JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1998, p. 2271–2278 Vol. 36, No. 8
0095-1137/98/$04.00+0

One-Step 2-Minute Test To Detect Typhoid-Specific Antibodies Based on Particle Separation in Tubes

PAK-LEONG LIM,[1]* FRANKIE C. H. TAM,[1] YUET-MENG CHEONG,[2]† AND M. JEGATHESAN[2]‡

Clinical Immunology Unit, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong,[1] and The Institute of Medical Research, Kuala Lumpur, Malaysia[2]

Received 17 November 1997/Returned for modification 4 March 1998/Accepted 6 May 1998

Typhoid fever is caused by *Salmonella typhi*. Detection of anti-*S. typhi* antibodies in the patient is a useful diagnostic aid. Among the various methods developed over the years for this purpose, the Widal test, based on bacterial agglutination, has remained the most widely used, even though it is neither specific nor sensitive. Its popularity stems from the fact that it is simple to use and inexpensive. We describe a new test which also uses a simple one-step procedure but is more rapid and accurate than the Widal. The new test (TUBEX) detects anti-*Salmonella* O9 (both immunoglobulin M [IgM] and IgG) antibodies in patients by inhibiting the binding between an anti-O9 IgM monoclonal antibody (MAb) conjugated to colored latex particles and *S. typhi* lipopolysaccharide (LPS) conjugated to magnetic latex particles. The reactants are mixed in a specially designed microtube for 2 min, and the result is read based on the resultant color of the supernatant following forced sedimentation of the magnetic beads. In the absence of inhibitory antibodies, there is a color change (from blue to red) due to cosedimentation of the indicator particles with the magnetic particles, whereas if these antibodies are present, they prevent such a change to a degree dependent on their concentration. Preliminary examination of TUBEX using the anti-O9 MAb and irrelevant MAbs as inhibitors revealed the test to be specific and reproducible, with an analytical sensitivity of 16 μg per ml of antibody. The reagents remained stable for at least 9 months when kept at 4°C. In the examination of 16 stored sera obtained from 14 patients with proven cases of typhoid fever and 78 serum samples from 75 subjects without typhoid fever, TUBEX was found to be 100% sensitive and 100% specific. The nontyphoid group comprised 26 healthy blood donors, 30 antinuclear antibody (ANA)-negative patients, 9 ANA-positive patients, of whom 1 was positive for anti-DNA antibody, 4 typhus patients, and 6 septicemic patients. In addition, the sera obtained from 11 patients clinically diagnosed as having typhoid fever were all positive in the test. The TUBEX results correlated to some extent, albeit insignificantly ($r = 0.38$, $P = 0.07$), with those of an enzyme-linked immunoassay (ELISA) which used a similar detection format (inhibition) and reagents (*S. typhi* LPS and anti-O9 antibody). TUBEX correlated very well with ELISAs which detected anti-*S. typhi* LPS IgM ($r = 0.58$, $P = 0.003$) or IgG ($r = 0.54$, $P = 0.006$) antibodies from the typhoid patients. There was no correlation with the Widal test. The TUBEX test, if performed on slides (instead of tubes) or with soluble antigen (instead of antigen-conjugated magnetic beads), suffered significantly in sensitivity. Direct agglutination tests using LPS-conjugated indicator particles performed either on slides or in microwells also failed to detect antibodies from the majority of typhoid patients. Thus, TUBEX appears to be well designed and well suited for use in the laboratory or by the bedside as a simple, rapid aid to the routine diagnosis of typhoid fever.

Typhoid fever remains a global health problem affecting an estimated 12.5 million people annually and is endemic in many countries, particularly those in Asia, Africa, and South America (1, 3, 16, 18, 20). Typhoid fever is a food-borne infection caused by *Salmonella typhi*. The infecting organisms invade the bloodstream of the host via the Peyer's patches in the intestine, causing an acute systemic disease characterized by high, spiking body temperatures. Clinically, it is sometimes difficult to differentiate typhoid fever from other fevers such as malaria and typhus. Laboratory tests thus provide a useful adjunct to the diagnosis of the disease.

The most definitive laboratory test is culture of the organism from the bone marrow, blood, or stool of the patient. This method, however, is long and cumbersome, and it is not always successful. Serological tests based on antibody detection provide a convenient alternative. In many countries, the method of choice is the Widal test, first employed a century ago. The Widal test uses a crude assay method to detect antibodies by their ability to agglutinate whole bacterial cells in test tubes. It is not surprising that the Widal test is not reliable, especially when used in areas endemic for typhoid fever (23), and results from different laboratories can vary more than fourfold (2). Many investigators have attempted to devise more-sensitive and more-specific tests for typhoid fever over the last two decades. The most common assay method used is the enzyme-linked immunosorbent assay (ELISA), and the detecting antigen used in these assays is a purified extract of *S. typhi* cells. The antigen is derived from various subcellular structures of the organism, including the lipopolysaccharide (LPS), the outer membrane (OM), the flagella (d-H), and the capsule (virulence [Vi] antigen) (8). As expected, LPS-based (15, 17, 18, 20, 22) and OM-based (3, 16, 24) ELISAs were found to be superior to the Widal test. The chemical structure of *Salmonella* LPS is, in fact, well-known (reviewed in references 6 and 8). It is composed of repeating units of an oligosaccharide (O) chain joined to a polysaccharide-lipid A backbone. The O

* Corresponding author. Mailing address: Clinical Immunology Unit, Prince of Wales Hospital, Rm. 34141, 1/F, Clinical Sciences Building, The Chinese University of Hong Kong, Shatin, Hong Kong. Phone: (852) 2632 3031. Fax: (852) 2645 0856. E-mail: pllim@cuhk.edu.hk.

† Present address: Pfizer Malaysia, Petaling Jaya 46200, Malaysia.

‡ Present address: Ministry of Health, Kuala Lumpur, Malaysia.

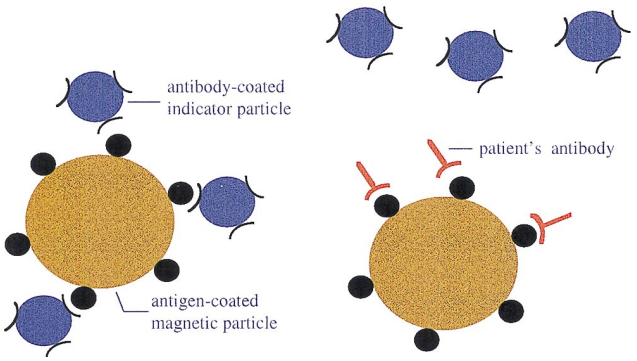

FIG. 1. Principle of TUBEX. See text for explanation. Left panel, negative test result; right panel, positive test result.

chain of *S. typhi*, which bears the O9 and O12 antigens, distinguishes this organism from >99% of other subtypes (serotypes) of *Salmonella*, and the organisms (serogroup D) that share this O chain can be further distinguished from *S. typhi* based on their flagellar and capsular antigens. The O9 antigen is highly specific to *Salmonella* serogroup D, as it contains a sugar that is extremely rare in nature, α-D-tyvelose.

