JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1987, p. 138–141
0095-1137/87/010138-04$02.00/0

Vol. 25, No. 1

Passive Hemagglutination Test for Enteric Fever

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Received 22 May 1986/Accepted 15 September 1986

A passive hemagglutination (PHA) test for serodiagnosis of enteric fever was developed by sensitizing glutaraldehyde-preserved erythrocytes with lipopolysaccharide from *Salmonella* serogroups A, B, C, and D singly or simultaneously. The lipopolysaccharide-sensitized erythrocytes were tested with sera from 200 blood donors, 100 patients whose hemoculture was positive for *Salmonella* species, and 10 patients septicemic for other members of the family *Enterobacteriaceae*. The PHA test was positive in 90% of 28 acute-phase serum samples from patients with enteric fever from one hospital and in 93% of 72 acute-phase serum samples from another hospital. It was also positive in 100 and 60% of early- and late-convalescent-phase sera, respectively. The PHA test was negative in all patients septicemic for other members of the *Enterobacteriaceae*. Absorption of sera from patients with enteric fever with lipopolysaccharide from other members of the *Enterobacteriaceae* did not reduce PHA titers, indicating the specificity of the PHA test. Simultaneous sensitization with lipopolysaccharide from *Salmonella* serogroups A, B, C, and D was useful as a screening test in a limited trial with 28 acute-phase sera, 10 early-convalescent-phase sera, and 17 late-convalescent-phase sera. The PHA test is indeed a simple, sensitive, specific, and rapid test supplementing hemoculture in laboratory diagnosis of enteric fever.

Enteric fever is prevalent in many developing countries and remains a risk to international travelers (16). Its diagnosis depends a great deal on a positive hemoculture for *Salmonella* species, which remains the standard. Serodiagnosis of enteric fever by the Widal test was once considered a useful alternative to hemoculture, but lately it has been found unreliable (6, 11, 14, 15), resulting in renewed effort to find improved methods for serodiagnosis. This search has led to the development of other methods for serodiagnosis of enteric fever which include microagglutination (2, 8), passive hemagglutination (PHA) (12, 13), counterimmunoelectrophoresis (9), enzyme-linked immunosorbent assay (1, 3, 5), and radioimmunoassay (17). However, counterimmunoelectrophoresis, the enzyme-linked immunosorbent assay, and the radioimmunoassay are technically too complicated. A simple, sensitive, and specific serological test for enteric fever will increase its practicality in many laboratories. The PHA test appeared to be practical but it had not been evaluated in a large number of hemoculture-positive patients with enteric fever. A PHA test for serodiagnosis of enteric fever which has already been subjected to such an evaluation is described in this presentation.

MATERIALS AND METHODS

Patients. Diagnosis of enteric fever was based on the isolation from blood of *Salmonella paratyphi* A, *S. paratyphi* B, *Salmonella cholerae-suis*, and *Salmonella typhi* in the presence of prolonged fever for at least 3 days in which no other etiology was demonstrated. Antisera used for the identification of *Salmonella* species were obtained from Difco Laboratories (Detroit, Mich.).

Twenty-eight patients with enteric fever admitted to Bamrasnaradura Hospital, the Thailand national hospital for infectious disease, were studied. The number of cases positive for *S. typhi*, *S. paratyphi* A, and *S. paratyphi* B were 20, 7, and 1, respectively. The sera collected comprised 20 acute-phase sera, 10 early-convalescent-phase sera, and 17 late-convalescent-phase sera. Among seven patients positive for *S. paratyphi* A, hemocultures were positive twice in four patients, whereas rectal swab cultures were all negative for *Salmonella* species. Among 20 patients positive for *S. typhi*, hemocultures were positive in all first specimens and positive twice in 9 patients, whereas rectal swab cultures were positive for *S. typhi* in 6 patients.

There were also acute-phase sera from 72 patients from the Faculty of Medicine, Siriraj Hospital, whose hemocultures were positive for *Salmonella* species. The numbers of specimens positive for *S. paratyphi* A, *S. paratyphi* B, *S. cholerae-suis*, and *S. typhi* were 11, 8, 5, and 48, respectively. Sera from 200 voluntary blood donors and 10 patients septicemic for other members of the family *Enterobacteriaceae* were used as controls.

Hemoculture. Blood (5 ml) was collected into a bottle containing 45 ml of tryptic soy broth, incubated at 37°C, and examined daily for 14 days. Any bottle showing evidence of growth was subcultured to blood agar and MacConkey agar plates. Salmonellae were then identified by the scheme of Edwards and Ewing (7). Serotypes were identified with appropriate antisera (Difco). Hemocultures were made within 2 days after the admission of the patients.

