JOURNAL OF CLINICAL MICROBIOLOGY, July 1987, p. 1165–1168
0095-1137/87/071165-04$02.00/0
Copyright © 1987, American Society for Microbiology
Vol. 25, No. 7

Detection of Group D Salmonellae in Blood Culture Broth and of Soluble Antigen by Tube Agglutination Using an O-9 Monoclonal Antibody Latex Conjugate

PAK-LEONG LIM* AND YIN-PING FOK

Department of Microbiology, Northcote Science Building, University of Hong Kong, Hong Kong

Received 22 September 1986/Accepted 23 March 1987

Latex particles sensitized with a *Salmonella* O-9 monoclonal antibody were used to detect group D salmonellae in blood culture broths. The system was found to be 100% accurate when used on 104 clinical specimens that included 13 *S. typhi* isolates. Enteric fever could thus be diagnosed as early as 1 day after the initiation of culture in some (18%) cases. The latex particles were also used in an experimental situation to detect soluble *S. typhi* lipopolysaccharide by a tube agglutination method. This was found to be more than four times as sensitive as the conventional slide agglutination method because at least 50 ng of antigen per ml in either buffer or urine was detected, albeit with less efficiency when serum was used.

Enteric fever is caused by a few serotypes of *Salmonella*, most notable of which is *Salmonella typhi*. The disease is endemic in many countries, especially those in the Third World, and poses a major health problem in those regions. Rapid laboratory diagnosis of it is essential both for patient management and for epidemiological reasons. In general, the most definitive and successful method of diagnosis is culturing of the causative organism from blood or, sometimes, stool. By conventional methods this requires 2 or more days for the identification of the organism, however, even after it is grown from the primary broth or agar. Consequently, various ways have been devised to simplify the identification procedure. A popular approach that has been used not only for *Salmonella* bacteria but also for *Staphylococcus aureus* (3) and *Streptococcus pyogenes* (18) is the direct identification of the organism in the primary culture with specific antibodies used in, for instance, agglutination methods. Thus, by using *Salmonella* A, D, Vi, and polyvalent antiserum-sensitized staphylococcal cells in coagglutination tests, Mikhail et al. (9) detected *Salmonella typhi* and *Salmonella paratyphi* A organisms in ox bile blood cultures as efficiently (95% agreement) as by conventional subculture methods.

We have recently produced a hybridoma antibody to the O-9 antigen of *Salmonella* that exhibited the expected specificity, namely, it bound only to those salmonellae in serogroup D, such as *Salmonella typhi* and *Salmonella panama* (6, 7). Moreover, it was shown to be diagnostically useful in an inhibition enzyme-linked immunosorbent assay for detecting antibodies in serum in patients with and carriers of typhoid fever (6, 7). We coupled this antibody to latex particles and show here the usefulness of this reagent in identifying group D salmonellae from primary blood broth cultures. Moreover, the latex conjugate was used in a novel approach (tube agglutination) to detect soluble antigen in buffer; the conditions of the assay and the potential of the system for diagnosing enteric fever are discussed.

MATERIALS AND METHODS

Clinical specimens. All blood specimens received and processed in our routine diagnostic laboratory during a 4-month period were included in the bacteriological study. These were derived from patients with various diagnoses, such as enteric fever, septicemia, meningitis, pyrexia of unknown origin, or other conditions.

Blood (5 to 10 ml) from each patient was inoculated into 80 ml (aerobic incubation) and 50 ml (anaerobic) of brain heart infusion broth and incubated at 37°C. After 1 day of culture (day 1), a loopful of the broth was used to make a Gram-stained smear, and subcultures were made on blood and MacConkey agar. Any growth that was obtained was identified by conventional methods described by Cowan and Steele (2) and, in difficult cases, by the AutoMicrobic system (Vitek Systems, Inc., Hazelwood, Mo.) as well; serotypes of *Salmonella* isolates were determined with polyvalent antisera obtained from Wellcome Diagnostics, Dartford, England. Similar subcultures were made on days 2 and 5. On other days, when the broth became turbid or suspect for other reasons, both smearing and subculturing were performed as well. To maintain this schedule as much as possible, latex agglutination was only performed (immediately) on those broths that showed the presence of organisms either by smearing or culturing.

