

Use of a DNA Probe To Detect *Salmonella typhi* in the Blood of Patients with Typhoid Fever

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A DNA probe was used to detect *Salmonella typhi* from blood samples from 14 of 33 patients with culture-confirmed typhoid fever, using the equivalent of 2.5 ml of blood. In contrast, *S. typhi* was detected in 17 of the same 33 patients by culture of 8 ml of blood. The probe hybridized to blood samples of 4 of 47 patients from whom *S. typhi* was not isolated.

Diagnosis of typhoid fever in a patient and assessment of the impact of typhoid fever in the community require accurate identification of *Salmonella typhi* in clinical specimens. *S. typhi* can be isolated from more than 90% of patients with typhoid fever if bone marrow, blood, stool, and intestinal secretions are all cultured (1, 2, 4-7). There are practical and technical limitations to obtaining bone marrow aspirates and intestinal secretions from patients in many endemic areas. Therefore, where bacteriology facilities are available, blood culture is generally the standard method for diagnosis of typhoid fever. Blood culture can detect 40 to 70% of actual typhoid patients, and this range of sensitivity probably reflects variations in the amount of blood cultured, the bacteremic level of *S. typhi*, the type of culture medium employed, and the length of the incubation period (1, 2, 4-7).

DNA probes offer a new approach to the detection and identification of *S. typhi* in the blood of typhoid patients. We have constructed a Vi antigen-specific DNA probe (8) and demonstrated its specificity and sensitivity with freshly isolated bacteria from febrile patients in Peru and in Indonesia (9). The current study was undertaken to determine whether the Vi probe could detect *S. typhi* directly in clinical specimens. Problems anticipated with the use of a DNA probe on blood samples were the low bacteremia in typhoid patients and the sensitivity of the probe. Patients with typhoid fever generally have less than 15 *S. typhi* per ml of blood (11), and the probe cannot detect less than 500 bacteria (8). Therefore, a method was devised to concentrate bacteria from the blood sample, followed by growth amplification of bacteria to increase total bacterial DNA.

Patients, older than 6 years of age, with fever admitted to the Infectious Disease Hospital, Jakarta, Indonesia, between August and December 1987 were included in the study. A bone marrow aspirate, a rectal swab, and 18 ml of venous blood were taken at the bedside of each patient. Bone marrow aspirates, rectal swabs, and 8 ml of blood were processed for microbiological identification of typhoid as previously described (6, 7). In addition, 10 ml of blood was placed in a DuPont Isolator tube (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.), mixed thoroughly, and centrifuged at $3,000 \times g$ at 22°C for 30 min without the brake.

The DuPont Isolator contains agents which lyse erythrocytes and block coagulation. The top 9 ml in the Isolator tube was removed and discarded. The remaining 1-ml concentrate, containing lysed blood cells and bacteria from the blood sample, was vortexed and transferred to a sterile tube. A total of 250 μ l of this concentrate was spotted in 25- μ l aliquots onto a nylon filter (Colony/Plaque Screen; Dupont, NEN Research Products, Boston, Mass.) which had been placed on top of a nutrient agar (Difco Laboratories, Detroit, Mich.) plate. Spots (5 μ l) of 18-h nutrient broth cultures of *S. typhi* Ty2 and *S. typhi* WR4226 were included on each filter as Vi-positive and Vi-negative controls, respectively, as in previous studies (8, 9). The pattern for placing bacterial control and blood sample spots on the filters is illustrated in Fig. 1b. Filters on agar plates were incubated at 37°C overnight and then processed for hybridization as described previously (8).

The Vi DNA probe is an 8.6-kilobase *Eco*RI fragment from plasmid pWR144, which was constructed by cloning a portion of the Vi antigen-encoding *viaB* locus of *Citrobacter freundii* (8). Plasmid pWR144 was derived from pWR141; the insert is the same 8.6-kilobase *Eco*RI fragment, but the vector of pWR144 is pBR329. The Vi probe was prepared and radiolabeled as previously described (8). Conditions for hybridization and autoradiography were the same as in earlier studies (8); autoradiographs were exposed for 18 h at -80°C. Interpretation of the autoradiographs was done without knowledge of the culture results. As described above, a filter was prepared for each patient that included positive and negative bacterial controls, as well as 10 spots of the blood concentrate of the patient. One hybridization signal within at least 1 of the 10 spots on a filter was considered a positive hybridization reaction for that patient. Each signal was thought to represent hybridization of a colony that grew from a single cell present in the blood concentrate that was spotted on the filter. Bacteremia was estimated by adding the total number of hybridization signals in 10 spots and dividing by 2.5 (i.e., 10 spots equal 250 μ l of the 1 ml of concentrate prepared from 10 ml of blood).