Preparation of LPS antigen. *S. paratyphi* A, *S. paratyphi* B, *S. cholerae-suis*, and *S. typhi* were used to prepare lipopolysaccharide (LPS) antigen of *Salmonella* serogroups A, B, C, and D, respectively. The method described by Neter (12) was used.

Sensitization of erythrocytes with LPS. Human group O erythrocytes were collected in Alsever solution and used 2 to 10 days after collection. Erythrocytes were washed three times in normal saline and packed by centrifugation. To 0.1 ml of packed cells, 1 ml of 0.15 M phosphate-buffered saline (pH 7.2) was added followed by 0.2 ml of 2.5% glutaraldehyde. The mixture was then rotated on a clinical rotator at

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TABLE 1. Breakdown of PHA test results among sera from patients from Bamrasnaradura Hospital and Siriraj Hospital

Serum[a] (n)	*Salmonella* group	% PHA positive	Mean reciprocal PHA titer	PHA titer range
S. paratyphi A[b]				
Acute phase (7)	A	86	383	40–1,280
	B	86	200	40–320
	C	0	43	20–80
	D	86	369	20–1,280
	Mixed	86	246	40–640
Early convalescent phase (1)	A	100	160	160
	B	100	320	320
	C	0	80	80
	D	100	160	160
	Mixed	100	160	160
Late convalescent phase (5)	A	20	36	20–80
	B	0	28	20–40
	C	0	20	<20–40
	D	0	32	20–40
	Mixed	0	28	20–40
S. paratyphi B[b]				
Acute phase (1)	A	0	20	20
	B	100	160	160
	C	0	20	20
	D	100	80	80
	Mixed	100	80	80
S. typhi[b]				
Acute phase (20)	A	55	102	<20–320
	B	50	82	<20–320
	C	0	26	<20–80
	D	90	374	<20–1,280
	Mixed	85	208	<20–640
Early convalescent phase (9)	A	78	164	<20–640
	B	67	93	<20–160
	C	0	20	<20–80
	D	100	480	80–1,280
	Mixed	100	267	80–1,280
Late convalescent phase (12)	A	17	33	<20–80
	B	25	47	<20–80
	C	0	45	<20–80
	D	83	200	40–640
	Mixed	83	140	40–320
S. paratyphi A[c]				
Acute phase (11)	A	91	467	20–>1,280
	B	91	282	40–1,280
	C	0	24	<20–80
	D	72	219	20–1,280
	Mixed	ND[d]		
S. paratyphi B[c]				
Acute phase (8)	A	75	269	20–1,280
	B	100	640	160–1,280
	C	0	23	<20–80
	D	75	246	<20–1,280
	Mixed	ND		
S. cholerae-suis[c]				
Acute phase (5)	A	0	15	<20–40
	B	0	23	20–40
	C	60	367	40–1,280
	D	0	17	<20–40
	Mixed	ND		
S. typhi[c]				
Acute phase (48)	A	68	131	<20–1,280
	B	73	147	<20–1,280
	C	4	22	<20–160
	D	96	586	20–>1,280
	Mixed	ND		

[a] Early- and late-convalescent-phase sera were collected 7 to 14 days and 41 to 222 days, respectively, after acute-phase sera.
[b] Serum from patients at Bamrasnaradura Hospital.
[c] Serum from patients at Siriraj Hospital.
[d] ND, Not done.

room temperature for 2 h. The glutaraldehyde-preserved erythrocytes were washed three times in normal saline and stored as a 10% suspension in 0.15 M phosphate-buffered saline (pH 7.2) containing 0.1% sodium azide.

Erythrocytes were sensitized to LPS by the method of Hirata and Brandriss (10). To 0.1 ml of packed glutaraldehyde-stabilized erythrocytes 10 ml of an optimal dilution of LPS diluted in 0.1 M acetate buffer (pH 4) was added; the erythrocytes were incubated at 37°C for 30 min, washed three times in normal saline, and suspended as a 0.5% suspension in 0.15 M phosphate-buffered saline (pH 7.2) containing 0.5% bovine serum albumin and 0.1% sodium azide.

