A MICROAGGLUTINATION TEST FOR *YERSINIA ENTEROCOLITICA* INFECTION: RECOGNITION OF MULTIPLE SEROTYPES IN EXPERIMENTS WITH STAINED SUSPENSIONS OF SEROTYPE O5,27

I. D. RICCIARDI*, A. D. PEARSON†, W. G. SUCKLING† AND C. KLEIN†

*Institute of Microbiology, Federal University of Rio de Janeiro, Brazil, and

†Department of Microbiology, Southampton University, Tremona Road, Southampton SO9 4XY

IN published reports, human beings infected with *Yersinia enterocolitica* usually had either abdominal pain or diarrhoea as their presenting symptom. During a 1-year study in Sweden, *Y. enterocolitica* was isolated from 22 (3·8%) of 581 patients with appendicitis and from 16 (5·6%) of 284 patients with abdominal pain in whom appendicitis was suspected (Niléhn, 1969). Investigation of these patients showed that positive cultures correlated with the presence of antibodies to serotype 3 while few of the culture-negative group had agglutinins. The serological diagnosis of *Y. enterocolitica* infection is usually made by carrying out tube agglutination tests to detect type-specific antibodies. Two schemes of antigenic classification of *Y. enterocolitica* are in use, namely (1) the Winblad scheme (Winblad, 1968) which comprises serotypes O1 to O9, and (2) Wauters' classification (Wauters *et al.*, 1972) which extends the O serotypes to 34. Because the detection of antibodies to all of these serotypes would require many tests, the search is usually limited to tests for antibodies to commonly recognised serotypes, for example O3 and O9 in Europe (Niléhn, 1969; Mollaret, 1971). This practice, of course, fails to detect clinical infection with a wide range of less common serotypes and non-typable strains (Niléhn, 1969; Mollaret, 1971; Aldová and Lím, 1974; Kapperud, 1977). A test based on an antigen common to all types would therefore be useful.

In attempting to assess the importance of strains other than those of the common serotypes Lysy and Knapp (1973) have used a haemagglutination test, but this has limited specificity (Maeland and Digranes, 1975*a* and *b*); and Carlsson, Hurvell and Lindberg (1976) have devised an experimental enzyme-linked immunosorbent assay.

This paper describes a simple microagglutination test, with a stained antigen preparation that appears to possess a species antigen that is specific.

MATERIALS AND METHODS

Bacterial strains. The following serotypes (Wauters' scheme) of *Y. enterocolitica* were used: O1 (O1, 2a, 3—I.P. 135); O2 (O2a, 2b, 3—I.P. 178); O4 (O4,32—I.P. 96); O5 (I.P. 123); O5 (O5,27—I.P. 885); O6 (O6,30—I.P. 102), and O7 (O7,8—I.P. 106), kindly supplied by Professor G. Wauters (Ecole Santé Publique, Brussels); serotypes O3 (I.P. 134) and O9 (I.P. 383) supplied by Dr N. S. Mair (Public Health Laboratory, Leicester, England); and serotype O8 (strain W. A.; NCTC10938), derived from a recent American isolate and virulent for mice (Carter, 1975).

Antigens for tube agglutination tests. Strains were grown on Nutrient Agar (CM-3; Oxoid) at 25°C for 24 h, harvested with phosphate-buffered saline (PBS) pH 7·3, boiled for 1 h, washed twice with PBS and then adjusted in a Unicam SP1800 spectrophotometer to an optical density (OD) of 0·3 at 540 nm.

Antigens for microtitre plate-agglutination tests. Strains were grown and harvested as above. Before boiling, the suspension was stained with 2 : 3 : 5 triphenyl-tetrazolium chloride (TTC, British Drug Houses, Poole, England) at a final concentration of 0·2% w/v. After incubation for 1 h at 25°C the suspension was boiled for 1 h, washed three times with PBS, resuspended in PBS containing 1% v/v glycerol and used at an OD of 1·6 at 680 nm.

Received 1 Nov. 1977; accepted 17 Feb. 1978.

Antisera. Reference antisera prepared in rabbits against Wauters' serotypes O1 to O9 of *Y. enterocolitica* were received from Professor Wauters. Additional antisera to serotypes O3 and O9 were prepared by immunising rabbits with heat-killed organisms in Freund's incomplete adjuvant; a total of 2.5 ml of a suspension containing 10^8 organisms per ml was injected subcutaneously in four doses over a period of 4 weeks and the antisera were collected during the 5th week. In addition, antisera to serotypes O3, O8 (strain WA) and O9 were obtained by inoculating Porton white mice intraperitoneally with a single dose containing 10^7 live organisms. Human sera from patients infected with serotype O3 were received from Dr N. S. Mair. Sera were examined from two additional groups of patients, namely (1) patients with fever of undetermined origin lacking antibodies to *Brucella abortus* and *Francisella tularensis* (with controls from patients with positive serological tests for *F. tularensis*), and (2) patients (mainly children) with abdominal pain.

