JOURNAL OF CLINICAL MICROBIOLOGY, June 1989, p. 1298–1302
0095-1137/89/061298-05$02.00/0

Vol. 27, No. 6

Indirect Immunoglobulin G (IgG) and IgM Enzyme-Linked Immunosorbent Assays (ELISAs) and IgM Capture ELISA for Detection of Antibodies to Lipopolysaccharide in Adult Typhoid Fever Patients in Pakistan

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Received 15 December 1988/Accepted 2 March 1989

Sera from 339 adult febrile patients in Pakistan were tested for antibodies to *Salmonella typhi* lipopolysaccharide by indirect immunoglobulin G (IgG) and IgM enzyme-linked immunosorbent assay (ELISA) and IgM capture ELISA. A total of 55 patients had *S. typhi* cultured from their blood, 20 had *S. typhi* cultured from their stool, 24 were blood or stool culture positive for *S. paratyphi* A, 41 were culture negative but clinically diagnosed as having enteric fever, 41 had gastrointestinal or urinary tract infections, 41 were clinically diagnosed as having malaria, 20 were smear-positive patients with malaria, 58 had respiratory infections, and the remaining 39 individuals were placed in a miscellaneous group who did not have *Salmonella* infection. The sensitivities of the indirect IgG ELISA, indirect IgM ELISA, and IgM capture ELISA determined with specimens obtained from the blood culture-positive patients with typhoid fever (positive controls) were 80, 64, and 62%, respectively. The specificities of the assays determined with sera from the patients with respiratory infections (negative controls) were 95, 95, and 97%, respectively. The percentage of smear-positive patients with malaria who were positive by these assays was lower than that in the negative control group. The percentages of individuals in the other patient categories who were positive by these tests were between those obtained with the positive and negative controls. Of the positive controls, 26 were positive by both IgM assays, 9 were IgM positive only by indirect ELISA, and 8 were IgM positive only by IgM capture ELISA. A total of 70% of the positive control patients who were tested for O agglutinins by the Widal tube agglutination assay were positive; however, 29% of the negative control patients were also positive. The indirect IgG ELISA was the single most effective test for the serodiagnosis of typhoid fever in this population.

Laboratory diagnosis of enteric fever is accomplished by culture of the organism from clinical specimens or demonstration of an elevated level of *Salmonella* antibodies, traditionally by the Widal tube agglutination assay. The efficacy of culture varies with the specimen being tested. Although bone marrow appears to be the most productive specimen (5), bone marrow culture is rarely a routine procedure. Even if blood, urine, and stool specimens are repeatedly cultured, some patients with active infections are not identified. In addition, the prehospital antibiotic therapy frequently used in developing countries (M. E. Kilpatrick, I. A. Mikhail, and N. I. Girgis, Trop. Geog. Med., in press) complicates the culturing of infectious agents from clinical specimens. Although an elevated titer by the Widal assay is suggestive of enteric fever, such titers can be found in noninfected individuals in regions endemic for this disease (6). A fourfold rise in the Widal assay titer is therefore generally required for a definite serological diagnosis, and a second serum sample is rarely obtained in regions where enteric fever is a major concern. The Widal assay has the additional disadvantages of being laborious and taking up to 48 h to complete.

Salmonella typhi lipopolysaccharide (LPS) cross-reacts with *Salmonella* O groups A and B, but not with the majority of *Salmonella* serogroups or with other enteric organisms. Thus, elevated levels of antibody to this antigen suggest an exposure to the agents of *Salmonella* enteric fevers (*S. typhi*, *S. paratyphi* A, and *S. paratyphi* B). Indirect enzyme-linked immunosorbent assay (ELISA) for immunoglobulin G (IgG) and IgM antibodies to *S. typhi* LPS was shown to be highly effective for the serodiagnosis of typhoid fever in a European population (7–9) and in Egyptian children (12). Since the serodiagnosis of typhoid fever is most difficult in adults in regions hyperendemic for the disease, we examined the efficacy of anti-LPS ELISA in an adult population in Rawalpindi, Pakistan.