Despite the fact that it is not reliable, the Widal test continues to be used. A reason for this is the simplicity of the test. A single step is involved, whereas multiple steps are required by the ELISA. The Widal test is also inexpensive and requires no instrumentation, whereas for the ELISA, enzyme conjugates and electronic readers are costly necessities. An ideal test is one that is not only reliable but, first and foremost, simple and affordable for the countries which need it most. Many of the affected countries are poor, and some places do not even have electricity.

We describe a new test (TUBEX) which has the advantages of the Widal test and the specificity normally accorded to ELISAs that utilize purified antigens for detection. (TUBEX is a proprietary name, and the test will shortly be marketed under this name by IDL Biotech, Sollentuna, Sweden.) Like the Widal test, TUBEX consists of one step, and the result is read visually based on the appearance of the liquid contents in the tube. On the other hand, a specially designed set of tubes is used in TUBEX, and, instead of whole bacterial cells, *S. typhi* LPS adsorbed to magnetic particles is used as the detecting reagent. To make the test more specific, a monoclonal antibody (MAb) which recognizes the immunodominant O9 determinant in *S. typhi* LPS is also used; the antibody is conjugated to colored latex particles. When the antibody-conjugated particles bind to the antigen-sensitized magnetic particles, and the latter are sedimented by use of a magnet, the color of the liquid (supernatant) in the tube changes. The infection-specific anti-O9 antibodies in typhoid patients are detected by their ability to block the binding between the two types of particles, hence, no change in color. The assay format is based on an ELISA we developed 15 years ago (12) which used the same antigen and antibody reagents and which proved to be highly sensitive and specific as a test for typhoid fever. Transformation of the ELISA to the TUBEX test was made possible by the demonstration that magnetic particles could be conveniently used to separate reacted from unreacted indicator latex particles (10, 13; Australian patent 640346) and, more importantly and more recently, by the development of a special tube which allows efficient reaction to take place.

MATERIALS AND METHODS

Sera. Sera were obtained from 14 patients in Malaysia with bacteriologically confirmed cases of typhoid (including one repeat specimen each from 2 patients) and were kept at −20°C. In addition, a serum sample from each of 11 patients clinically diagnosed as having typhoid fever but for whom culture was not done was included in the study. Control sera included 1 sample each from 26 healthy blood donors in Malaysia, 39 samples sent to the Prince of Wales Hospital, Hong Kong, for detection of antinuclear antibodies (ANA) by immunofluorescence on HEp-2 cells, and 6 samples from Hong Kong with positive serology in the Weil-Felix test (1 sample) or for *Rickettsia mooseri* (5 samples from 3 patients [including 1 repeat specimen each from 2 patients]), and 7 samples from 6 patients in Hong Kong with septicemia (3 with *Escherichia coli*, 1 with *Providencia stuartii*, 1 with *Staphylococcus aureus*, and 1 with *Aeromonas hydrophila*). The Widal titers of all sera were those originally determined in the regional laboratory by a standard procedure (12). Nine of the ANA samples were positive for ANA; of these, one was also positive for anti-DNA antibody and another was also positive for anti-SSA (Sjögren's syndrome)/Ro antibody.

Reagent antibody. The anti-O9 antibody used is an immunoglobulin M (IgM) mouse hybridoma antibody produced by one of us (P.-L.L.) previously (12). The antibody was produced in mouse ascitic fluid and purified by cryoprecipitation (11). The immunoglobulin content of the purified preparation was determined by single radial immunodiffusion (5) using goat anti-μ serum (Sigma Chemical Co., St. Louis, Mo.) and, as a standard, a mouse hybridoma IgM antibody (7C2C5). The latter, which is specific for *Trichinella spiralis* (4, 19), was purified by affinity chromatography (9), and its protein content was determined by the method of Lowry et al. (14). 7C2C5 was used because it was available in our laboratory in purified form. For biotinylation, 75 μl of *N*-hydroxysuccinimidobiotin (Sigma) was added to 1 ml of purified antibody (1 mg/ml) in 0.1 M $NaHCO_3$ (pH 8.4). After incubation for 4 h at room temperature, the antibody was dialyzed against phosphate-buffered saline (PBS) (pH 7.5).

Particle conjugation. White latex particles (diameter, 0.8 μm; Sigma) were sensitized with purified anti-O9 antibody or *S. typhi* LPS (Difco Laboratories, Detroit, Mich.) by passive adsorption as described previously (11).

TUBEX test. (i) Principle. When magnetic particles coated with antigen (*S. typhi* LPS) are mixed with blue latex particles coated with anti-*S. typhi* LPS (O9) antibody, the two types of particles will bind to each other. When the magnetic particles are sedimented to the bottom of the tube by use of a magnet at the end of the experiment, the blue latex particles are also brought down (Fig. 1). This would leave behind a clear supernatant if not for the fact that control (bovine serum albumin [BSA]-coated) red latex particles are also added to the reaction mixture and remain suspended in the solution throughout the experiment. This makes the supernatant red when the blue particles are sedimented, which is easier to see than a colorless supernatant. When a patient's anti-O9 antibodies are present in the reaction mixture, they will inhibit the binding of the blue particles to the magnetic particles (Fig. 1). Consequently, the supernatant remains purplish blue (unchanged from the beginning) due to the presence of blue particles and also, in a lower concentration, of red particles.

(ii) Materials. A main feature of the TUBEX test is the set of tubes used, which were specially designed and manufactured to ensure efficient mixing of the reactants (U.S. patent design 367,932; United Kingdom registered design 2,037,508; Australian patent 640346). As shown in Fig. 2, the set comprises six identical V-shaped tubes, each with a capacity of 1 ml. For reagent A, magnetic particles (Dynal, Oslo, Norway) were coupled with *S. typhi* LPS (Difco) by passive adsorption as described previously (11). The particles were suspended as 0.25% (wt/vol) solids in 0.1 M glycine-borate buffer (pH 8.2) containing 0.9% NaCl (GBS buffer) and 1% BSA (GBS-BSA buffer). For reagent B, blue carboxylated latex particles, 1 μm in diameter, obtained from Rhone-Poulenc, Paris, France, were coupled with purified anti-O9 MAb by using carbodiimide as described previously (13). The particles were suspended as 0.2% solids in GBS-BSA buffer. To this suspension, 0.06% BSA-coated red latex particles (Rhone-Poulenc) were added.

