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Virulence Evaluation of *Vibrio anguillarum* in Accordance with Hemolytic Activity on Human Red Blood Cells

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Vibriosis caused by *Vibrio anguillarum* has been intensively studied for the understanding of the virulence mechanism. High efficiency iron sequestering system, serum resistance, proteolytic enzyme, and so forth have been reported. It is found that virulent strain of *V. anguillarum* clearly lysed RBCs from human. The intensity of hemolysis was quite correlated to the degree of virulence. Among various bacterial media, the highest titre of extracellular hemolytic activity of *V. anguillarum* strain V7 (NCMB6; J01) was observed in BHI medium. The production of hemolysin was initially detected when the growth reached stationary phase. Hemolytic activity was detected using RBCs from various animals as well as murine splenocytes. We now report that presence and incidence of virulence could be detected by hemolysis using human RBCs, and this system, as an experimental model, could be applied to studies for understanding pathogenic mechanisms of vibriosis.

KEY WORDS □ ***V. anguillarum*, vibriosis, hemolysis, virulence, human RBC**

Vibriosis is caused by *Vibrio anguillarum*, Gram negative, halophilic bacterium. This is one of the most important fish disease since it was reported (3, 8). Its pathogenicity has been under intensive research suggesting various factors and products such as siderophore (5-7), outer membrane protein (1, 2), extracellular toxin (9, 10, 12). Nevertheless, the mechanism how this pathogenic microbe develops vibriosis is not clear.

After Crosa and his colleagues found that fish are dying due to iron deficiency in vibriosis (7), it seems that *V. anguillarum* turns on some unknown metabolic pathways under unfavourable condition sequestering iron from host fish. Therefore, it is reasonable to approach how iron can be released from host tissue. Searching for factor(s) responsible for hemolysis, we could find that human RBCs can be used for the measurement of virulence caused by *V. anguillarum*, since the virulence determined by *in vivo* injection into goldfish in laboratory aquariums is correlated with hemolytic activity. This study was, in fact, initiated on the observation of great hemorrhage in abdomen and humoral accumulation implying that this fish pathogen produces toxic material which in turn causes tissue destruction. We report that virulence of *V. anguillarum* can be evaluated by hemolytic activity.

MATERIALS AND METHODS

Strains

Vibrio anguillarum strain V-7 (NCMB6, serotype JO1) and V-104 (NOAA V-775, serotype JO3) were kindly provided by Professor Kimura in 1987 and maintained in our laboratory thereafter. For the maintenance of the virulence in both strains, a group of gold fish *Cyprinus caprio* L. weighing 4~5 g each were injected intraperitoneally with these strains cultured in tryptic soy broth supplemented with 1.5% NaCl (Merck) at 22~23°C. Media for bacterial culture were purchased from Difco, unless otherwise mentioned. Through *in vivo* passages, virulent bacteria were recovered from the peritoneal cavity and spleen of dead fish on tryptic soy agar (TSA) supplemented with final 1.5% NaCl at 22°C for 24~48 hrs, and they were kept at 4°C for stock. When it was necessary to diminish the virulence, bacteria in stock were cultured at 32~33°C for 18~24 hrs.

Confirmation of *V. anguillarum* by O/129 test and culture in TCBS medium

V. anguillarum had to be identified every time whenever *in vivo* passage had been conducted by O/129 test and culture in TCBS medium

(Thiosulfate-citrate-bile-sucrose agar, Acumedia). The growth of reisolated *V. anguillarum* spread on TSB agar media was inhibited around the paper disc which contains 2,4-diamino-6,7-diisopteridine, one of vibriostatic agents, at the concentration of 0.1 g/100 m*l* in acetone (O/129 test). Other bacteria, presumed to be normal flora of peritoneal cavity, were not affected their growth. Culture of reisolates on TCBS medium developed yellow colonies for *V. anguillarum* through characteristic sugar fermentation.

