J. Hyg., Camb. (1976), **77**, 97
Printed in Great Britain

Modified Vi tests in the screening of typhoid carriers

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(*Received* 16 *December* 1975)

SUMMARY

Two tests for the estimation of Vi antibody in sera were studied and their validity in the screening of typhoid carriers was assessed. One was a modified Vi haemagglutination test in which Vi-coated glutaraldehyde-fixed erythrocytes were used as the antigen and Vi antibody resistant to treatment with 2-mercaptoethanol was titrated. The other was a fluorescent Vi antibody test in which acetone-fixed bacterial Vi cells were used as the antigen and the total Vi antibody was titrated. In both tests, the antigens used were stable so that standardization of the tests would not be difficult. The modified Vi haemagglutination test was found equally sensitive but more specific in giving less false positives than the conventional Vi haemagglutination test. The fluorescent Vi antibody test was however found superior to both tests not only in giving less false positives but also in detecting more typhoid carriers.

INTRODUCTION

For the detection of typhoid carriers, Vi bacterial agglutination or haemagglutination tests have been suggested as a screen (Felix, 1938; Landy & Lamb, 1953) but with varied success (Public Health Laboratory Service Working Party, 1961; Bokkenheuser, Smith & Richardson, 1964; Forrest, Matthews, Robertson & Hanley, 1967). This might be attributed to (*a*) different frequency in the occurrence of false positives which seems to be variable from one place to the other (Wilson & Miles, 1975), (*b*) the instability of the Vi bacterial cell suspensions or the Vi-coated erythrocyte suspensions which renders the standardization of the Vi agglutination test difficult, and (*c*) that agglutination tests are known to favour greatly the detection of antibodies of the IgM immunoglobulin class (Robbins, Kenny & Suter, 1965; Pike, Schulze & Chandler, 1966) and Vi and O antibodies of this immunoglobulin class have been found deficient in typhoid carriers' sera (Chernokhostova *et al.* 1969).

To overcome these difficulties, two tests have been studied. One was a modified haemagglutination test in which sera were treated with 2-mercaptoethanol to inactivate antibodies of the IgM-type and the residual Vi agglutinins were titrated against a Vi-coated glutaraldehyde-fixed erythrocytic antigen (Eskenazy & Cohen, 1974). The other was a fluorescent antibody test in which unreduced sera were allowed to react with acetone-fixed Vi bacterial cell antigen, and the Vi antibody in dilutions of these sera was measured by staining with a fluorescein-conjugated

anti-human immunoglobulin antiserum. The validity of these two tests in the screening of typhoid carriers has been assessed and the results of such assessment are reported in this paper.

MATERIALS AND METHODS

Serum specimens

Sera were collected for antibody assay from 16 chronic typhoid carriers and 133 normal individuals. All the 16 typhoid carriers were confirmed chronic faecal carriers who had carried typhoid bacilli for more than one year without apparent symptoms. Two to three serum specimens were collected one month to one year apart from each of the carriers. The 133 'normal' individuals chosen as control subjects consisted of 41 prisoners, 40 orthopaedic patients and 52 patients with biliary tract infection. All of them had no apparent history of typhoid fever and TAB-vaccination, and their stool cultures were negative for *Salmonella typhi*. Bile cultures were also performed for the 52 patients with biliary tract infection and were found negative for *S. typhi* as well. One serum specimen was collected from each of these 133 normal controls. After collection, all the serum specimens were kept at $-20°$ C. until use.

Direct bacterial agglutination (DBA) test

Preparation of the antigen

The bacterial cell suspension used for the Vi-agglutination test was prepared from *S. typhi* strain NCTC 8222* according to the method recommended by Professor E. S. Anderson of the Enteric Reference Laboratory, Colindale, London (personal communication, 1962).

Procedure of the bacterial agglutination test

This was done according to Professor E. S. Anderson's recommendation. Twofold dilutions of serum, previously heated at 56° C. for 30 min., were made in saline from 1/2·5 to 1/80, and 0·5 ml. of the diluted serum was mixed with 0·5 ml. of the antigen suspension. After incubation at 37° C. in a water bath for 2 hr., the tubes were kept in a refrigerator at 4° C. overnight. The results were read the following day. Three criteria as recommended by the Public Health Laboratory Service Working Party (1961) were used for assessing the degree of agglutination: the clarification of the supernatant; the pattern of dispersion of the deposit; and the coarseness of the granularity of the suspension after shaking the tube.

