Kasetsart J. (Nat. Sci.) 34 : 248 - 253 (2000)

Effects of Temperature and Cell Number on the Production of Hemolysin by *Vibrio parahaemolyticus*

Siriporn Stonsaovapak

ABSTRACT

Two strains of *Vibrio parahaemolyticus*, ATCC 17802 Kanagawa negative (K-) and ATCC 27519, Kanagawa positive (K+) were used in this study. The effect of temperature on the production of hemolysin was studied in trypticase soy broth (TSB) with 3 % NaCl. Maximum hemolysin productions by both strains were obtained at 35°C with little or no production of hemolysin at 4°, 10° and 42°C. The K+ strain consistently produced higher titers of hemolytic activity than its K- counterpart. Hemolysin production at lower temperatures (4° and 10°C) could only be detected after 10 or 12 h in TSB with 3 % NaCl. At 20°, 25°, 30° and 35°C in this medium, production of hemolysin could be detected after 6 or 8 h of incubation. At these same temperatures, both K+ and K- strains produced comparable cell numbers by 12 h; however, the greatest hemolysin production occurred at 35°C. This indicates that temperature, rather than cell numbers, determines the total hemolysin produced during a controlled incubation time period.

Key words : *Vibrio parahaemolyticus*, temperature, hemolysin

INTRODUCTION

Diarrheal diseases may be caused by a variety of microorganisms such as *Salmonella* or *Shigella*. The obligate halophile, *Vibrio parahaemolyticus*, is also included in the list of microorganisms producing major foodborne diseases (Morris, 1995). Illnesses caused by this organism occur primarily throughout the coastal areas of the Far Eastern Pacific Perimeter, where seafood is a staple of the diet.

The hemolysin produced by this organism plays a significant role in the pathogenesis of enteric human disease (Nishibuchi *et al*., 1992). Strains of *V. parahaemolyticus* can be divided into two epidemiologically significant groups on the basis of their ability to exhibit hemolysis on a special blood agar–Wagatsuma agar. Strains which show β-type hemolysis on Wagatsuma agar are called Kanagawa positive (K+) and those which give no hemolysis or ∝–type hemolysis on Wagatsuma agar are called Kanagawa negative (K-) (Oliver and Kaper, 1997). Traditionally, only K+ strains have been considered virulent but a significant portion of *V. parahaemolyticus* food poisoning appears to be caused by K- strains. It has been reported that the production of hemolysins is affected by the NaCl content, heat sterilization and the pH of the basal medium (Miyamoto *et al*., 1969).

Since the illness almost always occur during the warm summer months, this study was

Institute of Food Research and Product Development, Kasetsart University, Bangkok 10900, Thailand.

undertaken to gain information on the conditions necessary for optimal yields of hemolysin. The objective of the study was to characterize the effect of temperature on the cell growth and hemolysin production by K+ and K- strains of *V. parahaemolyticus* under controlled conditions.

MATERIALS AND METHODS

Cultures

The two cultures of *V. parahaemolyticus* used in this study were obtained from the American Type Culture Collection, Rockville, Maryland.

1. *V. parahaemolyticus* ATCC 17802 (Kanagawa negative)

2. *V. parahaemolyticus* ATCC 27519 (Kanagawa positive)

Both cultures were originally isolated from Japanese food poisoning patients during food – borne gastroenteritis outbreaks in Japan.

Media

Trypticase soy agar (TSA) and trypticase soy broth (TSB) were obtained from BBL (Baltimore Biological Laboratories, Cockeysville, Maryland).

Standardization of Inocula

Cultures were grown in 180 ml bottles containing 50 ml of TSB with 3 % NaCl for 6 h at 35°C on a shaking incubator. The cultures were then centrifuged at 4,500 rpm for 20 min. The supernates were discarded and the pellets were washed twice with 3 % sterile NaCl. The pellets were resuspened in 3 % sterile NaCl to obtain densities of approximately 1×10^4 cells / ml. Spread plates were made to confirm inocula cell counts by using TSA with 3 % NaCl.

