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Rapid Detection of Salmonella enterica Serovar Choleraesuis in Blood Cultures by a Dot Blot Enzyme-Linked Immunosorbent Assay

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A dot blot enzyme-linked immunosorbent assay (ELISA) with a monoclonal antibody specific to phase1-c Salmonella was developed for the direct detection of Salmonella enterica serovar Choleraesuis in blood cultures. This system was applied to the identification of serovar Choleraesuis, and the results were compared with those obtained by a conventional biochemical method. It was revealed that all 12 samples identified to be infected with serovar Choleraesuis were positive on testing by the ELISA. In contrast, 77 samples infected with bacteria commonly isolated from the blood were not reactive by the ELISA. The calculated sensitivity and specificity of the established assay are 100%.

The genus Salmonella consists of gram-negative rod-shaped bacteria that cause salmonellosis in humans and animals. The disease is one of the most important public health problems in many countries, including Thailand. The infection is an important cause of diarrhea and, in some cases, septicemia. In general, Salmonella septicemia is due to Salmonella enterica serovar Typhi infection and is called typhoid fever (8). However, during the past decade, the incidence of septicemia due to nontyphoidal Salmonella has been increasing, especially among patients with human immunodeficiency virus infection (7, 10). The rate of isolation of Salmonella spp. from cultures of blood from this group of patients is 11.2%, and among these identifiable Salmonella spp., the most common isolate is S. enterica serovar Choleraesuis (P. Komolpis, S. Srifuengfung, C. Dhiraiputra, B. Pingwang, T. Wensantia, and P. Siripoonkiet, Program Abstr. Siriraj Sci. Congr., p. 311, 2000).

Conventional methods for the isolation and identification of Salmonella in blood cultures are laborious and time-consuming, and it takes at least 3 days to obtain a result. Since septicemia requires rapid treatment, a rapid technique for the detection and identification of this significant blood microorganism is urgently needed. Currently, molecular-biology-based techniques including PCR assays and DNA hybridization assays have been reported for the rapid, specific, and sensitive detection of microorganisms in blood samples (3, 9, 11). However, these methods require sophisticated laboratory equipment which is available in only a few large diagnostic laboratories. Here we report on the development of a simple dot blot enzyme-linked immunosorbent assay (ELISA) for the identification of S. enterica serovar Choleraesuis, a phase1-c Salmonella, directly from hemoculture bottles. Furthermore, cultures of blood obtained from septicemic patients were analyzed to evaluate the potential use of the established assay. By this assay, biochemical identification of pure serovar Choleraesuis cultures can be avoided. The identification can be finished within 1 day instead of the 2 to 3 days required by conventional biochemical identification methods.

MAb and bacterial strains. A monoclonal antibody (MAb) specific to phase1-c flagellin from Salmonella was developed by Ekpo et al. (4). Hybridomas producing the MAb were obtained by using the Barber protein of S. enterica serovar Paratyphi C as the immunogen. According to the ELISA and immunoblot results, the MAb gave positive reactions with Salmonella strains that express the phase1-c flagellin, including strains of serovars Paratyphi C and Choleraesuis, for a protein with a molecular mass of 61 kDa. There was no cross-reactivity with Barber proteins of other bacteria known to cause enteric fever and enteric fever-like illness (i.e., S. enterica serovars Typhi, Paratyphi A, Paratyphi B, Enteritidis, Krefeld, Panama, and Typhimurium; Escherichia coli; Burkholderia pseudomallei; and Yersinia enterocolitica). All bacterial strains examined in this study were obtained from the Department of Microbiology, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand.

Inoculation of blood culture broth and flagellin extraction. Five milliliters of blood donated by healthy subjects was added to a culture bottle containing 45 ml of sterilized Trypticase soy broth (TSB). This bottle was inoculated with S. enterica serovar Choleraesuis at final concentrations of 1, 10, 102, and 103 cells/ml of blood. The bacterial inoculum had been prepared by diluting the overnight culture of serovar Choleraesuis with TSB so that it contained 1.2 × 109 CFU/ml (corresponding to a no. 4 McFarland standard). Thereafter, further dilutions were undertaken to obtain the appropriate cell culture concentration for artificial inoculation into the blood culture. The inoculated blood culture bottles were then incubated at 37°C for 4 h or 20 h (overnight). To extract flagellin protein after incubation, 5 ml of broth from each blood culture bottle was centrifuged at 150 × g for 5 min to separate the red blood cells. The supernatant was subsequently centrifuged at 1,500 ×
g for 15 min to collect the bacterial cells. The bacterial cell pellet was then washed once with 0.85% NaCl before resuspension in 2 ml of 0.85% NaCl and separation into two tubes for comparison of two different flagellin extraction methods.

The first flagellin extraction method tested followed that described by Ibrahim et al. (6), with some modification. Essentially, the flagellin protein was extracted by exposure of the bacterial cells to 1 N hydrochloric acid at pH 2 for 20 min. The cellular debris was then separated by centrifugation at 1,500 × g for 15 min and the flagellin protein in the supernatant was collected. The pH of this supernatant solution was adjusted to 7.2 with 1 N sodium hydroxide before the dot blot ELISA was performed. The second flagellin extraction method tested followed that previously described by Chart et al. (2). Briefly, the flagellin protein was extracted by heating the bacterial cell suspension at 60°C for 30 min in a water bath, followed by centrifugation at 1,500 × g for 15 min. The supernatant containing the flagellin protein was collected for the dot blot ELISA.

