

## RAPID IDENTIFICATION OF *Vibrio cholera*-01 BY COAGGLUTINATION TEST USING MONO-SPECIFIC ANTIBODY

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### ABSTRACT

Mono-specific antisera against *Vibrio cholera* Ogawa NIH-43 and *Vibrio cholera* Inaba 35-A3 were prepared from rabbit hyperimmune sera by absorbing against a heterologous strain. Using ammonium sulphate precipitation procedure, gamma globulins were purified and concentrated.

To visualize antigen-antibody reaction, gamma globulins were conjugated to *Staphylococcus aureus* cowan-1 (NCTC: 8325) in the presence of 50% propanol-1. Then equal suspensions of each conjugated serum were mixed to prepare *V. cholera*.

Rectal swab samples from suspected choleric patients were inoculated in bile peptone broth for 5 hours at 37°C. One drop of each sample was mixed with one drop of VBCR and coagglutination was read at 2-3 minutes. The results were compared with corresponding results obtained from conventional culture methods. Specificity and sensitivity of coagglutination tests were found to be 98.03% and 95.1%, respectively.

Regarding the fact that rapid diagnosis of cholera is vital to save patients, our study reveals that coagglutination test, using bivalent mono-specific antisera, can be considered as a simple, rapid and reliable test to detect *V. cholera*-01 from stool samples of suspected patients.

**Keywords:** *Vibrio cholera*, *Vibrio cholera* bivalent mono-specific coagglutination reagent (VBCR), Classical culture method.

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### INTRODUCTION

Cholera still remains an important medical problem in developing countries.<sup>1</sup> Recent outbreaks of cholera in Iran proves this claim. Although infection in adults often causes

mild diarrhea, serious sequelae including rapid death may occur in children below six years.<sup>2</sup> Therefore, during the

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## V. Cholera Identification by Coagglutination

outbreaks, particularly in endemic areas lacking specialized laboratory facilities, rapid detection of etiological agents is of great diagnostic importance.

Routine diagnostic tests for cholera are time-consuming, irrespective of technological requirements. Thus a rapid and reliable test seems to play a vital role in the prevention and treatment of cholera, particularly in areas lacking specialized facilities. Kronvall et al. in 1973 demonstrated the potential usefulness of coagglutination test as a method for identification of *Streptococcus pneumoniae*. Then using polyvalent antisera, Rahman et al. in 1984 claimed that this method can be equally effective for rapid identification of *V. cholera*-01 in stool samples of suspected patients. Later this finding was accepted by many other investigators as well.<sup>3-13</sup> But serological cross-reactivities of *V. cholera*-01 with many members of *Enterobacteriaceae* limits its applications.<sup>14</sup> Therefore in this study, to overcome this drawback, purified mono-specific antisera against *V. cholera* Inaba 35-A3 and *V. cholera* Ogawa NIH-43 were used to conjugate *Staph. aureus* cowan-1. The coagglutination test showed 98.03% specificity and 95.1% sensitivity when compared with classical culture diagnostic tests.

### MATERIALS AND METHODS

#### Standard strains

Two strains of *V. cholera* Ogawa NIH-43, *V. cholera* Inaba35-A3 and *Staphylococcus aureus* cowan-1 (NCTC: 8325) were used to prepare mono-specific antisera and coagglutination antigen, respectively.

#### Maintenance of strains

All the strains used in this research were propagated in appropriate media such as BHIA (brain heart infusion agar) and CCYA (casein yeast extract agar). A thick suspension of each microorganism was lyophilized and kept as stock culture. Working stock cultures were prepared by growing the strains in medium in screw-capped tubes. All the stock cultures were kept at 4°C until use.

#### Lab animals

New Zealand white female rabbits, weighing 1.8-2 kgs and aged 4 months were used for production of antisera

Table I. Percentage binding capacity of antibodies to *Staph. aureus* cowan-1 subjected to different chemical fixatives.

Chemical fixatives	Time of treatment (hours)	Ogawa antiserum binding %	Inaba antiserum binding %
50% Propanol-1	0.50	*ND	78.50
	3.00	83.00	82.50
0.5% Glutaraldehyde	0.50	57.00	73.17
	3.00	59.25	73.70
0.5% Formaldehyde	0.50	ND	78.30
	3.00	ND	75.70

\*ND: not detected

against different serotypes of *V. cholera*-01.