Partial Purification of the Hemolysin

Hemolysin was concentrated and partially purified from the culture supernate by the method of Honda *et al.* (1976). A 3.5 g amount of $(NH_4)_2SO_4$ was added to 10 ml of culture supernate to give 55 % saturation. The mixture was chilled at 4°C for 12 h and centrifuged at 10,000 rpm at 4°C for 10 min. The pellet was dissolved in 2 ml of buffer (Phosphate Buffer Saline 0.01 M, pH 7.0) before being dialyzed overnight against at least 100 volumes of the same buffer.

Assay of Hemolytic Activity

In quantitative titrations of hemolysins, microtiter techniques were used(Honda *et al.*, 1976). One drop (ca. 0.025 ml) of PBS was added to each well of a microtitration multi–well plate with microtiter pipette droppers. The hemolysins were diluted in each row with a microtiter micro–diluter (ca. 0.025 ml). One drop (ca. 0.025 ml) of a 2 % suspension of washed rabbit blood in PBS was added to each well of the plate with the pipette dropper. The contents of the wells were mixed by shaking. The plate was incubated at 37°C for 4 h. The cells were allowed to settle for 12 h at 4°C, and the results of lysis were recorded. Control experiments included incubation of rabbit blood cell in the absence of hemolysin. The reciprocal of the highest dilution showing complete hemolysis was noted as the hemolytic titer under the conditions of the assay.

Production of Hemolysin at Different Temperatures

Bottles containing 10 ml of TSB with 3 % NaCl at pH 6.5 were inoculated with standardized inocula of each test strains to obtain a concentration ca. 1×10^4 cells / ml and allowed to stand under static conditions at 4°, 10°, 20º, 25°, 30°, 35°, and 42°C. After inoculation at 0, 2, 4, 6, 8, 10 and 12 h, viable cell counts were determined by spreading on TSA with 3 % NaCl and hemolytic activity monitored by using partial purification of the

hemolysin and assayed by microtiter techniques.

Statistical Analysis

A multiple regression analysis was used to determine whether strain, temperature, and time could predict hemolytic activity and bacterial counts. Data at 12 h were analyzed by ANOVA to compare the hemolytic activity and bacterial counts at different temperatures. Finally, if there were statistically significant differences in factorial analysis of variance, the Student– Newman–Keuls (SNK) test was used to compare treatment means (Steel and Torrie, 1980).

RESULTS

The data for hemolytic activity at different times and temperatures are shown in Table 1 and the relationships can be explained by the following regression analysis.

$$Y1 = -10.81 + 0.18\ X2 + 1.65\ X3$$

$$Y2 = -7.57 + 0.18\ X2 + 1.65\ X3$$

where Y1 = the hemolytic activity for ATCC 17802,

Y2 = the hemolytic activity for ATCC 27519,

X2 = temperature in °C,

and X3 = time in h

R^2 was 0.277 and indicates a weak relationship between the production of hemolysin and the variables time and temperature.

The data for bacterial counts at different times and temperatures are shown in Table 2. The regression analysis can be explained by the following equations :

$$Y1 = 3.235 + 0.028\ X2 + 0.317\ X3$$

Table 1 Production of hemolysin by *V. parahaemolyticus* strains at different times and temperatures in TSB + 3 % NaCl, pH 6.5 (n = 3).

