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Validation of Salmonellosis and Shigellosis Diagnostic Test Kits at a Provincial Hospital in Thailand

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SUMMARY **Rapid Diagnosis of salmonellosis and shigellosis was performed using six different diagnostic test kits which recently have been made available commercially. They were Salmo-Dot, Typhi-Dot, Shigel Dot A, B, C, and D test kits for detection of *Salmonella* spp., group D salmonellae, and groups A, B, C, and D *Shigella* spp., respectively. The principle of all test kits is a membrane (dot) ELISA using specific monoclonal antibodies to the respective pathogens as the detection reagents. The present study was designed to validate the accuracy of the test kits, at a laboratory in a provincial hospital in Thailand, in comparison with the conventional bacterial culture method alone or with the combined results of the culture and the Western blot analysis (WB) for detecting the respective bacterial lipopolysacchharides (LPS) in specimens. Five hundred rectal swab samples of patients with diarrhea who seeked treatment at the hospital, were evaluated. The diagnostic accuracy of the Salmo-Dot was 91.0% when compared with the conventional bacterial culture method alone but was 100.0% in comparison with the combined results of the culture and the WB. The Typhi-Dot and the Shigel-Dot A, B, C, and D showed 100%, 99.2%, 95.0%, 94.0% and 96.4%, respectively when compared with the culture alone and all were 100% in comparison with the combination of the results of the bacterial culture and the WB. The Shigel-Dot A revealed antigen of type 1 *Shigella dysenteriae* in several specimens in which the bacteria could not be recovered by the culture method. This difference is important as type 1 *Shigella dysenteriae* have high epidemic potential and often cause severe morbidity. Unawareness of their presence by the conventional culture may have great impact on disease surveillance for public health. The pathogen detection using the six diagnostic test kits is sensitive, specific, rapid, and relatively simple and less expensive. Several specimens can be tested at the same time without much increase in turn around time. Moreover, these kits produce no contaminated waste as compared with the bacterial culture method. The test kits should be used for rapid screening of specimens of patients with diarrhea especially in areas where culture facilities are inadequate.**

Enteric diseases caused by *Salmonella* spp. and *Shigella* spp. continue to be public health problems in most parts of the world. The Foodborne Diseases Active Surveillance Network (FoodNet) estimated that the incidence rates of the two infections in the US in 1999 were 17.7 and 4.0 cases per 100,000 population, respectively.[1] Salmonellosis and shigellosis in children of developing countries confer a high mortality especially when treatment is delayed.

Current diagnosis of salmonellosis and shigellosis is based primarily on the conventional bacterial culture, isolation and identification method. Stool or rectal swab are the specimens of choice for cases suspected suffering from shigellosis or *Salmonella* gastroenteritis. Hemocultures are usually performed in cases suspected of *Salmonella* septicemia or typhoid fever. The conventional bacterial culture method is laborious. It requires several culture media, complicated biochemical testings and technical skills. Besides, serogroup and serotype specific antisera are needed in the final step for the bacterial identification. However, not only are such polyclonal antisera expensive and variable in their specificities from lot-to-lot, they often are also not adequately and promptly supplied. The major drawbacks of the bacterial culture method are its low sensitivity and lengthy process. It takes 3-5 days before the results are known; thus most often it can not be used for treatment implication but only for

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verification and epidemiological record purposes. To overcome the cumbersome process of the bacterial culture method, alternatives have been sought and several diagnostic tests for salmonellosis and shigellosis have been developed. These include DNA techniques, *i.e.* DNA hybridization[2-7] and polymerase chain reaction (PCR).[8-15] However, DNA hybridization usually requires radioisotopes for higher sensitivity which are hazardous. Besides, it is not rapid. The PCR detection method has been claimed to be highly specific and sensitive for detecting and identifying the pathogens. However, it is still time-consuming and laborious. The method requires a laboratory setting with an expensive equipment, *i.e.* thermal cycler, and reagents. In addition, ethidium bromide used for staining the PCR amplicons in an agarose gel is a mutagen. Most of all, the PCR reagents are susceptible to the interfering factor(s)/ inhibitor(s) present in the specimens especially food and rectal swab/ stool samples which renders the test less sensitive.