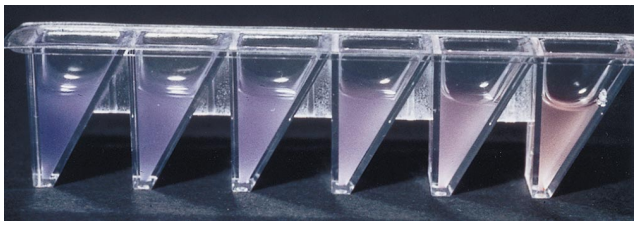

FIG. 2. Microtube set used in TUBEX, filled with solutions containing various amounts of indicator latex particles to demonstrate the range of colors obtainable in the test (used as a standard). Scores arbitrarily assigned to these colors range from 0 (reddest; shown at the extreme right of the set) to 10 (bluest; shown at the extreme left). The redness seen in the picture is duller than that in the actual sample because of the dark background used for the photograph.

(iii) Procedure. A drop (25 μl) of reagent A was placed in one of the tubes in the set. A drop (25 μl) of the unknown serum (undiluted) or control antibody (diluted in GBS-BSA buffer) was added and mixed instantly with the reagent. Two drops (50 μl) of reagent B were then added. When other tubes in the set were similarly filled (six tests per set), the mouth of the whole device was sealed with adhesive tape to provide closure. The tubes were placed on a flat-bed orbital shaker (Stuart Scientific, Redhill, United Kingdom) and shaken for 2 min at 250 rpm at ambient temperature. The tubes were then stood on a magnet, and the resultant color of the supernatant of the reaction mixture was noted visually and scored against a color standard (Fig. 2).

(iv) Interpretation of results. Figure 2 shows the range of colors obtainable in the TUBEX test. At the one extreme, red (arbitrarily given a score of 0) denotes the most negative reaction (no inhibition). At the other extreme, blue (with a score of 10) denotes the most positive reaction. In between these extremes can be found various degrees of blueness (or redness), which are given the corresponding scores 2, 4, 6, and 8. Odd-numbered scores are given to intermediate colors where appropriate.

Inhibition ELISA. The basic procedure described previously (12) was followed except that the reagent anti-O9 antibody was labeled with biotin rather than horseradish peroxidase. Briefly, Immunon-2 microtiter plates (Dynatech Laboratories, Alexandria, Va.) were coated with *S. typhi* LPS (Difco) at 5 μg/ml in 0.1 M bicarbonate buffer (pH 9.6). Fifty microliters of serum (1:10 dilution in PBS–1% BSA) and 50 μl of biotinylated anti-O9 antibody (1:500 dilution in PBS–1% BSA, previously titrated) were added to a well and incubated for 30 min at 37°C. After three washes with PBS, peroxidase-labeled ExtraAvidin (2 μg/ml; Sigma) was added. Following incubation at 37°C for 1 h and subsequent washing with PBS, the assay was developed with 0.01% *o*-phenylenediamine dihydrochloride (Sigma) and the results were read in a microplate reader (Dynatech MK 600).

Inhibition slide agglutination test. To 15 μl of GBS-BSA buffer, the following were added in sequential order: *S. typhi* LPS (4 μl in GBS-BSA, 1 μg/ml), unknown serum (4 μl in GBS-BSA, undiluted) or GBS-BSA buffer, and white anti-O9 antibody-conjugated latex particles (15 μl in GBS-BSA, 1% solids). The slide was rocked gently by hand for 2 min, and the results were read based on whether "clumps" (aggregated material) visible to the naked eye appeared in an otherwise "smooth" solution. Clump formation indicates a positive agglutination reaction and a negative result in this inhibition test.

Direct slide agglutination test. To 16 μl of GBS-BSA buffer on a slide, 4 μl of undiluted unknown serum or GBS-BSA buffer and 15 μl of 1% (wt/vol in GBS-BSA) LPS-coated latex particles were added and mixed for 2 min by rocking the slide gently. Results were read based on clump formation in the reaction fluid (see above).

Well agglutination test. To 25 μl of GBS-BSA buffer contained in a well (200-μl capacity) of a round-bottom microtiter plate (Costar), 25 μl of unknown serum (1:4 dilution in GBS-BSA) and 50 μl of 1% (wt/vol in GBS-BSA) LPS-coated latex particles were added. The plate was placed on a flat-bed shaker (MTS4; Ika-Schuttler, Staufen, Germany) and shaken at 800 rpm for 1 h at room temperature. After a further incubation for 15 min without agitation, the results were read based on the presence of clumps at the bottom of the well (the presence of clumps indicates a positive reaction). Unreacted particles remained suspended.

ELISA. Immunon-2 microtiter plates (Dynatech Laboratories) were coated with *S. typhi* LPS (Difco Laboratories) at 2.5 μg/ml in 0.1 M bicarbonate buffer (pH 9.6). The unknown serum (100 μl of 1:2,000 dilution in PBS–1% BSA, in duplicate) was added and incubated at 37°C for 2 h. Following a wash with PBS, biotin-labeled goat anti-human IgM (1:5,000 in PBS–1% BSA; TAGO, Burlingame, Calif.) was added and incubated for 1.5 h at 37°C. Following a wash with PBS, the assay was developed by using peroxidase-labeled ExtraAvidin (2 μg/ml; Sigma) as in the inhibition ELISA. IgG anti-LPS antibodies were detected in a similar way except that the unknown serum was used at 1:4,000 dilution in PBS–1% BSA and goat anti-human IgG (1:5,000 in PBS–1% BSA; TAGO) was used in place of the anti-IgM reagent.

Statistics. Regression analysis was performed by using linear regression programmed in Statview II (MacIntosh).

RESULTS

Preliminary examination of TUBEX. The performance of the TUBEX test using the anti-O9 hybridoma antibody as the inhibitor was examined. If score 2 in the color standard (Fig. 2) is arbitrarily chosen as the cutoff for negative reactions, in which the liquid contents are still more red than blue, then the test could detect as little as 16 μg of the antibody/ml (Fig. 3). Replicate testing revealed identical results (data not shown). The specificity of the test was shown by two irrelevant IgM hybridoma antibodies (TS2 and 7C2C5) which were specific for *T. spiralis* antigens (4, 19) but were completely unreactive in the test (Fig. 3). The test kit kept well at 4°C, as shown by the fact that only a slight loss of activity was detected after 9 months of storage (Fig. 3).

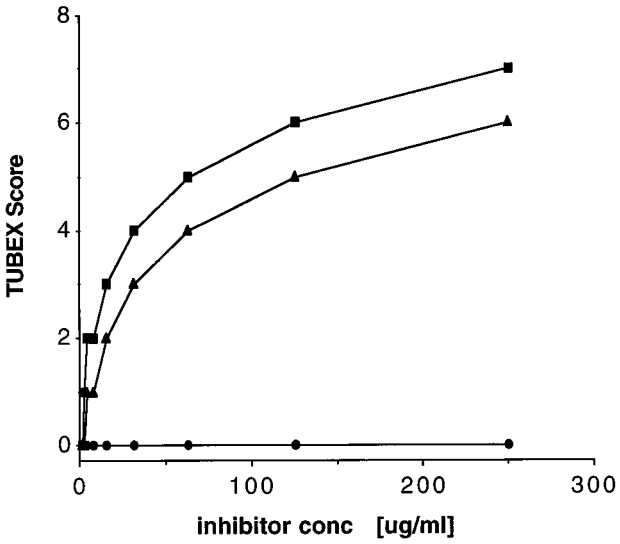

FIG. 3. Sensitivity, specificity, and stability of TUBEX. Symbols: ■, anti-O9 MAb (inhibitor) and freshly prepared reagents; ▲, anti-O9 MAb and 9-month-old reagents; ●, control MAbs and freshly prepared reagents.