Indirect haemagglutination (IHA) test

Preparation of the antigen

Purified Vi antigen prepared from *Citrobacter* 5396/38 by the ethanol-cetavlon mild precipitation method (Wong & Feeley, 1972) was used for the sensitization of sheep erythrocytes. Sheep blood was freshly collected in Alsever's solution. The

* This strain is no longer available from NCTC now.

https://doi.org/10.1017/S002217240005556X Published online by Cambridge University Press

erythrocytes were washed three times with phosphate buffered saline (PBS), pH 7·2, and suspended in 0·2 % glutaraldehyde for 10 min. After centrifugation, a 10 % erythrocyte suspension in PBS was prepared. To 9 ml. of the red cell suspension, 1 ml. of a 0·2 % glutaraldehyde and 10 ml. of a purified Vi antigen solution (10 μg./ml.) in PBS were added. The mixture was gently tumbled at room temperature for 1 hr. The erythrocytes were sedimented by centrifugation at 1500 rev./min. for 10 min. and washed twice with PBS. The erythrocytes thus sensitized were either used freshly as a 0·5 % suspension in PBS or preserved at $-10°$ C. as a 10 % suspension in 70 % glycerol. The glycerol was removed by washing with PBS before use and the agglutinability of the sensitized erythrocytes was checked in each test against a reference Vi antiserum.

Procedure of the haemagglutination test

To determine the Vi agglutination titre, serum was first heated at 56° C. for 30 min., 0·05 ml. aliquots of the serial twofold dilutions of the serum starting from 1/4 were then mixed in a V-type microtiter tray (Cooke Engineering Co., Alexandria, Va., U.S.A.) with equal volumes of a 0·5 % suspension of the sensitized sheep erythrocytes. Controls were similarly set up using a 0·5 % suspension of sheep erythrocytes not coated with Vi antigen. Agglutination titres were read after the mixtures were incubated at 37° C. for 1 hr. and allowed to stand at 4° C. overnight.

Treatment of sera with 2-mercaptoethanol

Sera were treated with 2-mercaptoethanol (ME) to inactive antibodies of the IgM immunoglobulin class as follows: equal volumes of serum and 0·2 M ME in PBS (pH 7·2) were mixed and left at room temperature for 2 hr. A weighed amount of iodoacetamide sufficient to make the solution 0·02 M was then added, and the mixture was left overnight at 4° C. The preparation was dialysed against PBS for 24 hr. with one change of fresh PBS after 6–8 hr. The ME-treated sera were tested again for their Vi haemagglutination titres as described above.

Fluorescent Vi antibody (FVA) test

Preparation of the Vi bacterial antigen

The Vi bacterial cell antigen was prepared from *S. typhi* strain NCTC 8222. Bacterial cells of the above strain grown in nutrient broth were washed 3 times with and suspended in saline. One loopful of the bacterial suspension was smeared evenly within a circle of 1 cm. in diameter on a clean slide. The concentration of the bacterial suspension was adjusted to such that after fixation about 100 bacterial cells could be seen per high dry field. After being dried in air and fixed with acetone, the antigen-loaded slides were either used immediately or kept at $-60°$ C. until use.

Procedure of the FVA test

Serial twofold dilutions of the serum specimen starting at 1/20 were made in PBS, pH 7·2, in microtitre trays. These serum dilutions were added in 0·03 ml. volumes to the circles containing bacterial antigen and incubated in a wet chamber at 37° C. for 30 min. The slides were rinsed with and washed in PBS with gentle stirring for 10 min. on a magnetic stirrer. Fluorescein isothiocyanate (FITC) conjugated anti-human immunoglobulin anti-serum (Wellcome Reagents Ltd, England), which had been diluted in PBS containing 0·5 % Nonidet P40 (BDH) to 1/32 and showed no reaction with the Vi antigen cells, was then added to each circle. The slides were incubated again in a wet chamber at 37° C. for 30 min., rinsed with and washed in PBS for 15 min. with gentle stirring and were examined under fluorescent microscopy. The FVA titres were taken as the highest dilutions of serum giving fluorescence for over 50 % of the bacterial cells.

