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# Diagnosis of Enteric Fever Caused by *Salmonella* spp. in Vietnam by a Monoclonal Antibody-Based Dot-Blot ELISA

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**SUMMARY** **Enteric fever caused by *Salmonella* spp. is prevalent in Vietnam. None of the currently available diagnostic methods meets the ideal criteria on rapidity, simplicity, sensitivity, specificity, cost-effectiveness and practicality for developing areas. In this study, a recently developed monoclonal antibody-based dot-blot ELISA was used in comparison with the hemoculture method and the classical Widal test for diagnosis of salmonellosis in 171 Vietnamese patients presenting with clinical features of enteric fever. Urine samples of 50 healthy counterparts were used as negative controls. *Salmonella* spp. were isolated from 77 of 171 patients (45%) while 98 and 111 patients were positive by dot-blot ELISA and Widal test, respectively. The diagnostic sensitivity, specificity, accuracy, positive predictive value and negative predictive value of the ELISA performed on three serial urine samples collected at 2 hour intervals of the 171 patients were 92.2%, 71.3%, 80.7%, 72.4% and 91.8%, respectively when compared with the culture method. The Widal test performed on acute and convalescence serum samples showed 87.0%, 46.8%, 68.4%, 60.4% and 83.3% diagnostic sensitivity, specificity, accuracy, and positive and negative predictive values, respectively when compared with the bacterial culture method. Kappa coefficience revealed very good agreement beyond chance between the MAb-based ELISA and the culture method. The ELISA was not reactive when tested on urine samples of 50 healthy individuals which indicates 100% specificity. The *Salmonella* antigenuria of the patients as detected by ELISA lasted 10.3 ± 3.9 days after initiating antibiotic treatment. The MAb-based dot-blot ELISA is easy to perform. It is rapid, sensitive, specific, inexpensive, and non-invasive and does not require equipment, thus is suitable for developing areas. It can detect acute/ recent infection and can be used for evaluation of the efficacy of the treatment.**

In the past, enteric fever caused by *Salmonella* spp. was easily recognized by virtue of its typical clinical features including prolonged fever, abdominal pain, hepatosplenomegaly, dull conciousness, diarrhea, dehydration and rose spots.[1,2] Since the 1970s, however, the diagnosis has become difficult as most cases are presented with pyrexia of unknown origin. In Vietnam, diagnosis of typhoid fever is frequently made solely on clinical grounds. Laboratory confirmation depends on the isolation of *Salmonella* spp. from blood, urine or stool of the patient. However, in small hospitals, facilities for culture and isolation are often not available. Moreover, even when appropriate cultures are performed, false negatives due to prior antibiotic treatment are not infrequent.[3] Presumptive bacteriological results often require two or three days and confirmation by biochemical and serological testing requires even longer. Although several serological assays for diagnosis of typhoid have been developed, the most widely used method is still the classical Widal test, which is an inexpensive and simple agglutination method. The sensitivity and specificity of the test is sometimes doubtful while the

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detection of rising antibody titers is too slow to allow quick decision for treatment by a clinician.[4,5]

Clearly, there is a need for simple, rapid, sensitive and specific test for diagnosis of typhoid fever in the developing areas. For this purpose, several rapid methods have been developed including radioimmunoassay,[6,7] enzyme-linked immunosorbent assay,[8-12] hemagglutination,[13] slide agglutination test,[14,15] counter immunoelectrophoresis test,[16-18] DNA probe[19,21] and PCR.[22,23] However, these tests have been hampered by the lack of specificity, technical complexity, high cost among various other drawbacks. Thus none of them has replaced the blood culture and the Widal test as yet. Recently, a dot-blot ELISA for diagnosis of typhoid fever using monoclonal antibodies specific to antigen 9 of *Salmonella* spp. as a diagnostic reagent was developed.[24] The test was found to have 95% sensitivity and 100% specificity when tested on serially collected urine samples of Indonesian patients suspected of typhoid fever. Monoclonal antibodies which reacted to core polysaccharide of salmonellae and did not react to other enteric pathogens were also produced.[25,26] In this paper, the two monoclonal antibodies, to *Salmonella* antigen 9 and to core polysaccharide were used in a dot-blot ELISA for rapid, sensitive and specific diagnosis of enteric fever in Vietnamese patients. The efficacy of the assay was evaluated in comparison with the hemoculture and the Widal methods.