Examination of sera by TUBEX. Serum samples from 14 patients (patients 1 through 14) with proven typhoid fever were examined by the TUBEX test. The scores obtained ranged from 3 (one patient) to 8 (two patients; the median score was 6 (Table 1; Fig. 4). A serum sample from each of 11 patients (patients 15 through 25) clinically diagnosed as having typhoid fever but not bacteriologically investigated was also examined. All were highly reactive, with scores ranging from 5 to 8 (median score, 7) (Table 1). In contrast, none of 65 control sera had scores higher than 2, and most (89%) had a score of 0 (Table 1; Fig. 4). These sera were obtained from healthy individuals (subjects N1 through N26) or patients suspected of having autoimmune disease (subjects N27 through N65; see Materials and Methods). In addition, six serum samples obtained from typhus fever patients who had positive serology in the Weil-Felix test (one sample; OX19 titer, >1:160) or for *R. mooseri* (five samples from three patients; titers ranged from 1:1,280 to 1:10,240) were unreactive in the TUBEX test (five had a score of 0 and one had a score of 1). Furthermore, seven sera obtained from septicemic patients infected with *E. coli* (three patients, one with a repeat specimen), *P. stuartii* (one patient), *A. hydrophila* (one patient) or *S. aureus* (one patient) were all negative in the test (six had a score of 0 and one had a score of 1).

Thus, if a score of 2 is taken as the upper limit for a negative reaction, then the test was 100% sensitive (16 of 16 samples) and 100% specific (78 of 78 samples). The 11 sera from patients diagnosed only clinically as having typhoid were all positive in the TUBEX test.

Examination of sera by other immunoassays. To validate the reliability of the TUBEX test, some of the serum samples were subsequently examined by several other immunoassays (Table 1). One of these, the inhibition ELISA, on which the TUBEX test is based and which detects anti-O9 antibodies, demonstrated a similarly high sensitivity (100%; 15 of 15 samples) and specificity (96.8%; 60 of 62 samples) (Tables 1 and 2).

The direct ELISA was used to measure objectively the

TABLE 1. Comparison of TUBEX with other immunoassays

Serum[a]	Result with the following test[b]:								
	TUBEX	Inh-ELISA	Inh-slide	IgM ELISA	IgG ELISA	Agg-well	Agg-slide	Widal (TO)	Widal (TH)
P1	8	0.04	+	1.10	1.20	−	−	400	400
P2a	6	0.02	+	1.40	0.22	+	ND	200	400
P2b	5	0.01	ND	1.30	0.88	+	ND	200	400
P3	4	0.06	−	0.42	0.84	−	ND	200	400
P4	6	0.16	−	0.67	0.33	+	+	400	200
P5	6	ND	+	0.93	1.40	−	−	200	100
P6	6	0.01	ND	ND	ND	−	ND	50	100
P7	7	0.02	−	0.41	1.10	−	−	100	100
P8	5	0.15	−	0.80	1.20	−	−	400	100
P9	3	0.14	ND	0.14	0.59	+	ND	400	100
P10	6	0.06	−	0.90	0.85	−	−	<50	<50
P11a	5	0.12	−	0.17	0.58	−	−	<50	<50
P11b	7	0.01	+	0.22	1.30	−	−	<50	<50
P12	5	0.01	−	0.90	1.00	−	−	50	100
P13	5	0.09	−	0.90	0.57	−	−	<50	50
P14	8	0.01	+	1.20	0.69	+	+	400	400
P15	8	0.08	ND	1.99	1.94	ND	+	200	100
P16	7	0.08	ND	1.38	1.31	ND	−	100	<50
P17	7	0.13	ND	1.64	1.60	ND	+	400	200
P18	6	0.20	ND	0.50	0.51	ND	−	50	100
P19	6	0.04	ND	0.41	0.72	ND	−	800	<50
P20	8	ND	ND	ND	ND	ND	ND	400	400
P21	7	ND	ND	ND	ND	ND	−	400	200
P22	5	0.23	ND	0.33	0.39	ND	−	<50	1,600
P23	5	0.27	ND	0.63	0.58	ND	−	<50	200
P24	5	0.13	ND	0.47	0.45	ND	−	<50	200
P25	7	0.04	ND	1.88	1.72	ND	+	400	800
N1	1	0.27	−	0.16	0.17	−	−	<50	<50
N2	2	0.24	−	0.29	0.62	−	−	200	<50
N3	0	0.40	ND	0.01	0.20	ND	ND	200	<50
N4	0	0.23	ND	0.03	0.15	−	ND	100	<50
N5	1	ND	ND	0.08	0.02	−	ND	50	<50
N6	0	0.27	ND	0.13	0.11	ND	ND	<50	<50
N7	0	0.28	ND	0.08	0.20	−	ND	<50	<50
N8	0	0.27	−	0.04	0.10	ND	−	<50	<50
N9	0	0.34	ND	0.03	0.04	ND	ND	50	<50
N10	0	0.24	ND	0.01	0.22	−	ND	<50	<50
N11	0	0.39	−	0.04	0.09	−	−	100	<50
N12	0	0.28	ND	0.07	0.07	ND	ND	<50	<50
N13	0	0.27	ND	0.06	0.27	ND	ND	<50	<50
N14	0	ND	ND	ND	ND	−	ND	50	<50
N15	0	0.22	ND	0.03	0.04	−	ND	<50	<50
N16	0	0.41	−	0.09	0.05	−	ND	50	<50
N17	1	0.26	−	0.06	0.26	−	ND	50	<50
N18	2	0.29	−	0.04	0.09	−	−	<50	<50
N19	0	0.15	ND	0.03	0.06	ND	ND	<50	<50
N20	0	0.24	ND	0.09	0.29	ND	ND	<50	<50
N21	0	ND	−	0.05	0.36	−	−	<50	100
N22	0	0.40	ND	0.02	0.04	ND	ND	<50	<50
N23	0	0.24	ND	0.08	0.03	−	−	50	50
N24	0	0.26	ND	0.02	0.01	−	ND	50	50
N25	0	0.41	ND	0.03	0.02	ND	ND	50	50
N26	0	0.31	−	0.08	0.19	−	+	50	50
N27	0	0.27	ND	0.01	0.07	−	−	50	50
N28	0	0.33	ND	0.06	0.08	−	−	50	50
N29	0	0.32	ND	0.03	0.23	−	−	50	800
N30	0	0.31	−	0.01	0.09	−	−	50	<50
N31	0	0.27	ND	0.05	0.10	−	ND	ND	ND
N32	0	0.30	−	0.09	0.07	−	−	ND	ND
N33	0	0.39	ND	0.03	0.17	−	ND	ND	ND
N34	0	0.36	ND	0.04	0.02	−	ND	ND	ND
N35	0	0.30	ND	0.02	0.05	−	ND	ND	ND
N36	0	0.33	ND	0.00	0.06	−	ND	ND	ND
N37	0	0.31	−	0.01	0.09	−	−	ND	ND

