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The Effects of Cultivation Factors for Hemolysin Production of Environmental *Vibrio cholerae* non-O1

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Abstract The growth and hemolysin production of *Vibrio cholerae* non-O1 was investigated under various pH and nutritional conditions. Addition of glucose or di-sodium hydrogenphosphate (Na_2HPO_4) into heart infusion (HI) broth significantly affected the growth and hemolysin production. Ten strains of *V. cholerae* non-O1 tested exhibited four groups in their behavior in growth and hemolysin production in the presence of glucose. These groups were: inhibition, suppression, promotion and no alteration in their growth and hemolysin production. Di-sodium hydrogenphosphate enhanced hemolysin production in five strains and caused suppression in one strain (CO-21). Four strains were not affected by the addition of this chemical. The influence of pH on the growth and hemolysin production was also investigated. The growth of four strains was inhibited at pH 6 but showed good growth at pH 7 and 8. Six strains were capable of growing at wide pH range (6 to 8). Three strains had their optimum pH for hemolysin production at 6 while the others had their optimum pH at 7 and 8.

INTRODUCTION

Vibrio cholerae non-O1 is frequently isolated from freshwater (KAPER *et al*; 1979, LEE *et al*; 1982, VENKATESWARAN *et al*; 1989) and estuarine (KAPER *et al*; 1979, LEE *et al*; 1982, VENKATESWARAN *et al*; 1989 (a), VENKATESWARAN; 1989 (b)) environment.

This bacterium is a recognized human pathogen among vibrios (BLACK *et al*; 1980). The pathogenic mechanisms involved has been well documented in clinical isolates (MADDEN *et al*; 1981), but little is known on the pathogenicity of environmental isolates of *V. cholerae* non-O1 (NAIR *et al*; 1988, NISHIBUCHI and SEIDLER; 1983).

Extracellular products such as proteases, hemagglutinin, enterotoxin, hemolysin, cytotoxin, and other uncharacterized virulence are associated with the pathogenicity of this organism (KAPER *et al*; 1979, SPIRA and FEDORKA-CRAY; 1983, YAMAMOTO *et al*; 1984, O' BRIEN *et al*; 1984, YAMAMOTO *et al*; 1982, MACCARDELL *et al*; 1985, NISHIBUCHI and SEIDLER; 1983). Hemolysin is recognized as one of the parameters in pathogenicity of *V. cholerae* (BARRET and BLACK; 1981) and *V. parahaemolyticus* (MIYAMOTO *et al*; 1969).

NISHIBUCHI and SEIDLER (1983) reported brain heart infusion (BHI) as the suitable medium in the release of enterotoxin in *V. cholerae*. Optimum conditions for the production of hemolysin (TISON and KELLY; 1984) and cytotoxin (R. RAJENDRAN, K. VENKATESWARAN, H. NAKANO and H. HASHIMOTO, communicated to Microbios Lett.) for *V. vulnificus* were established. Although production of extracellular hemolysin by *V. cholerae* non-O1 was well

documented, no studies were conducted on the optimal condition in the elaboration of this toxin.

The physiological mechanism for survival and production of extracellular products by vibrios are not yet clear. Hemolysin production by the environmental *V. cholerae* non-O1 under various growth media, pH and nutritional conditions was studied to understand the mechanism involved.

MATERIALS AND METHODS

Bacteria. Environmental strains of hemolytic *V. cholerae* non-O1 which were isolated earlier (VENKATESWARAN *et al*; 1989 (b)) were used during this study. These strains were numbered as CO-04, 06, 08, 18, 19, 21, 22, 23, 55 and 56.

Growth media. The following media were used during the present study: nutrient broth (NB, meat extract 0.5 %, polypeptone 1.0 %, pH 7), nutrient broth having 0.5 % NaCl (NB'), heart infusion (HI, Eiken, Tokyo), brain heart infusion (BHI, Eiken, Tokyo) and tryptosoy broth (TSB, Eiken, Tokyo).