Various immunological methods have been developed for *Salmonella* and *Shigella* detections, such as latex agglutination, immunodiffusion and enzyme-immunoassays, *i.e.* ELISA.[16-27] The immunomagnetic enrichment (IME) method has also been used in combination with the enzyme-immunoassay or the PCR to rapidly concentrate the target bacteria to the immunomagnetic beads before they were tested by the detection method.[23, 28] In 1988, Chaicumpa *et al.*[29] produced two hybridomas secreting specific monoclonal antibodies to lipopolysaccharides of all salmonellae and antigen 9 of group D salmonellae, *i.e.* clones $102B_2$ and $204D_3$, respectively. The monoclonal antibodies secreted by the two clones have been used as antigen detection reagents in both microplate and membrane (dot-bot) ELISA for rapid and specific detection of the bacterial contamination in foods and for diagnosis of *Salmonella* typhoid/septicemia by detection of the bacterial antigens in patients' urine samples.[29-31]

In 1999, Soisangwan[32] prepared four sets of monoclonal antibodies, namely MAbSD, MAbSF, MAbSB and MAbSS, which were specific to *S. dysenteriae* type 1, *S. flexneri* types 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b, 6, x and y, *S. boydii* types 2, 4, 5 and 12 and *S. sonnei* phases 1 and 2, respectively. The four sets of the monoclonal antibodies were used in a dot-ELISA to detect their respective antigens in stool/rectal swab samples of patients with diarrhea. The diagnostic specificity of all monoclonal antibodies *i.e.* MAbSD, MAbSF, MAbSB, and MAbSS were 99.0%, 97.60%, 93.33% and 99.29%, respectively, and the diagnostic sensitivity of MAbSF, MAbSB and MAbSS were 97.06%, 100% and 93.71%, respectively.

Recently, the monoclonal antibodies, *i.e.* MAb$102B_2$, MAb$204D_3$, MAbSD, MAbSF, MAbSB and MAbSS have been assembled individually into ready-to-use diagnostic test kits, namely, Salmo-Dot, Typhi-Dot, Shigel-Dot A, Shigel-Dot B, Shigel-Dot C and Shigel-Dot D, respectively. In this study, the six ready-to-use diagnostic test kits have been validated for their diagnostic accuracy in comparison with the result of the conventional culture method alone or with the combined results of the bacterial culture and the Western blot analysis at the Clinical Microbiology Laboratory Unit of Prachom-klao Hospital, Petchaburi province, southwest of Bangkok, Thailand. The results of such validation are reported herein.

MATERIALS AND METHODS

Clinical specimens

The clinical specimens used in this study were rectal swab samples of 500 patients with acute/ invasive diarrhea admitted to Prachom-klao Hospital, Petchaburi province, Thailand. The sample size of the study was initially calculated using the estimation formula of Daniel[33] with estimated positivity of the specimens for shigellosis. The formula used was:

$$\text{Sample size (n)} = Z^2pq/d^2$$

z = the confidence coefficient
p = the proportion of positive cases of shigellosis
q = 1-p = the proportion of negative cases of shigellosis
d = allowable error

As calculated by this formula, the appropriate sample size of specimens from Prachom-klao Hospital had to be at least 384 specimens.

Treatment of specimens

Five hundred samples of rectal swabs were collected from patients with diarrhea before any treatment and on the first day of hospital arrival. They were individually placed in Cary-Blair transport medium and sent to the Clinical Microbiological Laboratory Unit of the hospital. The swab was, then, either directly streaked onto the bacterial culture agar or enriched in a buffered peptone water (BPW) at 37°C for 18 hours. After incubation, the culture was divided into

two equal aliquots. One aliquot was boiled for 20 minutes and later tested for antigens of *Salmonella* spp. and *Shigella* spp. using the Salmo-Dot, Typhi-Dot and Shigel-Dot diagnostic test kits by a different scientist at the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Bangkok, Thailand. The second aliquot was kept frozen a -20°C. The results of both methods were revealed after all of the 500 samples had been tested. If culture and dot-ELISA results did not conform, the frozen aliquots of those samples were used for confirmation by Western blot analysis.

Bacterial culture, isolation and identification

The bacterial isolation was done by a laboratory technician of the Clinical Microbiology Laboratory Unit of the hospital. Rectal swab samples in the transport medium were either directly streaked onto semisolid media, *e.g.* MacConkey agar, SS agar, XLD agar and/or HE agar, or swirled in a 4 ml buffered peptone water (BPW) as an enrichment medium, incubated at 37°C overnight and then streaked onto the agar plates as mentioned above. Two or more suspicious colonies from each agar plate were picked and inoculated into triple sugar iron (TSI) agar, LIM (lysine-indole-motile) medium and urea agar for biochemical testings. Most *Salmonella* spp. show an alkaline over acid reaction with a gas and H_2S production on the TSI slant, although some may be negative for H_2S. *S.* Typhi do not produce gas but are weakly positive for H_2S.[34] Most salmonellae are motile, and decarboxylize lysine. They do not produce indole and urease. *Shigella* spp. produce an alkaline over acid reaction with no gas on TSI, except *S. boydii* which produce gas from glucose. All shigellae are non-motile, do not decarboxylize lysine and do not produce H_2S or urease. The results of the indole tests of shigellae are variable. After the biochemical testings, serogrouping/serotyping of the isolated salmonellae and shigellae were done using serogroup specific antisera of Serotest Reagent Inc., Thailand.