Continued on following page

TABLE 1—*Continued*

Serum[a]	Result with the following test[b]:								
	TUBEX	Inh-ELISA	Inh-slide	IgM ELISA	IgG ELISA	Agg-well	Agg-slide	Widal (TO)	Widal (TH)
N38	0	0.35	−	0.01	0.06	−	−	ND	ND
N39	0	0.33	ND	0.01	0.15	−	ND	ND	ND
N40	0	0.37	ND	0.04	0.12	−	ND	ND	ND
N41	0	0.36	ND	0.10	0.19	−	ND	ND	ND
N42	0	0.29	ND	0.03	0.10	−	ND	ND	ND
N43	0	0.33	−	0.04	0.06	−	−	ND	ND
N44	2	0.20	−	0.03	0.11	−	−	ND	ND
N45	0	0.24	ND	0.03	0.05	−	ND	ND	ND
N46	0	0.27	ND	0.09	0.07	−	ND	ND	ND
N47	0	0.32	ND	0.08	0.16	−	ND	ND	ND
N48	0	0.33	ND	0.01	0.02	−	ND	ND	ND
N49	0	0.27	ND	0.05	0.17	−	ND	ND	ND
N50	0	0.38	−	0.08	0.08	−	−	ND	ND
N51	0	0.26	ND	0.04	0.04	−	ND	ND	ND
N52	0	0.37	ND	0.06	0.13	−	ND	ND	ND
N53	0	0.31	ND	0.12	0.04	−	ND	ND	ND
N54	0	0.26	ND	0.06	0.14	−	−	ND	ND
N55	0	0.24	ND	0.08	0.04	−	ND	ND	ND
N56	0	0.29	ND	0.04	0.25	−	ND	ND	ND
N57	0	0.40	ND	0.01	0.11	−	ND	ND	ND
N58	0	0.21	−	0.14	0.06	−	+	ND	ND
N59	0	0.26	ND	0.07	0.32	−	ND	ND	ND
N60	0	0.27	ND	0.03	0.17	−	ND	ND	ND
N61	0	0.16	ND	0.09	0.12	−	ND	ND	ND
N62	0	0.24	−	0.01	0.27	−	−	ND	ND
N63	0	0.21	−	0.05	0.42	−	−	ND	ND
N64	0	0.28	ND	0.06	0.16	−	ND	ND	ND
N65	1	0.26	−	0.07	0.53	−	−	ND	ND

[a] Sources of sera were as follows: P1 through P14, 14 patients with bacteriologically confirmed cases of typhoid; P15 through P25, 11 patients clinically diagnosed as having typhoid fever but not bacteriologically investigated; N1 through N26, 26 healthy blood donors; N27 through N65, patients suspected of having autoimmune disease. In addition, the TUBEX text was performed on six Weil-Felix- or *R. mooseri*-positive serum samples (N66 through N71; five with a score of 0 and one with a score of 1) and on seven samples from six septicemic patients (N72 through N78; six with a score of 0 and one with a score of 1).

[b] Inh-ELISA, inhibition ELISA; Inh-slide, inhibition slide agglutination test; Agg-well, well agglutination test; Agg-slide, direct slide agglutination test. Results are expressed as scores (ranging from 0 to 10) for the TUBEX test (see Materials and Methods), as ODs at 490 nm for all ELISAs, as reciprocals of titers for the Widal test, and as positive (+) or negative (−) reactions for the inhibition slide agglutination, direct slide agglutination, and well agglutination tests. ND, not done. Positive results are ≥3 for TUBEX, ≤0.2 for the inhibition ELISA, ≥0.14 for the IgM ELISA, ≥0.38 for the IgG ELISA, and ≥1:50 for the Widal test.

amounts of IgM and IgG antibodies to *S. typhi* LPS (whole antigen) in the sera (Tables 1 and 2). Of the 15 samples from typhoid-proven patients that were examined, all (100%) had significant levels of IgM antibodies (albeit in 2 of the samples [P11a and P11b], they were just detectable) (mean absorbance = 0.76 ± 0.41), and all but 2 samples (P2a and P4) (86.7%) were positive for IgG antibodies (mean absorbance = 0.85 ± 0.35). All nine samples (100%) from the clinically diagnosed typhoid patients that were examined had high titers of IgM (absorbance = 1.03 ± 0.69) and IgG (absorbance = 1.02 ± 0.62) antibodies. In contrast, 96.9 and 95.3% of the 64 control samples examined had no IgM (absorbance = 0.06 ± 0.05) or IgG (absorbance = 0.14 ± 0.12) anti-LPS antibodies, respectively.

Since TUBEX is based to some extent on the slide latex agglutination test, the performance of TUBEX on slides, instead of tubes, was examined. The slide test required another modification, since LPS-coated magnetic particles could not be used on slides; free LPS was used instead. In this format, the test was found to be poorly sensitive (38.5%; 5 of 13 samples) (Tables 1 and 2). Direct slide agglutination tests using LPS-coated indicator particles were also examined. Again, the detection rate obtained was extremely poor whether the test was performed on slides (18.2%; 2 of 11 samples) or in microwells (31.3%; 5 of 16 samples) (Tables 1 and 2).

The Widal titers of some of the sera are shown in Table 1. These were obtained from another laboratory and were withheld from the person (F.C.H.T.) conducting the TUBEX test until after the study. If Widal titers of antibodies to the O antigens (TO) or the flagellar antigens (TH) of >1:50 are taken to be positive, as is normally the case for single-sample testing (2), the detection rate based on the 14 proven cases of typhoid is 81.3% (13 of 16 samples). All the sera from patients with clinical cases of typhoid were positive in the Widal test, although three of them had TO of <1:50. However, the specificity of the test was low (43.3%; 13 of 30 samples) when 30 healthy blood donors (subjects N1 through N30) were examined.