A total of 80 febrile patients, admitted to the Infectious Disease Hospital with a presumptive diagnosis of typhoid fever, were included in this study. Laboratory test results among patients with typhoid fever are summarized in Table

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TABLE 1. Laboratory test results from patients with typhoid fever^a

No. of patients	Test results ^b			
	Bone marrow	Blood culture ^c	Rectal swab	DNA probe ^d
5	+	+	+	+
8	+	+	-	+
4	+	+	-	-
2	+	-	+	-
10	+	-	-	-
3	-	-	+	-
1	-	-	-	+

^a All four laboratory tests were performed on each patient included in this study.

^b Positive bone marrow, blood culture, and rectal swab results are defined as the isolation and bacteriological identification of *S. typhi* by standard culture methods from these clinical specimens. A hybridization signal detected in at least 1 of the 10 spots processed with blood on a nylon filter and hybridized with Vi DNA probe was considered a probe-positive (+) result. Each filter was spotted with positive and negative bacterial control cultures, as well as the blood concentrate of a patient, as described in the text.

^c 8 ml of blood cultured.

^d Equivalent to probing 2.5 ml of blood.

1. *S. typhi* was isolated from 32 patients by standard microbiological methods. The blood culture was positive in 17 of 32 patients by culturing 8 ml of blood. The Vi probe detected typhoid DNA in 13 of 32 patients, using the equivalent of 2.5 ml of blood for DNA probing. Table 2 presents the hybridization data for probe-positive patients in this study. Examples of positive filters are shown in Fig. 1a and c.

The Vi probe was also positive in 5 of 48 patients in whom all specimen cultures were negative for *S. typhi* by standard methods. One of these five patients (patient 5) actually had typhoid fever since *S. typhi* was isolated from blood by a gradient centrifugation protocol that is being developed in our laboratory for concentrating bacteria from blood (submitted for publication). Patient 74 was probe positive, with

17 hybridization signals detected within the 10 spots on the filter and an estimated bacteremia of 7 CFU/ml. This patient probably had typhoid fever. Although a non-typhoid salmonella was isolated from a rectal swab, the bacterial isolate was not available for further probe analysis. Filters from the other three patients (patients 32, 75, and 82) had only a few reactive spots on the autoradiograph and an estimated bacteremia equal to or less than 1 CFU/ml (Table 2). Although one cannot rule out false-positives, we think it more likely that the probe was able to pick up low levels of *S. typhi* bacteremia that were not detected by culture. In patients with low numbers of typhoid bacilli in the blood, serum factors that inhibit bacterial growth (11) may account for the culture-negative results. In addition to concentrating bacteria in blood, the lysis centrifugation method probably removes inhibitory serum factors in the 9-ml portion of blood discarded during the preparation of the concentrate used for probe analysis.

In our earlier studies, the Vi probe was more than 99% specific when hybridized with colonies from approximately 1,000 non-typhoid salmonellae and other gram-negative bacterial isolates, including *Escherichia coli*, and *Klebsiella* and *Pseudomonas* spp. (8, 9). *S. paratyphi* A was isolated from the blood of 3 of the 80 patients included in this study. The Vi DNA probe did not hybridize with any of the spots of blood from these three patients, further demonstrating the specificity of the Vi probe for the detection of *S. typhi*.

