AN INVESTIGATION OF THE NORMAL AGGLUTININS FOR TYPHOID AND PARATYPHOID BACILLI IN HUMAN SERA IN VICTORIA, AND THE INTERPRETATION OF THE WIDAL TEST

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Introduction

During recent years the use and interpretation of the Widal test for the diagnosis of enteric fever have received much attention from serologists throughout the world. The results of some of the investigations of normal agglutinins for the causal organisms of enteric fever have been discussed by Topley & Wilson (1936) who state that "these surveys should be carried out by a standardized technique, and with a standard reagent". They pointed out that although the results which they discussed were not strictly comparable with one another on account of a lack of standardization, they provided a valuable basis for discussion, and these authors emphasized the need for further investigations in many localities. For the correct interpretation of the Widal test it is desirable to have information regarding the natural level of agglutinins among a random sample of the population in the particular area in which the test is being made. The work of several investigators has been directed toward the obtaining of such evidence, especially with regard to the agglutinins for the organisms which cause enteric fever in their own locality and, similarly, the object of our investigation has been primarily to enable us to interpret correctly the results of the tests in which agglutinins for *Bact. typhosum* are detected. Our experience over the last nine years suggests that typhoid has been the only enteric fever occurring in this State during that time as, in examinations at this laboratory on behalf of the Victorian Department of Health, only *Bact. typhosum* has been isolated from blood cultures and faeces from enteric patients and small epidemics investigated have always been caused by the same organism. Only those people who have travelled abroad or were on overseas military service during the Great War are likely to have been exposed to infection with paratyphoid bacilli to any great extent during the last twenty-five years and they also compose the group likely to have undergone inoculation with typhoid or T.A.B. vaccine. That the likelihood of the average individual being exposed to infection with the typhoid bacillus in this State is at present very small is indicated by the

report taken from the *Victorian Year Book* of 1930–1 which stated that: "The reported cases of typhoid fever for the whole State declined from 288 per 100,000 of population in 1895–9 to 53 per 100,000 in 1914–18 and 8 per 100,000 in 1930, or by 97 % in the intervening years." The figures since 1930 have been of the same low order.

The 500 samples of serum which we have tested in the course of this investigation were sent to this laboratory for the Wassermann test. We chose 300 of the samples at random and 200 from women attending an antenatal clinic. This latter group was included because we considered that the present age of expectant mothers would indicate that they had not been on military service abroad and, moreover, we were better able to obtain histories from these patients when required. Of the 500 samples tested 190 were from males and 310 from females. Samples for the agglutination tests were taken before the sera were inactivated for the Wassermann test.

Although we included suspensions of *Bact. paratyphosum* A and *Bact. paratyphosum* B in routine tests in this laboratory there is really little need to do so as paratyphoid fever seems to be non-existent. In this investigation, however, it was decided to include these suspensions as we thought the results obtained would be of interest to others by indicating the level of normal agglutinins in a population where the specific stimuli are lacking. Such results might thus serve as a basis for the interpretation of the test in countries where low-titre agglutination results from the presence of normal agglutinins not caused by a specific paratyphoid stimulus. Moreover, if low-titre agglutination was found to occur commonly in this State, the conclusion could be drawn that in a highly contaminated environment such as that described by Pasricha *et al.* (1936), it would be wrong to attribute all low-titre agglutination to subclinical infection.

TECHNIQUE

Suspensions. For the detection of H agglutinins formolized broth suspensions of motile *Bact. typhosum*, *Bact. paratyphosum* A, and *Bact. paratyphosum* B in the specific phase, were used; for the detection of O agglutinins a formolized broth suspension of *Bact. typhosum* 901 O and alcohol-treated suspensions of *Bact. paratyphosum* A and *Bact. paratyphosum* B. All six suspensions were carefully standardized by comparing their agglutinability with that of suspensions obtained from the Oxford Standards Laboratory. Five of the suspensions were of "standard" agglutinability, so that the observed titre with these could be taken as the true titre, but with the sixth suspension, that of *Bact. paratyphosum* B O, the suspension factor proved to be 0·25 when compared with the Oxford suspension of *Bact. aertrycke* O (for *Bact. paratyphosum* B O), so that all observed titres had to be subsequently multiplied by four to give the adjusted titre.