Measurements of growth and pH. Absorbance at 600 nm (A_{600}) to elucidate was measured using spectrophotometer (Hitachi Model 100-20) and pH at various time interval was detected using a pH meter (TOA Electronic Ltd. Type HM-10P).

Influence of glucose and Na_2HPO_4. Glucose and Na_2HPO_4 to a concentration of 0.2 and 0.25 % respectively were added to HI broth and incubated at 35℃ with shaking. The A_{600}, pH of the medium and hemolysin production at various time intervals were checked.

pH dependence. The HI broth adjusted to 6, 7 and 8 were used as growth media and incubated overnight at 35℃ with shaking. Growth, pH change and hemolysin production at various time intervals were measured.

Crude toxin. BHI broth was used throughout the period of the study as growth medium unless otherwise stated. Bacterial strains were streaked onto BHI agar and the overnight grown colonies were transfered into 2 ml of HI broth. One ml of the bacterial suspension (5.3×10^6 ml^{-1}) was transfered into 50 ml of the growth media and incubated for 24 to 32 h at 35℃ with rotary shaking (200 rpm).

The cultures were centrifuged (9,000×g, 30 min, 4℃) and the supernatants were treated as crude toxin.

Hemolysin assay. The hemolysin production was assayed as described earlier (VENKATESWARAN *et al*; 1989 (b)). One hemolysin unit (HU) was defined as the amount of hemolysin causing 50 % hemolysis of 1 ml of 1 % rabbit erythrocytes suspension.

RESULTS

Among various culture media tested for hemolysin production, BHI broth was the best followed by TSB and HI broth (data not shown). These broths were used frequently by various workers for the extracellular toxin production in vibrios. Six strains, CO-04, 06, 08, 19, 21 and 56 grew well and produced high hemolysin in BHI broth while four strains CO-18, 22, 23 and 55 showed good results in both BHI and TSB broths. In the experiments, HI broth did not support increased production of hemolysin compared to other media. This is in contrary to the published results where HI broth was found to be more

suitable for heat labile hemolysin production in *V. vulnificus* than in BHI and TSB (VENKATESWARAN *et al*; 1984 (a)). Growth, pH change and hemolysin production in NB, NB', HI and BHI broth were as presented in Table 1. The culture period was 10 h which corresponded with the late exponential growth phase of the bacterium (unpublished data). The BHI and HI broths were more suitable for luxuriant growth while HI and NB' showed high hemolysin production.

Table 1. Growth, pH and hemolysin production of *Vibrio cholerae* non-O1 on various media

Strain	Condition	Hemolytic units that produced in:			
		NB	NB'	HI	BHI
Control[a]	$A6_{00}$[b]	0.06	0.07	0.04	0.05
	pH[c]	6.80	6.90	7.50	7.50
CO-04	A_{600}	1.03	1.44	1.54	1.59
	pH	8.06	8.06	7.77	7.05
	H. U[d].	1.6×10^{1}	6.2×10^{2}	2.8×10^{2}	9.8
CO-06	A_{600}	0.73	1.57	1.73	1.76
	pH	7.93	8.18	8.20	7.60
	H. U.	5.7	1.4×10^{3}	$>3.8\times10^{3}$	1.8×10^{2}
CO-18	A_{600}	0.28	1.61	1.75	1.77
	pH	7.27	8.16	8.21	7.32
	H. U.	1.2	1.1×10^{2}	2.5×10^{2}	5.0
CO-19	A_{600}	0.28	1.39	1.72	1.74
	pH	7.17	7.63	8.08	7.36
	H. U.	1.5	3.0×10^{2}	8.9×10^{2}	1.3×10^{1}
CO-21	A_{600}	0.61	1.47	1.74	1.75
	pH	7.35	7.64	8.04	7.45
	H. U.	1.3×10^{1}	$>3.3\times10^{3}$	$>4.0\times10^{3}$	1.1×10^{3}
CO-22	A_{600}	0.31	1.66	1.64	1.69
	pH	7.31	8.20	8.15	7.36
	H. U.	2.4	3.6×10^{2}	6.0×10^{2}	2.1×10^{1}
CO-23	A_{600}	0.77	1.60	1.80	1.83
	pH	7.80	8.21	8.09	7.74
	H. U.	5.3	$>2.3\times10^{3}$	$>2.7\times10^{3}$	1.0×10^{2}
CO-55	A_{600}	0.26	1.63	1.79	1.80
	pH	7.31	8.02	8.09	7.36
	H. U.	1.1	$>3.9\times10^{1}$	2.5×10^{1}	<1
CO-56	A_{600}	0.27	1.54	1.67	1.70
	pH	7.35	8.03	7.97	7.46
	H. U.	5.7	1.6×10^{3}	4.2×10^{3}	2.6×10^{2}