For the experimental studies on antigen detection, the following fluids spiked with various amounts (0 to 6.4 μg/ml) of *Salmonella typhi* lipopolysaccharide (LPS; Difco Laboratories, Detroit, Mich.) (source of O-9 antigen) were used: (i) 0.1 M glycine–0.9% sodium chloride (pH 8.2) buffer containing 0.1% Tween 20 and 1% bovine serum albumin (GBS-tb); (ii) normal human urine obtained from four healthy adults, including a pregnant woman; (iii) human sera obtained from seven individuals suspected of having syphilis but found to be negative by the Venereal Disease Research Laboratory test and from four healthy adults; and (iv) cerebrospinal fluid obtained from three patients with meningitis but that were found to be sterile.

Latex suspensions. A 1% suspension of latex particles (diameter, 0.797 μm; Sigma Chemical Co., Ltd., Poole, United Kingdom) was sensitized in 0.1 M glycine–0.9% sodium chloride buffer (pH 8.2) with an equal volume of *Salmonella* O-9 monoclonal immunoglobulin M (IgM) antibodies (7) by a previously described method (5). The antibodies were obtained from mouse ascites fluid that was partially purified by precipitation with cold 50% saturated ammonium sulfate and subsequently by cryoprecipitation.

* Corresponding author.

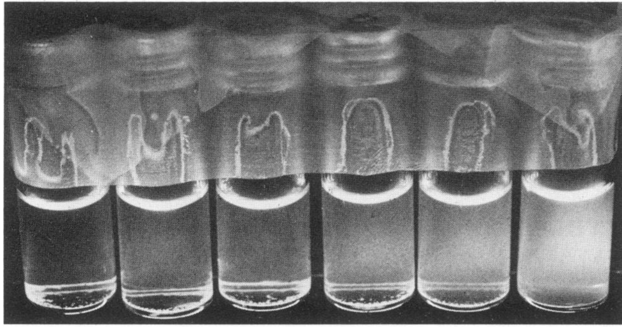

FIG. 1. Tube latex agglutination. Each vial contained 0.01% latex and various (400, 200, 100, 50, 25, and 0 ng/ml; in the tubes from left to right) amounts of LPS in 1 ml of GBS-tb buffer. Note the decrease in turbidity and the presence of agglutinated material at the bottom of the tube in the positive cases.

The latex-antibody mixture was incubated at room temperature for 2 h on a mixer (Coulter Electronic Ltd., Luton, Bedfordshire, England), after which 1% (final concentration) bovine serum albumin (Sigma) was added, and the incubation was continued for another hour. The latex particles were then washed (13,000 × *g*, 10 min) once in 0.1 M glycine–0.9% sodium chloride buffer and finally suspended as a 1% solution in the same buffer containing 1% bovine serum albumin and 0.02% sodium azide. (The O-9 monoclonal antibody used in this study can be obtained from Wellcome Diagnostics.)

The control latex suspension was prepared similarly by using a mouse ascites fluid fraction produced in our laboratory containing a monoclonal IgM antibody (MT46) to *Mycobacterium tuberculosis*.

Slide agglutination test. Test samples (30 μl) were placed on a blackened area of a reaction card (Wellcome), and 10 μl of a 1% latex suspension was added to it and mixed. For the detection of bacteria, the card was rocked gently by hand for 1 min. With soluble antigen, continuous mixing was allowed for 5 min by incubation of the mixture at room temperature (27°C) on a mechanical rotator (Arthur H. Thomas Co., Philadelphia, Pa.) at 120 rpm. The results were then read visually under a lamp. Positive agglutination by bacteria was indicated by the prompt and complete clumping of the latex particles. In the case of agglutination by the soluble antigen, the reaction was graded (±, +, ++, +++) according to the promptness and extent of the reaction.