The IgM response to successfully treated bacterial infections generally persists for only a few weeks or months. Demonstration of IgM antibodies to *Salmonella* antigen might therefore be of more diagnostic significance in an endemic population than detection of IgG is. However, there are theoretical limitations to the indirect ELISA for IgM. If the concentration of specific IgG in a sample is substantially greater than the concentration of IgM, it can produce false-negative results by competing for the antigen determinants on the plate. On the other hand, an IgM-class rheumatoid factor that may be present in the sample can react with antigen-IgG complexes and produce a false-positive result. ELISAs in which serum IgM is first captured on the plate and subsequently examined for specificity do not have these deficiencies and have been shown to be effective in the diagnosis of viral infections (2, 4, 10). However, few data are available for IgM capture ELISAs for bacterial infections.

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An IgM capture assay was therefore compared with an indirect ELISA for IgM in this study.

MATERIALS AND METHODS

Patients. Febrile patients admitted to the Number 14 Hospital, Rawalpindi, from October 1986 to June 1988 were evaluated by blood, urine, and stool cultures; the Widal tube agglutination assay; anti-LPS ELISA; and thick and thin blood smears for malaria. The subjects were males between 16 and 70 years of age; 62% were in their 20s. They were separated into the following categories according to confirmed or presumed etiologies (see Table 1): blood culture-positive typhoid; blood culture-negative, stool culture-positive typhoid; blood and/or stool culture-positive paratyphoid (*S. paratyphi* A); culture negative with clinical presentations consistent with enteric fever; noninvasive enteritis or urinary tract infections; smear-positive malaria; smear-negative but clinically suspected malaria; upper or lower respiratory infections; and etiologies that did not fit into any of the categories given above (miscellaneous fevers). Patients with blood culture-positive typhoid fever were considered as the positive control group, and since respiratory infections were the least likely to be confused with enteric fever, patients with respiratory illnesses were the negative controls.

Immunoassays. The indirect anti-LPS ELISAs for IgG and IgM antibodies were performed essentially as described previously (12). Briefly, polystyrene microtiter plates were coated with 10 μg of LPS (LPS 0901 *S. typhosa*; Difco Laboratories, Detroit, Mich.) per ml in carbonate buffer overnight at 4°C, after which patient sera diluted 1:100 in phosphate-buffered saline (PBS), followed by peroxidase-conjugated anti-human IgG (heavy and light chain specific; Cappel Laboratories, West Chester, Pa.) or peroxidase-labeled anti-human IgM (mu chain specific; Cappel Laboratories) in PBS, were incubated for 30 min at 37°C. The plates were flood washed three times between each step. Peroxidase substrate [2,2-azino-di(3-ethylbenzthiazoline sulfonate)] was then added and incubated at room temperature until the optical density (wavelength of 415 nm) with a positive control serum reached approximately 1.0 when read on an ELISA reader (Titertek Multiscan; Flow Laboratories, Inc., McLean, Va.).

The IgM capture ELISA was performed as follows. Polystyrene microtiter plates (Linbro Titertek; Flow Laboratories) were coated with affinity-purified goat anti-human IgM (mu chain specific; Tago, Inc., Burlingame, Calif.) that was diluted 1:1,000 in carbonate buffer (pH 9.6). After overnight incubation at 4°C, the plates were flood washed three times with PBS containing 0.05% Tween (PBS-Tween). Serum from patients diluted 1:100 in PBS was incubated in duplicate wells for 30 min at 37°C. The plates were washed, and LPS was incubated in wells at a concentration of 10 μg/ml in serum buffer (5% horse serum in PBS-Tween) for 30 min at 37°C. After the wells were washed, rabbit anti-*S. typhi* serum was incubated in the wells for 30 min at 37°C at a dilution of 1:500 in serum buffer. The plates were again washed, after which peroxidase-labeled anti-rabbit IgG (Miles-Yeda Ltd., Tel Aviv, Israel) was incubated in the wells at a dilution of 1:500 in serum buffer for 30 min at 37°C. After a final wash, peroxidase substrate was added and the plates were incubated at room temperature until the optical density (415 nm; Titertek Multiscan ELISA reader) obtained with a positive control serum reached approximately 1.0.