The optimal dilution of each LPS was determined by checkerboard titration. It was the highest dilution of antigen which could sensitize erythrocytes, yielding the highest PHA titer with positive serum, but which could be discriminated from negative sera. Control erythrocytes were a 0.5% suspension of unsensitized glutaraldehyde-preserved human group O erythrocytes in 0.15 M phosphate-buffered saline (pH 7.2)–0.5% bovine serum albumin–0.1% sodium azide. Simultaneous sensitization with LPS from all four serogroups was made by using the optimal dilution of each LPS as determined during the course of sensitization with LPS from a single serogroup.

Standardization of PHA test. Each lot of PHA reagent was standardized against positive and negative control sera. PHA titers obtained with the positive control sera, obtained from patients with positive hemocultures, must reach the known titers, whereas PHA titers obtained with the negative control sera must remain within the normal limit. Control sera were stored in small working samples at −40°C, and each sample was used only once. Consistency between lots was maintained by titration for the optimal concentration of LPS used in the preparation of every lot for the PHA test.

PHA test. Serial twofold dilutions of sera were made in U-shaped microtiter plates at 0.05 ml per well starting from 1:10 by using 0.15 M phosphate-buffered saline (pH 7.2)–0.5% bovine serum albumin as the diluent. LPS-sensitized erythrocytes were added to the test wells, 0.05 ml per well, starting from a 1:20 dilution, and 0.05 ml of control erythrocytes was added to the 1:10 serum dilution which served as the control well. For each serum, four serial dilutions were tested with erythrocytes sensitized with a single LPS from each of the four serogroups, and one serial dilution was tested with erythrocytes simultaneously sensitized with LPS from all four serogroups. The plate was left undisturbed at room temperature for 1 h, and then the PHA titer was read. The PHA titer was the highest serum dilution

TABLE 2. PHA titers among positive sera after absorption with LPS from members of the *Enterobacteriaceae*

Salmonella serogroup	Titer against corresponding serogroup	Titer after absorption by LPS from:								
		Salmonella species[a]	*E. coli*	*Shigella flexneri*	*Shigella boydii*	*Shigella sonnei*	*Klebsiella* sp.	*Enterobacter* sp.	*Proteus* sp.	*Citrobacter* sp.
A	1,280	<80	640	640	1,280	1,280	1,280	1,280	1,280	1,280
B	640	<80	640	640	320	640	640	640	640	640
C	640	<80	640	640	640	640	640	640	640	640
D	320	<80	320	640	640	640	320	320	320	320
D	640	<80	640	640	640	640	640	640	640	640
D	640	<80	640	640	320	320	640	320	320	320

[a] *Salmonella* species positive in hemoculture and used in PHA and absorption corresponded to each other.

showing hemagglutination with LPS-sensitized erythrocytes, which appear as a mat of erythrocytes on the bottom of the well. A negative result appeared as a button of erythrocytes on the bottom of the well.

Interpretation. The upper normal limit was established from the PHA test with 200 blood donors. Increased PHA titers of at least one dilution above normal with any sensitized erythrocytes were considered positive.

Absorption studies. Six sera from patients hemoculture positive for *Salmonella* species of groups A (one case), B (one case), C (one case), and D (three cases) were absorbed with LPS prepared from *Shigella flexeneri*, *Shigella boydii*, *Shigella sonnei*, *Klebsiella*, *Enterobacter*, *Proteus*, and *Citrobacter* species, *Escherichia coli*, and corresponding *Salmonella* species. LPS was prepared as described previously (12). Each serum was absorbed with 1/20 volume of LPS and incubated at room temperature for 1 h and at 4°C overnight. Sera were tested with erythrocytes sensitized with LPS from *Salmonella* species corresponding to those found in hemoculture of that particular patient. This study was carried out to check the specificity of the PHA reagents.

RESULTS

The upper normal limits obtained from 200 voluntary blood donors with erythrocytes sensitized with LPS from *Salmonella* groups A, B, C, and D and a mixture of four serogroups were 1:40, 1:40, 1:80, 1:40, and 1:40, respectively. All 200 blood donors had titers within the normal value. Blood donors and patients were residents of Bangkok and surrounding areas. Sera were collected during the same period.