The purpose of this study was to determine if *S. typhi* could be detected with a DNA probe in the blood of patients with typhoid fever by using methods for concentration of bacteria in the blood, followed by growth amplification of the bacteria. The filters were processed in Indonesia and hybridized in the United States; time was not a factor in this preliminary study. If the hybridizations were done immediately after processing the filters, the time of detection with the current radiolabeled Vi DNA probe would be about 3 days. The hybridization and detection time can be greatly

TABLE 2. Hybridization data of blood samples on filters^a

Patient no.	Hybridization-positive signals detected within the indicated spots of blood on each filter										Total no. of signals	Estimated bacteremia ^b (CFU/ml)
	1	2	3	4	5	6	7	8	9	10		
5 ^c	1	1	1	1	1	0	2	1	0	1	9	4
10 ^c	0	1	0	1	0	0	0	1	0	0	3	1
13 ^c	22	22	25	23	21	20	25	24	23	28	233	93
20 ^c	0	0	1	1	0	1	1	0	2	0	6	2
28 ^c	0	0	0	1	0	0	0	0	1	0	2	<1
42 ^c	13	12	9	10	17	13	13	18	17	15	137	55
47 ^c	3	3	2	2	1	3	3	2	2	3	24	10
61 ^c	0	0	0	1	0	0	1	0	0	0	2	<1
62 ^c	5	4	5	8	3	3	5	5	3	2	43	17
68 ^c	1	1	5	0	2	1	1	1	1	1	14	6
73 ^c	0	0	0	0	2	0	0	0	0	0	2	<1
81 ^c	1	0	0	1	0	0	0	0	0	0	2	<1
85 ^c	0	2	2	1	1	1	0	0	0	0	7	3
86 ^c	0	0	0	0	0	1	0	0	0	0	1	<1
32 ^d	0	0	0	1	0	0	0	2	0	0	3	1
74 ^d	1	3	1	4	0	1	3	1	3	0	17	7
75 ^d	0	0	0	0	0	0	1	0	0	0	1	<1
82 ^d	0	0	0	0	0	0	1	0	0	0	1	<1

^a Filters were spotted with a total of 250 µl of blood concentrate, incubated overnight, processed, and hybridized with the Vi DNA probe as described in the text. Positive and negative bacterial controls were included on each filter; no hybridization was detected with the negative controls.

^b Bacteremia estimated by dividing the total number of hybridization signals on 10 spots by 2.5, since the 10 spots are equivalent to probing 2.5 ml of blood (250 µl of 1 ml of concentrate prepared from 10 ml of blood).

^c Probe-positive, culture-positive.

^d Probe-positive, culture-negative.

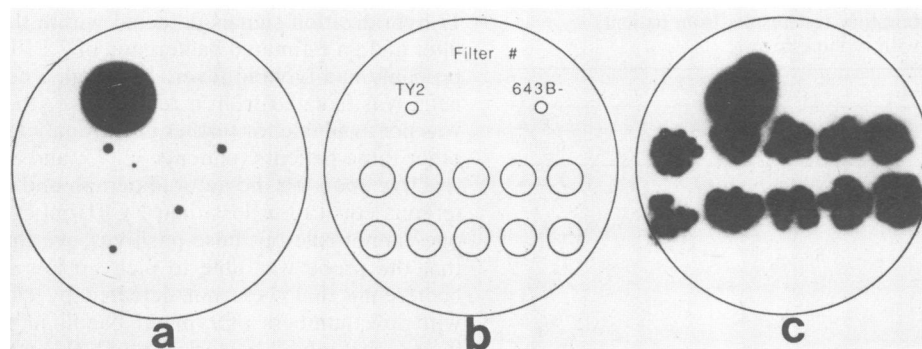


FIG. 1. Sample pattern for filter preparation and representative autoradiographs of filters containing processed blood samples from two bacteriologically confirmed typhoid fever patients. Processed filters were reacted with radiolabeled Vi probe; hybridization was detected by autoradiography as described in the text. Bacterial controls included the Vi-positive *S. typhi* Ty2 and the Vi-negative *S. typhi* WR4226 (643 B-). (a) Filter containing blood from patient 10; (b) example template showing placement of blood samples and bacterial controls; (c) filter containing blood from patient 13.

reduced by the use of oligonucleotide probes (3) and nonisotopic detection systems. In addition, bacterial DNA may be more easily amplified by employing the polymerization chain reaction (10). We are currently sequencing a portion of the large Vi DNA probe in an effort to test more-rapid procedures for detection of *S. typhi* in the blood of patients with typhoid fever.