## MATERIALS AND METHODS

### Subjects

A cross sectional study was conducted from April to October 1996 at Phuly Hospital and Institute for Clinical Research in Tropical Medicine (ICRTM) which are located in the north of Vietnam. Patients included in the study were 15 years or older and of both sexes. Individuals suspected of having enteric fever were interviewed and examined. Minimal criteria required for clinical diagnosis of enteric fever were: history of fever for at least five days, a body temperature at admission equal to or higher than 38.5°C and at least two of the following clinical features being presented, *ie.* abdominal pain, diarrhea, hepatomegaly, splenomegaly. One hundred and seventy-one patients meeting the clinical criteria were included in the study. Three serial urine samples collected at two hour intervals were obtained from each patient before commencing the antibiotic treatment. Hemocultures (3 times) were also performed accordingly. The Widal test was done on the acute and convalescent serum samples of all patients. The patients were subsequently treated with 500 mg ciprofloxacin orally twice daily for 5 days. When recovered, they were discharged from the hospital but were asked to return for follow up every week for a period of about one month. Three urine samples collected at 2 hour intervals were obtained from each patient every time they revisited the hospital. Single urine specimens from 50 healthy individuals who had no history of enteric fever and typhoid vaccination and who were culture negative for *Salmonella* served as negative controls. All urine samples were coded and sent to the laboratory at the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok where they were stored at -20°C until assayed for *Salmonella* antigens by monoclonal-antibody based dot-blot ELISA in November 1996 to January 1997.

### Antigen preparation

Lipopolysaccharide (LPS) and whole cell lysate (Ly) were prepared from *S. typhi* strain O901 as described previously.[25] They were used as positive controls in the MAb-based dot-blot ELISA.

### Preparation of monoclonal antibodies

Two hybridoma clones, *ie.* clones 102 $B_2$ and 204 $D_3$ were used in this study. The clone 102 $B_2$ secreted monoclonal antibodies which reacted with core polysaccharide of all salmonellae and did not react to other enteric bacteria,[25,26] while the clone 204 $D_3$ produced antibodies specific to antigen 9 of group D *Salmonella* spp.[24,25] These clones were derived from fusion of Balb/c mouse immune splenocytes (immunized with Barber antigen extracted from *S. typhi*) and Sp 2/0 myeloma cells. They were stored by the method of Patel and Brown[27] in the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University.

### Monoclonal antibody-based dot-blot ELISA (MAb dot-ELISA)

The MAb dot ELISA, which involved monoclonal antibodies from clones 102 $B_2$ and 204 $D_3$ (MAb 102 $B_2$, MAb 204 $D_3$), was used to detect the presence of *Salmonella* core polysaccharide and antigen 9 of group D salmonellae, respectively, in urine samples of the

patients and controls. The test was performed as previously described.[24] Aliquots of urine samples were boiled for about 20 minutes to eliminate endogenous enzyme, then 100 microliter samples were dotted in triplicate onto three different nitrocellulose (NC) strips using a slot-blot device. The Ly or LPS of *S. typhi* (adjusted to 1 mg per ml of PBS, pH 7.4) and urine of normal individual were included on the same NC strips. The blotted membranes were air dried then blocked with 1% (w/v) skimmed milk in Tris buffered saline (TBS), pH 7.5 at room temperature for 10 minutes. The NC strips were washed thoroughly with TBS containing 0.05% Tween-20 (TBST), then one strip each was put into a solution of 640 ELISA units per ml of MAb 102 $B_2$, and MAb 204 $D_3$ and in RPMI-1640 medium, respectively. The strips were kept at 26°C for 20 minutes with occasional shaking then they were washed thoroughly with TBST. All three strips were placed in a box containing anti-mouse immunoglobulin-alkaline phosphatase conjugate (diluted 1:1,000 in TBST; Dakopatt, Denmark) and allowed to react for 15 minutes, washed three times with TBST and finally washed once with 0.15 M Tris buffer, pH 9.6. The NC strips were then placed in a freshly prepared substrate solution (NBTP) for about 5 minutes with light protection. The enzyme-substrate reaction was stopped by rinsing the membrane with distilled water, then the strips were air dried. Positive results were interpreted by observing the areas dotted with specimens in comparison with the positive and negative controls and the corresponding results on the third strip, which was not reacted with the MAb. Positive reaction was the appearance of a purplish-blue or blue spot distinguishable from the negative control and from the reaction of the same specimen on the third strip.