Antisera to *Brucella abortus*, *Vibrio cholerae*, salmonellae (poly O, poly H and Vi), and *Escherichia coli* (poly I-O26, O55, O111, O119, O126; poly II-O86, O114, O125, O127, O128 and poly III-O18, O44, O112a, O124a c, O142) were kindly supplied by the Standards Laboratory, Central Public Health Laboratory, Colindale. Antisera to *Yersinia pseudotuberculosis* serotypes 1 to 6 were supplied by Dr N. S. Mair.

Tube agglutination tests. These were performed as described by Winblad (1975), but incubating at 37°C. Sera were diluted in PBS starting at a dilution of 1 in 10; the end-point was taken as the highest dilution producing naked-eye agglutination.

Microtitre plate-agglutination tests. These were carried out in Microtiter Plates, Model 1S-MRC-96 (Linbro Chemical Company, New Haven, Conn., USA). A dilution of serum of 1 in 2 or 1 in 10 was used. The cell suspension settled rapidly but reproducible end-points were observed only after overnight incubation at 37°C. The end-point was taken as the highest dilution of serum giving a mat of stained antigen. End-points were affected also by antigen concentration; the effect of this was investigated by testing suspensions of optical densities of 1.4, 1.6 and 2.0 against rabbit antiserum. Reproducible results could not be obtained with the antigen at OD 1.4. Titres of 320 and 1280 were obtained with antigen at OD values of 1.6 and 2.0 respectively. Antigen at OD 1.6 gave the clearest endpoints and was chosen for all further tests.

RESULTS

In experiments with human, rabbit and mouse sera the sensitivities of microtitre plate and tube agglutination tests were similar (table I), the slight variations observed being due to differences in the starting dilutions. When the type specificity of suspensions of serotypes O3 and O9 was tested in microtitre plates against homologous and heterologous antisera, strong cross-reactivity was observed with both TTC-stained suspensions, whereas unstained suspensions reacted specifically. TTC thus affected the specificity of the antigen in both the microtitre plate and tube agglutination tests (table II).

As a result of these findings, TTC-stained suspensions of serotypes O1 to O9 were tested in a "chessboard" pattern against antisera to a range of *Y. enterocolitica* serotypes; all cross-reacted. Results are presented in table III for the four serotypes most frequently associated with human infection, namely: O3; O5,27; O8; O9. The TTC-stained suspensions of serotype O5,27 consistently agglutinated with heterologous sera. In table IV the results obtained when TTC-stained suspensions of serotype O5,27 were tested in microtitre plates against sera from mice infected with serotypes O3, O8 or O9 are compared with the results obtained by testing the same sera against their homologous antigen by both the tube and microtitre plate methods. It will be seen that the reactivity of TTC-stained suspensions of serotype O5,27 differed little from that of the individual homologous antigens.

The cross-reactivity of TTC-stained antigens in microtitre plate tests was further investigated by testing them against antisera to *B. abortus*, *V. cholerae*, salmonellae (poly O, poly H and Vi), *Shigella flexneri*, *Shigella sonnei*, *E. coli* (polyvalent sera I, II and III) and *Y. pseudotuberculosis* serotypes 1 to 6. Cross-reactions were not observed with TTC-stained suspensions of serotype O3. TTC-stained suspensions of serotype O9 reacted with *B. abortus* to a titre of 128, and with antiserum to *V. cholerae* to a titre of 16. These cross-reactions are similar to

TABLE I

Titres obtained in tube and microtitre-plate agglutination tests of sera from patients and experimentally-infected animals

Serum source (and infecting serotype of <i>Y. enterocolitica</i>)	Titres* obtained in	
	tube test	microtitre plate test
Patient 1 (O3)	160	128
Patient 2 (O3)	80	64
Rabbit 1 (O3)	640	256
Rabbit 2 (O9)	1280	512
Mouse 1 (O3)	40	32
Mouse 2 (O3)	40	40
Mouse 3 (O3)	40	40
Mouse 4 (O8)	40	16
Mouse 5 (O9)	80	64
Mouse 6 (O9)	320	160

* The titres are the highest obtained against homologous antigen.

TABLE II

Effect of TTC staining on the specificity of antigen in tube agglutination tests

Rabbit antiserum to serotype	Titre of rabbit antiserum as shown by			
	stained antigen		unstained antigen	
	O3	O9	O3	O9
O3	640	320	640	40
O9	320	1280	40	1280

those described by Ahvonen, Jansson and Aho (1969) and Barua and Watanabe (1972). Suspensions of serotype O5,27 stained with TTC reacted with antisera to *Y. pseudotuberculosis*, serotypes 1, 3, 5 and 6, but the titres did not exceed 32.