Preparation of buffered peptone water (BPW)

BPW was used as an enrichment medium for *Salmonella* and *Shigella* spp. in rectal swab samples. It was prepared by dissolving 20 grams of buffered peptone powder (MAST Diagnostic Limited, UK or equivalent) in 1 liter of distilled water while heating it on a hot plate with continuous stirring. Four ml aliquots were placed individually in screw capped glass tubes (16 × 125 mm). The tubes were autoclaved at 15 lb/inch^2 at 121°C for 15 minutes, cooled down to room temperature before being kept at 4°C for use within 2 weeks.

Detection of *Salmonella* and *Shigella* antigens in rectal swab samples by using Salmo-Dot, Typhi-Dot and Shigel-Dot test kits

Salmo-Dot, Typhi-Dot and the four Shigel-Dot test kits namely Shigel-Dot A, Shigel-Dot B, Shigel-Dot C, and Shigel-Dot D, were used for detecting *Salmonella* lipopolysaccharide, group D *Salmonella* antigen 9 and *Shigella* antigens of groups A, B, C and D, respectively, in the first aliquots of the enriched BPW of individual samples which had been boiled for at least 20 minutes. Each test kit consist of two boxes. One is called "starter kit", the other "test kit". The "starter kit" contains solutions that have to be diluted and thus can serve four boxes of "test kits". These solutions are 0.3 ml of antibody-enzyme conjugate and 40 ml of "substrate". It also contains two plastic boxes labeled "Test (T) Box" and "Control (C) Box". The "test kit" boxes holds 4 bottles with 100 ml each containing the respective monoclonal antibodies, a blocking solution, washing buffer A and washing buffer B. Further contents are a small capped plastic vial containing 20 control pieces, 2 pieces of nitrocellulose membrane, each divided in 100 small squares, and 2 pieces of filter paper. The test procedures as given in the instruction manual of the test kits were followed throughout.

Western blot analysis

Western blot analysis was performed on the second aliquots of those rectal swab samples that revealed ambiguous results between the culture method and the antigen tests, Salmo-Dot or Shigel-Dot. The second aliquots of these individual rectal swab samples were dialyzed extensively against distilled water at 4°C overnight and lyophilized. The dried preparations were individually restored to about 50 µl of SDS-PAGE sample buffer. The samples were subjected to SDS-PAGE as previously described.[35] The SDS-PAGE separated antigens in the polyacrylamide gel were electrotransblotted onto the nitrocellulose membrane (NC). The NC was cut vertically into strips; the strips were allowed to react individually with either MAb $102B_2$ or the group specific MAb preparations to *Shigella* serogroups A, B, C or D, *i.e.* MAbSD, MAbSF, MAbSB or MAbSS using spent culture medium of the P3x-63-Ag 8.653 myeloma cells as a control. The

Western blot analysis was proceeded to completion as previously described.[36,37] Samples that contained the lipopolysaccharide of the target organism revealed a ladder or diffuse pattern against the monoclonal antibody preparation.

Statistical analysis

The statistical analysis for the diagnostic sensitivity, specificity, predictive values and efficacy of the Salmo-Dot, Typhi-Dot and Shigel-Dot test kits for diagnosis of salmonellosis and shigellosis at Prachom-klao Hospital were calculated by the method of Galen[38] in comparison with the results of culture method alone or the combined results of the culture method and the Western blot analysis. Kappa coefficient value (κ) was calculated in order to determine a degree of agreement between the methods.[39]

RESULTS

Detection of *Salmonella* antigens in the samples using the Salmo-Dot test kit

By using the Salmo-Dot test kit for the detection of *Salmonella* spp. antigens (lipopolysaccharide) in the 500 rectal swab samples, 89 samples were found positive. Among them, *Salmonella* spp. could be isolated only from 49 samples by the culture method. *Salmonella* organisms were also recovered from 5 other samples of the 411 antigen negative-specimens. Thus, the perceived diagnostic sensitivity, diagnostic specificity, positive and negative predictive values and diagnostic accuracy of the antigen detection using the Salmo-Dot test kit in comparison with the results of the culture method were 90.74%, 91.03%, 55.06%, 98.78% and 91%, respectively. The kappa coefficient was 0.6364 which indicate very good agreement between the two methods for *Salmonella* detection beyond chance (Table 1A). The second BPW aliquots of the 40 Salmo-Dot antigen positive-culture negative-samples were subjected to Western blot analysis against the MAb102B_2 and all samples revealed the *Salmonella* lipopolysaccharide (Fig. 1) which indicated that the dot-ELISA correctly identified these 40 samples. Thus, the diagnostic sensitivity, specificity, positive and negative predictive values and accuracy of the *Salmonella* antigen detection using the Salmo-Dot test kit in comparison with the combined results of the bacterial culture method and the Western blot analysis were 94.68%, 100%, 100%, 98.78% and 99%, respectively (Table 1B).