Comparison of TUBEX with other immunoassays. The TUBEX test results correlated to some extent with those of the inhibition ELISA based on the antibody activities of the typhoid-proven and typhoid-suspected patients, although the association was not significant ($r = 0.38$, $P = 0.07$) (Fig. 5). Excellent correlation was seen between the TUBEX results and those of the IgM ELISA ($r = 0.58$, $P = 0.003$) and between the TUBEX results and those of the IgG ELISA ($r = 0.54$, $P = 0.006$) (Fig. 5). In contrast, there was no correlation between the TUBEX results and the Widal TO ($r = 0.25$, $P = 0.20$) (Fig. 5) or TH ($r = 0.01$, $P = 0.97$). Specifically, for patient P9, who had a low TUBEX score (3), low activities were also found in the various ELISAs but not in the Widal test (Table 1). Two patients (patients 1 and 14) who had the highest TUBEX score (8) also had high activities in the ELISAs and the Widal test. Sera from three of the clinically diagnosed typhoid patients

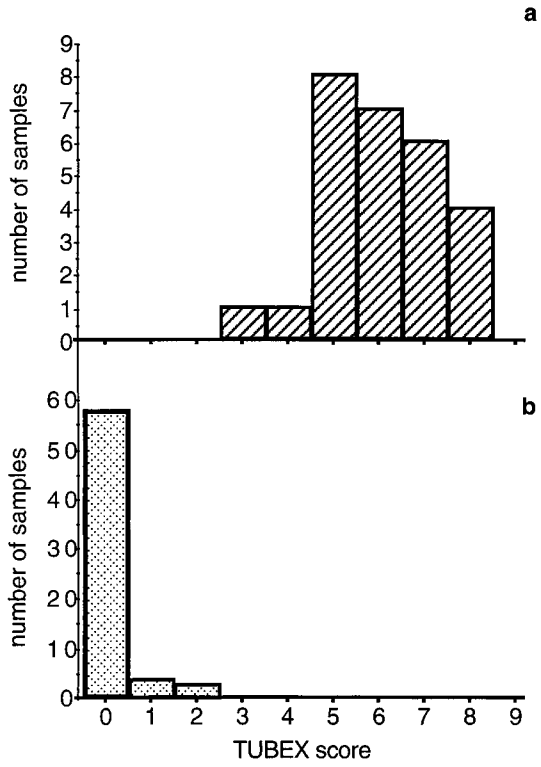

FIG. 4. Frequency distribution of serum samples obtained from typhoid-proven and typhoid-suspected patients (a) and control subjects (b) according to their TUBEX scores.

(patients 18, 22, and 23) that were negative in the inhibition ELISA also showed low activities in the TUBEX and Widal (TO) tests and in the IgM and IgG ELISAs.

There was no correlation between the results of the inhibition ELISA and those of the IgM ($r = 0.31$, $P = 0.14$) or IgG ($r = 0.37$, $P = 0.08$) ELISA. There was also no correlation between the results of the Widal (TO) test and those of the IgM ($r = 0.21$, $P = 0.33$) or IgG ($r = 0.18$, $P = 0.40$) ELISA. In contrast, the results obtained for the IgM and IgG ELISAs were strongly correlated with each other ($r = 0.59$, $P = 0.002$).

TUBEX detected both IgM and IgG antibodies. Thus, it was positive for sera which had low IgM but high IgG (P9, P11a, P11b) or low IgG but high IgM (P2a, P4) anti-LPS antibodies (Table 1). Although the low IgM levels in the serum samples from patient 11 could account for the negative Widal (TO) result, this was not the case with two other Widal (TO)-negative samples (P10 and P13), which had high IgM levels.

The value of repeat specimen testing is shown in the case of patient 11, where an increase in antibody activity was noted in the TUBEX and the ELISAs, although the Widal results remained negative (Table 1). Among the control subjects, only one (subject N2) had slightly elevated IgM and IgG anti-LPS activities as well as a strongly positive Widal (TO) result (Table 1). However, the TUBEX (score 2) and inhibition ELISA results were negative for this individual.

DISCUSSION

TUBEX appears to be an ideal test to help in the diagnosis of typhoid fever. The test is fast, simple, and easy to use. TUBEX can be used in developing countries where the disease is especially prevalent and where the cost of ELISA readers and other forms of instrumentation is prohibitive. Although we have used a mechanical shaker in the present study, the test can also be performed entirely by hand (just like slide agglutination tests). TUBEX may thus be used by the bedside instead of in the laboratory. Since multiple tests can be performed simultaneously, TUBEX may be useful for mass screening.

There are drawbacks in the system, however, as a result of its simplicity. Firstly, the results can be subjective, since they are read by eye. While there was no problem in noting strong reactions (scores of 5 and higher) and scoring them as positives, some deliberation was required with the weaker reactions (scores of 3 or 4). Another drawback of TUBEX is that the analytical sensitivity is not very high (Fig. 3). Although this means that early cases of typhoid may be missed, the clinical sensitivity of TUBEX was found to be very good (100%) in the present study, comparable to those of the inhibition and direct ELISAs (Table 2). TUBEX detected anti-O9 antibodies in three of the serum samples which were negative by the Widal test. An advantage of TUBEX over the Widal test and other agglutination assays is that, unlike these tests, which favor IgM detection, TUBEX detects both IgM and IgG antibodies equally well by virtue of the inhibitory activities of the antibodies rather than their ability to agglutinate. Inhibitory function is easier to detect; this is possible even with low-affinity antibodies. This is one of the reasons why the slide agglutination test examined in our study, which used LPS-coated indicator particles, failed to detect antibodies in the majority of the typhoid cases. On the other hand, since some of the missed cases did have high levels of IgM anti-LPS antibodies, there may be other problems associated with the slide technique. Increasing the incubation period and performing the assay in microwells instead of slides, however, did not improve the sensitivity. Since the Widal test result was positive for most of the typhoid cases, presumably success in detection by direct agglutination requires prolonged (overnight) incubation so that large antigen-antibody aggregates can form. TUBEX does not require such aggregate formation, since the antigen-sensitized magnetic particles it uses will cosediment any antibody latex particles associated with them.

Despite the fact that the TUBEX test is subjective and semiquantitative, the results obtained from it correlated very well with those obtained by a more objective and quantitative method, the IgM or IgG ELISA, which detects antibodies to *S. typhi* LPS (Fig. 5). This implies that TUBEX detects "real" antibodies rather than some nonspecific inhibitor and that anti-O9 antibodies form an integral part of the anti-LPS response. We expected the TUBEX results to be very similar to those of the inhibition ELISA, since both assays used the same principle of detection and the same reagent antibody and antigen. Al-

TABLE 2. Comparison of TUBEX with other immunoassays (summary)

Assay[a]	Sensitivity[b]	Specificity[b]
TUBEX	100 (16)	100 (69)
Inh-ELISA	100 (15)	96.8 (62)
Inh-slide	38.5 (13)	100 (20)
IgM ELISA	100 (15)	96.9 (64)
IgG ELISA	86.7 (15)	95.3 (64)
Agg-well	31.3 (16)	100 (55)
Agg-slide	18.2 (11)	91.3 (23)
Widal (TO + TH)	81.3 (16)	43.3 (30)

[a] Inh-ELISA, inhibition ELISA; Inh-slide, inhibition slide agglutination test; Agg-well, well agglutination test; Agg-slide, direct slide agglutination test.
[b] Expressed as percent (sample size).