Medium for the optimal growth

Inoculum of V7 cultured for over night in TSB, 50 μl each, was seeded to 50 m*l* of nutrient broth (NB), LB broth (LB), tryptic soy broth (TSB), brain heart infusion broth (BHI), and chemically defined media (CDM: K_2HPO_4 2.3 g, KH_2PO_4 0.78 g, $(NH_4)_2SO_4$ 1.0 g, $MgSO_4$ 0.1 g, $Na_3C_6H_5O_7$ 0.6 g, and glucose 2.0 g/*l*) (14) and incubated at 23°C shaking at 140 rpm. Culture supernatant collected at 6, 24, 48, and 72 hr had been examined for growth of bacteria and hemolytic activity. The increase of total bacterial cells was measured in biomass by A_{600}, and hemolytic activity was estimated by the method described below after removing bacteria by centrifugation (Eppendorf Centrifuge 5415, 6,000×*g*, for 2 min). Each medium was supplemented with 0.85% NaCl.

Preparation of RBCs

Human RBCs with blood type O were very useful to evaluate the hemolytic activity of *V. anguillarum*. Heparinized normal peripheral blood from healthy volunteers with blood type O was diluted in equal volume of Hanks' balanced salt solution (HBSS) and loaded onto lymphocyte separation medium (LSM p=1.077, Flow Laboratory). After centrifugation at 1,000×*g* for 20 min, RBCs were recovered from the sediment. RBCs were washed twice in Alserver's solution at final concentration of 40% and kept at 4°C until use. This RBC preparation was washed twice again before use in 10 mM phosphate buffered saline (PBS) solution and finally adjusted to 1% solution (v/v, 1.1×10^8 cells/m*l*) or 1.5%. The solution of 1% RBC gave the A_{540} value of 1.0 when mixed with equal volume of PBS supplemented with 0.02% SDS. Fresh RBCs were stable even after a week at room temperature.

Hemolytic activity

The method of Cavalieri and Snyder (4) was slightly modified to evaluate the hemolytic activity from growing bacteria. In brief, bacteria were cultured in TSB and human RBC suspension was added to estimate the different hemolytic activities between bacteria passed three time *in vivo* and those passed four times *in vitro* and between bacteria grown at 23°C and those grown at 32°C. Bacterial cultures were adjusted to $A_{600}=0.5$ and added to equal volume of 1.5% human RBC suspension. Reaction mixture was further kept at 25°C until maximum hemolysis was completed under the given conditions. After centrifugation, lysed RBCs were quantified by measuring absorbency of supernatant at 540 nm (Shimadzu Model UV-265).

Hemolytic activity in the culture supernatant was measured by Short's method (13) also with some modification. Each of serially diluted hemolysin preparation in double fold was added to equal volume of 1% RBC suspension. They were gently stirred at 30 min, and tested hemolytic activity in an hour by measuring absorbency at 540 nm. The maximum dilution to lyse 50% RBCs was converted to 50% hemolysis unit (HU_{50}). RBCs from other animals were compared for the hemolytic activity of *V. anguillarum*; goldfish, rabbit, mouse, sheep as well as human. RBCs in 750 μl PBS at 5×10^7 cells/m*l* were mixed with 250 μl of hemolytic supernatant (64 HU_{50}, therefore, finally 16 HU_{50}/m*l*) at room temperature, and the intact RBCs were counted on hemocytometer after 20 min reaction. The count of RBCs in control group treated with PBS only was not decreased during the experiment.

Hemolytic activity has been tested even on cultured fish cells such as CHSE-214 and EPC which were originally provided by Professor Fryer at Oregon State University in 1982 and maintained in our laboratory thereafter. These cells were grown in EMEM (Flow Laboratory) supplemented with 10% fetal bovine serum (Hyclone) at 18°C. Filtered culture supernatant was added to the cultures to 10% v/v after the cells grew to make monolayer in the 96 well culture plate.

Preparation of hemolysin

Supernatant of shaking culture at 23°C for 48 to 72 hrs in BHI broth was slowly saturated up to 60% with ammonium sulfate (Oriental Chemical Inc.). Precipitate was resuspended in PBS and dialysed against sufficient volume of PBS. After filtration through 0.22 μm cellulose acetate filter (Millipore, GS type), this preparation was kept at 4°C until use.