Table 1 Diagnostic sensitivity, specificity, predictive values and accuracy of the dot-ELISA using Salmo-Dot for the diagnosis of salmonellosis caused by *Salmonella* spp. at Prachom-klao Hospital in comparison with the bacterial culture method (A) and the combined results of the culture method and the Western blot analysis (B)

	A			B		
Dot-ELISA results of Salmo-Dot	**Bacterial culture**		**Total**	**Culture and Western blot analysis**		**Total**
	Positive	**Negative**		**Positive**	**Negative**	
Positive	49	40	89	89	0	89
Negative	5	406	411	5	406	411
Total	54	446	500	94	406	500
	Perceived values for dot-ELISA			**True values for dot-ELISA**		
Diagnostic sensitivity	90.74%			94.68%		
Diagnostic specificity	91.03%			100%		
Positive predictive value	55.06%			100%		
Negative predictive value	98.78%			98.78%		
Diagnostic accuracy	91.00%			99%		
Kappa coefficient	0.6364			0.9666		
Efficiency	91.00%			99.00%		
Strength of agreement	very good			excellent		

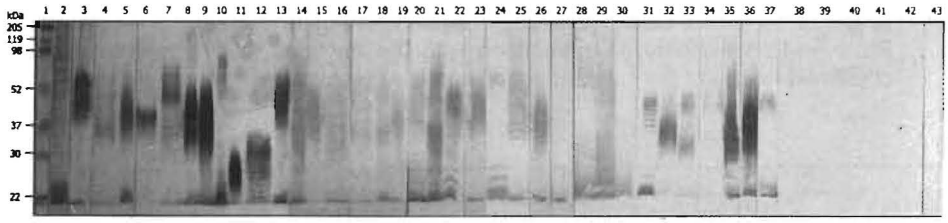

Fig. 1 Western blot (WB) results of the 40 culture-negative, antigen-positive specimens against MAb102B$_2$.
Lane 1 = standard molecular weights
Lane 2 = positive control, Ly of *Salmonella* Typhi
Lanes 3 to 42 = positive WB patterns of the 40 culture-negative, antigen-positive samples
Lanes 43 = negative control, Ly of *E. coli* K-12

Detection of group D salmonellae by Typhi-Dot test kit

Among the *Salmonella* spp. isolated from the 49 culture positive specimens, 4 isolates were found to be *Salmonella* Typhi by the culture method. These four samples were also positive for antigen detection by both Salmo-Dot and Typhi-Dot. Thus, the diagnostic sensitivity, specificity, predictive values and accuracy of the Salmo-Dot and the Typhi-Dot for group D *Salmonella* spp., *i.e.*, *Salmonella* Typhi were all 100% which indicated complete agreement between the bacterial culture method and the antigen test using both of the diagnostic test kits.

Detection of *Shigella* spp. by Shigel-Dot test kits

Shigel-Dot A

None of the 500 rectal swab samples was positive for *Shigella dysenteriae* (*Shigella* group A) by the conventional culture method. However, 4 of the 500 samples tested positive by Shigel-Dot A. Although the diagnostic sensitivity and positive predictive value could not be calculated (as none of the samples was positive by the culture method), the diagnostic specificity, negative predictive value and accuracy of the Shigel-Dot A were 100%, 100% and 100%, respectively (Table 2A).

The 4 culture-negative samples that were positive by the Shigel-Dot A were subjected to Western blot analysis against the MAbSD. The second aliquots of the 4 samples were individually dialyzed extensively against distilled water overnight at 4°C then lyophilized. The dried samples were individually restored in a small volume of a sample buffer and subjected to SDS-PAGE. The SDS-PAGE-separated samples were electro-transblotted onto a nitrocellulose membrane (NC). Then the blotted NC was cut vertically into strips. Whole cell lysates (Ly) of *S. dysenteriae* type 1 and *Escherichia coli* strain K-12 were included as the positive and negative control, respectively. The strips were blocked, reacted with MAbSD and the Western blot was continued to completion. It was found that all of the 4 culture-negative, dot-ELISA positive samples revealed *Shigella dysenteriae* lipopolysaccharide (Fig. 2). Thus, the percentage diagnostic sensitivity, specificity, predictive values and accuracy of the Shigel-Dot A in comparison with the combined results of the culture method and the Western blot analysis were all 100% (Table 2B).