### Hemoculture method

Blood cultures were performed for the 171 patients with suspected clinical enteric fever. Whole blood (10 ml) from each patient was collected aseptically by venipuncture and inoculated into 80 ml (aerobic incubation) or 50 ml (anaerobic incubation) of brain heart infusion broth and incubated at 37°C for 14 days with inspection daily for growth. After 1 day of culture (day 1), a smear for Gram staining and subcultures onto blood and MacConkey agar plates were made. Any growth obtained on these plates was identified by the scheme of Edwards and Ewing.[28] Serogroups of *Salmonella* were determined with polyvalent antisera obtained from Wellcome Diagnostics, England. Subcultures were also made on days 2, 5 and on other days when the broth became turbid.

### Widal test

Widal agglutination was performed on paired sera of the 171 patients using O and H antigens obtained from Wellcome Diagnostics (Dartford, UK). A dilution series of 1:50 to 1:800 of each serum in 0.85% saline was made and 0.5 ml of each dilution was added with 0.5 ml of appropriate antigens. Positive and negative control sera were included in the test. The agglutination titers were determined against the controls after 18 hour incubation at 56°C. The significant rises of antibody titers between the acute and convalescence sera or the O antibody titers higher than 1:200 were classified as positive enteric fever.

The bacterial culture, the Widal test and the ELISA were performed by different individuals and the results were revealed only after all tests were completed.

### Data analysis

The diagnostic sensitivity, specificity, accuracy and positive and negative predictive values of the MAb-based dot-blot ELISA and the Widal test were compared with the blood culture method using the method of Galen.[29] Kappa coefficience ($k$) and kappa probability (Z) were calculated by the method of Cohen[30] and Fleiss.[31] The $k$ and Z were used for determining correlation of the results of *Salmonella* antigen detection and Widal agglutination with the hemoculture method. The degrees of agreement beyond chance between the serological assays and the blood culture method were given according to the classification of Landis and Koch.[32]

## RESULTS

Of the 171 patients, 96 were males and 75 were females with the mean age of 34.7 $\pm$ 11.3 years (range from 15 to 68 years). At the time of admission when hemocultures and urine samples were collected for the first time, 97 patients (56%) had been treated with one or more chemotherapeutic agent(s), *ie.* 17, 33, 5, and 11 patients had received ampicillin, chloramphenicol, tetracycline and thrimethoprim-sulphamethoxazole, respectively; 15 had received ampicillin and trimethoprim-sulphamethoxazole, 7 received chloramphenicol and trimethoprim-

sulphamethoxazole, 3 had received ampicillin and chloramphenicol and 6 had received chloramphenicol, ampicillin and trimethoprim-sulphamethoxazole. All patients presented with high fever with the body temperature of 39.6 ± 0.4°C (range 38.5 to 40.2°C) at the time of admission. Other associated symptoms included anorexia, malaise, fatigue, nausea, vomiting, abdominal pain and diarrhea which were present in 100% (171/171), 100% (171/171), 93% (156/171), 41.2% (72/171), 31.6% (54/171), 29.2% (50/171) and 39.2% (67/171), respectively. Rose spots, rhonchi, hepatomegaly, splenomegaly and meningeal signs were present in 38 (22.2%), 28 (16.4%), 84 (49.1%), 70 (40.9%) and 1 (0.6%) of the patients, respectively.