The reactivity of TTC-stained suspensions of serotype O5,27 with human sera was assessed in microtitre plate tests with sera from three groups of individuals. The first group comprised 261 patients with pyrexia of undetermined origin whose sera lacked antibodies to brucella and *F. tularensis*. The second group comprised 10 people whose sera contained agglutinins for *F. tularensis*; none of these sera agglutinated TTC-stained suspensions of serotype O5,27 at a dilution of 1 in 4. The third group, from which 190 sera were tested,

TABLE III

Cross-reactivity of TTC-stained antigen suspensions of four serotypes with rabbit antisera to serotypes O1 to O9 of Yersinia enterocolitica

Antigen suspension of serotype	Titre demonstrated in antiserum to serotype									
	O1, 2a, 3	O2a, 2b, 3	O3	O4,32	O5	O5,27	O6,30	O7,8	O8	O9
O3	32	1024	1024	64	256	2048	64	32	256	1024
O5,27	128	128	128	128	256	4096	64	32	128	2410
O8	16	64	512	16	4	4096	128	512	512	256
O9	64	64	512	32	32	4096	128	16	256	2048

TABLE IV

Titres of mouse sera tested by tube agglutination against unstained homologous antigens and by microtitre plate agglutination against homologous and O5,27 TTC-stained antigens

Serotypes of <i>Y. enterocolitica</i> against which antisera were produced (in mice no. 1-6)	Titres demonstrated in mouse antisera by		
	tube test with unstained homologous antigen	microtitre plate test with stained	
		homologous antigen	O5,27 antigen
O3 (mouse 1)	40	32	8
O3 (mouse 2)	40	40	16
O3 (mouse 3)	40	40	16
O8 (mouse 4)	40	16	16
O9 (mouse 5)	80	64	32
O9 (mouse 6)	320	160	128

consisted of patients, mostly children, admitted to hospital with abdominal pain. Sera from 10 of these patients agglutinated TTC-stained suspensions of serotype O5,27 to a titre of 8; one patient had a titre of 64. Two of these 10 sera had higher titres with other antigens: one had a titre of 16 with TTC-stained antigen of serotype O3, and a second had a titre of 32 with TTC-stained antigen of serotype O9.

DISCUSSION

In the experiments described above the specificity and cross-reactivity of suspensions of *Y. enterocolitica* serotype O5,27 stained with TTC were established by testing sera from experimentally infected animals. The results also showed that sera from patients from whom *Y. enterocolitica* had been isolated gave titres that were similar in both microtitre-plate and tube-agglutination tests. Non-specific agglutination was not detected in tests of sera from 261 patients with pyrexia of undetermined origin, nor was cross-reactivity demonstrated when TTC-stained suspensions of serotype O5,27 were tested against antisera to organisms of five

other genera. However, TTC-stained suspensions of serotype O5,27 reacted almost as well as the homologous antigens with antisera to other serotypes of *Y. enterocolitica*. Thus, in these experiments, microtitre plate-agglutination tests with TTC-stained suspensions of serotype O5,27 appeared to offer a method for detecting antibodies to a wide range of *Y. enterocolitica* serotypes that was more satisfactory and simple than multiple tube agglutination tests.

To detect antibody to *Y. enterocolitica* Lysy and Knapp (1973) have used a haemagglutination test with a supernate of an autoclaved bacterial suspension as antigen. This preparation may contain common enterobacterial antigen (CEA), thus explaining the lack of specificity of haemagglutination tests reported by Maeland and Digranes (1975*a* and *b*). Recently Makela and Mayer (1976) have described a technique for extracting lipopolysaccharide antigens free from CEA; this technique might improve the specificity of *Y. enterocolitica* antigens in haemagglutination tests.

It is not clear why suspensions of *Y. enterocolitica*, when stained with TTC, should have altered antigenic specificity. TTC has been used to stain brucella antigens and its use as an antigen stain in microtitre plate tests for clinical, serological and epidemiological investigations of brucellosis is well established (Mello and Mello, 1969; Ricciardi *et al.*, 1976). To our knowledge antigen modification has not been recognised in TTC-stained brucella preparations. An understanding of the action of TTC on the bacterial cell might lead to the identification of a species-specific and possibly even a genus-specific diagnostic antigen. Further, it is possible that an antiserum to TTC-stained suspensions of serotype O5,27 would be of use in the detection of biochemically aberrant strains of *Y. enterocolitica*.

Evaluation is now needed of the usefulness of TTC-stained suspensions of serotype O5,27 as the antigen in a species-specific serological test for antibodies to *Y. enterocolitica* by testing many more sera from patients from whom different serotypes of *Y. enterocolitica* have been isolated. Such sera are not currently available to us.

SUMMARY

When suspensions of *Yersinia enterocolitica* were stained with triphenyl-tetrazolium chloride (TTC) their antigenic specificity, as determined by tube and microtitre-plate agglutination tests, was altered.

Thus, a TTC-stained suspension of serotype O5,27 detected antibodies to nine O serotypes of *Y. enterocolitica* in sera from experimentally infected animals but did not cross-react with antisera to organisms of five other unrelated genera including *Brucella*. The same suspension did, however, cross-react weakly with antisera to four serotypes of *Yersinia pseudotuberculosis*.

The value of this antigen preparation in detecting antibodies to the wide range of *Y. enterocolitica* serotypes likely to cause infection in man should now be assessed.

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