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Quantitation of Hemolysin Produced by *Vibrio cholerae* Using Reversed Passive Latex Agglutination*

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Abstract: A simple, sensitive and specific technique for the detection of hemolysin of *Vibrio cholerae* O1 and non−O1 was developed by using the reversed passive latex agglutination method to quantitate the amount of hemolysin in the culture supernatant and accumulated fluid by the De test. Purified O1 hemolysin was titrated with latex sensitized with the anti non−O1 hemolysin antibody as well as purified non−O1 hemolysin, and no cross−reaction with cholera toxin was observed. Hemolysin in the culture supernatant of *V. cholerae* O1 and non−O1 was detected but not in those of *V. cholerae* biotype Classic or other bacterial enteropathogens except for one strain of *Shigella.*

A high titer of hemolysin was also detected in the accumulated fluid by the rabbit ileal loop test.

Key words: Vibrio cholerae, Hemolysin, Reversed passive latex agglutination

INTRODUCTION

Vibrio cholerae non−O1 as well as *Vibrio cholerae* O1 have been recognized as causative agents of diarrheal diseases (Blake *et al.*, 1980). *Vibrio cholerae* non−O1 produce cholera toxin (CT) −like toxin in the culture supernatant (Craig *et al.*, 1981; Ohashi *et al.*, 1972) and its toxin was characterized to be identical (Yamamoto *et al.*, 1983b) or similar (Yamamoto *et al.*, 1983a) to the CT of *Vibrio cholerae* O1. However, all the

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isolated strains of *V. cholerae* non−O1 do not necessarily produce CT−like enterotoxin (Nishibuchi & Seidler, 1983; Nishibuchi *et al.*, 1983; Spira *et al.*, 1978). The clinical features of gastroenteritis due to *V. cholerae* non−O1 are different from those of *V. cholerae* O1 (Blake *et al.*, 1980; Hughes *et al.*, 1978). Abdominal cramp, fever, and mucous and bloody stool frequently observed in the gastroenteritis due to *V. cholerae* non−O1 are not common in cholera. Gastroenteritis due to *V. cholerae* non−O1 may be associated with not only CT−like toxin but also other diarrheagenic factors. Yamamoto *et al.* (1984) reported the purification of hemolysin from *V. cholerae* non−O1 and showed that non−O1 hemolysin is identical to El Tor hemolysin immunologically, biologically and physicochemically (Yamamoto *et al.*, 1986). We also found that non−O1 hemolysin causes fluid accumulation in the ligated rabbit ileal loop and suckling mice (in press). This suggests that hemolysin produced by *V. cholerae* is an important diarrheagenic factor especially among the strains producing less CT and the strains not encoding the CT gene. It is very important to quantitate the amount of hemolysin in diarrheal stool specimens to investigate the role of hemolysin on the pathogenesis of vibrio infections. Thus, we report a simple and specific quantitative method of hemolysin using reversed passive latex agglutination as applied for CT (Oda, 1981).

Materials and Methods

Polystylene latex

Polystylene latex (SDL 59 Lot. L−3003) was purchased from Takeda Pharmaceutical Co. Ltd.

Preparation of purified hemolysin

Non−O1 hemolysin was purified as previously described (Yamamoto *et al.*,1984) from *V. cholerae* non−O1 strain S7 kindly given by Y. Zinnaka, Toho University, School of Medicine, Tokyo, Japan. O1 hemolysin was purified from *V. cholerae* O1, biotype El Tor, serotype Inaba, strain N86 obtained from Toshio Miwatani, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.

Preparation of cholera toxin, subunit A and B

Cholera toxin was purified from *V. cholerae* O1, 569B through aluminum hydroxide precipitation and gel filtration through Sephadex G−100, Bio−Gel A5m and Sephadex G−75, superfine as previously described (Yamamoto *et al.*, 1983b). Subunits A and B were purified from cholera toxin by column chromatography using Sephadex G−75 equilibrated with 0.1 M propionic acid containing 6 M urea (pH 4).

