Immunological Methods for Detection of Kanagawa Phenomenon of *Vibrio parahaemolyticus*

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A modified Elek test and an immuno-halo test were developed for detection of the thermostable direct hemolysin produced by Kanagawa phenomenon-positive strains of *Vibrio parahaemolyticus*. The sensitivity of the modified Elek test was almost the same as that of the Kanagawa phenomenon on Wagatsuma medium, and the results of both reactions correlated very well. The immuno-halo test was a little less sensitive than the modified Elek test. Since the Kanagawa phenomenon on Wagatsuma medium sometimes gives false-positive results due to instability of the blood used in the medium, we recommend the immunological techniques described here as reproducible methods for identification of the thermostable direct hemolysin by *V. parahaemolyticus*.

Vibrio parahaemolyticus is a causative bacterium of food poisoning and has been isolated in many countries (7). This organism is divided into two groups based on hemolysis in a special blood agar medium (Wagatsuma medium); the hemolysis observed in this medium has been called the Kanagawa phenomenon (7). It is well recognized that Kanagawa phenomenon positivity is closely associated with human pathogenicity (8, 11). Among several hemolysins produced by *V. parahaemolyticus* (15, 18), a thermostable direct hemolysin was found to be responsible for the Kanagawa phenomenon (6, 8, 9, 14, 15, 20).

Although Wagatsuma medium is routinely used in clinical laboratories to test for hemolysin production by isolated organisms, false-positive hemolytic reactions occasionally occur (1) due to pH changes around the colonies, fragility of erythrocytes, or hemolysis caused by hemolysins other than the thermostable direct hemolysin.

Since it is well recognized that the thermostable direct hemolysin is produced only by Kanagawa phenomenon-positive strains, and since the hemolysin is available as a highly purified preparation (6), it is possible to develop immunological methods to identify this hemolysin. This paper reports the development of such methods to detect Kanagawa phenomenon-positive strains of *V. parahaemolyticus*. These methods are more reliable and simple to perform than the method with Wagatsuma medium, which has been routinely used in clinical laboratories.

MATERIALS AND METHODS

Bacterial cultures. All cultures were from the Laboratory for Culture Collections, Research Institute for Microbial Diseases, Osaka University. The strains used were 29 strains of Kanagawa phenomenon-positive *V. parahaemolyticus*, 14 strains of Kanagawa phenomenon-negative *V. parahaemolyticus*, and 2 strains of *V. parahaemolyticus* with indeterminate reactions on Wagatsuma medium.

Medium. Tryptic soy agar, heart infusion agar, brain heart infusion agar, and nutrient agar were purchased from Difco Laboratories (Detroit, Mich.). Wagatsuma medium was obtained from Nissui-Seiyaku (Tokyo). Peptone (Difco)-NaCl agar was an agar-solidified medium prepared from a liquid medium used for preparation of the thermostable direct hemolysin of *V. parahaemolyticus* (6). It contained 1% peptone, 3% NaCl, 0.5% Na₂HPO₄, and 0.5% glucose.

Preparation of antihemolysin antiserum. The thermostable direct hemolysin was purified by the method described previously (6). Twenty micrograms of the purified thermostable direct hemolysin in 2.5 ml of 0.01 M phosphate buffer (pH 7.0) was emulsified in an equal volume of Freund incomplete adjuvant (Difco). The emulsion was inoculated intramuscularly (5 ml per rabbit) into young rabbits weighing about 2 kg. After two booster injections at days 14 and 29, antisera were obtained from three rabbits at day 45 and pooled. This antiserum gave single precipitation lines with crude hemolysin preparations of *V. parahaemolyticus* in Ouchterlony gel diffusion plates (16). A 1:16 dilution of serum gave a precipitation line with 50 μ l of a solution of 60 μ g of the purified thermostable direct hemolysin per ml in Ouchterlony gel diffusion plates. When tested in this manner, the serum was considered to have an antihemolysin titer of 1:16.

Modified Elek test. To examine the production of the thermostable direct hemolysin by *V. parahaemolyticus* strains, the classical Elek test (2) was applied with some modifications. Strains to be examined were

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inoculated on agar plates and incubated overnight at 37°C. After colony formation occurred, 50 µl of anti-hemolysin serum, diluted twofold and containing 1% NaN₃, was placed into 4-mm wells made at a distance of about 5 mm from the colony to be tested. In some cases, 25 µl of undiluted antihemolysin serum absorbed onto paper disks (Whatman AA, 6.0 mm in diameter) was placed on the agar plate near the test colony. The plate was kept at room temperature overnight and examined for precipitation line formation.