[a] Control is the medium where no organism was added.

[b] Growth is measured spectrophotometrically at 600 nm after 10 h of incubation.

[c] pH of the growth medium.

[d] Hemolysin activity.

Strain CO-08 is not tested.

The influence of glucose and Na_2HPO_4 in hemolysin production. The discrepancy noted between BHI and HI broths and the published data (TISON and KELLY; 1984) in their effect on hemolysin production was assumed to have been caused by the differences in their composition. The effect of glucose and Na_2HPO_4 added to the HI broth on the hemolysin production is shown in Table 2. Glucose inhibited hemolysin production of 3 strains (CO-04, 08 and 19) completely while causing one log reduction in the production of hemolysin of strain CO-21 compared to control HI broth. Three strains (CO-23, 55 and 56) had their hemolysin production capacity increased significantly by the addition of glucose but in the strains CO-06, 18 and 22, hemolysin production were not affected as compared to HI broth control.

Addition of Na_2HPO_4 altered hemolysin production significantly by increasing the activity in 5 strains (CO-04, 06, 23, 55 and 56) in which strains CO-23 and55 were highly stimulated. The strain CO-21 was suppressed by this chemical as much as glucose did. Strains CO-08, 18, 19 and 22 were not affected as compared to HI broth control. Addition of both glucose and Na_2HPO_4 to HI broth increeased the hemolysin production in all the 10 strains. The hemolysin production of CO-08, 19 and 55 was highly increased by the addition of both agents. Strains CO-08 and 19 were inhibited by the presence of glucose alone.

All the 10 strains can be divided into 5 groups in terms of hemolysin production: group 1, CO-4 (inhibited by glucose but stimulated by Na_2HPO_4); group 2, CO-08 and 19 (inhibited by glucose but influenced by Na_2HPO_4); group 3, CO-21 (hemolysin production was suppressed by both agents); group 4, CO-23, 55 and 56 (hemolysin production increased by both agents) and group 5, CO-06, 18 and 22 (not affected by both chemicals).

pH dependence. The pH change reflected the situation of the bacterial growth. The hemolysin production in various pH conditions for *V. cholerae* non-O1 was as shown in Fig. 1 through 5. As depicted in Fig. 1 and 2 at initial pH of 6, the strains CO-04, 08, 23 and 56 could not grow but at pH 7 and 8 these strains exhibited growth and produced hemolysin. The optimum pH for strain CO-23 was 8, but the pH at the peak of hemolysin production

Table 2. Effect of glucose and di-sodium hydrogenphosphate for the hemolysin production of *Vibrio cholerae* non-O1[a]