Preparation of antiserum

Non−O1 hemolysin (100 μg) in 1 ml of 10 mM phosphate−buffered saline, pH 7.0 (PBS) emulsified with an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) was injected into the footpad of rabbits. The rabbits were rechallenged intravenously with 100 μg of the purified non−O1 hemolysin in PBS at 2 and 4 weeks

after the first injection. Antiserum was obtained 5 weeks after the first injection.

Monospecific polyclonal antiserum evoked by non−O1 hemolysin was purified by immuno−affinity chromatography of whole antiserum against non−O1 hemolysin over the column of the purified antigen immobilized on activated CH−Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) by methods previously described (Tsukamoto *et al.*, 1980).

Preparation of sensitized latex with anti non−O1 hemolysin antibody

For the sensitization of latex, five different concentrations (5, 10, 20, 40 and 80 μg/ml) of affinity purified anti non−O1 hemolysin antibody were compared to find the optimal concentration. Each purified antiserum was mixed with an equal volume of diluted latex solution (0.25%) and incubated at room temperature for 1 h in a shaking water bath. The pellet obtained by high speed−centrifugation (10,000 rpm, 10 min.) was suspended with 5 ml of PBS. The suspension solution was further centrifuged (10,000 rpm, 10 min.) and resuspended with latex diluent (PBS : 5% BSA : 1% PVP, 200 : 40 : 1). The resuspension was again centrifuged (10,000 rpm, 10 min) and resuspended in the diluent at a final concentration of 0.025%. Latex sensitized with 10 μg/ml of anti hemolysin antibody gave a maximal sensitivity (0.5 ng/ml). No agglutination was seen when non−sensitized latex (as control) was used. Thus, the latex sensitized with 10 μg/ml of the antibody was used in the following experiment.

Reversed passive latex agglutination (RPLA)

Hemolysin preparations were diluted in two−fold series in round−bottomed microtiter plates (Cooke Engineering Co.) in 25 μl of latex diluent. Sensitized latex was added in 25 μl, vortexed by a micromixer (Kowakizai Co. Ltd) and incubated overnight at room temperature. The titer was defined as the reciprocal of the highest dilution in which agglutination was visible to the naked eye. Two−fold diluted non−O1 hemolysins were titrated to check the reproducibility. Purified El Tor hemolysin, cholera toxin, subunits A and B were checked for the cross−reaction. The concentration of samples used was 1 μg/ml.

Cholera toxin assay

Cholera toxin assay was performed by VET−RPLA (Denka Seiken).

Strains and culture conditions

Five strains each of *V. cholerae* non−O1 and *V. cholerae* biotype El Tor, which showed complete or marked hemolysis in the modified method of Feeley & Pittman (1963) were used for the titration of hemolysin produced in the culture supernatant. The strains were cultured in heart infusion broth (HIB) containing 3% glycerol at 37℃ overnight in a resting state. The hemolytic property of these strains was checked by the hemolytic zone on blood agar by spotting 15 μl of the culture supernatant. For the detection of hemolysin in the culture supernatant of other diarrheagenic pathogens, five strains of *Vibrio cholerae* biotype Classic, five strains of *Vibrio parahemolyticus,* nine strains of enterotoxigenic *E. coli,* five strains of enteroinvasive *E. coli,* five strains of enteropathogenic *E. coli,* 20 strains of *Shigella* and 13 strains of *Salmonella* were used.

The strains of *Vibrio cholerae* biotype Classic were cultured in HIB and Syncase medium containing 3% glycerol. The strains of *V. parahemolyticus* were cultured in alkaline-peptone water containing 0.5% of sodium chloride at 37℃ overnight in a shaking water bath. The strains of *E. coli* were cultured in CAYE medium and the strains of *Shigella* and *Salmonella* were cultured in heart infusion broth in the same manner.

Quantitation of hemolysin in the accumulated fluid in rabbit ileal loop test

The fluid accumulating in the intestine was obtained by the De test as previously described (De & Chatterjee, 1953). Four strains each of *V. cholerae* non-O1 and biotype El Tor were cultured in HIB at 37℃ overnight.