The overall results of MAb-based dot-blot ELISA, culture method and Widal test performed on the specimens of the 171 patients are shown in Table 1. Hemocultures were positive for *Salmonella* spp. in 77 of the 171 patients (45%). The MAb 102 $B_2$ based dot-blot ELISA was positive in 98 patients (57.3%). For the 77 blood culture positive cases, the MAb 102 $B_2$ ELISA was positive in 71 (92.2%) while the MAb 204 $D_3$ ELISA was positive in 48 cases. There were 6 patients whose blood cultures were positive but in which the dot-ELISA gave doubtful results and thus interpreted as negative (Table 2). *S. typhi, ie.* group D were isolated from blood of the 48 patients whose urine samples were positive for both antigen 9 as detected by the MAb 204 $D_3$ ELISA and also for *Salmonella* core polysaccharide by the MAb 102 $B_2$ ELISA. The other 23 patients which gave positive results only by the MAb 102 $B_2$ ELISA were found to be infected by other groups of salmonellae by bacterial isolation. Monoclonal antibody based dot-blot ELISA was negative for all of the urine samples of the 50 healthy individuals.

Statistical analysis revealed that the diagnostic sensitivity, specificity and accuracy of the MAb-based dot-blot ELISA using the culture method as a standard were 92.2%, 71.3% and 80.7%, respectively. The positive and negative predictive values of the test were 72.4% and 91.8%, respectively.[29] The kappa coefficience ($k$) was 0.62 (Table 2) which indicates very good degree of agreement of the ELISA and the culture beyond chance.[32] The kappa probability (Z) was 8.35 (Table 2) which indicates reliability of the ELISA ($p < 0.0001$).[30,31]

Of the 77 culture proven enteric fever patients, 67 of them were positive by the Widal test. Among the remaining 94 clinically diagnosed enteric fever patients whose blood cultures were negative, 44 of them gave significant Widal titers. Thus the Widal test was positive in 111 of the 171 cases. The diagnostic sensitivity, specificity, accuracy, and positive and negative predictive values of the Widal test using the bacterial culture method as a standard were 87%, 46.8%, 68.4%, 60.4% and 83.3%, respectively.

**Table 1.** The overall results of hemocultures, dot-blot ELISA and Widal tests of the 171 patients suspected of enteric fever

| Hemoculture | | No. of patients who gave positive (%) by | | |
|---|---|---|---|---|
| | | Widal | MAb-based ELISA | |
| | | | 102 $B_2$ | 204 $D_3$ |
| Positive | 77 patients | 67 (87.0) | 71 (92.2) | 48 (62.3) |
| Negative | 94 patients | 44 (46.8) | 27 (28.7) | 16 (17.0) |
| **Total** | **171** | **111** | **98** | **64** |

**Table 2.** The results of MAb 102 $B_2$-based dot-ELISA compared with the hemoculture

| MAb-102 $B_2$ ELISA | Hemoculture | | Total |
|---|---|---|---|
| | Positive | Negative | |
| Positive | 71 | 27 | 98 |
| Negative | 6 | 67 | 73 |
| **Total** | **77** | **94** | **171** |

| | | |
|---|---|---|
| Diagnostic sensitivity | = | 92.2% |
| Diagnostic specificity | = | 71.3% |
| Accuracy | = | 80.7% |
| Positive predictive value | = | 72.4% |
| Negative predictive value | = | 91.8% |
| Kappa coefficience (*k*) | = | 0.62 |
| Kappa probability (Z) | = | 8.35 |
| P-value | = | < 0.0001 |

**Table 3.** *Salmonella* antigenuric period of the patients after commencing treatment

| Duration after treatment (days) | No. of patients with antigenuria as detected by MAb-102 $B_2$ dot blot ELISA [positive/total (%)] |
|---|---|
| 0 | 98/171 (57.3) |
| 3 | 98/171 (57.3) |
| 7 | 92/171 (53.8) |
| 14 | 50/171 (29.2) |
| Thereafter | 0/171 (0) |

The 171 patients were treated orally with 500 mg of ciprofloxacin twice daily in the hospital for 5 days. They were discharged when all the signs of illness disappeared. It was found that no patient failed to respond to the treatment clinically. No relapse occurred among them during the one month follow-up period. The mean duration of fever was 5.18 ± 0.78 days (range 3-6) after commencing the treatment. The patients did not have any complications: no eosinophilia, no rashes, and no other side effects.