Tube agglutination test. Test samples (1 to 4 ml) were placed in screw-cap glass vials (Flow Laboratories, Uxbridge, Middlesex, United Kingdom), and 10 to 40 μl of a 1% latex suspension was added to each sample. The mixture was then incubated on a mixer (Coulter Electronic Ltd.) for 1 to 3 h at room temperature. Following this, unless otherwise specified, the vials were held at 4°C for 2 h before the results were read. Positive reactions were interpreted as the decrease in turbidity of the solution compared with that of the control and the presence of agglutinated material at the bottom of the tube; these were graded (±, +, ++, +++) according to the prominence of these features (see Fig. 1).

RESULTS

Detection of group D salmonellae in blood culture broths. Of the 104 blood culture broths yielding pure cultures of various organisms that were examined, all those containing group D salmonellae (13 *Salmonella typhi*, 1 *Salmonella panama*) agglutinated the O-9 latex particles in a slide test. On the other hand, none of the other 90 broths reacted. These contained the following organisms: *Escherichia coli* (28 strains), *Bacillus* spp. (16 strains), *Klebsiella pneumoniae* (13 strains), *Acinetobacter anitratus* (7 strains), *Staphylococcus epidermidis* (5 strains), *Staphylococcus aureus* (4 strains), *Klebsiella aerogenes* (3 strains), *Salmonella typhimurium* (2 strains), *Salmonella* group C (2 strains), *Salmonella san diego* (1 strain), *Candida* sp. (1 strain), *Flavobacterium* sp. (1 strain), *Fusobacterium* sp. (1 strain), *Pseudomonas aeruginosa* (1 strain), *Pseudomonas putida* (1 strain), *Streptococcus agalactiae* (1 strain), *Streptococcus faecalis* (1 strain), and *Streptococcus pyogenes* (1 strain). (With both of the *Salmonella typhimurium* isolates, slight agglutination was observed, but this was delayed and incomplete.)

The frequencies of detection by the latex conjugate were 18.2, 36.4, 72.8, 81.9, and 100% of cases after 1, 2, 3, 4, and 5 days of culturing, respectively.

Detection of soluble antigen in buffer: optimization of conditions for tube latex agglutination. Reaction mixtures containing 400 ng of LPS per ml and 0.01% latex in 2 ml of GBS-tb buffer were incubated for 1, 2, or 3 h either with continuous mixing (on a mixer) at room temperature or without shaking (still) at 37°C. At the end of the incubation or after further incubation (without shaking) at 4°C for 2 h or overnight, the tubes were examined for agglutination. This was indicated by a decrease in turbidity in the suspension, the presence of agglutinated material at the bottom of the tube, or both (Fig. 1). It was found that continuous mixing of the reactants was absolutely required because without shaking even prolonged (3 h) incubation at 37°C followed by overnight incubation at 4°C did not result in agglutination. On the other hand, a positive reaction could be seen immediately if the reaction mixture was incubated with continuous mixing for as short as 1 h. Thus, to ensure the completeness of the reaction, especially with weaker antigen solutions, the protocol adopted in later studies was to use 2 h of continuous mixing at room temperature followed by an additional 2 h of still incubation at 4°C.

Other conditions for obtaining maximal sensitivity in the assay were investigated. The total reaction volume (1 to 4 ml) and the concentration of latex (final concentration, 0.0025 to 0.04%) in the mixture were varied. In general, the sensitivity of the assay increased with a decreasing concentration of latex (Table 1). However, it was difficult to

TABLE 1. Effect of various reaction volumes and latex concentrations on agglutination

Reaction vol (ml)	Concn of latex (final %)	Effect of the following concn of LPS (ng/ml)[a]:					
		400	200	100	50	25	0
1	0.01	+++	++	++	+	±	−
	0.02	+++	++	+	±	−	−
	0.04	+	±	−	−	−	−
2	0.005	+++	+++	+++	++	+	−
	0.01	+++	+++	++	+	±	−
	0.02	+++	++	±	−	−	−
4	0.0025	+++	+++	+++	++	+	−
	0.005	+++	+++	++	+	±	−
	0.01	+++	++	+	+	−	−

[a] Key: −, No agglutination; ± to +++, increasing agglutination.