One tenth milliliter of organisms (approximately 5×10^7) was inoculated in adult rabbit ileal loops. The intestinal loops were taken out 8 h after inoculation and intestinal fluids of two strains of *V. cholerae* biotype El Tor (A88, C128) and one strain of *V. cholerae* non-O1 (S7) giving an FA ratio of more than 0.63 were kept and stored at −30℃ until use. The supernatants of intestinal fluid were obtained by centrifugation (18,000 g, 20 min).

Protein determination

The protein content was assayed by the method of Bradford (1976) using bovine serum albumin as a standard.

RESULTS

Sensitivity of latex sensitized by anti hemolysin antibody

Two-fold diluted non-O1 hemolysin (1 μg/ml) was titrated.

The limit of detection for hemolysin was about 0.97 ng/ml (Table 1).

Table 1. Sensitivity of diluted hemolysin

Dilution/2^n	1	2	3	4	5	6	7	8	9	10	11	12
1 μg/ml	+	+	+	+	+	+	+	+	+	+	−	−
x2	+	+	+	+	+	+	+	+	+	−	−	−
$x2^2$	+	+	+	+	+	+	+	+	−	−	−	−
$x2^3$	+	+	+	+	+	+	+	−	−	−	−	−
$x2^4$	+	+	+	+	+	+	−	−	−	−	−	−
$x2^5$	+	+	+	+	+	−	−	−	−	−	−	−
$x2^6$	+	+	+	+	−	−	−	−	−	−	−	−
$x2^7$	+	+	+	−	−	−	−	−	−	−	−	−

Cross-reactivity with cholera toxin

Purified El Tor hemolysin (1 μg/ml) was agglutinated with the latex quite the same as the non-O1 hemolysin at a titer of 1024. However, no agglutination was observed in the samples of CT, subunits A and B, and no cross-reaction with cholera toxin was observed.

Titration of hemolysin produced in the culture supernatant

The hemolysin in the culture supernatant of *V. cholerae* was titrated to check whether this system could detect hemolysin and correlate with the hemolytic property of the culture supernatant.

The supernatant of *V. cholerae* non-O1 in HIB containing 3% glycerol showed relatively higher titers, ranging from 2^4 to 2^9, than those of *V. cholerae* El Tor. No hemolysin was detected in the culture supernatant of one strain of *V. cholerae* El Tor which showed marked hemolysis.

Moreover, the hemolytic zone around the colony was seen in the culture supernatant of *V. cholerae* non-O1 in accordance with the high titer of hemolysin in RPLA (Table 2).

Table 2. Titration of hemolysin produced in the culture supernatant

Strains	Latex/2^n	1	2	3	4	5	6	7	8	9	ND
Non-O1 V. cholerae	Sensitized				1		1	1	1*	1*	
	Non sensitized										5
V. cholerae El Tor	Sensitized	2		2							1
	Non sensitized										5

ND : Not detected

* : Hemolytic zone around the colony could be seen on the blood agar

Table 3. Titration of culture supernatant of other diarrheal pathogens

Strains	No. of tested	No. of positive
V. cholerae, Classic	5	0
V. parahemolyticus	5	0
Enterotoxigenic E. coli	9	0
Enteroinvasive E. coli	5	0
Enteropathogenic E. coli	5	0
Shigella dysenteriae	5	0
Shigella flexneri	5	0
Shigella boydii	5	1(4)
Shigella sonnei	5	0
Salmonella	13	0
Total	62	1

( ) : The titer of agglutination

Hemolysin was not detected with the latex in the culture supernatant of *V. cholerae*, biotype Classic. No agglutination of the culture supernatant of other diarrheal pathogens against the latex sensitized with anti non−O1 hemolysin antibody was observed except for one strain of *Shigella boydii*, which gave a titer of 4 (Table 3).