*Salmonella* antigenuria among the 171 patients were studied. During the first 7 days after admission, *ie.* at days 0, 3 and 7, 98 (57.3%), 98 (57.3%) and 92 (53.8%) patients were found to have *Salmonella* antigen in their urine samples as detected by the MAb 102 $B_2$ dot-blot ELISA. However, the number of antigenuric patients reduced markedly at day 14 (50 from 171 patients; 29.2%) and none of them were antigenuric thereafter (Table 3). The mean of the antigenuric period was 10.3 ± 3.9 (range 3-14) days.

## DISCUSSION

Currently, two methods form a basis for the confirmation of enteric fever caused by *Salmonella* spp. in an individual with a compatible clinical symptoms. Direct bacteriological confirmation resulting from the isolation of *Salmonella* from clinical specimens represents the preferred means. However, this method has relatively low sensitivity, time consuming and is frequently not available in areas of high endemicity due to economic and logistic reasons. The Widal test which depends upon the demonstration of a four fold or greater rise in agglutinin titers between acute and convalescence specimens has also been used. However, convalescence specimens are rarely obtained. Single specimens collected during the acute phase of illness are frequently used alone and reliance on the results of a single Widal test is common in many countries even though disputed reliability of the method has been reported.[4,5] The Widal test should be interpreted in the light of baseline titer in healthy residents of the same geographical area as the patient. In a patient strongly suspected to have enteric fever, it may be useful to perform the Widal test only if two blood cultures are negative.[33] Certainly, the Widal test, by itself can never

provide more than a presumptive diagnosis of enteric fever.[14,34]

Recently, we produced two MAbs which are specific to core polysaccharide component of the genus *Salmonella* (MAb 102 $B_2$) and antigen 9 of the group D *Salmonella* spp. (MAb 204 $D_3$). The usefulness of the dot-blot ELISA using the MAb 102 $B_2$ has been demonstrated for the detection of *Salmonella* contamination in foods,[26] while the MAb 204 $D_3$ was used for the detection of *S. typhi* antigen 9 in urine specimens from patients for diagnosis of typhoid.[24,25] These attempts were successful because: firstly, LPS is major cell surface antigen of gram negative bacteria; secondly, the O-antigenic polysaccharide chains are formed by repeating units and are also present on the bacterial surface, providing a sufficient level of antigens for detection; thirdly LPS are stable to heat and proteinases, thus allowing the possibility of detection in a variety of conditions and situations. Therefore, the MAb-based dot-blot ELISA was used in the present study.

In this study, *Salmonella* spp. were isolated from blood of 77 from 171 patients suspected to have enteric fever. The isolation rate was similar to those of the previous reports.[16,35] This low percentage (45%) of culture positive cases may be due to prior antibacterial therapy. Of the 171 cases studied, 97 (56.7%) patients had been treated with one or more chemotherapeutic agent(s). The persistent illness may be due to an incomplete treatment or resistance of the organisms to the drug(s) or both. In Vietnam, normally, the people seek treatment from general medical practitioners for their illnesses, and in most instances,. without proper laboratory investigations they are treated with antibiotics. Once fever or illness apparently diminishes, the patients do not complete the antibiotic course, which allow the illness to recur and persist for prolonged periods. Be-sides, most patients had late ad-mission to the hospitals, *ie.* in 2nd or 3rd week of illness. These factors probably render hemoculture less sensitive. The 94 clinically suspected patients who were culture negative might be explainable for the above mentioned reasons. The MAb-based dot-blot ELISA gave a higher positivity (57.3%) of LPS detection in the urine samples of the patients in comparison with the culture method (45%). The finding is similar to data previously reported.[24,25]