TABLE 2. Antigen detection in biological fluids: comparison of tube and slide agglutination methods[a]

Medium	Agglutination method	Antigen detection at the following concn of LPS (ng/ml)[b]:											
		6,400	3,200	1,600	800	400	200	100	50	25	12.5	0	400[c]
Buffer	Tube	ND	ND	ND	ND	+ + +	+ +	+	±	±	–	–	–
	Slide	+ + +	+ + +	+ + +	+ +	+	±	–	–	ND	ND	–	ND
Urine (neat)	Tube	ND	ND	ND	ND	+ + +	+ +	+	±	–	–	–	–
	Slide	+ + +	+ + +	+ +	+ +	+	+	–	–	ND	ND	–	ND
Urine (diluted 1:2)	Tube	ND	ND	ND	ND	+ + +	+ +	+ +	+	±	–	–	–
	Slide	+ + +	+ + +	+ + +	+ +	+	+	–	–	ND	ND	–	ND
Serum (neat)	Tube	ND	ND	ND	ND	–	–	–	–	–	–	–	–
	Slide	+ +	+	±	–	–	–	ND	ND	ND	ND	–	ND
Serum (diluted 1:4)	Tube	ND	ND	ND	ND	±	–	–	–	–	–	–	–
	Slide	+ +	+	±	–	–	–	ND	ND	ND	ND	–	ND

[a] All reaction mixtures contained 0.01% latex in a 1-ml volume.
[b] Key: –, No agglutination; ± to + + +, increasing agglutination; ND, not done.
[c] Control latex was used.

observe the endpoint with latex concentrations of less than 0.01%. At a 0.01% latex concentration, which was consequently chosen for later studies, the sensitivity of the assay was 25 to 50 ng of LPS per ml, regardless of the reaction volume (Fig. 1 and Table 1). Thus, for convenience, 1-ml reaction mixtures were employed in later studies.

Antigen detection in biological fluids: comparison with the slide method. The applicability of the tube latex agglutination method to the detection of antigen in fluids other than plain buffer was investigated. Normal human urine was found to behave like buffer in all four cases examined, and the sensitivity of detection in the pooled urine that had been spiked with antigen was very similar to that obtained in buffer (Table 2). On the other hand, all seven serum samples obtained from individuals that were negative by the Venereal Disease Research Laboratory test and the four serum samples obtained from healthy adults nonspecifically agglutinated the latex particles; this, however, could be removed by pretreatment (continuous mixing for 2 h) of the serum sample with the latex control. By using the pooled, absorbed serum sample in either the tube or the slide test, the sensitivity of antigen detection observed was at least eightfold less than the corresponding result obtained in buffer or urine (Table 2). In all cases, whether in buffer, urine, or serum, the tube agglutination test was found to be at least fourfold more sensitive than the conventional slide agglutination test (Table 2). The specificity of the latex agglutination test was shown by the ability of the soluble homologous antibody (O-9), but not an irrelevant antibody (TS2), to inhibit the reaction in a slide test.