Quantitation of hemolysin in the accumulated fluid in the ileal loop test

Hemolysin was detected with a fairly high titer, ranging from 1024 to 128 in three strains. No agglutination was observed with non sensitized latex. The supernatant of accumulated fluid was thought to contain hemolysin, ranging from 2 μg/ml to 0.25 μg/ml. Cholera toxin was also detected in all three fluids, but no particular relationship was found between the production of CT, hemolysin and FA ratio (Table 4).

Table 4. Titration of hemolysin in the accumulated fluid in the ileal loop test

Supernatant	Latex	Hemolysin	Cholera Toxin	FA ratio
S7	Sensitized	2^7	2^2	1.06
	Non sensitized	ND	ND	
A88	Sensitized	2^{10}	2^6	0.89
	Non sensitized	ND	ND	
C128	Sensitized	2^8	2^7	0.63
	Non sensitized	ND	ND	
Control		2^9	2^9	

Control : 1 μg/ml of non−O1 hemolysin and CT were used.
ND : Not detected

DISCUSSION

In this study, a technique for the detection of hemolysin of *V. cholerae* O1 and non−O1 was developed by using the reversed passive latex agglutination method to quantitate the amount of hemolysin in the culture supernatant and accmulated fluid in the De test.

Six conclusions can be made from this study. 1)Ten micrograms of anti non−O1 hemolysin antibody was an optimal concentration for sensitization which gave a maximal sensitivity of 0.5 ng/ml. 2)Purified El Tor hemolysin identical to non−O1 hemolysin immunologically, biologically and physicochemically, was agglutinated with latex in the same way as non−O1 hemolysin of a high titer, but 3)no cross−reactivity with cholera toxin was observed. 4)Hemolysin in the culture supernatant of *V. cholerae* O1 and non−O1 was detected and the titer was in good agreement with the results obtained by the modified method of Feeley & Pittman (1963). 5)A fairly high titer of hemolysin as well as CT was detected in the accumulated fluid in the rabbit ileal loop test. 6)No agglutination of the culture supernatant of other diarrheal pathogens against the latex sensitized with the anti

non−O1 hemolysin antibody was observed except for one strain of *Shigella boydii*, which gave a titer of 4. Non−specific agglutination of latex appeared hardly occurred.

Therefore, the reversed passive latex agglutination method using the anti non−O1 hemolysin antibody is considered to be simple, specific and sensitive for the detection of hemolysin. Hemolysin was detected in the accumulated fluid in the De test as well as the culture supernatant. This method enables us to quantitate the amount of hemolysin and to clarify the conditions and the mechanisms of its production and the role of hemolysin on the pathogenesis of vibrio infections. Moreover, a fairly large amount of hemolysin is required for the hemolytic reaction on blood agar.

However, further examination is needed since the end point of agglutination is relatively unstable (2^9 to 2^{10}) and other bioactive factors reactive with sensitized latex might exist when the culture condition of the organism is changed.

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抗 non−O1 溶血素抗体を用いた逆受身ラテックス凝集反応法によるコレラ菌，及び non−O1 コレラ菌が産生する溶血素の定量法

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コレラ菌（Vibrio cholerae O1），及び non−O1 コレラ菌（Vibrio cholerae non−O1）が培養上清中に産生する，またウサギ腸管ループ貯留液中の溶血素を定量するために抗 non−O1 溶血素抗体を用いた，簡便で特異的，かつ感受性の高い逆受身ラテックス凝集法を開発した．精製した O1 溶血素は精製した non−O1 溶血素と同程度の感受性を示したが，コレラ毒素との交差反応は認められなかった．エルトール型コレラ菌，及び non−O1 コレラ菌の培養上清からは溶血素が検出されたが，溶血性を示さないクラシック型コレラ菌の培養上清からは検出されなかった．またボイド型赤痢菌の1株を除いて他の下痢原因菌の培養上清ともラテックス凝集は認められなかった．ウサギ腸管ループ貯留液も非特異的凝集は認められず，高い溶血素価を示し定量性が確認された．

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