It has been our experience that alcohol-treated suspensions are less sensitive than those not treated with alcohol, and we consider that in the

case of *Bact. paratyphosum* A the titres obtained are lower than would be the case if an O variant were available for use without such treatment.

Agglutination tests. For the detection of H agglutination the final serum dilutions were from 1 : 10 to 1 : 80, but in order to determine the end-titre the range was extended if agglutination occurred in the last tube. For the detection of O agglutination a similar series of serum dilutions was used with the *Bact. typhosum* O and *Bact. paratyphosum* A O suspensions, but with the *Bact. paratyphosum* B O suspension an extra tube containing a final serum dilution of 1 : 5 was included on account of the low suspension factor. As this proved to be 0·25 we were unable to state the result which would be obtained with a suspension of "standard" agglutinability at any serum dilutions below 1 : 20. The racks were incubated in a water-bath at 55° C., the results for H agglutination being read after 2 hr. and those for O agglutination after incubation overnight. The end-titre was taken as that dilution of serum showing well-marked agglutination without complete sedimentation when the tubes were observed through a hand lens in front of an artificial light with a dark background.

Results

In expressing the results of investigations of this nature most workers have calculated the percentage of sera which gave a positive reaction at various dilutions, against any one suspension. From the point of view of the immunologist, however, it is of special interest to note the combinations of agglutinins which occur at each serum dilution. In using the six suspensions mentioned above there are sixty-three possible combinations, but in our tests only twenty-three were found to occur and these are shown in Table I. It is generally admitted that at the point where the titre for a single agglutinin becomes significant in diagnosis the titre for O agglutination would generally be about twice that for H agglutination. There would be great difficulty, however, in arranging a table for combined agglutinins where such a relation between H and O titres was maintained. We have, therefore, attempted to picture the different results as they would appear for the various combinations of agglutinins if the sera were diluted first at 1 : 10, then at 1 : 20 and so on up to 1 : 1280. The results, then, should be read for each serum dilution in the vertical columns of Table I, and it should be realized that a single serum which, for example, agglutinated the three suspensions *Bact. typhosum* H and O and *Bact. paratyphosum* B H to end-titres of 1 : 40, 1 : 20, and 1 : 10 respectively would appear in the table showing the combination of agglutinins for *Bact. typhosum* H and O and *Bact. paratyphosum* B H at serum dilution of 1 : 10, but only for the first two at 1 : 20, and for *Bact. typhosum* H only at 1 : 40. When agglutination occurred with any H suspension at a titre of 1 : 40 or higher or with any O suspension at 1 : 80 or higher we attempted to obtain the history of the persons from whom such sera were obtained, and in most cases this was supplied. We were not able to show at serum dilutions

of 1 : 10 those combinations containing *Bact. paratyphosum* B O agglutinin, as the suspension factor, as explained above, was low and the lowest adjusted titre obtainable was 1 : 20.

Table I. *Showing the number of sera out of* 500 *examined which, at each dilution stated, showed the presence of one or more of the H and O agglutinins for* Bact. typhosum, Bact. paratyphosum *A and* Bact. paratyphosum *B*

Bracketed numerals show the number of sera which came from persons with a definite history of enteric infection or inoculation. Thus 16 (5) indicates that 16 sera were positive at a certain dilution and of these 5 came from persons with such histories.