Detection of serovar Choleraesuis flagellin by dot blot ELISA. Optimal incubation conditions and reagent concentrations for the dot blot ELISA were predetermined by checkerboard titration. One hundred microliters of flagellin protein extracted from artificially inoculated blood cultures was applied to a nitrocellulose membrane with a dot blot apparatus (Bio-Rad Laboratories, Richmond, Calif.). The blotted nitrocellulose strip was subsequently blocked with 3% bovine serum albumin in phosphate-buffered saline (PBS; 0.15 M [pH 7.2]) for 20 min at room temperature. After washing with PBS containing 0.02% Tween 20, the strip was incubated with the MAb specific to phase1-c flagellin for 1 h at room temperature. The strip was washed once and was incubated with alkaline phosphatase-conjugated goat anti-mouse immunoglobulins (Dakopatt, Copenhagen, Denmark) at room temperature for 1 h. The substrate, o-dianisidine tetratozitated (6 mg per ml) and beta-naphthyl acid phosphate (1 mg per ml) (Sigma Chemical Company, Saint Louis, Mo.), diluted with substrate buffer (0.05 M carbonate buffer [pH 9.8]), was then added and the reaction was stopped by rinsing the strip with distilled water. A red-purple dot indicated a positive reaction, and a pale brown dot indicated a negative one. The same donated blood sample into which serovar Choleraesuis was not inoculated was used as a negative control.

Figure 1 shows the sensitivity of the established assay. Positive reactions were observed when 1, 10, 102, and 103 cells/ml of serovar Choleraesuis were present in 1 ml of a blood sample. The incubation time required to obtain such positive results was 4 h (Fig. 1A). At that time, the viable cell counts before the assay were 1 × 102, 5 × 102, 1 × 103, and 1 × 104 CFU/ml, respectively. These concentrations gave no turbidity of the culture broth. In addition, when the incubation time was extended to overnight (20 h), stronger signals were obtained when the viable cell count for each concentration exceeded 108 CFU/ml (Fig. 1B). Comparison of the two flagellin extraction methods tested revealed that both heat and acid extraction gave the same sensitivity for serovar Choleraesuis detection (Fig. 1A, lanes I and II, and Fig. 1B, lanes I and II). However, the heat extraction method described by Chart et al. (2) was superior because it was simpler and required less equipment.

The specificity of the dot blot ELISA was tested by artificially inoculating bacteria known to cause bacterial septicemia (i.e., Enterobacter spp., Klebsiella pneumoniae, E. coli, Staphylococcus aureus, Pseudomonas aeruginosa, and two other closely related S. enterica serovars, serovars Typhi and Paratyphi C) into culture bottles containing blood from healthy donors. The results showed that all samples except those containing serovar Paratyphi C gave negative results (Fig. 2). This finding confirmed the phase1-c-specific property of the MAb used. The phase1-c Salmonella strains that have been reported to be causative agents of septicemia are of serovars Choleraesuis and Paratyphi C only. Serovar Paratyphi C has not been reported in Thailand over the last 10 years (1). As a result, positive detection by this dot blot ELISA is highly suggestive of serovar Choleraesuis infection.

Testing of clinical samples. Identification of S. enterica serovar Choleraesuis, a phase1-c Salmonella species, by the established assay was compared with identification by the conventional biochemical identification method. Eighty-nine unknown blood culture samples from the Microbiology Section of
the Rajavithi General Hospital were received for testing by the dot blot ELISA. All these samples showed turbidity and were randomly selected by the staff in the Microbiology Section. In brief, 5 ml of each unknown blood culture sample was centrifuged to separate the red blood cells from the cultured fluid, and the supernatant was heated to 60°C to extract the flagellin protein from the bacterial cells, followed by the dot blot ELISA. After comparison of this result with the result obtained by the conventional biochemical identification method (done by the Microbiology Section), it was revealed that all 12 samples identified by the conventional biochemical identification method to be infected with serovar Choleraesuis were also positive by the ELISA (Table 1). No false-positive result was observed for blood cultures positive for other gram-positive and gram-negative bacteria. The calculated sensitivity and specificity (5) obtained by using the conventional biochemical identification method as the “gold standard” were 100%. In addition, the advantage of the established assay over the conventional method is that identification of the bacteria, after blood culture, can be achieved within 3 h, whereas at least 2 days was required to obtain the result by the conventional method.

In conclusion, the results of the immunological technique described here were in very good agreement with those of the conventional biochemical test for the identification of S. enterica serovar Choleraesuis directly from blood cultures, and the immunological technique has the advantage of being simple and rapid. Large numbers of blood culture samples could be dotted onto a membrane and microorganisms could be detected within a few hours. Therefore, the technique described here offers the benefit of allowing proper treatment earlier than the time of treatment allowed as a result of the use of the conventional method. Furthermore, the dot blot ELISA could be used as a model for the development of methods for the detection of other microorganisms in blood cultures.

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