The PHA test was quite sensitive for serodiagnosis of enteric fever. In sera from patients from Bamrasnaradura Hospital, PHA was positive in 90% of 28 acute-phase serum samples, 100% of 10 early-convalescent-phase serum samples, and 60% of 12 late-convalescent-phase serum samples. The PHA titers remained elevated above normal for at least 222 days after the acute phase. PHA was also positive in 93% of acute-phase sera from hemoculture-positive patients from Siriraj Hospital, which confirmed the sensitivity of the PHA test. The mean reciprocal PHA titers in acute-phase sera for each serogroup were in the range of 160 to 374 (Table 1), which was much higher than the upper normal limit of 40. The results from Siriraj Hospital were similar to the results from Bamrasnaradura Hospital (Table 1). PHA titers from all five patients who were hemoculture positive for *S. choleraesuis* showed increased PHA titers specific to group C, agreeing with the fact that LPS from *Salmonella* group C does not share a common LPS antigen with *Salmonella* groups A, B, and D.

Absorption studies with six sera whose hemoculture and PHA were positive for *Salmonella* species showed that a positive reaction in the PHA test could be prevented only by LPS from the corresponding *Salmonella* species (Table 2). Absorption by other members of the *Enterobacteriaceae*, including *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei*, *E. coli*, and *Klebsiella*, *Enterobacter*, *Proteus*, and *Citrobacter* species, did not reduce PHA titers against *Salmonella* LPS antigen. The PHA test also showed excellent specificity by being negative for all sera whose hemoculture was positive for members of the *Enterobacteriaceae* other than *Salmonella* species.

PHA reagents could be easily prepared and standardized. However, bacterial contamination of the LPS antigen is the most common cause of failure in preparing sensitized erythrocytes. The PHA test prepared by simultaneous sensitization with the optimal concentration of LPS from all four serogroups was also found to be a very convenient screening test. It was positive in 86, 100, and 60%, respectively, of acute-phase and early- and late-convalescent-phase sera collected from Bamrasnaradura Hospital. The PHA reagent remained stable in solution at 4°C for at least 2 months or lyophilized for longer storage.

DISCUSSION

It was demonstrated by enzyme-linked immunosorbent assay (1, 3, 5) and radioimmunoassay (17) that considerable improvement in serodiagnosis of enteric fever was possible. It was shown by radioimmunoassay (17) and PHA (12) that antibody to LPS of the immunoglobulin M (IgM) class (17) is commonly present in enteric fever. Based on the presence of IgM antibody to LPS in enteric fever and the sensitivity of PHA to IgM antibody (4), the ground was already prepared for the use of LPS in this PHA test.

The PHA test displayed considerable specificity through the absorption study (Table 2) and the negative results in patients septicemic for other members of the *Enterobacteriaceae*. The absorption study showed that the PHA reaction with erythrocytes sensitized with LPS from *Salmonella* species could not be absorbed with LPS from other members of the *Enterobacteriaceae*. In addition, its specificity among serogroups of *Salmonella* species was strictly defined by the LPS antigens they share; e.g., cross-reaction occurred only among serogroups A, B, and D (Table 2), whereas antibody to serogroup C in patients was specific for serogroup C (Table 1). Among 95 patients with positive hemocultures for serogroups A, B, and D, only two sera were positive with erythrocytes sensitized with LPS from serogroup C.

The PHA test also showed good sensitivity in acute-phase sera from Bamrasnaradura Hospital and Siriraj Hospital (90 and 93%), early-convalescent-phase sera (100%), and late-convalescent-phase sera (60%). Besides the good sensitivity, specificity, rapidity, and simplicity demonstrated, PHA did

not require frequent preparation and was suitable for testing each specimen on arrival, which increased its practicality even further. The PHA test is quite simple to prepare; however, the test demands extensive titration with sera from known hemoculture-positive patients with enteric fever from *Salmonella* serogroups A, B, C, and D. A centralized laboratory with access to control sera and other quality control would be suitable to prepare the PHA reagent for distribution.

Simultaneous sensitization of LPS antigens from the main serogroups of *Salmonella* species offers the possibility for a better screening test, and the present PHA test is the only one that has this feature. In areas such as Thailand, where enteric fever is mostly due to *Salmonella* groups A, B, and D, simultaneous coating of erythrocytes with LPS from the three serogroups could be adequate for screening. However, the evaluation of the screening test was limited to only 28 patients, and further trials are needed to establish its value. The PHA test, on account of its simplicity, sensitivity, and specificity, will prove its value in practice as a procedure for the early recognition of enteric fever. PHA will also be a valuable supplement to hemoculture.

ACKNOWLEDGMENTS

Prakorb Boonthai kindly permitted this study to be done at Bamrasnaradura Hospital. Associate Professor Pranee Singhprasert and Associate Professor Benjawan Rungpitarangsi, Division of Clinical Pathology, Department of Pathology, Faculty of Medicine, Siriraj Hospital, supported this study at Siriraj Hospital.

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