RESULTS AND DISCUSSION

Hemolytic activity of *V. anguillarum* hemolysin on human RBCs

Hemolytic activity was detected on human RBCs when supernatant of V7 and V104 culture was loaded on to 2% blood agar plates. As shown in Fig. 1, human RBCs were lysed producing clear area around each colony, but it was not as clear as those produced by *Pseudomonas* (11). Human RBCs, in comparison with RBCs from other animals, were valuable for the estimation of hemolytic activity of *V. anguillarum*.

Several strains of *V. anguillarum*, including strains V7 and V104, had been tested if their

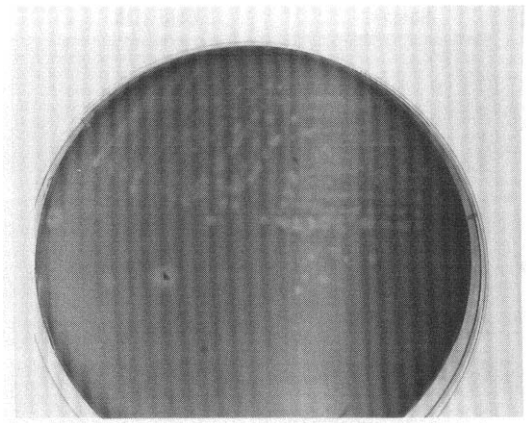

Fig. 1. *Hemolysis by V. anguillarum.*
Hemolysin produced by *V. anguillarum* strain V7 lysed human RBCs prepared in 2% blood agar. Clarity of hemolysis could be correlated with the different virulence.

Table 1. *Susceptibility of RBCs to Vibrio hemolysin*[a].

Source[b]	Number of RBCs not lysed[c] ($\times 10^6$ cells /ml)	% of non response
Rabbit	complete lysis	0
Mouse	16.7± 2.13[d]	33.4
Human	20.3± 1.56	40.6
Fish[e]	24.5± 1.79	49.0
Sheep	36.5± 2.46	73.0

[a] Hemolytic activity was estimated by direct counting with hemacytometer 20 min after *Vibrio* hemolysin was added to each RBCs in PBS.
[b] Obtained freshly.
[c] Initial concentration of RBCs was adjusted to 1×10^8 cells/ml, and equal volume of 4 HU_{50}/ml was added to each RBC.
[d] Mean± S.E.M.
[e] Goldfish.

difference in virulence could be detected on human RBCs. The hemolytic activity of strain V7 was evaluated very much potent than that of V104. Such difference in virulence could be detected at least by time of synthesis.

RBCs from other animals were also tested if they could be applied for evaluation of bacterial hemolytic toxicity (Table 1). Rabbit RBCs were mostly sensitive to *Vibrio* hemolysin. Susceptibility of RBCs from mouse, human, and fish was still usable for hemolytic test, meanwhile RBCs from sheep was not so sensitive. This result is consistent with other experiments such as one with the *Aeromonas hydrophila* hemolysin, and the incidence of susceptibility decreased in order of rabbit, mouse, human, fish, and sheep (personal communication with Prof. J.S. Chang, Inha University Medical School). It implies that surface components on RBCs are important for hemolysin attachment, which must be different from animal to animal.

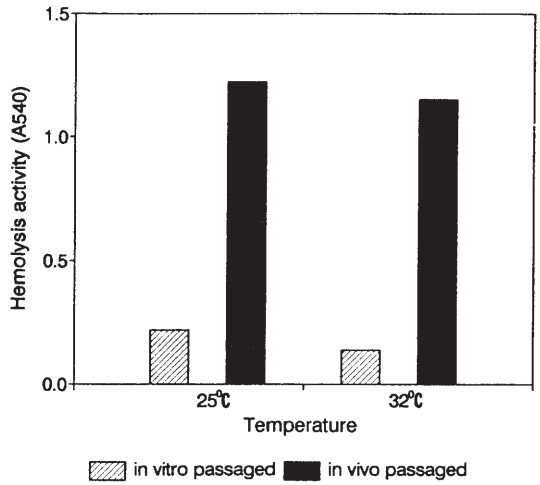

Fig. 2. *Virulence decrease by repeated in vitro passage.*
When strain V7 was passaged repeatedly either in *in vitro* or *in vivo* culture, for 3 times and 4 times, respectively, there was great difference in virulence between them and such difference could be easily detected on hemolysis of human RBCs.

