Asian Pacific Journal of Allergy and Immunology (1994) 12 : 155-159

A Monoclonal Antibody-Based Dot-Blot ELISA Diagnostic Kit for the Detection of *Vibrio cholerae* 01 in Stools of Diarrheic Patients and Household Contacts

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SUMMARY **A "cholera diagnostic kit" was developed for sensitive, specific, rapid, and inexpensive detection of *Vibrio cholerae* 01. The monoclonal antibody specific to antigen A of *Vibrio cholerae* 01 was used as an antigen detection reagent and the principle of dot-blot ELISA was adopted. The kits were used in seven Regional Medical Sciences Centres, Ministry of Public Health, located at various regions of Thailand where diarrhea occurs frequently. Diagnostic efficiency of the kits in the detection of *Vibrio cholerae* 01 from rectal swabs of the diarrheic patients and their household contacts was evaluated in comparison with the conventional culture method. The two methods were found to have excellent degree of agreement (kappa values >95%). The dot-blot ELISA has several advantages over the culture methods, ie rapid (dot-blot ELISA takes 1-2 hours while the culture method takes at least two days) and inexpensive. It requires no sophisticated equipment. The procedure is not complicated thus it is easy to train personnel. The diagnostic kits are recommended for use in the detection of severe diarrhea caused by *V.cholerae* 01 not only in hospitals and health centres where adequate treatment of the patients is required as a life-saving measure but also for early recognition of cholera cases and their contacts so that other action, ie prevention and control of outbreaks and surveillance can be promptly implemented.**

Diarrhea caused by *Vibrio cholerae* serogroup 01 remains a major public health problem in developing countries. However, epidemics of the disease occur also in developed part of the world.[1] The clinical spectrum of the disease is broad, ranging from inapparent infection, mild to severe diarrhea.[2] The inapparent infection or the mild form is important epidemiologically as the infected individuals may be unrecognized and may serve as a source of an explosive outbreak or even a pandemic of the disease.[3] For severe cases, prompt and adequate treatment is required or shock and death of the patients may ensue.

The conventional method for diagnosis of diarrhea caused by *V.cholerae* serogroup 01 is usually culture.[4] The technique is laborious, time consuming, expensive, insensitive and can be performed only in a well-equipped microbiological laboratory. The culture facilities are often not available in remote areas of developing countries where severe diarrhea occurs frequently among the populations. In this situation, faeca. specimen must be put in a suitable transport medium, ie Cary-Blair transport medium and sent to an available bacteriology laboratory. The sample from the transport medium would then be enriched with a suitable enrichment medium, ie alkaline-peptone solution for at least 6-8 hours before plating onto a selective medium and incubated overnight. Growing colonies are subsequently tested biochemically and serologically using various reagents and media and specific antisera, respectively. The whole process of the culture method takes at least 36 to 72 hours and by that

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time the disease may have spread widely if the clinicians or other personnel involved are inexperienced. Thus, a rapid, specific, simple and economic diagnostic method is required for the prevention and control of the disease and for disease surveillance.

Recently, a monoclonal antibody (MAb) specific to the antigen A of *V.cholerae* serogroup 01 has been produced.[5] The MAb reacted with all *V.cholerae* serogroup 01 and did not react with other serogroups of *V.cholerae,* all other vibrios, bacteria or intestinal parasites.[5] This MAb was used in a dot-blot ELISA for detecting *V. cholerae* serogroup 01 in rectal swab specimens, enriched in the alkaline-peptone solution, of diarrheic patients. Specificity of the assay was found to be 100% and sensitivity was 96.0%. The lowest number of *V.cholerae* serogroup 01 which could be detected by the assay was as low as 3,000 vibrios (10^6/ml). Since this number of vibrios was frequently found in the stools of most patients with severe diarrhea, the dot-blot ELISA would be able to directly detect the bacteria in the clinical specimens. For the few patients excreting lesser number of the vibrios,[7] the specimens can be put in alkaline-peptone solution during transportation and upon arrival, the number of vibrios would be sufficient to give a positive dot-blot ELISA.[5] Furthermore, the whole process of the immunological test did not take longer than 2 hours and the cost per specimen was a lot less than the culture method.[5]

In this report, a cholera diagnostic kit based on the specific MAb to antigen A of *V.cholerae* serogroup 01 and the dot-blot ELISA was developed.[5] The kits were sent to seven Regional Medical Sciences Centres (RMSCs), Department of Medical Sciences, Ministry of Public Health, Thailand, located at various regions of the country, ie Northern region (Chiang Rai, Chiang Mai, and Phitsanulok Provinces), Northeastern region (Nakorn Ratchasima Province), Central region (Chon Buri Province), and Southern region (Trang and Songkhla Provinces). The efficiency of the kits for rapid and specific detection of *V.cholerae* serogroup 01 in stool specimens of diarrheic patients and household contacts was evaluated under field conditions in comparison to the conventional culture method.