These results are the first demonstration of the use of a DNA probe to detect bacteria from blood. Bacteria in blood samples were concentrated by lysis-centrifugation of blood with Isolator tubes, and total bacterial DNA was amplified by overnight incubation of the bacteria on nylon filters. Only 25% of the blood sample was actually processed for hybridization. It is probable that probing the entire blood sample of patients with low bacteremia will yield better results. Currently, a study is being conducted in Lima, Peru, in which the entire blood concentrate is being plated onto nutrient agar after lysis-centrifugation, and resulting colonies are being analyzed by standard bacteriological methods, as well as Vi probe analysis (F. A. Rubin, M. Finch, and E. Gotuzzo, work under way). In addition, efforts are focused on sequencing the Vi probe to develop a hybridization system using the polymerization chain reaction and an oligonucleotide probe, to provide a rapid, specific, sensitive, and reliable test for the diagnosis of typhoid fever.

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LITERATURE CITED

1. Avendano, A., P. Herrera, I. Horwitz, E. Duarte, I. Prenzel, C. Lanata, and M. L. Levine. 1986. Duodenal string cultures:

practicality and sensitivity for diagnosing enteric fever in children. *J. Infect. Dis.* **153**:359-361.

2. Benavente, L., E. Gotuzzo, J. Guerra, O. Grados, H. Guerra, and N. Bravo. 1984. Diagnosis of typhoid fever using a string capsule device. *Trans. R. Soc. Trop. Med. Hyg.* **78**:404-406.
3. Bryan, R. N., J. L. Ruth, R. D. Smith, and J. M. Le Bon. 1986. Diagnosis of clinical samples with synthetic oligonucleotide hybridization probes, p. 113-116. *In* L. Leive, P. F. Bonventre, J. A. Morello, S. D. Silver, and H. C. Wu (ed.), *Microbiology—1986*. American Society for Microbiology, Washington, D.C.
4. Gilman, R. H., M. Terminel, P. Hernandez-Mendoza, and R. B. Hornick. 1975. Relative efficiency of blood, urine, rectal swab, bone-marrow and rose-spot cultures for recovery of *Salmonella typhi* in typhoid fever. *Lancet* **i**:1211-1213.
5. Guerra-Caceres, J. G., E. Gotuzzo-Herencia, E. Crosby-Dagnino, J. Miro-Quesada, and C. Carillo-Parodi. 1979. Diagnostic value of bone marrow culture in typhoid fever. *Trans. R. Soc. Trop. Med. Hyg.* **73**:680-683.
6. Hoffman, S. L., D. C. Edman, N. H. Punjabi, M. Lesmana, A. Cholid, S. Sundah, and J. Harahap. 1986. Bone marrow aspirate culture superior to streptokinase clot culture and 8 ml 1:10 blood-to-broth culture for diagnosis of typhoid fever. *Am. J. Trop. Med. Hyg.* **35**:836-839.
7. Hoffman, S. L., N. H. Punjabi, R. C. Rockhill, A. Sustomo, A. R. Rivai, and S. P. Pulungsih. 1984. Duodenal string-capsule culture compared with bone-marrow, blood and rectal swab cultures for diagnosing typhoid and paratyphoid fever. *J. Infect. Dis.* **149**:157-161.
8. Rubin, F. A., D. J. Kopecko, K. F. Noon, and L. S. Baron. 1985. Development of a DNA probe to detect *Salmonella typhi*. *J. Clin. Microbiol.* **22**:600-605.
9. Rubin, F. A., D. J. Kopecko, R. B. Sack, P. Sudarmono, A. Yi, D. Maurta, R. Meza, M. A. Moechtar, D. C. Edman, and S. L. Hoffman. 1988. Evaluation of a DNA probe for identifying *Salmonella typhi* in Peruvian and Indonesian bacterial isolates. *J. Infect. Dis.* **157**:1051-1053.
10. Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**:1350-1354.
11. Watson, K. C. 1959. Isolation of *Salmonella typhi* from the blood stream. *J. Lab. Clin. Med.* **47**:329-332.