Calculations. Sera obtained from several blood culture-positive patients with typhoid fever were pooled for use as the positive control and added at a dilution of 1:100 in PBS to duplicate wells on each plate. PBS was used as the plate control and was tested in duplicate on each plate. The optical density obtained with the positive control was arbitrarily given an ELISA value of 10.0, and the values obtained with the other specimens were calculated accordingly. The value obtained with the PBS control was generally about 1.0. ELISA values which gave the highest specificities with the specimens from patients with respiratory infections (negative controls) and the highest sensitivities with the sera from blood culture-positive patients with typhoid fever (positive controls) were arbitrarily chosen as the minimum positive readings; the values were 3.6 for IgG ELISA, 4.0 for IgM ELISA, and 2.6 for IgM capture ELISA. They were approximately the means of the values with the negative controls plus 1 standard deviation.

Widal assay. The standard Widal tube agglutination assay with locally prepared antigen was used for measuring *S. typhi* O agglutinins.

RESULTS

The ages of the patients and the ELISA results for the different patient groups are summarized in Table 1. The mean ages for the groups were similar, ranging from 24.8 to 32.8 years.

The specificities of the assays, as determined with the sera from patients with respiratory infections, were 95% with the IgG ELISA, 95% with the indirect IgM ELISA, and 97% with the IgM capture ELISA. Specimens from four of the negative controls were positive by at least one of the ELISAs; sera from two of these patients were strongly positive, suggesting that they were definite false positives and not sera from individuals with equivocal results because of the selection of positive values. The patients had no known history of enteric fever or immunization with typhoid vaccine. Specificities of 100% for the IgG ELISA and IgM capture ELISA would have been obtained if they were calculated with the specimens from the smear-positive patients with malaria.

The sensitivities with blood culture-positive sera from patients with typhoid fever were 80% with the IgG ELISA, 64% with the indirect IgM ELISA, and 62% with the IgM capture assay. Of the 55 specimens from the positive controls, 50 (91%) were positive by at least one of the three tests. The sensitivities with stool culture-positive patients with typhoid fever were only slightly less than those in the blood culture-positive group. The results with the specimens from patients with paratyphoid and enteric fever were similar, with approximately 57% of each group being positive for IgG and 29 to 39% being positive for IgM. Approximately 20% of the patients who were diagnosed as having malaria but who were smear negative for the parasite had positive anti-LPS ELISA values.

The anti-LPS IgM results obtained by the indirect and the IgM capture assays were similar, with the average values being slightly lower with the latter method (Table 1). A total of 88 specimens were positive by each test, but 19 were positive by the indirect ELISA and negative by IgM capture ELISA, and 19 were positive by the IgM capture ELISA and negative by the indirect ELISA. Sixty-nine specimens were positive by both assays. The values obtained by the two methods with the 55 positive control serum specimens are illustrated in Fig. 1. Of these specimens, 35 were positive by indirect ELISA and 34 were positive by IgM capture ELISA; 26 were positive by both assays, and 43 were