Strain	Hemolysin units that produced in:			
	HI	HI+glucose	HI+Na_2HPO_4	HI+glucose+Na_2HPO_4
CO-04	3.0×10^2	0.0	1.9×10^3	8.9×10^2
-06	4.6×10^2	3.9×10^2	1.8×10^3	9.3×10^2
-08	1.6×10^2	0.0	6.1×10^2	2.9×10^3
-18	2.1×10^2	2.2×10^2	4.9×10^2	2.0×10^3
-19	5.1×10^1	0.0	8.1×10^1	1.9×10^3
-21	5.5×10^2	5.2×10^1	3.9×10^1	4.8×103
-22	2.3×10^2	6.7×10^2	5.5×10^2	1.6×10^3
-23	7.5×10	3.2×10^3	3.7×10^3	4.1×10^3
-55	4.9	2.3×10^2	1.2×10^2	1.3×10^3
-56	1.2×10^2	3.4×10^3	3.6×10^3	3.4×10^3

[a] Hemolysin activity was measured after 16 h incubation.

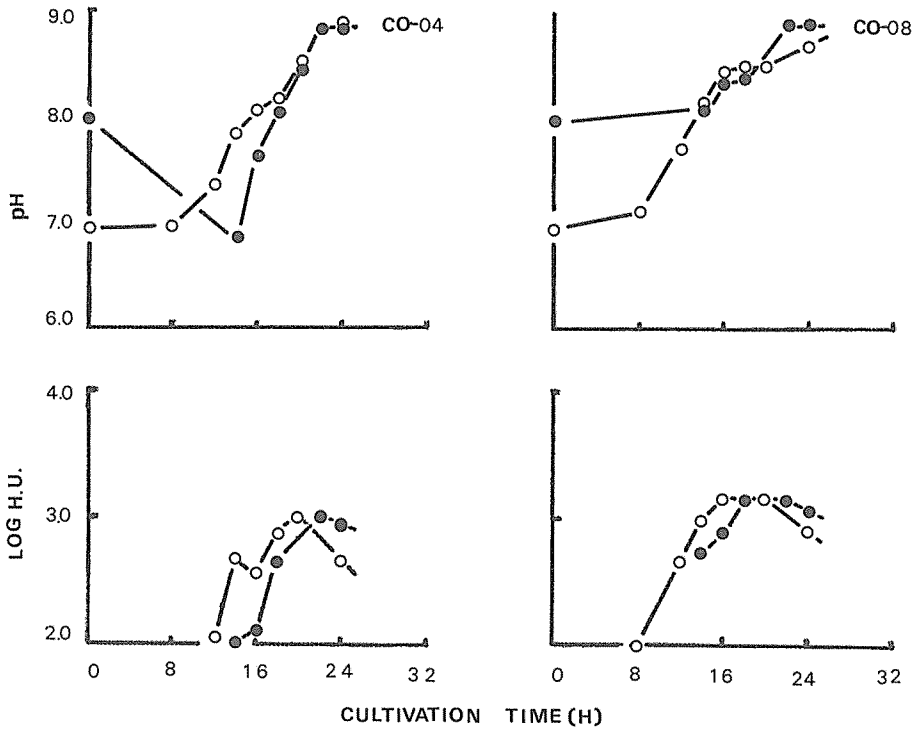

Fig. 1. Changes in hemolysin production and pH of *Vibrio cholerae* non-O1 (CO-04 and 08) under various pH conditions. -▲-: pH 6.0, -○-:pH 7.0, -●-:pH 8.0,