Since RBCs from rabbit was so sensitive, it is suggested to use rabbit RBCs to hemolysin detection in place of human cells. Rabbit RBCs, however, was not so good to measure the difference in virulence. It is much likely that RBCs from mouse, human, and fish fulfils our requirement exerting correlated response to hemolysin.

Correlation between hemolytic activity and virulence

There was correlation between hemolytic activity and virulence. It is the well known fact that repeated *in vitro* passage of bacteria reduces virulence of pathogenic microorganisms. Fig. 2 shows that RBCs were lysed in a few hours when culture of V7 prepared by *in vivo* passage for three times before activity test. The same strain kept in *in vitro* culture for four passages hardly lysed RBCs when examined even after 48 hrs. It was observed that hemolysis was much higher when *in vitro* passage was cultured for 2 days, probably due to the induction of hemolysin production.

The difference between V7 and V104 in hemolytic activity was confirmed again, from the observation that hemolysin from V7 could lyse more quickly, 2~5 hrs, than that from V104, 6~8

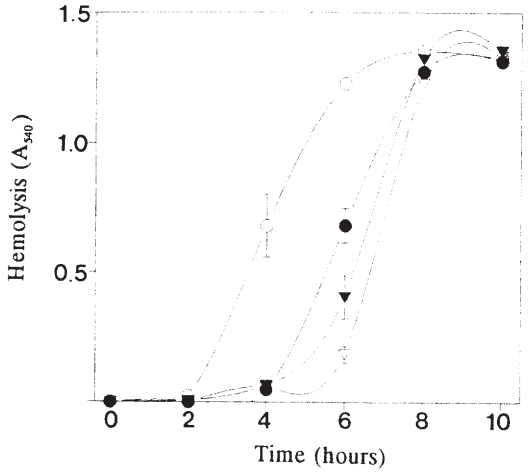

Fig. 3. *Temperature effect on hemolytic activity of strains V7 and V104.*
Hemolysin was induced through *in vivo* passage more than three times. They were grown at 23°C and 32°C, and evaluated hemolytic activity by measuring optical density at 540 nm of culture supernatant after reaction with 3% human RBCs. Error bar stands for standard error of the mean. ○, V7 at 23°C; ▽, V7 at 32°C; ●, V104 at 23°C; ▼, V104 at 32°C.

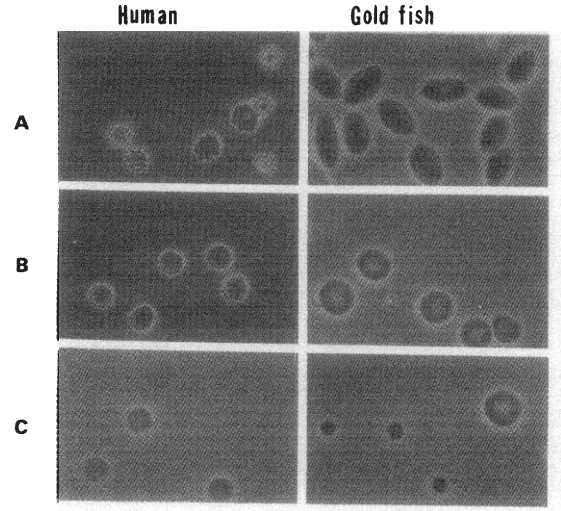

Fig. 4. *Hemolysis of human and fish RBCs.*
Upon addition of *Vibrio* hemolysin (50 HU_{50}/ml) to the equal volume of RBC suspension at 5×10^7 cells/ml, morphological change could be observed from both RBCs. A, normal morphology of RBCs; B, swelling and rounding, sometimes even darkening, occurs with hemolysin addition; C, ruptured ghost cells and exposed nuclear particles, even with still intact RBCs.