RESULTS

Comparison of the conventional Vi IHA and DBA tests in the screening of typhoid carriers

The Vi titres in sera of the 16 typhoid carriers and 133 non-carriers were measured concomitantly by the DBA and the IHA tests. The results presented in Table 1 indicate that IHA is of comparable specificity but more sensitive than DBA in the detection of Vi agglutinins: the IHA titres were 4-8 times higher than the corresponding DBA titres. By arbitrarily choosing an IHA titre of 1/64 as the significant level, 28 (82 %) out of the 34 sera of typhoid carriers and 11 (8 %) out of the 133 sera of normal individuals would give positive results, i.e. give titres equal to or greater than 1/64. On the other hand, if a DBA titre of 1/10 is taken as the significant level, the corresponding figures would be 80 % (27 out of 34) and 9 % (12 out of 133), respectively.

Stability of the glutaraldehyde-fixed Vi-coated erythrocytes

Samples of 28 selected sera, 14 from carriers and 14 from non-carriers, with Vi IHA titres up to 1/32 were titrated against the same batch of Vi-coated erythrocytes 3 months apart, i.e. immediately after preparation and after subsequent storage in 70 % glycerol at −10° C. for 3 months. The results as shown in Fig. 1 indicate that the agglutinability of the sensitized red cells remained stable after storage for at least 3 months.

Titration of Vi antibody on ME-treated sera by IHA test

Thirty-four sera from the 16 typhoid carriers and 133 sera from the normal individuals were subjected to ME treatment and their residual titres were measured. The results in Table 1 show that ME-treatment appeared to have rendered the Vi IHA test more specific in the detection of typhoid carriers: 27 (80 %) of the 34 sera of carriers but only 4 (3 %) of the 133 sera of normal individuals showed Vi titres equal to or greater than 1/32 after both groups of sera were treated with ME before titration of the Vi agglutinins.

Table 1. *Vi IHA and DBA titres in sera of typhoid carriers and normal individuals*

		Total no. of sera examined	No. of sera with reciprocal IHA titre of						No. of sera with reciproca DBA titre of				
			⩽ 16	32	64	128	256	⩾ 512	⩽ 5	10	20	40	⩾ 160
Un-rec carrie	sera of typhoid	34	4	2	2	5	9	12	7	4	7	7	4
		(100%)*	6 (18%)		28 (82%)				(20%)	27 (80%)			
Norma	viduals	133	115	7	6	4	1	0	121	5	5	1	0
		(100%)	122 (92%)		11 (8%)				(91%)	12 (9%)			
ME-re carrie	sera of typhoid	34	7	3	3	8	9	4					
		(100%)	(20%)	27 (80%)									
Norma	viduals	133	129	3	1	0	0	0					
		(100%)	(97%)	4 (3%)									

Not done

* Figures in parentheses indicate percentages to the total number of sera examined.

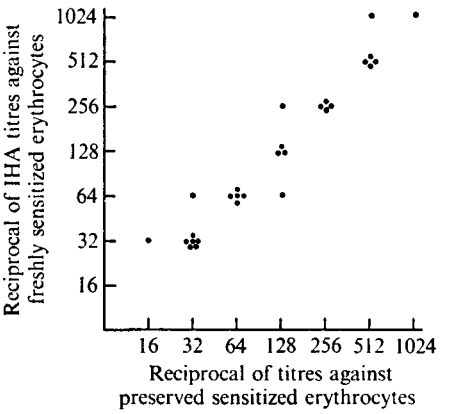

Fig. 1. Comparison of the agglutinability of freshly prepared and glycerol preserved Vi-coated erythrocytes.

Table 2. *Fluorescent Vi antibody titres in sera of typhoid carriers and normal individuals*

	No. of sera examined	No. of sera with reciprocal FVA titres of ⩽ 80	160	320	640	1280	⩾ 2560
Typhoid carriers:							
1st sera	16	1	2	6	6	1	0
2nd and 3rd sera	18	0	3	6	7	1	1
Total	34	1	5	12	13	2	1
	(100%)*	(3%)	33 (97%)				
Normal individuals:							
With Vi IHA titres of 1/32 or greater	18	17	1	0	0	0	0
With Vi IHA titres below 1/32	115	114	1	0	0	0	0
Total	133	131	2	0	0	0	0
	(100%)	(98·5%)	2 (1·5%)				

* Figures in parentheses indicate percentages to the total number of sera examined.