Immuno-halo test. For the immuno-halo test for thermostable direct hemolysin produced by Kanagawa phenomenon-positive strains of *V. parahaemolyticus*, a method described previously for the detection of cholera enterotoxin (4) was applied. Undiluted anti-hemolysin serum was mixed with an agar medium at a final serum concentration of 2.5%. *V. parahaemolyticus* strains to be examined were inoculated on the plate, which was then incubated overnight at 37°C. A precipitation zone was observed as a halo around colonies which produced thermostable direct hemolysin.

Test for Kanagawa phenomenon (Kanagawa test). *V. parahaemolyticus* was inoculated on Wagatsuma medium which contained 5% washed human erythrocytes. The plate was incubated at 37°C for 24 h, and hemolytic zones around the colonies were observed. Strains producing a clear hemolytic zone around the colonies were identified as Kanagawa phenomenon-positive, those with no hemolytic zone were identified as Kanagawa phenomenon-negative, and those with a very narrow hemolytic zone were identified as Kanagawa phenomenon-indeterminate.

RESULTS

Production of the thermostable direct hemolysin on various agar media. The amount of thermostable direct hemolysin produced by Kanagawa phenomenon-positive strains varied with different culture media. Among the various agar media examined, peptone-NaCl agar yielded maximal hemolysin (Table 1) when examined by the modified Elek test.

To examine optimal conditions for the production of thermostable direct hemolysin by Kanagawa phenomenon-positive strains, various concentrations of the components of peptone-NaCl agar were tested. As shown in Table 2, optimal concentrations of NaCl, Na₂HPO₄, peptone, and glucose were 4, 3, 3, and 0.5%, respectively. Thus, an agar medium containing 4% NaCl, 3% Na₂HPO₄, 3% peptone, 0.5% glucose, and 1.5% Noble agar (Difco) (pH 7.3) was designated as "Biken agar" in this paper.

Modified Elek test for *V. parahaemolyticus* thermostable direct hemolysin on Biken agar. Various strains of *V. parahaemolyticus* including both Kanagawa phenomenon-positive and -negative strains were tested with the modified Elek test on Biken agar. As shown in Table 3, the results of the modified Elek test

TABLE 1. Production of the thermostable direct hemolysin on various agar media

Medium	Kanagawa phenomenon-positive strains ^a	Kanagawa phenomenon-negative strains ^b
Wagatsuma medium	— ^c	—
Tryptic soy agar	—	—
Nutrient agar	±	—
Heart infusion agar	±	—
Brain heart infusion agar	—	—
Peptone-NaCl agar	+	—

^a Ten strains were tested. All 10 strains were alike in each medium.

^b Five strains were tested.

^c +, ±, and —, Strong, weak, and negative reactions, respectively, of each strain in precipitation reactions with the modified Elek test.

TABLE 2. Effect of various concentrations of medium components on the production of thermostable direct hemolysin^a

Component	Concn (%)	Reaction at serum dilution:		
		1:2	1:4	1:8
NaCl	1	— ^b	—	—
	2	±	±	—
	3	+	±	—
	4	+	+	±
	5	±	—	—
Na ₂ HPO ₄	0	—	—	—
	1	±	—	—
	2	+	+	—
	3	+	+	+
	4	+	+	—
Peptone	1	—	—	—
	2	±	—	—
	3	+	+	±
	4	+	±	—
Glucose	0	—	—	—
	0.5	+	+	±
	1	—	—	—

^a Composition of peptone-NaCl agar was 3% NaCl, 0.5% Na₂HPO₄, 1% peptone, 0.5% glucose, and 1.5% Noble agar; the concentration of each component alone was varied independently as indicated.

^b Ten strains of Kanagawa phenomenon-positive strains were tested with each medium. +, ±, and —, Strong, weak, and negative precipitation reactions, respectively, using the modified Elek test.

correlated well with those of the Kanagawa test. All of 29 Kanagawa phenomenon-positive strains gave positive Elek tests and all 14 Kanagawa phenomenon-negative strains gave negative Elek tests. Of the two Kanagawa phenom-

enon-indeterminate strains, one was Elek-positive and the other was Elek-negative. Typical appearances of the modified Elek test are shown in Fig. 1.