#### Preparation of antigens

**Injecting antigen:** This was prepared from a thick suspension of each *V. cholera* strain in 5% carbolic acid saline solution heated one hour at 60°C. The inactivated cells were washed and diluted to the final concentration of  $9 \times 10^8$  cells per mL (540 nanometer wavelength in spectrophotometry).

**Titrating antigen:** This was prepared by the same procedure as the injecting antigen, but using 0.5% formaldehyde to inactivate bacteria.

**Absorbing antigen:** The absorbing antigen was prepared by suspending *V. cholera* strain in PBS (pH= 7.2) containing 20% ethanol and 0.5% formaldehyde, kept at 60°C for one hour, washed and used for absorption of contaminated immunoglobulins.

**Coagglutination antigen:** *Staphylococcus aureus* (NCTC: 8325) was harvested from CCYA medium after 24 hours at 37°C by 0.15 mol PBS (pH= 7.2). To increase the fixation frequency, a variety of fixatives including 0.5% formaldehyde, 0.5% glutaraldehyde and 50% propanol-1, were added to the suspension, and kept at 80°C on a rotary shaker for 5 min. Using Hopkins' tube, a cell suspension of 10% cpv\* was prepared on PBS (pH= 7.2), then stained by

\*Cell-packed volume

Table II. Comparison of the results obtained from classical culture method and coagglutination test.

Test	Total No. of samples	Negative cases	Positive cases	Not detected -ve cases	Not detected +ve cases	% Specificity	% Sensitivity
Classical culture	206	102	104	Nil	Nil	100	100
Coagglutination test	206	100	99	2	5	98.03	95.1

Lugol's solution and 1% methylene blue for better visualization of agglutination.

**Negative control:** 2% suspension of *Staphylococcus aureus* cowan -1 (NCTC: 8325) was fixed by propanol-1 and heated at 80°C for 5 min, and then subjected to normal rabbit gammaglobulin to block the mono-specific sites on the cell surface. The same suspension lacking the coating procedure was used as a second negative control.

**Positive control:** A cell suspension containing  $5 \times 10^8$  *V. cholera*, Inaba & Ogawa strains in PBS (pH= 7.2) was prepared as positive control.

#### Preparation of antisera

Before immunization, rabbits were examined for probable presence of natural antibodies against *V. cholera* and *Staph. aureus*. Those with a titer of less than 40 were selected for antiserum preparation.

#### Schedule of immunization

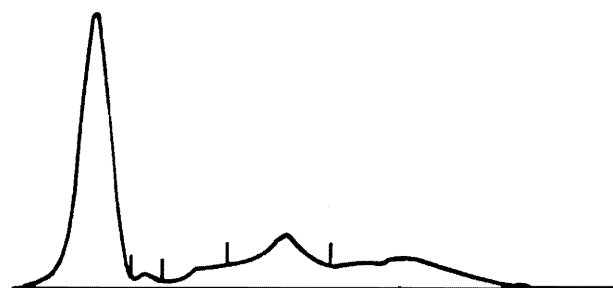
Selected rabbits were injected via marginal ear vein on the first, third, fifth and seventh days with 0.5, 1, 1.5 and 2 mL of  $9 \times 10^8$  cells/mL of antigens followed by the injection of 2.5 and 3 mL of the same antigens on days 14 and 16 respectively, as boosters. The sera were obtained after 10 days of rest by terminal bleeding from the heart. Sera showing titers more than 600 were pooled and stored at -20°C.

#### Absorption of contaminated antibodies

The titers\* of homologous antibodies were 2560 and 640 against *V. cholera* Ogawa and *V. cholera* Inaba strains, and after absorption with heterologous serotype, the titers dropped to 1280 and 320, respectively. There was no titer against any different *V. cholera* serotype. Therefore by absorption, 100% of non-specific antibodies were removed from each polyclonal antibody. The mono-specific antibodies were further purified and concentrated with 45% saturated ammonium sulphate precipitation technique.