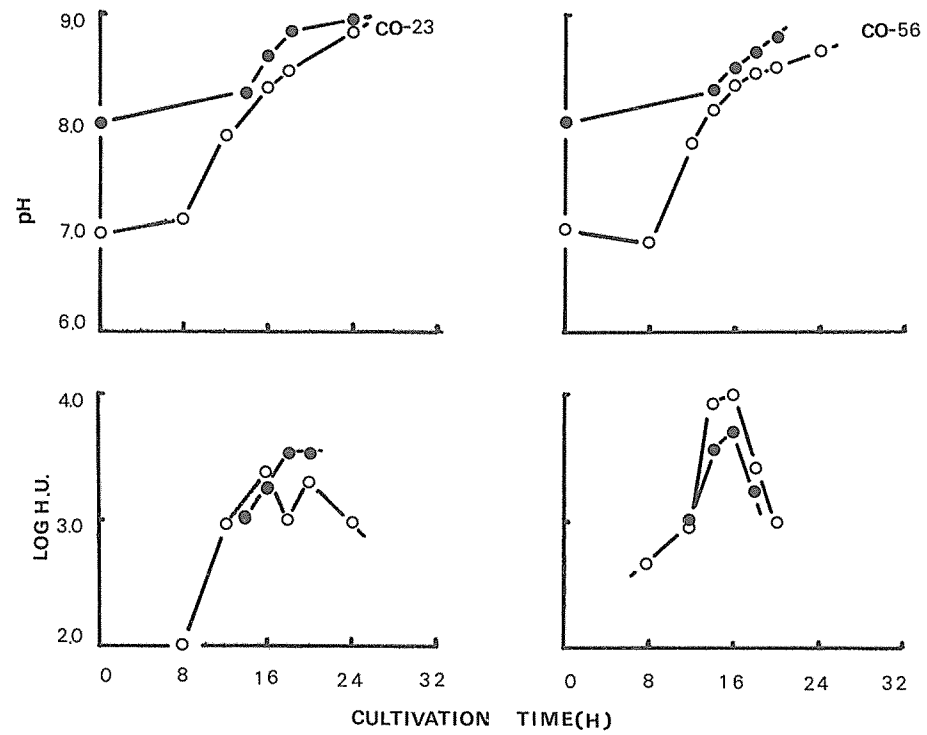

Fig. 2. Changes in hemolysin production and pH of *Vibrio cholerae* non-O1 (CO-23 and 56) under various pH conditions. -▲-: pH 6.0, -○-:pH 7.0, -●-:pH 8.0.

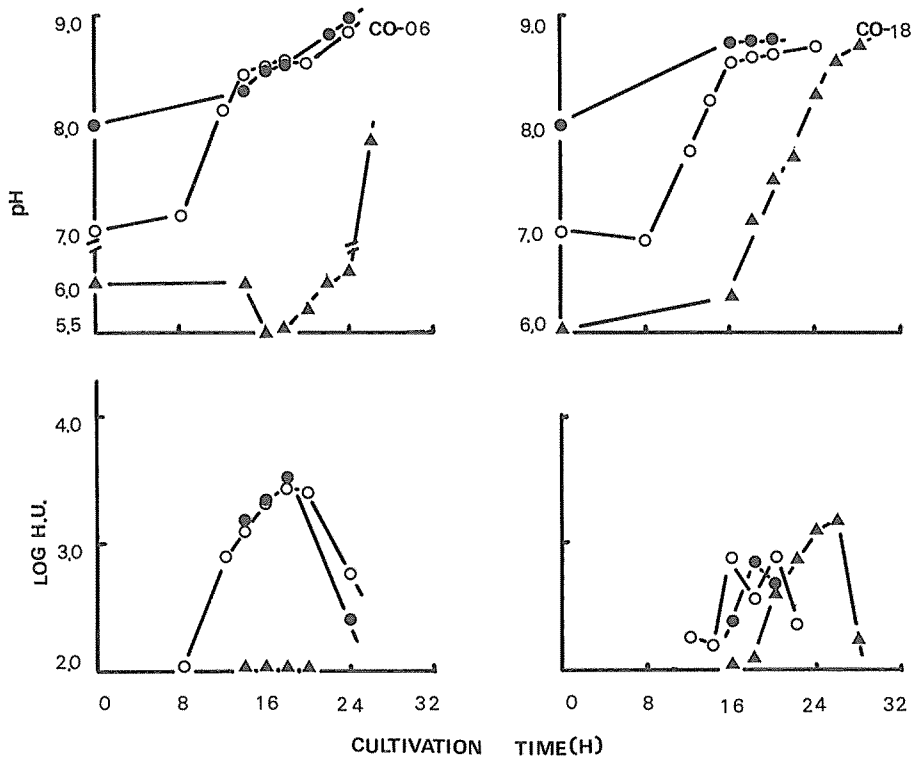

Fig. 3. Changes in hemolysin production and pH of *Vibrio cholerae* non-O1 (CO-06 and 18) under various pH conditions. -▲-: pH 6.0, -○-:pH 7.0, -●-:pH 8.0.