TABLE 1. ELISA results

Patient category	No. of serum samples tested	Age (yr) (mean ± SD)	IgG		IgM			
					Indirect ELISA		Capture ELISA	
			Mean ELISA value ± SD	No. (%) positive[a]	Mean ELISA value ± SD	No. (%) positive[b]	Mean ELISA value ± SD	No. (%) positive[c]
Typhoid (BP)[d]	55	24.8 ± 5.6	7.3 ± 7.8	44 (80)	5.9 ± 3.4	35 (64)	4.5 ± 3.7	34 (62)
Typhoid (SP)[e]	20	26.5 ± 7.9	6.3 ± 2.5	15 (75)	5.9 ± 3.4	12 (60)	3.5 ± 1.9	11 (55)
Paratyphoid	24	26.1 ± 8.0	4.8 ± 2.9	14 (58)	4.2 ± 3.2	7 (29)	4.0 ± 4.0	9 (38)
Enteric fever[f]	41	27.5 ± 11.0	5.1 ± 3.5	23 (56)	5.0 ± 4.0	16 (39)	3.5 ± 3.2	15 (37)
GI, UTI[g]	41	30.5 ± 10.7	2.9 ± 2.7	8 (20)	2.8 ± 2.0	5 (12)	2.2 ± 1.7	8 (20)
Miscellaneous fevers[h]	39	32.8 ± 12.4	2.0 ± 1.3	3 (8)	2.0 ± 1.1	2 (5)	1.6 ± 0.6	2 (5)
Malaria (CL)[i]	41	26.7 ± 8.1	2.9 ± 2.2	9 (22)	3.1 ± 2.4	7 (17)	2.3 ± 1.6	7 (17)
Malaria	20	27.5 ± 4.8	1.8 ± 0.7	0 (0)	2.4 ± 0.9	1 (5)	1.5 ± 0.5	0 (0)
Respiratory infections	58	28.7 ± 9.4	1.9 ± 1.7	3 (5)	2.1 ± 1.2	3 (5)	1.9 ± 0.7	2 (3)
Total	339			119		88		88

[a] Number with ELISA values of >3.5.
[b] Number with ELISA values of >3.9.
[c] Number with ELISA values of >2.5.
[d] Blood culture-positive patients with typhoid fever.
[e] Blood culture-negative, stool culture-positive patients with typhoid fever.
[f] Culture-negative patients who were suspected of having enteric fever.
[g] GI, UTI, Gastrointestinal or urinary tract infections.
[h] Febrile patients who did not fall into one of the other categories.
[i] Blood smear-negative patients suspected of having malaria.

positive by at least one IgM assay. Of the 58 negative control specimens, 3 were positive by indirect IgM ELISA, and 2 of these were positive by IgM capture ELISA.

Of the sera from 119 patients who were positive for IgG (Table 1), 9 were positive for IgM only by the IgM capture ELISA, and therefore these samples were likely candidates for having false-negative indirect IgM ELISA results because of competing IgG. Four of these patients were from the positive control group. Ten patients had sera which were positive for IgG but positive for IgM only by the indirect