hrs (Fig. 3) in accordance with their significant difference of virulence, which had been discussed previously. But their final hemolysis evaluated 10 hrs after initiation of reaction showed no difference. For strain V7, hemolysin production was significantly delayed when cultured at 32°C in comparison with the culture at 23°C. When LD_{50} was measured for cultures at 23°C and at 32°C, that for V7 increased around 200 times, meaning decrease in virulence, while that for V104 did not vary much (10). It is believed that temperature in natural habitat for *V. anguillarum* is not so high as 30°C and at the temperature above 30°C this bacterium would turn on some physiological metabolism for survival instead of hemolysin production. Temperature for culture, therefore, comparatively affected hemolysin production significantly regardless of passage pattern (Fig. 3).

Lysis of RBCs by *Vibrio* hemolysin

When disruption of RBC with culture supernatant was observed under microscope, morphological transformation could be defined such as appearance of multiple projection, spherocyte formation, and ghost cells, in order, as was previously reported (13). RBC from fish was not much different as compared in Fig. 4. Bacterial cells did not attach to RBCs even with active motility when culture supernatant was added before centrifugation. When filtered supernatant was added to CHSE-214 cells in culture, cells were destroyed immediately.

V. anguillarum in live form could be recovered from ascites of goldfish which died of vibriosis. Some intact RBCs from this ascites also disappeared very quickly.

Production of hemolysin

After culture of *V. anguillarum* in BHI broth, supernatant was concentrated 25 times and had been analyzed if any component in medium may interrupt experiments. As shown in Fig. 5, medium itself was completely inert for hemolysis. Other media used for hemolysin production (Table 2) did not show hemolytic activity of *V. anguillarum* in assays.

Other bacteria normally isolated when *V. anguillarum* was recovered were also examined if they could produce any hemolysin. It was very clear that they did not produce any hemolysin-like toxin (data not shown).

V. anguillarum V7 secreted hemolysin only after stationary phase of culture (Fig. 6), and total amount of hemolysin produced was variable according to the media used (Table 2). Synthesis of hemolysin continued until day 4 or 5. No hemolysin was estimated in CDM, supplemented with 0.2% glucose and ammonium sulfate, where

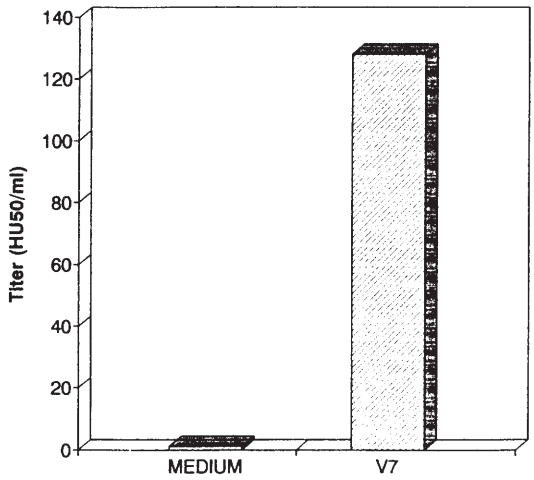

Fig. 5. *Hemolytic activity of concentrated culture supernatant.*
Strain V7 was cultured in BHI broth at 23°C for 24 hrs. Culture supernatant was concentrated with 80% ammonium sulfate and dialysed overnight against PBS. BHI broth as well as other culture media did not exhibit any significant hemolytic activity. Culture of other bacteria isolated along with V7 as normal enterobacteria on the occasion of reisolation after *in vivo* passage was not of hemolytic activity either.