MATERIALS AND METHODS

Specimens

Rectal swabs obtained from 211 diarrheic patients and 415 household contacts were put individually in separate tubes each containing 5 ml of alkaline-peptone solution and sent back to the local RMSCs immediately. This usually took less than 4 hours. Upon arrival, small aliquots of the alkaline-peptone solution from each tube were boiled for 15 minutes and these samples were tested by the dot-blot ELISA using the cholera diagnostic kits. The untreated samples were inoculated onto Thiosulphate Citrate Bilesalt Sucrose (TCBS) agar for *V.cholerae* serogroup 01 isolation and identification using the conventional method.[4]

Monoclonal antibody

The MAb specific for antigen A of *V.cholerae* serogroup 01 derived from the clone 27E10 were produced and maintained at the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok.[5] The culture supernate (2,560 indirect ELISA units per ml)[6] was diluted to 80 indirect ELISA units/ml in 0.5 M Tris-HCl pH 7.5 containing 0.15 M NaCl and 0.05% Tween 20. This diluted MAb preparation was used as a specific probe in the cholera diagnostic kits.

Cholera diagnostic kit

Each cholera diagnostic kit consists of: (1) washing buffer (0.1 M Tris-HCl, pH 7.5); (2) blocking reagents (0.5% bovine serum albumin in phosphate bufferred saline, pH 7.4); (3) 50 ml of specific MAb (80 ELISA units/ml); (4) rabbit anti-mouse immunoglobulin-alkaline phosphatase conjugate (Dakopatts, Denmark) diluted 1:1,000 in PBS, pH 7.4 containing 0.2% bovine serum albumin and 0.2% gelatin; (5) substrate solution which contained 5 mg of nitroblue tetrazolium and 2.5 mg of 5-bromo-4-chloro-3-indolyl phosphate in 20 ml of 0.1 M Tris-HCl containing 0.1 M NaCl and 50 mM $MgCl_2$; (6) a plastic box (8 cm × 11 cm × 3 cm) which contained the following; (a) a nitrocellulsoe sheet with 50 circles with 7 mm in diameter; (b) one sheet of Whatman filter paper no. 3 (8 cm × 11 cm); (c) 500 µl of whole cell lysate of *V.cholerae* serogroup 01 at a concentration of 1 mg per ml prepared as previously described[6] which served as a positive control antigen; and (d) 0.5 ml of medium used for culturing the Sp 2/0 myeloma cells, which served as a negative control antigen. An instruction manual for performing the dot-blot ELISA was also included in each kit.

MAb-based dot-blot ELISA

The test was performed as previously described by Chaicumpa *et al.*[5] using the reagents and materials supplied in the cholera diagnostic kit. In brief, three µl of the boiled samples were placed individually on each circle of the provided nitrocellulose sheet. Three µl of the positive and negative controls were placed on circles no. 49 and 50, respectively. The sheet was air-dried and then soaked in the blocking solution in the supplied plastic box for 10 minutes with occasional shaking. The blocking solution was poured off and the sheet was washed three times with

the washing solution in the same box. After the last wash, the sheet was allowed to react with the specific MAb to antigen A of *V.cholerae* serogroup 01 for 10 minutes, washed as above and then put in the rabbit anti-mouse immunoglobulin-alkaline phosphatase conjugate for 10 minutes. After washing to remove the excess conjugate, the sheet was allowed to react with the substrate solution for 10 minutes. The enzyme-substrate solution was stopped by rinsing the nitrocellulose sheet with distilled water. The sheet was placed on the Whatman filter paper and the reaction at all circles was examined. Positive reaction appeared as a purplish blue spot while the negative reaction appeared as clear area on the circle.

Statistical analysis

Sensitivity, sepcificity, efficacy and predictive values of the dot-blot ELISA were evaluated in comparison with the culture method using the method of Galen.[8] Kappa coefficient[9] was used to measure the degree of agreement of the association between the outcome of cholera diagnosis using the dot-blot ELISA and the culture method. The probability of kappa introduced by Fleiss[10] was also used to indicate the extent of agreement beyond chance of the culture and the dot-blot ELISA. In the case that the probability of the kappa was statistically significant, the degree of agreement of the two tests (culture *vs* dot-blot ELISA) did not occur by chance (beyond chance). In other words, a correlation between the outcomes of the two tests exists. Landis and Kock[11] have characterized different ranges of values for kappa with respect to degree of agreement. They suggest for most purposes, kappa values greater than 75% or so may be taken to represent "excellent" agreement beyond chance while values below 40% may be taken to represent "poor" agreement and values between 40% and 75% represent "fair" to "good" agreement beyond chance of the two tests.