Immuno-halo tests of *V. parahaemolyticus* on Biken agar. Immuno-halo tests were carried out on Biken agar containing 2.5% antihemolysin serum as shown in Table 3. This method proved to be slightly less sensitive than the modified Elek test; thus, some Elek-positive strains (one of Kanagawa phenomenon-positive and one of Kanagawa phenomenon-indeterminate) gave no haloes around their colonies. Typical appearances of the immuno-halo reactions are shown in Fig. 2. When 0.5% instead of 2.5% antihemolysin serum was used, the results were not clear.

TABLE 3. Comparison of the results of Kanagawa test, modified Elek test, and immuno-halo test for various strains of *V. parahaemolyticus*

Condition	No. of strains
Kanagawa phenomenon (+)	
Elek (+), immuno-halo (+)	28
Elek (+), immuno-halo (−)	1
Kanagawa phenomenon (−)	
Elek (−), immuno-halo (−)	13
Elek (−), immuno-halo (±)	1
Kanagawa phenomenon (±)	
Elek (+), immuno-halo (−)	1
Elek (−), immuno-halo (−)	1

FIG. 1. The modified Elek test for detection of the thermostable direct hemolysin of *V. parahaemolyticus*. *V. parahaemolyticus* strains were grown on Biken agar. After the colonies were formed, antihemolysin serum was placed either into a well cut in the agar plate or absorbed in a paper disk and placed on the agar surface. First row (W), wells containing antihemolysin serum; second row, colonies of *V. parahaemolyticus* (+ and − represent Kanagawa phenomenon-positive and -negative, respectively); bottom row (d), Paper disks containing antihemolysin serum. Precipitation lines were observed between the colonies of Kanagawa phenomenon-positive strains and the antihemolysin serum.

DISCUSSION

The thermostable direct hemolysin produced by *V. parahaemolyticus* shows hemolytic activity (19), cytotoxicity in various cultured cells (12, 13), and lethal toxicity to mice and rats due to cardiotoxicity (3, 5, 6). The role of these biological activities of the thermostable direct hemolysin in human pathogenicity during *V. parahaemolyticus* infection has been discussed by several workers (5, 6, 10, 12, 20). It has been established that the ability to produce this hemolysin is closely correlated with the capacity for *V. parahaemolyticus* to cause human disease. Thus, the test for the Kanagawa phenomenon has been considered to be highly important from the clinical and epidemiological viewpoints, since the Kanagawa phenomenon is produced by the thermostable direct hemolysin (6, 8, 9, 15, 20).

The method routinely used for the detection of the Kanagawa phenomenon sometimes gives false-positive results (1) due to the instability of erythrocytes or to the production of other hemolysins by these organisms. Thus, the determination of the Kanagawa phenomenon is difficult, and sometimes yields misleading results. In this paper, we described two new immunological methods for the detection of the production of the thermostable direct hemolysin by *V. parahaemolyticus* strains.

The Elek test (2) was first developed for the detection of extracellular toxins produced by *Corynebacterium diphtheriae* and *Staphylococcus aureus*. Thereafter, it was applied to various

FIG. 2. The immuno-halo test for detection of the thermostable direct hemolysin of *V. parahaemolyticus*. *V. parahaemolyticus* was inoculated on Biken agar containing the antihemolysin serum. Colonies of Kanagawa phenomenon-positive strains gave haloes around the colonies. Ten positive and six negative reactions could be identified.

kinds of bacterial products such as cholera enterotoxin (17). Using the modified Elek test described in this paper, determination of the production of thermostable direct hemolysin was reproducible and simple to carry out and the results were easy to read. The sensitivity of the modified Elek test appeared to be almost equal to that of the Kanagawa phenomenon on Watatsuma medium and no false-positive results were observed.

The immuno-halo test was also useful for detection of the thermostable direct hemolysin, but it was slightly less sensitive than the modified Elek test. However, this method might be useful for testing many strains at once on the same plate.

ACKNOWLEDGMENTS

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture, Japan, and by Yamada Science Foundation. We thank J. P. Craig for help in preparing this manuscript.

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