DISCUSSION

The direct use of antibodies as a rapid means of identifying *Salmonella* spp. in culture (8, 9, 11, 13, 14), clinical specimens (8, 14), and foods (10) has been investigated previously; variable success, which seemed to depend on the specificity of the (polyclonal) antibody and technique used, was seen in these studies. Svenungsson et al. (14), for example, demonstrated the high (100%) specificity that is achievable with a monospecific O-9 antiserum sample when used with immunofluorescence to detect *Salmonella enteritidis* organisms in fecal cultures. Under these circumstances an O-9 monoclonal antibody would be expected to be just as specific, but it has the advantage that its continual production is ensured without any change in its characteristics (quality). Indeed, we have shown here that by using such an antibody in a simple and popular technique (latex agglutination; for a review, see reference 16), a useful and reliable system can be obtained that can save at least 3 days time (and much cost and labor) compared with the conventional methods of detection of group D salmonellae from blood (or other sources). Positive cases of enteric fever were revealed by the antibody-latex conjugate throughout the 5-day study period, with most (73%) being noted after 3 days of culturing (Table 2). Only 18%, however, were found after 1 day; this is similar to the findings (22% positive) of Rockhill et al. (11) but differs from those (60% positive) of Mikhail et al. (9). The latter attributed their early detection success to the fact that blood clots were used in place of whole blood, although it may be just as important that the culture medium that they used was threefold smaller in volume (15 ml) than ours. Because all cases subjected to latex agglutination in our study were preselected by smear or culture, detection of some of these perhaps could be made 1 or 2 days earlier if latex examination had been used daily from the start of the culture. On the other hand, only those broths containing more than 10^8 organisms per ml would have been detected, a limit that has been determined previously from simulated blood cultures of *Salmonella typhi* organisms (C. W. K. Pang and P. L. Lim, unpublished observations). Earlier detection may also have been possible if the tube latex agglutination test (see below) had been employed instead of the slide agglutination test used in this study.

A limitation of the O-9 system is that infections caused by *S. typhi*, the most important cause of enteric fever, cannot be distinguished from those of the other group D salmonellae; however, this may not be clinically important. Differentiation requires additional tests, such as examining for the presence of d-H and Vi antigens in *S. typhi* organisms.

In addition to culturing, enteric fever can also be diagnosed in the laboratory by serological means, based on the detection of circulating antibodies or antigens in the patient (for a review, see reference 6). Of the two methods, antigen detection is used less often, but theoretically, it offers an earlier and more reliable marker of the disease. Results that have been obtained so far by the use of this approach have been conflicting. For example, a claim that *S. typhi* antigens

(O-9, d-H, and Vi) are present in the urine of most (97%) patients with typhoid fever (12) could not be substantiated by a later study (15), in which Vi antigen in urine was detected in only 34% of the cases. The three techniques most commonly used for antigen detection in these studies were coagglutination, counterimmunoelectrophoresis, and enzyme-linked immunosorbent assay (5, 6). Thus, it occurred to us that the O-9 latex conjugate could perhaps be used to diagnose enteric fever. Rather than using it in a slide test, we investigated whether it could be used to trap antigen in large volumes of fluid to enhance the chance of detection in voluminous materials such as urine and serum (hence, the tube method). The results were disappointing, because the sensitivity of the test that was obtained was the same regardless of the volume of fluid used (Table 1). Moreover, the addition of soluble antigen O-9-specific antibodies to the mixture following reaction of the latex and antigen did not enhance the sensitivity of the tube test (data not shown). Nevertheless, better (>fourfold) sensitivities were observed when the latex particles were reacted with antigen in the tube than on the slide (Table 2). Consequently, the tube agglutination method alternative may be preferred (as with bacterial agglutination [4]) for the detection of antigen in biological fluids, especially urine (1, 17); we are presently investigating this possibility in patients with typhoid fever. However, with some clinical specimens, such as cerebrospinal fluid, the slide test should be used first, owing to its rapidity and the seriousness of the disease; the tube method can then be used for confirmation or quantitation or in cases considered negative by the other method. While urine (Table 2) and cerebrospinal fluid (data not shown) had no inhibitory effect on our system, serum was found to be unsuitable because it interfered with agglutination in both the tube and slide methods (Table 2).