Strains	T (°C)	Incubation time (h)						
		0	2	4	6	8	10	12
ATCC 17802	4	0.00[a]	0.00	0.00	0.00	0.00	0.00	0.00
ATCC 27519	4	0.00	0.00	0.00	0.00	0.00	1.67	2.00
ATCC 17802	10	0.00	0.00	0.00	0.00	0.00	0.00	1.33
ATCC 27519	10	0.00	0.00	0.00	0.00	0.67	1.33	4.00
ATCC 17802	20	0.00	0.00	0.00	1.67	2.00	5.33	16.00
ATCC 27519	20	0.00	0.00	0.00	0.00	2.00	8.00	26.67
ATCC 17802	25	0.00	0.00	0.00	2.00	2.00	10.67	32.00
ATCC 27519	25	0.00	0.00	0.00	0.00	2.67	16.00	42.67
ATCC 17802	30	0.00	0.00	0.00	0.00	2.00	12.00	21.33
ATCC 27519	30	0.00	0.00	0.00	0.00	2.67	32.00	53.33
ATCC 17802	35	0.00	0.00	0.00	0.00	2.00	10.67	42.67
ATCC 27519	35	0.00	0.00	0.00	0.00	4.00	26.67	85.33
ATCC 17802	42	0.00	0.00	0.00	0.00	0.00	1.67	2.00
ATCC 27519	42	0.00	0.00	0.00	0.00	1.33	5.33	8.00

a = hemolytic activity

Y2 = 3.541 + 0.028 X2 + 0.317 X3

where Y1 = log Y of bacterial counts for ATCC 17802,

Y2 = log Y of bacterial counts for ATCC 27519,

X2 = temperature in °C,

and X3 = time in h

There were significant differences among strains, temperatures, and times for the growth of the organisms. R^2 was 0.591 and indicates a slight relationship between the growth and the variable times and temperatures. In general, at lower temperature (4° and 10°C) productions of hemolysin occurred slowly and could be detected only after 10 or 12 h, whereas, at higher temperatures (20°, 25°, 30° and 35°C), production of hemolysin could be detected after 6 or 8 h of incubation.

The 2–way ANOVA's conducted on the 12 h data for hemolytic activity and bacterial counts showed significant interaction terms (strain x temperature) in both cases. Maximum production of hemolysin was obtained at 35°C for both strains with the lowest production of hemolysin occurring at 4°, 10°, and 42°C. The K+ strain showed higher hemolytic activity than the K- strain. At 20°, 25°, 30° and 35°C, both the K+ and K- strains produced statistically equal viable cell number by 12 h (Figure 1). This indicates that maximum production of hemolysin at 35°C is strictly a temperature phenomenon and is not a result of larger bacterial populations.

DISCUSSION

Maximum production of hemolysin by both strains of *V. parahaemolyticus* was observed at 35° C with little or no production of hemolysin at 4°, 10° and 42°C. A similar effect of temperature on

Table 2 Bacterial counts (log_{10} CFU/ml) of *V. parahaemolyticus* strains at different times and temperatures in TSB + 3 % NaCl, pH 6.5 (n = 3).

Strains	T (°C)	Incubation time (h)						
		0	2	4	6	8	10	12
ATCC 17802	4	4.49	4.30	4.34	4.43	4.61	4.89	5.08
ATCC 27519	4	4.43	4.51	4.61	4.83	4.57	5.18	5.65
ATCC 17802	10	4.34	4.34	4.53	4.88	5.38	5.60	5.81
ATCC 27519	10	4.41	4.59	4.72	5.28	5.77	6.15	6.62
ATCC 17802	20	4.40	4.58	4.85	7.83	8.51	8.79	8.89
ATCC 27519	20	4.38	4.65	6.82	7.76	8.81	8.82	9.00
ATCC 17802	25	4.38	4.70	4.92	6.56	8.20	8.80	8.93
ATCC 27519	25	4.23	4.67	5.51	6.23	8.61	8.92	9.00
ATCC 17802	30	4.41	4.65	4.92	5.34	8.11	8.67	8.89
ATCC 27519	30	4.26	4.67	6.58	6.66	8.26	8.81	8.97
ATCC 17802	35	4.18	4.68	4.90	4.97	7.51	8.40	8.72
ATCC 27519	35	4.43	4.62	4.74	4.96	8.58	8.80	8.99
ATCC 17802	42	4.28	4.58	4.80	4.95	5.34	5.62	6.53
ATCC 27519	42	4.26	4.54	4.88	5.15	5.48	6.83	7.23