	Agglutinins						Serum dilutions							
Row	*Bact. typhosum*		*Bact. paratyphosum* B		*Bact. paratyphosum* A		1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1280
1	H	.	.	.	.	.	16 (5)	20 (6)	14 (7)	10 (7)	8 (7)	4 (4)	2 (2)	2 (2)
2	H	O	.	.	.	.	10 (1)	5 (1)	2 (1)	—	—	—	—	—
3	H	.	H	.	.	.	8 (3)	10 (6)	7 (6)	5 (5)	2 (2)	—	—	—
4	H	.	.	O	.	.	—	5 (1)	1 (1)	—	—	—	—	—
5	H	O	H	.	.	.	4 (1)	—	—	—	—	—	—	—
6	H	O	.	O	.	.	—	1 (0)	1 (1)	—	—	—	—	—
7	H	O	.	.	.	O	1 (0)	—	—	—	—	—	—	—
8	H	.	H	O	.	.	—	1 (1)	—	—	—	—	—	—
9	H	.	H	.	H	.	3 (3)	1 (1)	1 (1)	—	—	—	—	—
10	H	O	H	O	.	.	—	2 (2)	1 (1)	—	—	—	—	—
11	.	O	.	.	.	.	85 (0)	51 (0)	26 (0)	8 (0)	3 (0)	—	—	—
12	.	O	H	.	.	.	1 (0)	1 (0)	1 (0)	—	—	—	—	—
13	.	O	.	O	.	.	—	38 (0)	11 (0)	3 (0)	1 (0)	1 (0)	—	—
14	.	O	.	.	.	O	5 (0)	1 (0)	—	—	—	—	—	—
15	.	O	H	O	.	.	—	2 (1)	1 (1)	—	—	—	—	—
16	.	O	.	O	.	O	—	1 (0)	—	—	—	—	—	—
17	.	O	H	O	H	.	—	1 (0)	—	—	—	—	—	—
18	.	.	H	.	.	.	6 (0)	6 (1)	7 (1)	5 (2)	4 (2)	1 (1)	—	—
19	.	.	H	O	.	.	—	5 (0)	1 (0)	2 (1)	1 (1)	—	—	—
20	.	.	H	O	H	.	—	0 (0)	1 (0)	—	—	—	—	—
21	.	.	.	O	.	.	—	32 (0)	32 (0)	7 (0)	3 (0)	2 (1)	1 (0)	—
22	.	.	.	.	H	.	1 (0)	1 (0)	—	—	—	—	—	—
23	.	.	.	.	.	O	2 (0)	1 (0)	1 (0)	—	—	—	—	—

Considering the results shown in Table I first from the point of view of the diagnosis of typhoid fever by the Widal test on sera obtained in Victoria, the significance of H agglutinins for *Bact. typhosum* may be determined by examining the figures obtained by adding the results shown in rows 1–10, for each serum dilution excluding 1 : 10. These figures are shown in Table II together with those obtained for O agglutinins for *Bact. typhosum* by adding the results of rows 11–17 to those for rows 2, 5, 6, 7 and 10.

For purposes of comparison the results for single agglutinins for the two paratyphoid organisms have been similarly compiled and are also shown in Table II.

Our inquiries regarding the history of those people whose sera agglutinated at the titres mentioned above, revealed that at least twenty of the 500 sera examined came from those who had suffered from enteric or had been inoculated. In this group of twenty it was found that thirteen were men who had been inoculated while on military service abroad, some of them also having a history of enteric, whilst five of the remaining seven had been inoculated and

Table II

Suspension	Number of sera out of 500 examined agglutinating at or above 1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1280
Bact. typhosum H	45 (18)	27 (18)	15 (12)	10 (9)	4 (4)	2 (2)	2 (2)
Bact. typhosum O	103 (4)	32 (4)	11 (0)	4 (0)	1 (0)	—	—
Bact. paratyphosum B H	29 (12)	20 (10)	12 (8)	7 (5)	1 (1)	—	—
Bact. paratyphosum B O	88 (5)	49 (4)	12 (1)	5 (1)	3 (1)	1 (0)	—
Bact. paratyphosum A H	3 (1)	2 (1)	—	—	—	—	—
Bact. paratyphosum A O	3 (0)	1 (0)	—	—	—	—	—

The numerals in brackets again indicate the number of persons giving a positive reaction at the titre shown who had a definite history of enteric fever or inoculation. These are included in the numbers preceding the brackets.

two had suffered from enteric fever. It may be concluded, therefore, that although nearly twenty years have elapsed since the close of the Great War it is desirable to have details of such service included in any history if a reliable diagnosis is to be made.

By removing the sera of this group of twenty from further consideration we are left with a group of 480 sera from apparently normal members of the population.

In order to determine the percentage of normal sera which contain any one agglutinin at a given dilution the number of positive reactions with sera from presumably normal people may be first derived from Table II, and then calculated as a percentage of 480. Thus for agglutinins for *Bact. typhosum* H at serum dilution 1 : 40 there were twenty-seven sera positive, but as eighteen were patients with special histories, only nine were from members of the normal group of 480. Therefore, 1·9 % of sera from normal people contained agglutinins for *Bact. typhosum* H at or above the serum dilution of 1 : 40. Similar results for other agglutinins have been calculated and are shown in Table III.