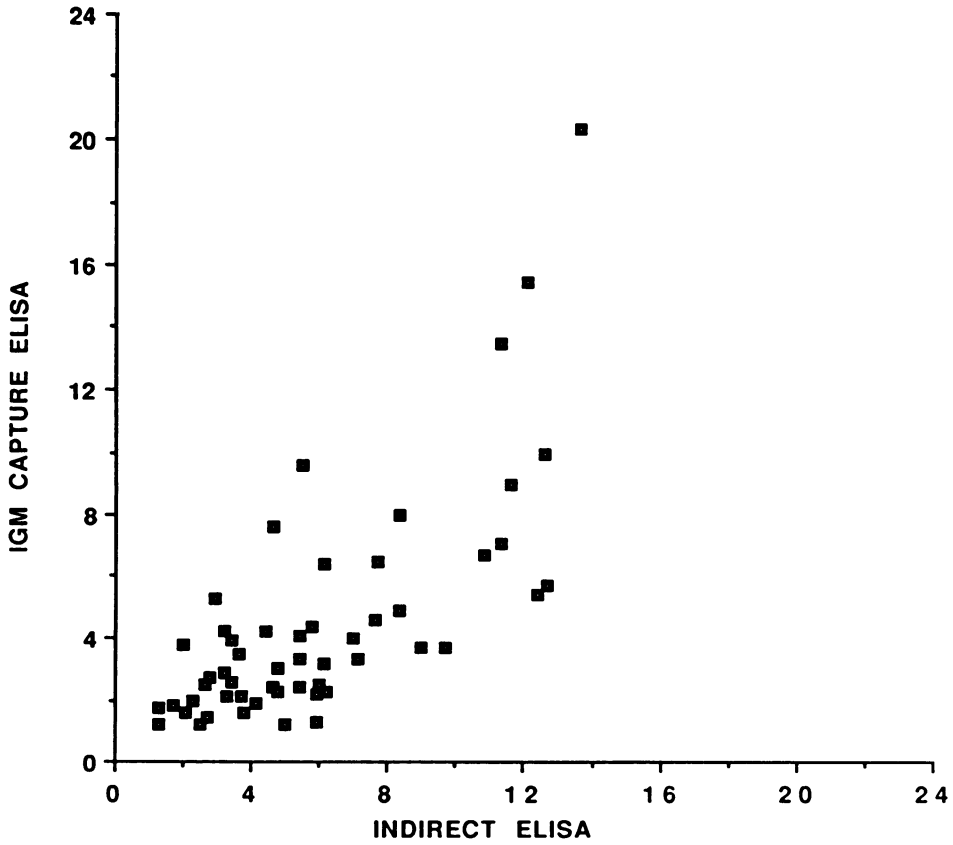

FIG. 1. Correlation between IgM ELISA values obtained by indirect and capture ELISAs with sera from blood culture-positive patients with typhoid fever (r = 0.72).

TABLE 2. Widal assay results

Patient category	No. of serum samples tested	No. (%) of samples with titers of:	
		≥1:160	≥1:80
Typhoid (BP)[a]	44	31 (70)	41 (93)
Typhoid (SP)[b]	19	13 (68)	17 (90)
Paratyphoid	16	10 (63)	15 (94)
Enteric fever[c]	41	33 (81)	37 (90)
GI, UTI[d]	25	7 (28)	17 (68)
Miscellaneous fevers[e]	24	3 (13)	9 (38)
Malaria (CL)[f]	27	6 (22)	16 (59)
Malaria	11	2 (18)	5 (45)
Respiratory infections	28	8 (29)	20 (71)

[a] Blood culture-positive patients with typhoid fever.
[b] Blood culture-negative, stool culture-positive patients with typhoid fever.
[c] Culture-negative patients who were suspected of having enteric fever.
[d] GI, UTI, Gastrointestinal or urinary tract infections.
[e] Febrile patients who did not fall into one of the other categories.
[f] Blood smear-negative patients suspected of having malaria.

ELISA; these could have been falsely positive for macroglobulin because of rheumatoid factor. Six of these patients were from the blood culture-positive group with typhoid fever.

The anti-O Widal agglutination assay results are presented in Table 2. If a titer of 1:160 was selected as the positive cutoff value, 8 (29%) of the 28 negative control specimens and 31 (70%) of the 44 positive control specimens were positive. If a titer of 1:80 was chosen, the percentages with positive results in the two groups would be 71 and 93%, respectively.

DISCUSSION

The results obtained in this study by indirect ELISAs in samples from adults in Pakistan were similar to those found for the same assays in a pediatric population in Egypt (12). In both studies, the negative controls were febrile patients not suspected of having enteric fever; the specificities in the present investigation were 95% for both IgG and IgM ELISAs, while those in the Egyptian study were 100 and 95%, respectively. The sensitivities obtained with blood culture-positive patients with typhoid fever in the present study were 80% with the IgG assay and 64% with the IgM assay, while the corresponding sensitivities in blood culture-positive patients with typhoid fever in Egypt were 92 and 77%. Considering that these investigations involved different populations and were performed with different batches of reagents and different conjugates by different technologists, the results are in excellent agreement. They suggest that anti-LPS ELISAs, especially for IgG antibodies, are effective methods for the serodiagnosis of enteric fevers in endemic populations, regardless of age.