ACKNOWLEDGMENTS

We thank M. McIllmurray and the Wellcome Foundation, Dartford, England, for introducing the latex system and their interest in this work. We are also grateful to M. H. Ng and Y. K. Leung for interest and support and to Goretti Tse for help with the manuscript.

LITERATURE CITED

1. **Coonrod, J. D.** 1983. Urine as an antigen reservoir for diagnosis of infectious diseases. Am. J. Med. **75**:85–92.
2. **Cowan, S. T., and K. J. Steele.** 1974. Manual for the identification of medical bacteria, 2nd ed. Cambridge University Press, Cambridge.
3. **Doern, G. V., and L. I. Robbie.** 1982. Direct identification of *Staphylococcus aureus* in blood culture fluid with a commercial latex agglutination test. J. Clin. Microbiol. **16**:1048–1051.
4. **Freter, R.** 1980. Agglutination titration (Widal) for the diagnosis of enteric fever and other enterobacterial infections, p. 460–463. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology, 2nd ed. American Society for Microbiology, Washington, D.C.
5. **Fung, J. C., and R. C. Tilton.** 1985. Detection of bacterial antigens by counterimmunoelectrophoresis, coagglutination, and latex agglutination. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed., American Society for Microbiology, Washington, D.C.
6. **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.
7. **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.
8. **McRill, C. M., T. T. Kramer, and R. W. Griffith.** 1984. Application of the peroxidase-antiperoxidase immunoassay to the identification of salmonellae from pure culture and animal tissue. J. Clin. Microbiol. **20**:281–284.
9. **Mikhail, I. A., W. R. Sanborn, and J. E. Sippel.** 1983. Rapid, economical diagnosis of enteric fever by a blood dot culture coagglutination procedure. J. Clin. Microbiol. **17**:564–565.
10. **Mohr, H. K., H. L. Trenk, and M. Yeterion.** 1974. Comparison of fluorescent-antibody methods and enrichment serology for the detection of *Salmonella*. Appl. Microbiol. **27**:324–328.
11. **Rockhill, R. C., M. Lesmana, M. A. Moechtar, and A. Sutomo.** 1980. Detection of *Salmonella* C_1, D, and Vi antigens by coagglutination, in blood cultures from patients with *Salmonella* infections. Southeast Asian J. Trop. Med. Public Health **11**:441–445.
12. **Rockhill, R. C., L. W. Rumans, M. Lesmana, and D. T. Dennis.** 1980. Detection of *Salmonella typhi* D, Vi, and d antigens, by slide coagglutination, in urine from patients with typhoid fever. J. Clin. Microbiol. **11**:213–216.
13. **Sanborn, W. R., M. Lesmana, and E. A. Edwards.** 1980. Enrichment culture coagglutination test for rapid, low-cost diagnosis of salmonellosis. J. Clin. Microbiol. **12**:151–155.
14. **Svenungsson, B., H. Jorbeck, and A. A. Lindberg.** 1979. Diagnosis of *Salmonella* infections: specificity of indirect immunofluorescence for rapid identification of *Salmonella enteritidis* and usefulness of enzyme-linked immunosorbent assay. J. Infect. Dis. **140**:927–936.
15. **Taylor, D. N., J. R. Harris, T. J. Barrett, N. T. Hargrett, J. Prentzel, C. Valdiviese, C. Palomino, M. M. Levina, and P. A. Blake.** 1983. Detection of urinary Vi antigen as a diagnostic test for typhoid fever. J. Clin. Microbiol. **18**:872–876.
16. **Thomas, M.** 1986. Agglutination methods for rapid analysis. Nature (London) **320**:289–290.
17. **Weinberg, G. A., and G. A. Storch.** 1985. Preparation of urine samples for use in commercial latex agglutination tests for bacterial antigens. J. Clin. Microbiol. **21**:899–901.
18. **Wetkowski, M. A., E. M. Peterson, and L. M. de la Maza.** 1982. Direct testing of blood cultures for detection of streptococcal antigens. J. Clin. Microbiol. **16**:86–91.