.
7. Fang JS, Chen JB, Chen WJ & Hsu KT. 1999. Haemolytic-uraemic syndrome in an adult male with *Aeromonas hydrophila* enterocolitis. *Nephrol Dial Transplant*. **14**: 439-440.
8. Robson WL, Leung AK & Trevenen CL. 1992. Haemolytic-uraemic syndrome associated with *Aeromonas hydrophila* enterocolitis. *Pediatr Nephrol*. **6**: 221.
9. Daily OP, Joseph SW, Coolbaough JC, Walker RI, Merrell BR, Rollins MD, Seidler RJ, Colwell RR & Lissner CR. 1981. Association of *Aeromonas sobria* with human infection. *J Clin Microbiol.* **13**: 769-777.
10. Sanyal SC, Singh SJ & Sen PC. 1975. Enteropathogenicity of *Aeromonas hydrophila* and *Plesiomonas shigelloides*. *J Med Microbiol*. **8**: 195-198.
11. Wadstrom T, Ljungh A & Wretlind B. 1976. Enterotoxin, haemolysin and cytotoxic protein in *Aeromonas hydrophila* from human infections. *Acta Pathol Microbiol Scand [B]*. **84**: 112-114.
12. Boulanger Y, Lallier R & Cousineau G. 1977. Isolation of enterotoxigenic *Aeromonas* from fish. *Can J Microbiol.* **23**: 1161-1164.
13. Dobrescu L. 1978. Enterotoxigenic *Aeromonas hydrophila* from a case of piglet diarrhoea. *Zentbl Vet Med B.* **25**: 713-718.
14. Ljungh A, Retlind B & Molby R. 1981. Separation and characterization of enterotoxin and two haemolysins from *Aeromonas hydrophila*. *Acta Pathol Microbiol Scand [B]*. **89**: 387-397.
15. Ljungh A, Enroth P & Wadstrom T. 1982. Steroid secretion in adrenal Y1 cells exposed to *Aeromonas hydrophila* endotoxin. *FEMS Microbiol Lett*. **15**: 141-144.
16. James C, Dibley M, Burke V, Robinson J & Gracey M. 1982. Immunological cross-reactivity of enterotoxins of *Aeromonas hydrophila* and cholera toxin. *Clin Exp Immunol*. **47**: 34-42.
17. Honda T, Sato M, Nishimura T, Higashitsutsumi M, Fukai K & Miwatani T. 1985. Demonstration of cholera toxin-related factor in cultures of *Aeromonas* species by enzyme-linked immunosorbent assay. *Infect Immun*. **50**: 322-323.
18. Schultz AJ & McCardell BA. 1988. DNA homology and immunological cross-reactivity between *Aeromonas hydrophila* cytotonic toxin and cholera toxin. *J Clin Microbiol*. **26**: 57-61.
19. Chopra AK, Peterson JW, Xu XJ, Coppenhaver DH & Houston CW. 1996. Molecular and biochemical characterization of a heat-labile cytotonic enterotoxin from *Aeromonas hydrophila*. *Microb Pathog*. **21**: 357-377.
20. Chopra AK, Houston CW, Peterson JW & Jin G-F. 1993. Cloning, expression and sequence analysis of cytotoxic enterotoxin gene from *Aeromonas hydrophila*. *Can J Microbiol*. **39**: 513-523.
21. Rose JM, Houston CW, Coppenhaver DH, Dixon JD & Kurosky A. 1989. Purification and characterization of a cholera toxin-cross reactive cytolytic enterotoxin produced by a human isolate of *Aeromonas hydrophila*. *Infect Immun.* **57**: 1165-1169.
22. Rose JM, Houston CW & Kurosky A. 1989. Bioactivity and immunological characterization of a cholera toxin-cross reactive cytolytic enterotoxin from *Aeromonas hydrophila*. *Infect Immun*. **57**: 1170-1176.
23. Ferguson MR, Xu XJ, Houston CW, Peterson JW, Coppenhaver DH, Popov VL & Chopra AK. 1997. Hyperproduction, purification and mechanism of action of the cytotoxic enterotoxin produced by *Aeromonas hydrophila*. *Infect Immun.* **65**: 4299-4308.
24. Asao T, Kinoshita Y, Kozaki T, Uemura T & Sakaguchi G. 1984. Purification and some properties of *Aeromonas hydrophila* haemolysin. *Infect Immun.* **46**: 122-127.
25. Richardson SH. 1969. Factors influencing *in vitro* skin permeability factor production of *Vibrio cholerae*. *J Bactriol*. **100**: 113-116.
26. Hoben HJ & Somasegaran P. 1982. Comparison of the pour, spread and drop plate methods for enumeration of *Rhizobium* spp. in inoculants made from presterilized peat. *Appl Environ Microbiol*. **44**: 1246 1247.
27. Tsai GJ, Tsai FC and Kong ZL. 1997. Effect of temperature, medium composition, pH, salt and dissolved oxygen on haemolysin and cytotoxin production by *Aeromonas hydrophila* isolated from oyster. *Int J Food Microbiol*. **38**: 111-116.
28. Karunakaran T & Devi BG. 1994. Characterization of haemolytic activity from *Aeromonas caviae*. *Epidemiol Infect*. **112**: 291-298.
29. Sha J, Lu M & Chopra AK. 2001. Regulation of cytotoxic enterotoxin gene in *Aeromonas hydrophila*: Characterization of an iron uptake regulation. *Infect Immun*. **69**: 6370-6381.
30. Neilands JB. 1981. Microbiology of iron compounds. *Ann Rev Biochem*. **50**: 715-773.
31. Nishibuchi M, Doke S, Toizumi S, Umeda T, Yoh M & Miwatani T. 1988. Isolation from a coastal fish of *Vibrio hollisae* capable of producing a haemolysin similar to the thermostable direct haemolysin of *Vibrio parahemolyticus*. *Appl Environ Microbiol*. **54**: 2144-2146.
32. Tison DL & Kelly MT. 1984. Factors affecting hemolysin production by *Vibrio vulnificus*. *Curr Microbiol*. **10**: 181-184.
33. Rahim Z & Aziz KMS. 1996. Factors affecting production of haemolysin by strains of *Vibrio fluvialis*. *J Diarrhoeal Dis Res*. **14**: 113-116.