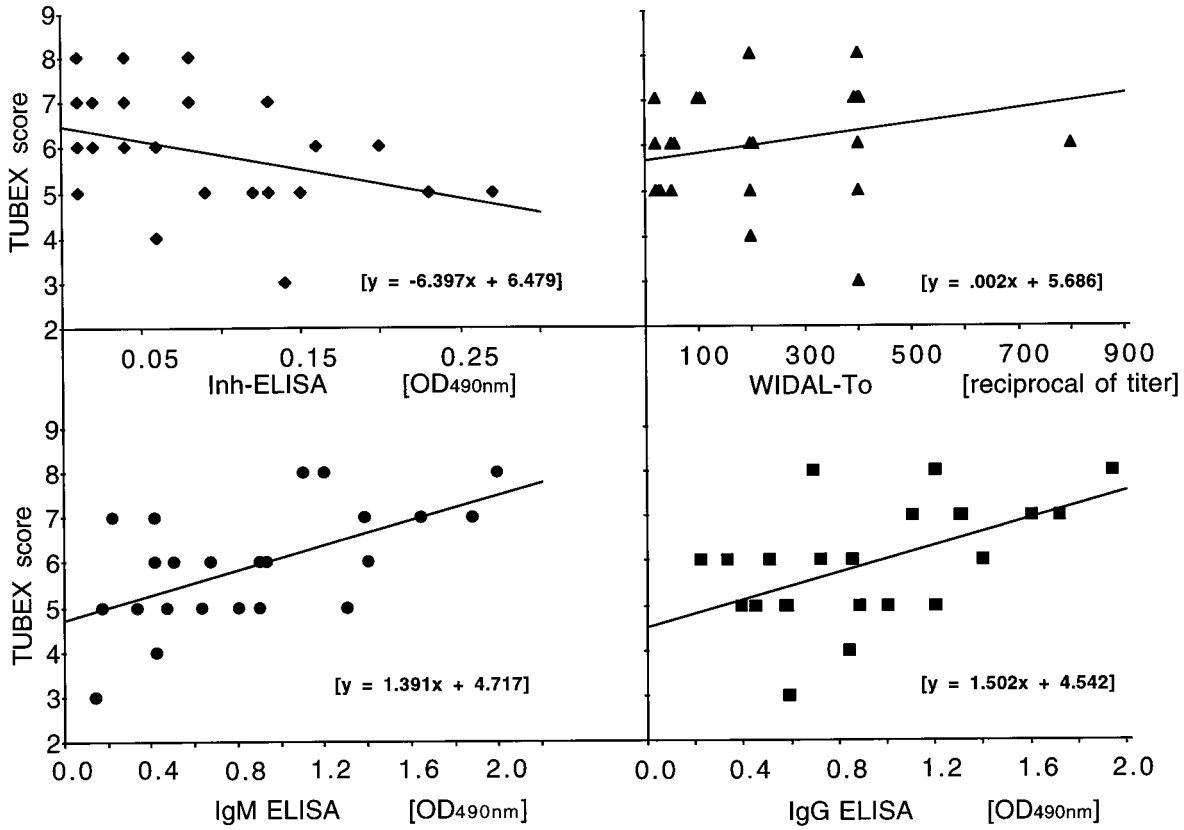

FIG. 5. Regression analysis of results obtained by TUBEX for the typhoid-proven and typhoid-suspected cases compared with those of other immunoassays. Inh-ELISA, inhibition ELISA.

though there was some correlation, the association was not significant. The probable reason for the discordance is that a very narrow window (0.0 to 0.2 optical density [OD] units) was used to score the positive reactions in the inhibition ELISA, which made readings rather inaccurate. Consequently, correlation was not seen between the inhibition ELISA results and those of the IgM or IgG ELISA, either. As expected, there was also no correlation between the TUBEX and Widal TO (or TH) results or between the Widal TO (or TH) and IgM or IgG ELISA results, implying that the Widal (TO) test possibly detects other reactants besides anti-O9 antibodies.

The study using a slide version of the TUBEX test is informative. The slide test uses the same antibody-coated indicator particles but uses unconjugated reagent antigen, and slides instead of tubes. The test worked poorly. The most probable reason for the failure is that, in this modification, much larger amounts of reagent antibody and antigen are used to make the reaction visible, and the formation of large antigen-antibody aggregates is required in the absence of the magnetic particles.

The specificity of TUBEX was extremely good (100%). This finding is not surprising, since previous investigators found *S. typhi* LPS to be very specific (18, 20, 21). Narrowing this antigen to the immunodominant O9 determinant would, in theory, increase the specificity of the assay. Indeed, using an inhibition ELISA to measure anti-O9 antibodies in patients, we observed very good specificity with the test previously (8, 12). The high specificity resulting from measuring anti-O9 antibodies may explain why a healthy subject (subject N2) in the present study who had elevated levels of IgM and IgG anti-LPS antibodies and a high Widal TO had negative results in both the TUBEX test and the inhibition-ELISA.

α-D-Tyvelose is the immunodominant sugar of the O9 determinant. An extremely rare sugar in nature, α-D-tyvelose is antigenically different from the β-D-tyvelose found in *T. spiralis* (19) or the L-tyvelose in *Ascaris lumbricoides* (7). One of the *T. spiralis*-specific MAbs (7C2C5) used in the present study recognizes the tyvelose moiety in the nematode (4) but is clearly not cross-reactive with the *S. typhi* antigen (Fig. 3). (The other control antibody used, TS2, is specific for phosphorylcholine [4].) However, the O9 determinant is present not only in *S. typhi* but also in several other serotypes of *Salmonella* (serogroup D) such as *S. enteritidis* and *S. sendai* (6). However, many of these bacteria are not invasive and may not stimulate a systemic antibody response. The extent to which TUBEX detects infection caused by these salmonellae or the paratyphoid serotypes (8, 12) remains to be investigated. Previously, using the ELISA equivalent of TUBEX (12), we found that serum samples from septicemic patients infected with *Salmonella* organisms not belonging to serogroup D (one with *S. choleraesuis*, one with *S. johannesburg*, and one with *S. senftenberg*) were negative in the test, whereas that from a patient infected systemically with *S. sendai* (serogroup D) was weakly positive. Interestingly, serum samples from two patients infected with *S. paratyphi A*, a non-serogroup D organism, were strongly positive in the test. The reactivity was due to the presence in the patients of anti-O12 antibodies, which bound to the O12 determinant in the detecting antigen (LPS) and consequently blocked, by steric hindrance, the binding of the reagent MAb

to the adjoining O9 determinant in the LPS. Consequently, TUBEX will potentially give positive results for infections caused by any invasive *Salmonella* bacteria which bear the O9 or O12 antigen. Thus, to make TUBEX more specific for typhoid fever, supplementary tests such as those detecting anti-Vi, anti-dH, or anti-OM antibodies, can be included.