Shigel-Dot B

Using Shigel-Dot B in the detection of antigens of *S. flexneri* in the first BPW aliquots of the 500 rectal swab samples revealed 27 positive samples. However, among these 27 dot-ELISA-positive specimens, there were only 2 samples of which *S. flexneri* could be isolated. The remaining 473 samples were both dot-ELISA-negative and culture-negative. The perceived diagnostic sensitivity, specificity, positive and negative predictive values and accuracy of the Shigel-Dot B in diagnosis of shigellosis in comparison with the conventional culture method were 100%, 94.98%,

Table 2 Diagnostic sensitivity, specificity, predictive values and accuracy of the dot-ELISA using Shigel-Dot A for the diagnosis of shigellosis caused by *Shigella dysenteriae* (group A) at Prachom-klao Hospital in comparison with the bacterial culture method (A) and the combined results of the culture method and the Western blot analysis (B)

	A			B		
Dot-ELISA results of Shigel-Dot A	**Bacterial culture**		**Total**	**Culture and Western blot analysis**		**Total**
	Positive	**Negative**		**Positive**	**Negative**	
Positive	0	4	4	4	0	4
Negative	0	496	496	0	496	496
Total	0	496	500	4	496	500
	Perceived values for dot-ELISA			**True values for dot-ELISA**		
Diagnostic sensitivity	undeterminable			100%		
Diagnostic specificity	99.20%			100%		
Positive predictive value	0%			100%		
Negative predictive value	100%			100%		
Diagnostic accuracy	99.20%			100%		
Kappa coefficient	0			1.00		
Efficiency	99.20%			100%		
Strength of agreement	poor			perfect		

Table 3 Diagnostic sensitivity, specificity, predictive values and accuracy of the dot-ELISA using Shigel-Dot B for the diagnosis of shigellosis caused by *Shigella flexneri* (group B) at Prachom-klao Hospital in comparison with the bacterial culture method (A) and the combined results of the culture method and Western blot analysis (B)

	A			B		
Dot-ELISA results of Shigel-Dot B	**Bacterial culture**		**Total**	**Culture and Western blot analysis**		**Total**
	Positive	**Negative**		**Positive**	**Negative**	
Positive	2	25	27	27	0	0
Negative	0	493	473	0	473	473
Total	2	478	500	27	473	500
	Perceived values for dot-ELISA			**True values for dot-ELISA**		
Diagnostic sensitivity	100%			100%		
Diagnostic specificity	94.98%			100%		
Positive predictive value	7.41%			100%		
Negative predictive value	100%			100%		
Diagnostic accuracy	95.00%			100%		
Kappa coefficient	0.1315			1.00		
Efficiency	95.00%			100%		
Strength of agreement	poor			perfect		

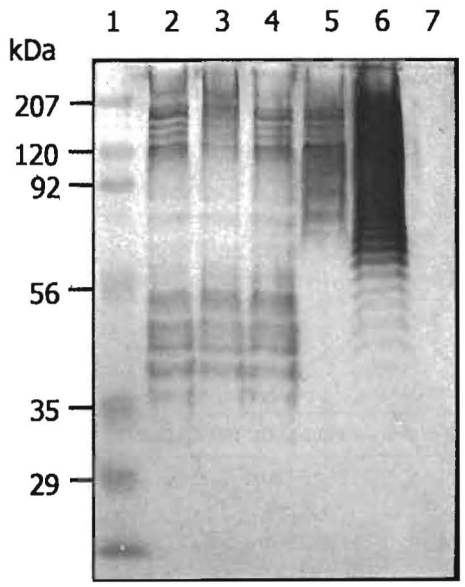

Fig. 2 Western blot results of the culture-negative, Shigel-Dot A-positive samples against MAbSD
Lane 1 = molecular weight markers.
Lane 2 = positive control, Ly of *S. dysenteriae* type 1
Lanes 3-6 = culture-negative, Shigel-Dot A-positive samples which were Western blot positive for *S. dysenteriae* LPS
Lane 7 = negative control, Ly of *E. coli* K-12

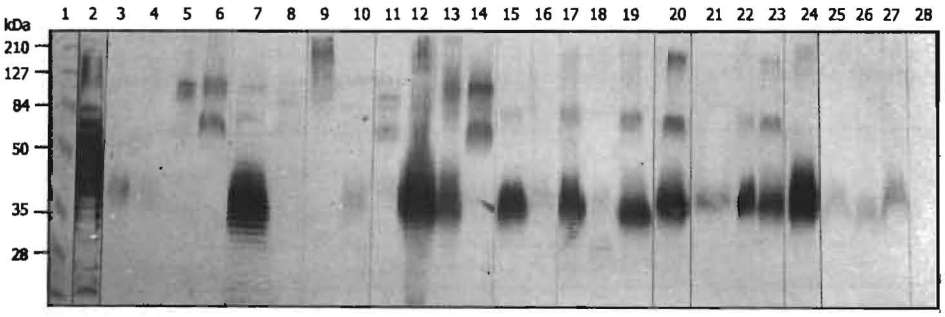

Fig 3. Western blot patterns of the 25 culture-negative, dot-ELISA-positive samples
Lane 1 = molecular weight markers
Lane 2 = positive control, Ly of *S. flexneri* 2a
Lanes 3-27 = WB of the 25 culture-negative, dot-ELISA-positive specimens which revealed *S. flexneri* LPS
Lane 28 = negative control, Ly of *E. coli* K-12