Titration of Vi antibody by the FVA test

Table 2 summarizes the results. 33 out of the 34 carriers' sera showed FVA titres of 1/160 or greater, including 5 sera from the 2 carriers with Vi IHA titres repeatedly below the significant titre, i.e. less than 1/64. By contrast, only 1 out of 18 sera of normal individuals with Vi IHA titres up to 1/32 and 1 from the remaining 115 sera with Vi IHA titres below 1/32 showed FVA titres of 1/160. Thus, if the FVA test is used for the screening of typhoid carriers, the positive rate would be 97% while the false positive rate would be 1·5% only.

https://doi.org/10.1017/S002217240005556X Published online by Cambridge University Press

DISCUSSION

As is generally recognized, the Vi agglutination titres in a population of normal individuals depend upon, among other factors, the endemicity of typhoid fever in the given locality. Titres that would be regarded as significant in a non-endemic area like Great Britain may not be regarded as significant in an endemic area like Hong Kong. Thus, for the screening of typhoid carriers, the significant titre of Vi agglutination should be established individually in different parts of the world according to their own local situation. There is, however, a dilemma in the establishment of such a titre. A high significant titre would cut down the number of false positives but would very likely reduce the number of genuine positives in the known carriers at the same time. The opposite would be true for a low significant titre. In the present study a Vi bacterial agglutination titre of 1/10 was chosen as the significant titre which appeared to have given best differentiation between typhoid carriers and normal individuals, because 80% of the sera of typhoid carriers and 9% of the sera of normal individuals were found to give titres up to this figure. The false positive rate was however still too high and the search for a Vi antibody test more specific than the conventional bacterial agglutination was therefore the purpose of this study.

Haemagglutination with the Vi-coated erythrocytes as the antigen has been well documented as a more sensitive test than bacterial agglutination for the estimation of Vi agglutinins (Landy & Lamb, 1953; Cooper, 1965; Kolyubakina & Karal'nik, 1965). The Vi-coated red cells are however unstable, losing their agglutinating capacity rapidly after preparation. The glutaraldehyde-fixed erythrocytes coated with purified Vi antigen appeared to have good agglutinating capacity and could be stored for a prolonged period without apparent loss of agglutinating capacity.

The specificity of Vi haemagglutination performed on unreduced sera was similar to that of the bacterial agglutination test. Titration of Vi antibodies in unreduced sera showed that 82% of the sera of carriers and 8% of the sera of normal individuals were with Vi titres equal to or greater than 1/64. The corresponding figures for ME-reduced sera with Vi titres equal to or greater than 1/32 were 80% and 3%, respectively. The 133 'normal' individuals examined in these studies were most probably non-carriers because their stool and urine cultures were repeatedly negative for *S. typhi*, and in 52 persons bile cultures were also performed which were also negative for *S. typhi*. Thus the false positive rate with the former test was 8% while that with the latter test was 3%. The decrease in the frequency of false positives from 8% to 3% was statistically significant ($P < 0{\cdot}05$). Titration of Vi agglutinins on ME-reduced sera by the haemagglutination test therefore appeared to be more specific than the conventional Vi haemagglutination test in the screening of typhoid carriers.

Best results, however, were obtained by the fluorescent Vi antibody test. By this test, 97% of the sera of typhoid carriers but only 1·5% of the sera of normal individuals gave titres equal to or greater than 1/160. It was also observed that the fluorescent antibody test was capable of detecting Vi and O antibodies at high

titres in five convalescent sera from two bacteriologically confirmed typhoid patients whose sera were repeatedly negative for O and Vi agglutinins by the conventional Widal test (unpublished data). The capability of the FVA test to detect the so-called 'non-agglutinating', or more precisely, the less agglutinating, antibodies might be responsible for its higher positive rate. The reduction in the number of false positives, on the other hand, was presumably due to a relatively high significant titre set for this test.

We are grateful to Dr W. K. Chang, Institute of Pathology, Government of Hong Kong, for supplying the bacterial Vi antigen, to Dr K. H. Wong, Division of Bacterial Products, Bureau of Biologics, Food and Drug Administration, U.S.A., for supplying the purified Vi antigen, and to Dr F. W. T. Wong, Department of Microbiology, University of Hong Kong, for supplying part of the serum specimens collected from patients with biliary tract infections. This work was supported by a research grant from the University of Hong Kong.

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