was 8.8 (Fig. 2). The strains CO-06, 18, 19, 21, 22 and 55 produced hemolysin at wider pH range (pH 6 to 8). The strains CO-06, 22 and 55 lowered the initial pH in the medium from 6 to 5.5 during their growth and produced hemolysin when the pH of the medium attained alkalinity (Fig. 3 and 4). The suitable initial pH for strain CO-19 was 7, while peak hemolysin production occurred when pH of the growth medium was 8.3. Strains CO-18, 21 and 22 favored initial pH of 6 and peak hemolysin production was observed at pH 7.5 for the later 2 strains, however CO-18 increased the pH of the medium to 8.6 when high hemolysin production was noticed. Strains CO-06 and 55 showed peak hemolysin production at pH 8.5 to 8.6. Initial pH of the medium seemed to affect hemolysin production and the peak in hemolysin production occurred when the pH of the cultured broth was higher. Strains other than CO-21 and 22 had their peak hemolysin production at pH 8.3 to 8.5 while strains CO-21 and 22 exhibited high hemolytic activity when the growth medium attained pH 7.0 to 7.5. Hemolysin production decreased rapidly after the peak. *V. cholerae* non-O1 strains grown at pH 7 exhibited peak hemolytic activity sooner (16 h) followed by the cultivation at pH 8 (20 h) and at pH 6 (24 h) except the strain CO-21 where high hemolysin production was seen at 14 h of incubation when grown at pH 6.

DISCUSSION

V. cholerae non-O1, the ubiquitous microflora of the estuarine environment (KAPER *et al*; 1979, Lee et al; 1982, VENKATESWARAN *et al*; 1984 (a)) was found to be less in freshwater en-