Table 4 Diagnostic sensitivity, specificity, predictive values and accuracy of the dot-ELISA using Shigel-Dot C for the diagnosis of shigellosis caused by *Shigella boydii* (group C) at Prachom-klao Hospital in comparison with the bacterial culture method (A) and the combined results of the culture method and the Western blot analysis (B)

	A			B		
Dot-ELISA results of Shigel-Dot C	**Bacterial culture**		**Total**	**Culture and Western blot analysis**		**Total**
	Positive	**Negative**		**Positive**	**Negative**	
Positive	2	30	32	32	0	32
Negative	0	468	468	0	468	468
Total	2	498	500	32	468	500
	Perceived values for dot-ELISA			**True values for dot-ELISA**		
Diagnostic sensitivity	100%			100%		
Diagnostic specificity	93.38%			100%		
Positive predictive value	6.25%			100%		
Negative predictive value	100%			100%		
Diagnostic accuracy	94.00%			100%		
Kappa coefficient	undeterminable			1.00		
Efficiency	94.00%			100%		
Strength of agreement	undeterminable			perfect		

Table 5 Diagnostic sensitivity, specificity, predictive values and accuracy of the dot-ELISA using Shigel-Dot D for the diagnosis of shigellosis caused by *Shigella sonnei* (group D) at Prachom-klao Hospital in comparison with the bacterial culture method (A) and the combined results of the culture method and the Western blot analysis (B)

	A			B		
Dot-ELISA results of Shigel-Dot D	**Bacterial culture**		**Total**	**Culture and Western blot analysis**		**Total**
	Positive	**Negative**		**Positive**	**Negative**	
Positive	31	18	49	49	0	49
Negative	0	451	451	0	451	451
Total	31	469	500	49	451	500
	Perceived values for dot-ELISA			**True values for dot-ELISA**		
Diagnostic sensitivity	100%			100%		
Diagnostic specificity	96.16%			100%		
Positive predictive value	63.27%			100%		
Negative predictive value	100%			100%		
Diagnostic accuracy	96.40%			100%		
Kappa coefficient	0.7565			1.00		
Efficiency	96.40%			100%		
Strength of agreement	very good			perfect		

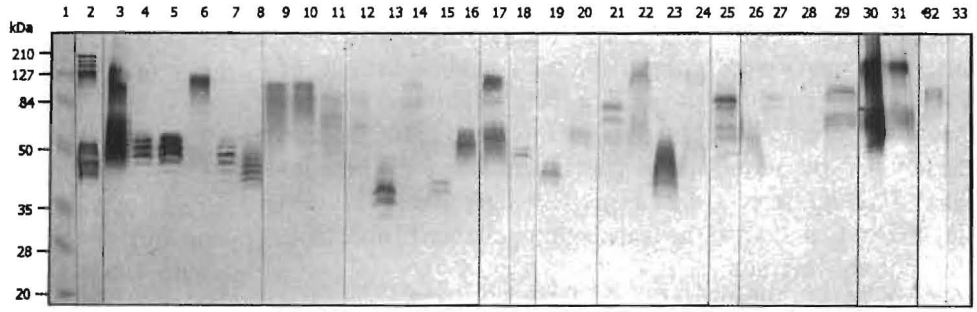

Fig. 4 Western blot (WB) results of the 30 culture-negative, dot-ELISA-positive samples.
Lane 1 = molecular weight markers
Lane 2 = positive control, Ly of *S. boydii* type 12
Lanes 3-32 = WB patterns of the 30 Western blot positive, culture-negative, dot-ELISA-positive specimens which revealed *S. boydii* LPS
Lanes 33 = negative control, Ly of *E. coli* K-12

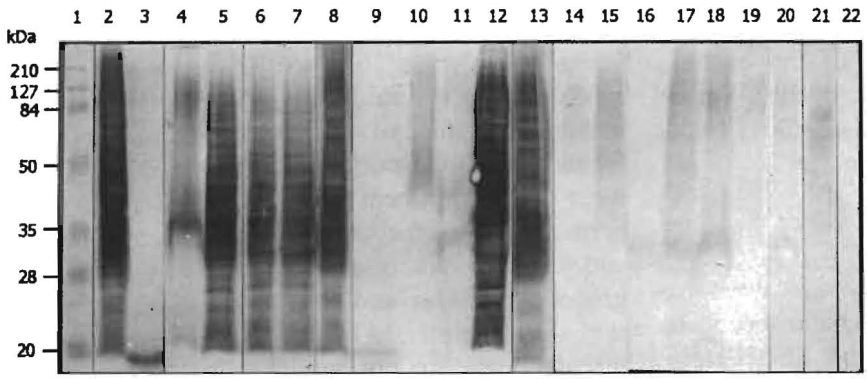

Fig. 5 Western blot (WB) patterns of the 18 specimens which were culture-negative, dot-ELISA-positive by the Shigel-Dot D.
Lane 1 = molecular weight markers
Lane 2 = positive control, Ly of *S. sonnei* phase I
Lanes 3-21 = WB patterns of the 18 samples which were culture-negative, dot-ELISA-positive which revealed *S. sonnei* LPS
Lane 22 = negative control, Ly of *E. coli* K-12