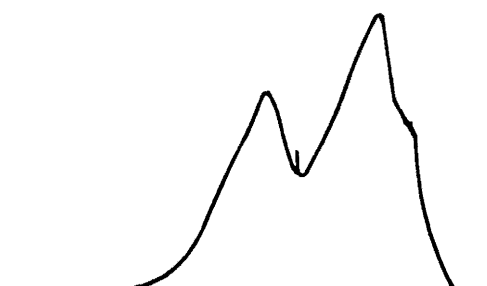
In Figs. 1 and 3, five peaks of albumin and alpha-1, alpha-2, beta and gamma globulin are observed from mono-specific antisera, while after purification peaks of albumin were absent and gamma globulin levels were 67.6% and 71.3% for *V. cholera* Ogawa and *V. cholera* Inaba mono-specific antibodies, respectively (Figs. 2 and 4).

To increase binding capacity of the antibodies to surface protein-A of *Staph. aureus*, different chemical fixatives such as formaldehyde, glutaraldehyde and propanol-1 were used in different concentrations and timing. As observed in Table I, the best conjugation level was achieved by subject-



	%	g/dL
Albumin	55.7	3.0
Alpha 1	1.5	0.1
Alpha 2	5.3	0.3
Beta	19.1	1.0
Gamma	18.4	1.0
Total		5.4

Fig. 1. Curve showing the principal proteins of rabbit serum, injected by Ogawa antigen after absorption by Inaba antigen.



	%	g/dL
Beta	32.4	1.0
Gamma	67.6	2.2
Total		3.2

Fig. 2. Curve showing the Ogawa mono-specific globulins after purification.

ing the reagent to 50% propanol-1 at room temperature for 3 hours which yielded more than 83% and 82% of binding for *V. cholera* Ogawa and *V. cholera* Inaba antibodies, respectively. Moreover, the test was performed with different concentrations of VBCR and it was found that 2% cell packed volume of the stained reagent suspended in 0.15 M PBS (pH= 7.2) is the most suitable concentration of VBCR for coagglutination test.

Rectal swab samples of 206 choleric patients and normal persons were collected from 4 recognized health centers in Tehran and Qom. The swabs were incubated in 2 mL of bile peptone broth (pH= 8.6) at 37°C for 5 hours. The cultures were examined by classical culture method and coagglutination test as well. As observed in Table II, out of

\*Titer is expressed as reciprocal of the highest dilution of antisera showing +2 agglutination with the antigen, using tube agglutination test.

## V. Cholera Identification by Coagglutination

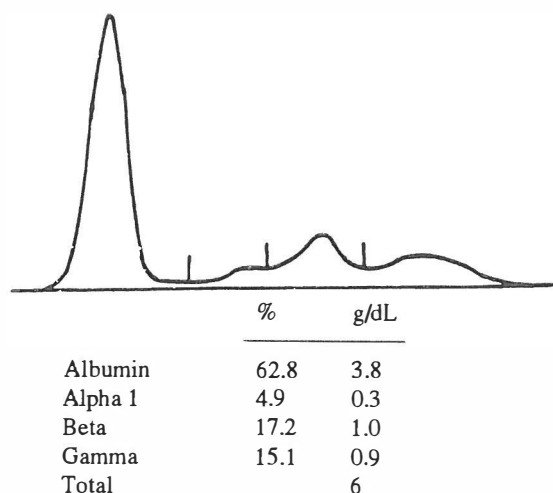


Fig. 3. Curve showing the principal proteins of rabbit serum, injected by Inaba antigen after absorption with Ogawa antigen.

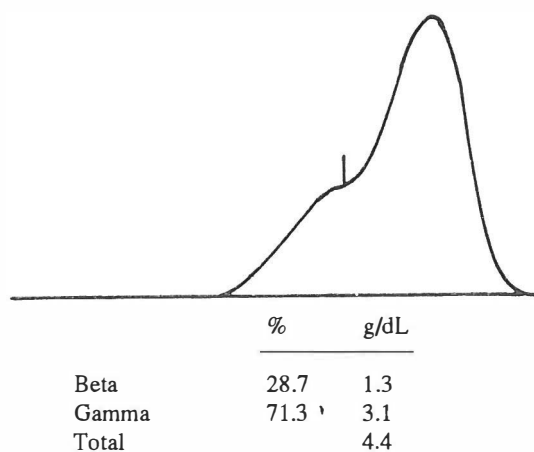


Fig. 4. Curve showing Inaba mono-specific globulins after purification.