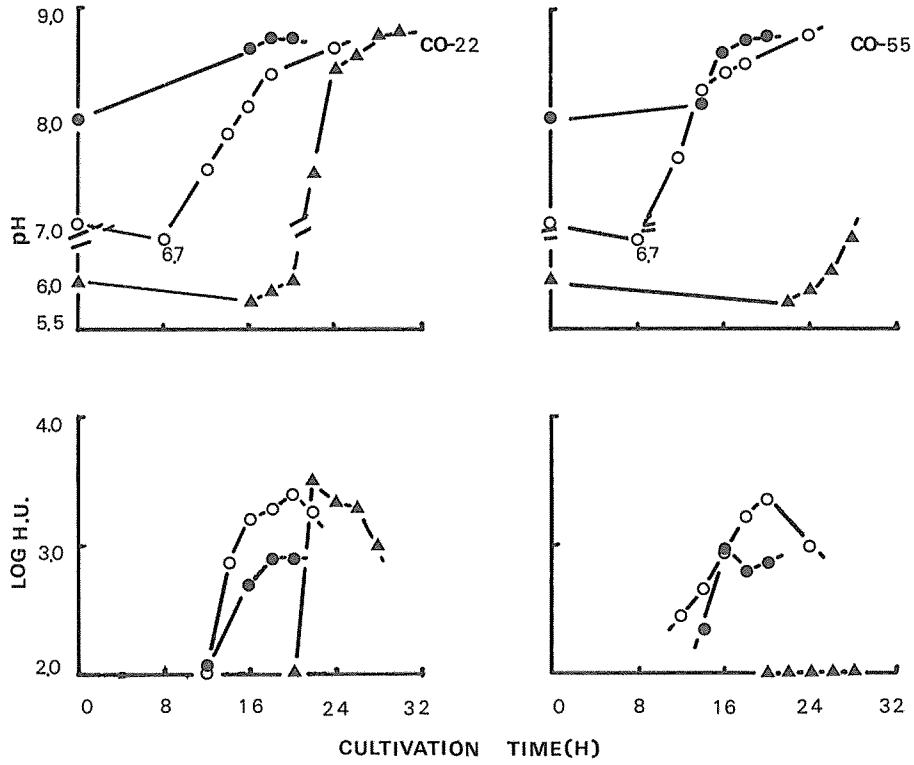

Fig. 4. Changes in hemolysin production and pH of *Vibrio cholerae* non-O1 (CO-22 and 55) under various pH conditions. -▲-: pH 6.0, -○-:pH 7.0, -●-:pH 8.0.

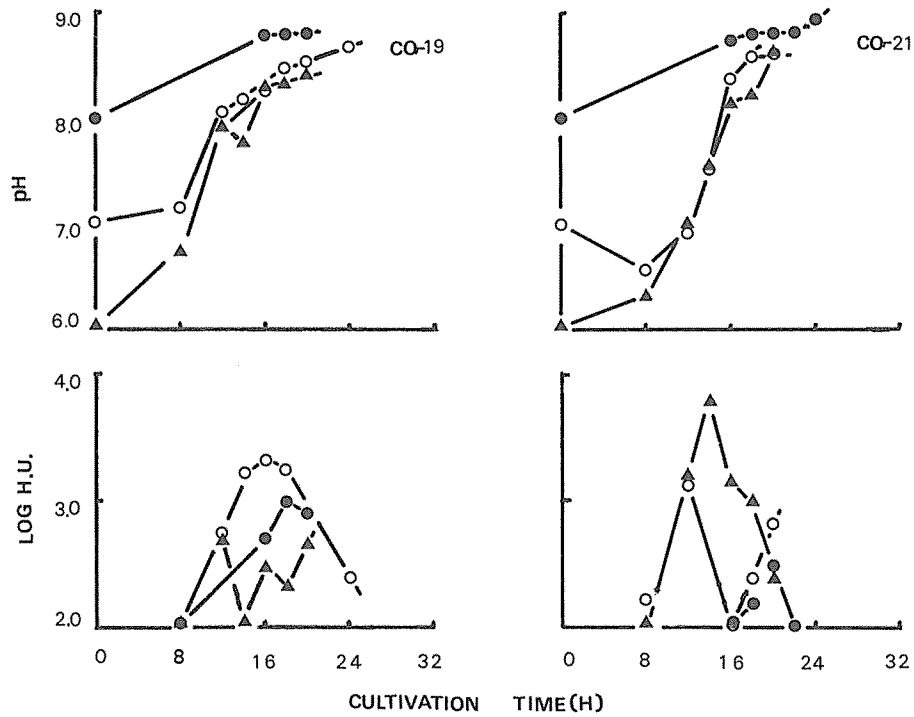

Fig. 5. Changes in hemolysin production and pH of *Vibrio cholerae* non-O1 (CO-19 and 21) under various pH conditions. -▲-: pH 6.0, -○-:pH 7.0, -●-:pH 8.0.

vironment. It is well documented that *V. cholerae* serotype O1, non-O1 and other vibrios cause human gastroenteritis. Some environmental and clinical isolates of *V. cholerae* non-O1 were capable of producing cholera toxin (MADDEN *et al*; 1981, NAIR *et al*; 1988, SPIRA *et al*; 1979). Other extracellular toxins produced by this organism includes hemolysin, enterotoxin, cytotoxin and fluid accumulating factors (MACCARDELL *et al*; 1985, SPIRA and FEDORKA-CRAY; 1983, YAMAMOTO *et al*; 1982, YAMAMOTO *et al*; 1984). Hemolysin is recognized as one of the toxins involved in cholera disease and food poisoning cases (MIYAMOTO *et al*; 1969). Although these vibrios are recognized as important water and food borne pathogens, there are some confusion still existing on the carriage of the pathogenic properties during the infection cycle of environment-animal-human-environment (NAIR *et al*; 1988).