7.41%, 100% and 95.00%, respectively (Table 3A). The second aliquots of the 25 dot-ELISA positive, culture-negative samples were subjected to Western bot analysis against the MAbSF preparation using *S. flexneri* type 2a and *E. coli* K-12 as positive and negative controls. All of the 25 samples revealed *S. flexneri* LPS (Fig. 3). The diagnostic sensitivity, specificity, positive and negative predictive values and accuracy of the dot-ELISA using Shigel-Dot B test kit in comparison with the combined results of the bacterial culture method and the Western blot analysis were all 100% (Table 3B).

Shigel-Dot C

From the 500 samples, there were 2 samples from which *Shigella boydii* could be isolated.

However, dot-ELISA using the Shigel-Dot C was positive not only for the 2 samples but also for 30 of the remaining 498 culture-negative specimens. Thus, the perceived diagnostic sensitivity, specificity, positive and negative predictive values and accuracy of the dot-ELISA for group C *Shigella* detection using the Shigel-Dot C in comparison with the culture method were 100%, 93.98%, 6.25%, 100%, and 94.00%, respectively (Table 4A). The second aliquots of the 30 culture-negative, dot-ELISA-positive specimens were subjected to SDS-PAGE and Western blotting using MAbSB and all of them revealed *S. boydii* LPS (Fig. 4). The re-calculated figures of diagnostic sensitivity, specificity, positive and negative predictive values and accuracy of the antigen detection by dot-ELISA compared with the combined results of the bacterial culture method and the Western blot analysis were all 100% (Table 4B).

Shigel-Dot D

Shigella sonnei were isolated from 31 of the 500 specimens. Dot-ELISA using the Shigel-Dot D was positive not only for the 31 culture-positive samples but also for 18 more samples of the remaining 469 culture-negative ones. Thus, the perceived diagnostic sensitivity, specificity, positive predictive and negative predictive values and accuracy of the dot-ELISA in comparison to the culture method were 100%, 96.16%, 63.27%, 100%, and 96.40%, respectively (Table 5A). The second aliquots of the 18 culture-negative, dot-ELISA-positive specimens were tested by Western blot analysis for the presence of *S. sonnei* lipopolysaccharide using the MAbSS preparation and all were positive (Figure 5). Thus, the diagnostic sensitivity, specificity, positive and negative predictive values and the accuracy of the dot-ELISA using Shigel-Dot D in comparison with combined results of the culture method and the Western blot analysis were all 100%. The kappa coefficient was 1.0 which indicates perfect agreement between the tests beyond chance (Table 5B).

DISCUSSION

Currently, the bacterial culture, isolation and identification is the standard method for diagnosis of salmonellosis and shigellosis. Most laboratories around the world follow the guideline set by the World Health Organization.[40,41] Even with the same guideline, however, the ability to recover the organisms from the patients' specimens, *e.g.* stool or rectal swab, varies from one laboratory to another due to several factors which include the method of specimen collection and transportation, treatment of the specimens, sources and preparations of the culture media and reagents, quality of the typing/grouping antisera and the laboratory skill. Frequently bacterial culture facilities are not available in areas of high endemicity of the diseases. The bacterial culture method is also a lengthy process. It takes several days before the results are known and thus play little, if any, role in early treatment implication.

In the present study, rapid diagnosis of the two entities was performed using six different diagnostic test kits which recently have been available commercially. They were Salmo-Dot, Typhi Dot and Shigel-Dot A, B, C and D test kits for the detection of *Salmonella* spp., group D salmonellae (typhoid group), and groups A to D *Shigella* spp., respectively. The principle of all test kits was a membrane (dot)-ELISA using specific monoclonal antibodies to the respective pathogens as the detection reagents. A similar test design has been used successfully for diagnosis of cholera O1 and O139[37-43] and typhoid fever.[44,45] The present study was designed to validate the efficiency (accuracy) of the six test kits at a laboratory in a provincial hospital.