102 negative cases and 104 positive cases with cultural method, only two false positives and 5 false negatives were encountered using coagglutination test. Therefore, the specificity and sensitivity of coagglutination test was calculated to be as high as 98.03% and 95.1%, respectively.

Stability of the reagent was checked by keeping VBCR at 4°C and performing coagglutination test at 0, 1, 2, 3, 4, 5 and 6 month intervals using standard *V. cholera*-01. In this regard, no autoagglutination or reduction of titer levels was observed.

## DISCUSSION

The coagglutination test seems to be highly valuable in the diagnosis of many infectious diseases,<sup>5-9</sup> including the rapid detection of cholera.<sup>4,12,13</sup>

The present research aims to increase the diagnostic

potential of this test by using purified pooled mono-specific antisera of *V. cholera* Ogawa NIH-41 and *V. cholera* Inaba

2% Cell-packed volume of *V. cholera* Ogawa & Inaba were mixed with heterologous antisera separately, and kept at 37°C for 4 hrs, then shaken at 140 rpm for 16 hours at 4°C. The cells were centrifuged and the supernatant was used as mono-specific serum.

## Purification of mono-specific gammaglobulins

Mono-specific gammaglobulins were purified from the sera by 45% ammonium sulphate.<sup>15</sup> After centrifugation, the sediment was dissolved in a minimum volume of PBS (pH=7.2) and dialyzed against distilled water by three changings for 48 hours at room temperature.

## Measurement of proteins

The protein was measured quantitatively and qualitatively using biurate and cellulose acetate electrophoresis technique (Helena, France 95320).

## Antiserum-staphylococcus conjugate

In 1 mL of 10% suspension of (v/v) fixed *Staph. aureus*, 0.2 mL *V. cholera* Ogawa or *V. cholera* Inaba mono-specific antisera was added (titer: 160) and kept on a shaker at 120 rpm for 3 hrs at room temperature, rinsed twice with 0.15 mol PBS containing 0.1% sodium azide, then a suspension of 2% (v/v) was made. This suspension was denoted as *V. cholera* coagglutination reagent. The suspension was incubated at 37°C, 30 min, with normal rabbit gammaglobulin to increase the test specificity and the non-occupied spaces on the cell surface of staphylococci. After washing the reagents with PBS, equal suspensions of both Ogawa and Inaba VBC reagents were mixed and used as bivalent VBCR.

## Coagglutination test

To enhance the growth of *V. cholera* in the specimens, rectal swabs were cultured in tubes of peptone water containing sodium thiochololate. The tubes were sustained for 5 hrs at 37°C. The presence of *V. cholera*-01 was examined by classical culture method and coagglutination test as well. One drop of each positive and negative control was transmitted to a clean slide and mixed with a drop of VBCR. The tests were observed for agglutination after 2-3 minutes. A test was considered positive if agglutination appeared clearly with positive control and no reaction occurred with negative control.

## RESULTS

Antigens used in preparation of homologous antisera and their absorption were heat-killed whole cells of *V. cholera* Ogawa NIH-41 and *V. cholera* Inaba 35-A3 accord-

ing to WHO recommendations, and coagglutination antigen was prepared from *Staph. aureus* cowan-1 (NCTC: 8325). 35-A3 (Figs.1-4) to bind *Staph. aureus* cowan-1 for preparation of *V. cholera* bivalent mono-specific coagglutination reagent (VBCR). Besides, the percentage rate of conjugation was increased by treatment of the reagent with different chemical fixatives such as formaldehyde, glutaraldehyde and propanol-1 at different concentrations and time intervals.<sup>17</sup> As shown in Table I, 82.8% binding efficacy was achieved by subjecting the reagent to 50% propanol-1 for 3 hours. This finding was evaluated by single radial immunodiffusion technique.

To standardize the technique, the coagglutination test was performed with different concentrations of coagglutination reagent and it was found that 2% cell packed volume of the stained VBCR suspension in 0.15 M PBS (pH= 7.2) is the most appropriate concentration of reagent.