Using positive blood culture as a standard diagnosis of enteric fever in the 171 patients, the dot-blot ELISA revealed 92.2%, 71.3%, 80.7%, 74.2% and 91.8% diagnostic sensitivity, specificity, accuracy, and positive and negative predictive values, respectively. In addition, the kappa coefficience (0.62) revealed very good agreement satisfactory beyond chance between the ELISA and the culture methods. The sensitivity of the ELISA in this study was similar to a study performed on 52 Indonesian patients, of which the diagnostic sensitivity was 95.5%.[24] The positive and negative predictive values of the MAb-based dot-blot ELISA were satisfactory within the ranges of reported prevalence of enteric fever among patients with prolonged fever in Southeast Asia. The diagnostic efficacy of the ELISA was better than the Widal test which gave 87%, 48.8%, 68.4%, 60.4% and 83.3% diagnostic sensitivity, specificity, accuracy, and positive and negative predictive values, respectively.

In this study, 27 patients were ELISA positive but blood cultures were negative. It is possible that the patients might have been infected with *Salmonella* spp., but the organisms could not be grown in the hemocultures. The appearance of the antigen in urine of these 27 patients were reproducibly detected at least two times (urine samples collected at days 0 and 3) and in some of them antigenuria was reproducibly detected for three or four times. Similarly, Chaicumpa *et al.*[24] reported 13 cases which were positive for antigen by the ELISA from whom organism was not isolated by culture method of 30 clinically diagnosed enteric fever patients. Our PCR results (data to be published) compared with the MAb-based dot-ELISA in the detection of *Salmonella* antigen in rectal swabs of patients were found to have excellent agreement. Thus, there are enough reasons to believe that the 27 patients were really suffering from salmonellosis.

It was found in the present study that there were urine samples of 6 patients whose blood cultures were positive for *Salmonella* spp. but the ELISA was negative. In fact, the ELISA results were doubtful because of the interfering brown color background, thus they were interpreted as being negative. To resolve this disputation, the ELISA should be performed on another set of specimens from the patients. Unfortunately, the patients were admitted to the hospitals in Vietnam since April to October, 1996 but the ELISA was performed in Bangkok during November 1996, to January,

1997, thus new specimens could not possibly be obtained. The discrepancy of ELISA negative, culture positive of patients may be due to an uneven distribution of the antigen in urine specimens after being frozen and thawed or may be due to the intermittent release of the *Salmonella* antigen into the urine of the patients.

McNemar's test was used to assess whether there was any significance in the disagreement between the culture method and MAb-based dot-blot ELISA (false positive and false negative). This study showed that the number of false positive (27) patients was significantly higher than the number of false negative (6) patients ($p < 0.001$). The results confirm the usefulness of the MAb-based dot-blot ELISA in the diagnosis of enteric fever. The bacterial culture process is time consuming with well documentation on its less sensitivity. Clearly, the results of blood culture would be more seriously affected by prior intake of anti-bacterial agents than those of the ELISA while the Widal titer is affected by previous exposure, unrelated febrile illness and treatment.

Ninety-eight from 171 patients were antigenuric on the day of admission (day 0). Reproducible results were obtained when the urine samples collected on day 3 were tested. The number of the patients with antigenuria reduced to 92 (93.9%), 50 (51%) and 0 (0%) at days 7, 14 and thereafter, respectively. The antigenuric period was $10.3 \pm 3.9$ days after commencing the treatment. Thus, the test has a high prognostic value. The results agreed with those reported by Jacob *et al.*[36] in 1984 of which the antigen could be detected up to 18 days and Banchuin *et al.*[8] in 1987 which the antigen was detected until 16 days after treatment. Most of all, the MAb-based dot-blot ELISA is inexpensive and simple which make the test appropriate for the areas where enteric fever is of high prevalence.

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