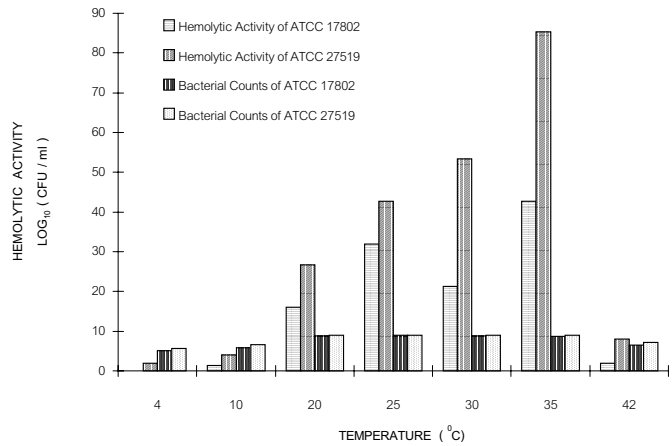


Figure 1 Hemolytic activity and bacterial counts at 12 h by *V. parahaemolyticus* strains in TSB with 3% NaCl, pH 6.5 at various temperatures (n = 3).

the production of hemolysin by *Escherichia coli* was observed by Springer and Goebel (1980). They reported that maximal release of hemolysin occurred at 30°C, with only about 50 % and 25 % of the maximum levels detected at 20° and 10°C, respectively. Secretion of hemolysin into the medium supernate was totally inhibited at 43°C.

At 20°, 25°, 30°, and 35°C each static culture contained almost the same total cell numbers at 12 h; however, the greatest hemolysin production was obtained at 35°C. This indicates that production of hemolysin is strictly a temperature phenomenon and is not a function of total cell numbers. On the basis of these findings, the results correlate well with the high incidence of food poisoning outbreaks in summer. Foods contaminated with *V. parahaemolyticus*, through inadequate preparation or handling, may be held under conditions favoring the production of hemolysin, thereby increasing the risk of foodborne illness.

It is recommended that in future studies with this organism, lower inocula of the organisms and food homogenate (instead of TSB) should be used so that one could establish parameters for prediction of the potential for food poisoning in products contaminated with *V. parahaemolyticus*.

ACKNOWLEDGEMENT

I would like to thank Japan International Cooperation Agency for financial support.

LITERATURE CITED

Honda, T., S. Taga, T. Takeda, M.A. Hasibuan, Y. Takeda, and T. Miwatani. 1976. Identification of lethal toxin with the thermostable direct hemolysin produced by *Vibrio parahaemolyticus*, and some physicochemical properties of the purified toxin. Infect. Immun. 13 : 133 – 139.

Miyamoto, Y., Y. Obara, S. Akiyama, K. Takizawa, and S. Yamai. 1969. *In vitro* hemolytic characteristic of *Vibrio parahaemolyticus* : its close correlation with human pathogenicity. J. Bacteriol. 100 : 1147 – 1149.

Morris, J.G., Jr. 1995. "Noncholera" Vibrio species, pp. 671-685. *In* M.J. Blasser, P.D. Smith, J.I. Ravdin, H.B. Greenberg, and R.L. Guerrant (eds.). Infections of the Gastrointestinal Tract.

Raven Press, Ltd., New York.

Nishibuchi, M., A. Fasano, R.G. Russell, and J.B. Kaper 1992. Enterotoxigenicity of *Vibrio parahaemolyticus* with and without genes encoding thermostable direct hemolysin. Infect. Immun. 60 : 3539-3545.

Oliver, J.D. and J.B. Kaper. 1997. Vibrio species, pp. 228 – 264. *In* M.P. Doyle, L.R. Beuchat and T.J. Montville (eds.). Food Microbiology : Fundamentals and Frontiers. ASM Press, Washington D.C.

Springer, W. and W. Goebel. 1980. Synthesis and secretion of hemolysin by *Escherichia coli*. J. of Bact. 144 : 53 – 59.

Steel, R.G.D. and J.H. Torrie. 1980. Principles and Procedures of Statistics, A Biometrical Approach. McGraw – Hill Book Company, New York. 633 p.

Received date : 12/11/99
Accepted date : 1/03/00