RESULTS

Results of tests performed on rectal swabs of 211 diarrheic patients and 415 household contacts by conventional method and dot-blot ELISA are shown in Tables 1 and 2. It was found that 13 of 211 rectal swab samples from diarrheic patients were positive by both the dot-blot ELISA and the culture method. One of the 198 culture negative samples was positive by the dot-blot ELISA. However, subculture of the specimen after 8 hours of enrichment in alkaline-peptone solution yielded positive results by both methods. Thus, the diagnostic sensitivity and specificity, positive and negative predictive values, and efficacy of dot-blot ELISA[8] were the same, ie 100%. The kappa coefficient value[9] was also 100% indicating "excellent" agreement (beyond chance) of the two tests. However, 1 of 21 culture positive rectal swabs of 415 household contacts was negative by dot-blot ELISA. The remaining 394 specimens were negative by the two methods. Thus, the diagnostic sensitivity and specificity, positive and negative predictive values and efficacy

Table 1. Results of *V.cholerae* 01 detection from rectal swabs of diarrheic patients by dot-blot ELISA and culture method.

	Dot-blot ELISA		Total
	Positive	Negative	
Culture positive	14*	0	14
Culture negative	0	197	197
Total	14	197	211

*one sample was culture positive after subculture at 8 hours of enrichment in the alkaline peptone solution.

Table 2. Results of *V.cholerae* 01 detection from rectal swabs of household contacts by dot-blot ELISA and culture method.

	Dot-blot ELISA		Total
	Positive	Negative	
Culture positive	20	1	21
Culture negative	0	394	394
Total	20	395	415

Table 3. Statistical interpretations of the dot-blot ELISA and the culture method.

	Rectal swabs	
	Diarrheic patients	Household contacts
Sensitivity	100.0%	95.2%
Specificity	100.0%	100.0%
Positive predictive value	100.0%	100.0%
Negative predictive value	100.0%	99.7%
Efficacy	100.0%	99.8%
Kappa value	100.0%	95.1%
Statistical interpretation	"excellent" degree of agreement between the two tests beyond chance	"excellent" degree of agreement between the two tests beyond chance

p value <0.001 at $\alpha = 0.05$

of the dot-blot ELISA[8] were 95.2%, 100%, 100%, 99.7%, and 99.8%, respectively. The two methods showed "excellent" agreement (beyond chance) as the kappa coefficient value[9] was 95.1%. The overall statistical interpretations of the dot-blot ELISA and the culture method are shown in Table 3.

DISCUSSION

The present study revealed that the dot-blot ELISA can be used for rapid, sensitive, and specific detection of *V.cholerae* 01 in rectal swabs of diarrheic patients as well as their household contacts in the field condition, ie at the RMSCs. The dot-blot ELISA results have shown statistically excellent agreement with the culture method. This study confirmed and extended our previous work[5] which utilized the dot-blot ELISA for detection of the organism in the stools and rectal swabs of patients with acute diarrhea and in seafood samples. It was found that the kappa value of the previous study was 94.7% compared with 100% in the present study. This may be attributable to the readiness and standardization of the reagents in the test kit in the present study. The possible explanation for the dot-blot ELISA positive result in one of the pre-enriched, culture negative rectal swab sample from a diarrheic patient is that too few living *V. cholerae* 01 were present at the time of culture. These few vibrios then multiplied during the 8 hours of enrichment, thus giving a positive result in the subsequent cultivation. The dot-blot ELISA could detect both living and dead *V.cholerae* 01 cells, indicating that the test has a high level of sensitivity.

The present study showed that the dot-blot ELISA diagnostic procedure is sensitive and specific and has high potential to be used under field conditions. The whole procedure takes less than two hour period compared to a few days required for the culture method. It is inexpensive, ie its cost is approximately one-fourth of that of the culture method. It should also be noted that the detection of *V.cholerae* using the dot-blot ELISA diagnostic kit was carried out by local technicians at seven RMSCs in various parts of Thailand. These technicians received training only during a two day workshop in Bangkok. Thus, it can be said that the dot-blot ELISA procedure is relatively simple to perform but highly reproducible.

Currently several monoclonal antibodies specific to various human pathogens, ie *Salmonella typhi, Opisthorchis viverrini, Bordetella pertussis,* are being produced in our laboratory. These monoclonal antibodies will be further developed and built into diagnostic test kits for detection of the corresponding pathogens in the near future.

ACKNOWLEDGEMENTS

The authors thank the scientists of the Regional Medical Sciences Centres for their cooperation. Our appreciation is extended to the organizing committee of the "Workshop on rapid detection of cholera" held during 21-22 March 1994 which introduced the application of the test kit.

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