From this study, the numbers of salmonellosis and shigellosis cases caused by *Salmonella* spp. and *Shigella* spp. of groups A, B, C and D among the 500 diarrheic patients, based on the results of the conventional bacterial culture method, were 54 (10.8%), 0 (0%), 2(0.4%), 2(0.4%) and 31 (6.2%), respectively. The Salmo-Dot correctly identified 49 of the 54 samples from which the *Salmonella* spp. were isolated; thus the diagnostic sensitivity was 90.74%. Among the 54 isolates of *Salmonella* spp., 4 isolates from 4 specimens were *S.* Typhi. These 4 samples were positive also for antigen test by the Typhi-Dot (100% diagnostic sensitivity, specificity and accuracy). From the 500 specimens, none of them revealed group A *Shigella* spp. by the culture; thus the diagnostic sensitivity of the Shigel-Dot A could not be calculated in comparison with the culture method. The other three Shigel-Dot test kits correctly identified 2, 2 and 31 specimens from which the *S. flexneri*, *S. boydii* and *S. sonnei* were isolated; thus their diagnostic sensitivities compared with the culture method were all 100%.

The Salmo-Dot also gave positive results to other 40 samples from which *Salmonella* spp. could not be recovered. Similarly, there were 5, 25, 30 and 18 specimens which were tested positively by the

Shigel-Dot A, Shigel-Dot B, Shigel-Dot C and Shigel-Dot D, respectively, but from which *Shigella* spp. could not be isolated. Thus the perceived diagnostic specificities of the Salmo-Dot and the four Shigel-Dot test kits in comparison with the culture method were 91.03%, 99.00%, 94.98%, 93.38% and 96.16%, respectively. The antigen positive results of the test kits might be considered "false positive" if compared with the results of the culture method alone. However, using Western blot analysis, it was found that all of the dot-ELISA positive, culture negative samples contained the lipopolysaccharides of the respective bacteria which confirmed that the results of the dot-ELISA were correct, and the culture method was "less sensitive".

In this study, we did not use DNA technology for the detection of the respective DNA of the pathogens in the samples that yielded ambiguous results by the culture and the dot-ELISA. The reason is that it is known and generally accepted that the PCR is insensitive for the human fecal samples due to the presence of *Taq* DNA polymerase inhibitor(s). DNA hybridization is laborious and expensive and is not a "rapid" assay. We have chosen to use the Western blot analysis to reveal the respective lipopolysaccharide by using genus specific monoclonal antibodies to *Salmonella* spp., *i.e.* MAb $102B_2$[29] and serogroups A, B, C and D *Shigella* spp. specific monoclonal antibodies recently produced in our laboratories. The Western blot analysis was found to be sensitive and the results correlated with- and confirmed all of the dot-ELISA results. However, there were several studies using other antigen-antibody systems to show that the Western blot analysis may be less sensitive than the ELISA.[46] With the dot-ELISA, the antigen is directly applied onto the nitrocellulose membrane while in the Western blot analysis, the antigen in small quantity and volume is subjected to the SDS-PAGE before the separated antigens are electro-transblotted onto the nitrocellulose membrane. An unsuccessful transfer of the antigens due to any reason which can be either from a technical problem or from the physical properties of the antigen and the membrane or other attributes would render Western blot results negative.

The combined results of all tests, *i.e.* the culture method, the dot-ELISA using the diagnostic test kits and the Western blot analysis performed on the 500 specimens confirmed the causative importance of the *Salmonella* spp., group D salmonellae, and *Shigella* spp. of all serogroups, as etiologic agents of diarrhea. The incidence of most pathogens was higher when the specimens were tested by dot-ELISA and Western blot analysis than when the bacterial culture method was used alone. Moreover, the dot-ELISA and the Western blot analysis could detect several samples which contained *S. dysenteriae* type 1 antigen (the MAbSD is specific to type 1 *S. dysenteriae*) while the culture method could not recover the *S. dysenteriae* from any of the 500 samples. This difference is important as the *S. dysenteriae* type 1 organisms are known to produce Shiga-toxin which may complicate the infection causing a more severe morbidity, *i.e.* hemolytic uremic syndrome (HUS) especially in the young children and the elderlies. Moreover, it is known that this serotype of group A *Shigella* spp. has a high epidemic potential and a propensity to cause explosive outbreaks. Thus, unawareness of the presence of this *Shigella* serogroup/serotype bacteria because the conventional bacterial culture method used routinely in most microbiological laboratories could not recover them, might have a great impact, not only to the individual patients in terms of disease severity and the complication, but also on the disease surveillance, as far as public health is concerned.

Besides being sensitive and specific, the disease diagnosis using the dot-ELISA ready-to-use diagnostic test kits reduces the test time from several days by the culture method to only one day. Moreover, several specimens can be tested at a single time without much increase of the turn-around time. It is cost effective and simple. Most of all, it produces much less contaminated waste than the conventional method. We recommend, therefore, that this kind of diagnostic test kits should be used for routine screening of the specimens of patients with diarrhea.

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