It was obvious that BHI broth was superior than HI broth for hemolysin production contrary to the findings of Tison and Kelly (TISON and KELLY; 1984). But our published data further reiterated that *V. vulnificus* cultured in BHI broth was producing cytolysin comparable to the amount produced in HI medium (R. RAJENDRAN, K. VENKATESWARAN, H. NAKANO and H. HASHIMOTO, communicated to Microbios Lett.). Medium-dependent production of extracellular enterotoxins by *V. cholerae* and allied vibrios was established (NISHIBUCHI and SEIDLER; 1983). Clinical isolates showed high toxic activity when cultured in BHI supplemented with 0.5 % NaCl, however NaCl amended TSB exhibited good enterotoxicity for the environmental strains (NISHIBUCHI and SEIDLER; 1983). Both TISON and KELLY (1984) and NISHIBUCHI and SEIDLER (1983) showed that added glucose in the growth medium lowered toxic activity for *V. vulnificus* and *V. cholerae* non-O1, respectively as seen in the present study. Change in the growth medium will drastically affect the extracellular secretions of the cultured organisms. Failure in the detection of enterotoxin in the studies of HUQ *et al.* (1980) and MADDEN *et al.* (1981) might be attributed to the selection of growth medium and in another report, TSB without added NaCl yielded no toxic activity in suckling mouse assay (NISHIBUCHI and SEIDLER; 1983). However these clinical strains when grown in BHI broth (NISHIBUCHI and SEIDLER; 1983) exhibited fluid accumulation in suckling mice. Simillar observations were seen during the present study for hemolysin production. Besides this, the addition of chemical elements in the required combination to HI showed increase in hemolytic activity which was equal in composition to the amount shown for BHI. Strains tested in this study demonstrated characteristics preference of initial pH of the culture medium, and high alkalinity for peak hemolysin production. All strains required lower initial pH for the establishment of growth, however high hemolysin production was noticed when the pH of the growth medium was higher.

The relationship between high hemolysin production and alkaline conditions of the growth medium exhibited by majority of *V. cholerae* non-O1 strains is interesting and the mechanism involved should be investigated further. Characterization of hemolysin under various pH conditions in different growth media is under progress. The differences, if any, between clinical and environmental strains in the hemolytic activity at various growth conditions are to be studied to understand the pathogenesis involved in *V. cholerae* non-O1 infections.

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水系環境由来の *Vibrio cholerae* non-O1 による ヘモリシン産生に及ぼす培養条件

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いろいろな培地条件および pH の下で，*Vibrio cholerae* non-O1 の増殖と本菌によるヘモリシン産生の状態を調べた。基本培地として用いたハートインヒュージョンブイヨン培地にグルコース，Na_2HPO_4 を加えると，増殖，ヘモリシン産生は大きく影響を受けた。グルコース存在下では，試験に供した10菌株は4グループに分類された。すなわち，グルコースによって，阻害されるもの，阻害は受けないがある程度抑制されるもの，反対に活性化されるもの，そして特に影響を受けないものが存在した。Na_2HPO_4 は CO-21 株，1菌株に対してヘモリシン産生を抑制したほかは，促進するか，あるいは，なにも影響しなかった。一方，pH に対しては，4菌株は pH 6 で増殖が阻害され，pH 7 と8でよく増殖した。残る6菌株はいずれも pH 6-8 の幅広い pH 領域で増殖した。ヘモリシンの産生は pH 6 が良いもの3菌株，pH 7 および8が良いもの6菌株であった。