Regarding the clinical evaluation of our modified coagglutination reagent, 206 rectal swab samples were collected from choleric patients and normal persons and were examined through both classical culture method and coagglutination test. As seen from Table II the coefficient of confidence using our highly sensitive VBCR is as high as 98.03% specificity and 95.1% sensitivity. This result is quite close to the culture method with 100% specificity and sensitivity, but with the advantage that the coagglutination test is an easy technique, giving results in only 5 hours after collection of the samples.

Since rapid detection of *V. cholera*-01 in stool samples of suspected choleric patients is an important factor to prevent mortality and morbidity, the present research reveals that by using our highly sensitive reagent (VBCR), the coagglutination test can be regarded as a simple, reliable and rapid method to detect *V. cholera*-01 among stool samples of suspected patients.

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#### REFERENCES

- Murray PR: Vibrionaceae and campylobacter. In: Drow WL, Kobayashi GS, Thompson JH, (eds.), Medical Microbiology. St. Louis: The C.V. Mosby Company, p. 128, 1990.
- Charles CJ: Cholera. In: Hoeprich PD, Colin JM, (eds.), Infectious Diseases. J.B. Lippincott Company, p. 725, 1989.
- Kronvall GA: Rapid slide-agglutination method for typing pneumococci by means of specific antibody adsorbed to protein A-containing staphylococcus. J Med Microbiol 6: 187-190, 1973.
- Jesudasan MV, Thangavelu CP, Lalitha MK: Rapi screening of fecal sample for *Vibrio cholera* by coagglutination technique. J Clin Microbiology 19: 712-713, 1984.
- Ram S, Khurana S, Khurana SB: Coagglutination test for detection of heat-labile enterotoxin-producing *Escherichia coli* strains. Indian J Med Res 97: 64-66, 1993.
- Gottschalk M, Higgins R, Boudreau M: Use of polyvalent coagglutination reagents for serotyping of *Streptococcus suis*. J Clin Microbiol 31(8): 2192-4, 1993.
- Katsukawa C, Harada K, Makino M: M typing of *Streptococcus pyogenes* by latex agglutination using monoclonal antibody. Kansenshogaku Zasshi 68(5): 706-11, 1994.
- Dhanalakshmi D, Mallika M, Rumaravel BM, Lashminarayana CS: Detection of *Salmonella typhi* antigens by slide coagglutination in urine from patients with typhoid fever. Indian J Pathol Microbiol 27: 33-36, 1984.
- Edwards EA, Hildebrand RL: Method for identifying salmonella and shigella directly from primary isolation plate by coagglutination of protein A-containing staphylococci sensitized with specific antibody. J Clin Microbiol 3: 339-343, 1976.
- Lesmana M, Rockhill RC, Sutani D, Sutomo A: A coagglutination test to detect *Vibrio cholera* in feces alkaline peptone water cultures. J Trop Med 13: 337-339, 1982.
- Lalitha MK, Sridharan G, Mercy J: Coagglutination for diagnosis of bacterial infection. Indian J Pediatr 56: 327-333, 1989.
- Mahbubar R, Daivid A, Salehmahmood S, Anowar H: Rapid diagnosis of cholera by coagglutination test using 4-h fecal enrichment cultures. J Clin Microbiology 25: 2204-2206, 1987.
- Rahman M, Sack DA, Wadood A, Yasminmandlatif A: Rapid identification of *Vibrio cholera* serotype 01 from primary isolation plates by coagglutination test. J Med Microbiol 28: 39-41, 1989.
- Winkle S, Refac M, Rode R: On the antigenic relationship of *Vibrio cholera* to *Enterobacteriaceae*. Ann Inst Pasteur 123: 775-781, 1972.
- Hudson L: The basic technique. In: Hay FC, (ed.), Practical Immunology. Blackwell Scientific Publications, pp. 1-2, 1989.
- Koneman W, Allen D, Dowell VR, Winn C, Lisa A: Diagnostic Microbiology. J.B. Lippincott Company, pp. 209-214, 1988.
- Gaganth C, Shegl S: Enhancement of the antigen binding capacity of incomplete IgG antibodies to *Brucella melitensis* through Fc region interactions with staphylococcal protein A. J Immunological Methods 124: 251-257, 1989.