In the present study, the specificity of the TUBEX test was examined mainly by using sera from healthy blood donors and patients suspected of having autoimmune disease. These were all negative. Although sera from four patients with typhus fever and six with bacterial septicemia were also examined and all found to be negative, more such specimens, including specimens from patients suffering from other febrile illnesses such as malaria and dengue fever, need to be studied in the future in order to obtain a more thorough evaluation. TUBEX is currently being evaluated in several countries, including Papua New Guinea.

ACKNOWLEDGMENTS

This study was partly supported by a grant from the Biotechnology Research Institute, the Hong Kong University of Science and Technology, Hong Kong.

We thank Willina Lim, Virus Unit, Queen Mary Hospital, Hong Kong, for the gift of the typhus sera. We also thank Ng Kui Hin, Wong Kai Yau, and Lawrence W. K. Ng for technical help in various parts of this work and Victoria Li and Peggy Fung for help in typing the manuscript.

REFERENCES

1. **Chaicumpa, W., Y. Ruangkunaporn, D. Burr, M. Chongsa-Nguan, and P. Echeverria.** 1992. Diagnosis of typhoid fever by detection of *Salmonella typhi* antigen in urine. J. Clin. Microbiol. **30:**2513–2515.
2. **Cheong, Y. M., and M. Jegathesan.** 1989. Standardisation of the Widal test. Med. J. Malays. **44:**267.
3. **Choo, K. E., S. J. Oppenheimer, A. B. Ismail, and K. H. Ong.** 1994. Rapid serodiagnosis of typhoid fever by dot enzyme immunoassay in an endemic area. Clin. Infect. Dis. **19:**172–176.
4. **Choy, W. F., M. H. Ng, and P. L. Lim.** 1991. Trichinella spiralis: light microscope monoclonal antibody localization and immunochemical characterization of phosphorylcholine and other antigens in the muscle larva. Exp. Parasitol. **73:**172–183.
5. **Hudson, L., and F. C. Hay.** 1989. Practical immunology, 3rd ed. Blackwell Scientific Publications, Oxford, United Kingdom.
6. **Jann, K., and O. Westphal.** 1975. Microbial polysaccharides, p. 1–25. *In* M. Sela (ed.), The antigens, vol. 3. Academic Press, New York, N.Y.
7. **Jezyk, P. F., and D. Fairbairn.** 1967. Ascarosides and ascaroside esters in Ascaris lumbricoides (Nematoda). Comp. Biochem. Physiol. **23:**691–705.
8. **Lim, P. L.** 1986. Diagnostic uses of monoclonal antibodies to Salmonella, p. 29–75. *In* A. J. Macario and E. C. de Macario (ed.), Monoclonal antibodies against bacteria, vol. 3. Academic Press, Inc., New York, N.Y.
9. **Lim, P. L.** 1987. Isolation of specific IgM monoclonal antibodies by affinity chromatography using alkaline buffers. Mol. Immunol. **24:**11–15.
10. **Lim, P. L.** 1990. A one-step two-particle latex immunoassay for the detection of Salmonella typhi endotoxin. J. Immunol. Methods **135:**257–261.
11. **Lim, P.-L., and Y.-P. Fok.** 1987. Detection of group D salmonellae in blood culture broth and of soluble antigen by tube agglutination using an O-9 monoclonal antibody latex conjugate. J. Clin. Microbiol. **25:**1165–1168.
12. **Lim, P. L., and M. Y. Ho.** 1983. Diagnosis of enteric fever by inhibition assay using peroxidase-labelled monoclonal antibody and Salmonella typhi lipopolysaccharide. Aust. J. Exp. Biol. Med. Sci. **61:**687–704.
13. **Lim, P. L., and K. H. Ko.** 1990. A tube latex test based on colour separation for the detection of IgM antibodies to either one of two different microorganisms. J. Immunol. Methods **135:**9–14.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
15. **Mekara, Y., N. Maneekam, V. Vithayasai, and S. Makonkawkeyoon.** 1990. Determination of antibody from typhoid patients against lipopolysaccharide and protein antigens of Salmonella typhi. Asian Pac. J. Allergy Immunol. **8:**95–101.
16. **Nandakumar, K. S., V. Palanivel, and V. Muthukkaruppan.** 1993. Diagnosis of typhoid fever: detection of Salmonella typhi porins-specific antibodies by inhibition ELISA. Clin. Exp. Immunol. **94:**317–321.
17. **Nardiello, S., S. 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.
18. **Quiroga, T., T. Goycoolea, R. Tagle, F. Gonzalez, L. Rodriguez, and L. Villarroel.** 1992. Diagnosis of typhoid fever by two serologic methods. Enzyme-linked immunosorbent assay of antilipopolysaccharide of Salmonella typhi antibodies and Widal test. Diagn. Microbiol. Infect. Dis. **15:**651–656.
19. **Reason, A. J., L. A. Ellis, J. A. Appleton, N. Wisnewski, R. B. Grieve, M. McNeil, D. L. Wassom, H. R. Morris, and A. Dell.** 1994. Novel tyvelose-containing tri- and tetra-antennary N-glycans in the immunodominant antigens of the intracellular parasite Trichinella spiralis. Glycobiology **4:**593–603.
20. **Shaheen, H. I., N. I. Girgis, G. R. Rodier, and K. A. Kamal.** 1995. Evaluation of the response of human humoral antibodies to Salmonella typhi lipopolysaccharide in an area of endemic typhoid fever. Clin. Infect. Dis. **21:**1012–1013.
21. **Sippel, J., N. Bukhtiari, M. B. Awan, R. Krieg, J. F. Duncan, K. A. Karamat, I. A. Malik, L. M. Igbal, and L. Legters.** 1989. 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. J. Clin. Microbiol. **27:**1298–1302.
22. **Srivastava, L., and V. K. Srivastava.** 1986. Serological diagnosis of typhoid fever by enzyme-linked immunosorbent assay (ELISA). Ann. Trop. Paediatr. **6:**191–194.
23. **Tsang, R. S. W., and P. Y. Chau.** 1981. Serological diagnosis of typhoid fever by counterimmunoelectrophoresis. Br. Med. J. **282:**1505–1507.
24. **Verdugo-Rodriguez, A., L. H. Gam, S. Devi, C. L. Koh, S. D. Puthucheary, E. Calva, and T. Pang.** 1993. Detection of antibodies against Salmonella typhi outer membrane protein (OMP) preparation in typhoid fever patients. Asian Pac. J. Allergy Immunol. **11:**45–52.