Over half of the patients in the present study who were culture negative but suspected of having enteric fever were IgG positive, suggesting that the infections in most of these patients were accurately diagnosed. Twenty percent of the patients suspected of having nontyphoidal gastrointestinal and urinary tract infections, a group that could be confused with patients with enteric fever, were also IgG positive; undoubtedly, some of these patients were also suffering from *Salmonella* infections. Another group of non-typhoid fever patients that had a significant incidence (22%) of IgG-positive samples was the smear-negative patients with malaria who were diagnosed clinically; either *Salmonella* infection is common with malaria or, more likely in that all of the smear-positive patients with malaria were IgG negative, typhoid is sometimes misdiagnosed as malaria.

The IgM capture ELISA was as effective as the indirect IgM ELISA for the detection of antibodies to LPS in patients with typhoid fever. The sensitivities and specificities of the two methods were almost identical. The correlation coefficient when sera from positive control patients were tested was 0.72. The finding that patients who had elevated levels of IgG were sometimes negative by one of the two IgM tests suggests that incorrect IgM results caused by competing IgG or IgM rheumatoid factor occurred in this patient population and that both methods should be used to ensure that all IgM-positive patients are correctly identified. However, all of the patients that were IgM positive only by indirect ELISA may not have had rheumatoid factor. Only 2 of 78 serum samples from the negative control and smear-positive patients with malaria were found to be positive only by the indirect IgM assay (data not shown). This indicates that the prevalence of IgM rheumatoid factor in this population is not more than 3%. Of the 55 patients in the positive control group, 9 (16%) were IgM positive only by the indirect IgM assay. Since it is unlikely that the prevalence of rheumatoid factor would be five times greater in one patient group than another, some serum samples from the positive control patients that were IgM positive only by indirect ELISA were probably true IgM positives. A test for the presence of rheumatoid factor might also be included to provide additional evidence that the specimens that are IgM positive only by indirect ELISA are false positives.

Just as many positive control samples were positive for IgG as for IgM, even when all of the positive results with both IgM assays were included. The average IgG ELISA value in this group (7.8) was 117% higher than the positive cutoff value for IgG, and therefore was considerably higher than the value obtained by indirect IgM ELISA (average value of 5.9, which was 48% above the cutoff value) and IgM capture ELISA (average value of 3.7, which was 42% above the cutoff value). The predominance of IgG suggests that many of these patients had previous experience with *S. typhi* LPS or a related antigen, a phenomenon that might be expected in a region highly endemic for *Salmonella* infections. Strong IgG responses to *S. typhi* LPS (3, 9, 12, 13), protein (3, 11, 13), and crude antigen (1) preparations have been observed in other studies.

In tropical developing countries where enteric fevers are endemic, hospital laboratory resources are generally limited. Frequently, the only attempt to confirm a clinical diagnosis of typhoid fever is to measure agglutinins in admission sera by the Widal assay, a method that this and other (6, 9, 13) studies have shown is unreliable for sera from patients in such populations. Since the incubation period of this disease is usually sufficient for a humoral response to be elicited by the time clinical symptoms develop, a serological assay with adequate specificity should identify most cases of infection by the time of hospital admission. The measurement of IgG antibodies directed against LPS determinants by indirect ELISA is an easy and highly effective method for accomplishing this. Measuring IgM antibodies to LPS is also effective; however, both indirect and capture ELISAs must be used to equal the efficacy of the IgG assay. Although antibody against *S. typhi* LPS does not differentiate between typhoid and paratyphoid fevers, this is primarily only of epidemiological significance, as clinical management of the two infections is generally the same.

ACKNOWLEDGMENTS

We thank Shahid Nabi and the medical staff on the medical wards, Number 14 Hospital, Rawalpindi, for providing the specimens and clinical data from the patients included in this study and James Meegan for helpful suggestions regarding the IgM capture assay.