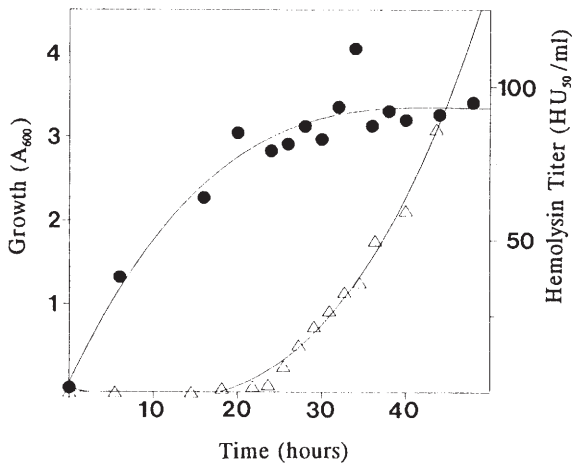

Fig. 6. *Hemolysin production in V. anguillarum strain V7 culture in BHI broth.*
As bacterial growth reached stationary phase, hemolysin production could be detected and increasing dramatically thereafter. Hemolytic activity was continuously accumulated even 50 hrs after initiation of culture. ●, bacterial growth measured in biomass, A_{600}; △, hemolytic activity expressed in HU_{50}/ml.

Table 2. *Hemolysin production and growth.*

Media[a]	Culture time (hrs)			
	6	24	48	72
CDM	−[b], 0.10[c]	−, 1.40	−, 1.55	−, 1.41
NB	−, 0.27	8.2, 1.60	−, 1.34	−, 2.34
LB	−, 0.83	14.5, 2.49	12.6, 3.49	<2, 4.98
TSB	−, 1.13	10.5, 2.88	224.5, 3.26	307.1, 4.56
BHI	−, 1.32	6.6, 3.17	549.7, 3.66	804.3, 4.95

[a] CDM, chemically defined medium, NB, nutrient broth; LB, Luria-Bertani broth; TSB, tryptic soy broth; BHI, brain heart infusion broth.
[b] Not detected. Hemolytic activity evaluated as described in Materials and Methods.
[c] Biomass as the evaluation of bacterial growth was measured as absorbency at 600 nm. Samples were diluted if necessary.

E. coli can produce hemolysin (14). It is suggested that pathogenic bacteria may produce toxin when their environmental condition is not suitable for their survival as a kind of overcoming response to the environment. For *V. anguillarum*, depletion of nutrient could have induced bacteria to initiate hemolysin production. It was reported previously that production of hemolytic activity in *E. coli* could be detected at logarithmic growth phase gradually disappearing at stationary stage (13, 14, 15). In culture of *Pseudomonas aeruginosa*, hemolysin could be identified only with 3~4 week culture (11). Young culture was reported not to produce any.

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초 록: 사람의 적혈구를 이용한 비브리오균의 용혈소 측정

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비브리오 균에 의한 어류의 대량폐사 등의 감염피해가 세계적으로 문제가 되고 있음에도 불구하고 지금까지 이들 병원성 세균에 의한 발병기작에 대하여 자세히 알려진 바가 없이 철분을 흡수하는 물질을 위시하여 외막단백질, 단백질 분해효소, 혹은 용혈독 등의 여러 가지 가능성 있는 인자들에 대한 보고가 있어왔다. 비브리오 균의 감염에 의하여 복강 내의 장기가 파괴되고 심한 출혈이 있음에 기초하여 철분 흡수가 발병의 중요한 요인이라고 판단하고, 용혈소를 측정하는 방법을 모색하였다. 적혈구의 용혈 정도와 비브리오 균의 병독 사이에 어느 정도의 상관관계가 있다고 판단되어, 사람의 적혈구를 용혈소 측정에 이용할 수 있는 방법을 연구하였다. 사람의 적혈구가 비브리오 균의 병독정도에 따라 파괴되는 정도에 뚜렷한 차이를 보임으로써, 토끼, 생쥐, 금붕어 등의 적혈구에 비하여 병독의 차이를 가장 적절하게 구분할 수 있는 적혈구라고 확인하였다. 비브리오 균은 생장 조건이 좋지 않게 되면서 부터 용혈소를 내기 시작하고, 용혈소의 생산은 꾸준히 계속되었으며 생장 온도와 배지에 따라 커다란 차이를 보였다. 용혈소는 적혈구의 표면에 흡착하여 삼투압의 변화를 일으키며 용혈을 유도하는 것으로 관찰되었다. 따라서 용혈소의 측정방법의 개발은 용혈소의 작용기작 연구에도 유용하게 사용될 수 있다고 기대된다.