This study was supported by grant 3M162770A870AA.122 from the U.S. Naval Medical Research and Development Command Work Unit and grant DAMD17-83-G-9521 from the U.S. Army Medical Research and Development Command.

LITERATURE CITED

1. **Beasley, W. J., S. W. Joseph, and E. Weiss.** 1981. Improved serodiagnosis of *Salmonella* enteric fevers by an enzyme-linked immunosorbent assay. J. Clin. Microbiol. **13**:106–114.
2. **Briantais, M., L. Grangeot-Keros, and J. Pillot.** 1984. Specificity and sensitivity of the IgM capture immunoassay: studies of possible factors inducing false positive or false negative results. J. Virol. Methods **9**:15–26.
3. **Calderon, I., S. R. Lobos, H. A. Rojas, C. Palomino, L. H. Rodriguez, and G. C. Mora.** 1986. Antibodies to porin antigens of *Salmonella typhi* induced during typhoid infection in humans. Infect. Immun. **52**:209–212.
4. **Gut, J.-P., C. Spiess, S. Schmitt, and A. Kirn.** 1985. Rapid diagnosis of acute mumps infection by a direct immunoglobulin M antibody capture enzyme immunoassay with labeled antigen. J. Clin. Microbiol. **21**:346–352.
5. **Hoffman, S. L., D. C. Edman, N. H. Punjabi, M. Lesmana, A. Cholild, S. Sunday, and J. Harahap.** 1986. Bone marrow aspirate culture superior to streptokinase clot culture and 8 ml 1:10 blood-to-broth ratio blood culture for diagnosis of typhoid fever. Am. J. Trop. Med. Hyg. **35**:836–839.
6. **Levine, M. M., O. Grados, R. H. Gilman, W. E. Woodward, R. Solis-Plaza, and W. Waldman.** 1978. Diagnostic value of the Widal test in areas endemic for typhoid fever. Am. J. Trop. Med. Hyg. **27**:795–800.
7. **Nardiello, S., T. Pizzella, S. Miglieresi, and B. Galanti.** 1985. Performance characteristics of an enzyme-linked immunosorbent assay for the determination of immunoglobulin M anti-*Salmonella typhi* lipopolysaccharide antibodies. Boll. Ist. Sieroter. Milan **64**:155–159.
8. **Nardiello, S., T. Pizzella, M. Russo, and B. Galanti.** 1983. ELISA determination of IgM anti-LPS in the early phase of typhoid fever. Boll. Ist. Sieroter. Milan **62**:372–375.
9. **Nardiello, S., T. Pizzella, M. Russo, and B. Galanti.** 1984. Serodiagnosis of typhoid fever by enzyme-linked immunosorbent assay determination of anti-*Salmonella typhi* lipopolysaccharide antibodies. J. Clin. Microbiol. **20**:718–721.
10. **Sakata, H., M. Tsurudome, M. Hishiyama, Y. Ito, and A. Sugiura.** 1985. Enzyme-linked immunosorbent assay for mumps IgM antibody: comparison of IgM capture and indirect IgM assay. J. Virol. Methods **12**:303–311.
11. **Sarasombath, S., N. Banchuin, T. Sukosol, B. Rungpitarangsi, and S. Manasatit.** 1987. Systemic and intestinal immunities after natural typhoid infection. J. Clin. Microbiol. **25**:1088–1093.
12. **Sippel, J. E., H. M. Hanafy, A. S. Diab, C. Prato, and R. Arroyo.** 1987. Serodiagnosis of typhoid fever in pediatric patients by anti-LPS ELISA. Trans. R. Soc. Trop. Med. Hyg. **81**:1022–1026.
13. **Tsang, R. S. W., P. Y. Chau, S. K. Lam, J. T. LaBrooy, and D. Rowley.** 1981. Antibody response to the lipopolysaccharide and protein antigens of *Salmonella typhi* during typhoid infection I. Measurement of serum antibodies by radioimmunoassay. Clin. Exp. Immunol. **46**:508–514.