Table III

Suspensions	Percentage of normal sera agglutinating at or above 1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1280
Bact. typhosum H	5·6	1·9	0·6	0·2	0	0	0
Bact. paratyphosum B H	3·5	2·1	0·8	0·4	0	0	0
Bact. paratyphosum A H	0·4	0·2	0	0	0	0	0
Bact. typhosum O	20·8	7·7	2·3	0·8	0·2	0	0
Bact. paratyphosum B O	17·3	9·4	2·3	0·8	0·4	0·2	0
Bact. paratyphosum A O	0·6	0·2	0	0	0	0	0

Interpretation of results

Agglutinins for Bact. typhosum

These are the most important from the point of view of our diagnostic methods on account of the apparent absence of paratyphoid fevers from this State. The low incidence of typhoid fever itself leads to the conclusion that the agglutinins found in apparently normal people do not result from the specific stimulus of contact with *Bact. typhosum* but probably result from contact with the same chemical antigenic component, possibly present in

other organisms. The fact that there is very little difference in the incidence of agglutinins for *Bact. typhosum* and for *Bact. paratyphosum* B further supports this argument, as it is quite certain that agglutinins for the latter organism could not have resulted from contact with the organism itself, and it is most unlikely that they have been produced by contact with any other *Salmonella* organism sharing the specific H antigen of *Bact. paratyphosum* B.

It may be seen from Table III that so far as H agglutination is concerned a positive result at serum dilution 1 : 80 or more occurs only in 0·6 % of apparently normal sera. Consequently, in the absence of a history of previous infection or inoculation such a titre may be taken as diagnostic in this community. Furthermore, agglutination at end-titres of 1 : 20 or 1 : 40 would be significant in the presence of suggestive symptoms, as there would be only 1 chance in 20 and 1 chance in 50 respectively of obtaining such a titre with a normal serum. The significance of these titres is greatly enhanced if O agglutinins are also present, and if the figures in rows 2, 5, 6, 7 and 10 of Table I are examined it will be found that only five of the 480 normal sera showed such a combination of H and O agglutinins at serum dilution 1 : 20 and 1 at 1 : 40. In other words the chances of obtaining such a result were 1 in 96 and 1 in 480 respectively. This observation may be very important in the diagnosis of early cases of typhoid fever where the result would be in doubt if judgement were based on the titre shown for either agglutinin singly.

With regard to O agglutination of *Bact. typhosum* the diagnostic titre may be fixed at 1 : 160, at which point only 0·8 % of normal sera gave a positive result, but a titre of 1 : 80 will still be significant and calls for further investigation. It was noted that O agglutination of both *Bact. typhosum* and *Bact. paratyphosum* B occurred at a higher dilution with the sera obtained from women attending the antenatal clinic and to such an extent that the diagnostic titre for normal sera was raised from 1 : 80 to 1 : 160. It would appear then that during pregnancy the normal level of O agglutinins was raised; there was no similar effect on H agglutinins. As indicated above, O agglutinins are of much greater significance when combined with H agglutinins.

Agglutinins for Bact. paratyphosum *B*

Although it might have been expected that there would be fewer normal agglutinins for this organism (which has not been detected as a cause of enteric fever for many years) than for *Bact. typhosum*, it may be seen that there is very little difference in the figures shown in Table III for the H or O agglutinins of either organism. Moreover, reference to rows 8, 10, 15, 17, 19 and 20 of Table I shows a very similar probability of obtaining a combination of H and O agglutinins in normal sera diluted 1 : 20 and 1 : 40.

With *Bact. paratyphosum* B the chances of obtaining such a result at these respective titres were 1 in 69 and 1 in 240, as against 1 in 96 and 1 in 480 with *Bact. typhosum*.

As we still include a type H suspension of *Bact. paratyphosum* B in our routine tests in this laboratory any positive results obtained may be interpreted in the light of this evidence. Reference to Table I will further show that in this community a combination of an agglutinin for *Bact. paratyphosum* B H with one or more of the other agglutinins investigated, at a serum dilution of 1 : 40 rarely occurs unless the serum comes from an inoculated person.

Agglutinins for Bact. paratyphosum *A*

These have rarely been present, and a positive result for either H or O agglutination at or above a serum dilution of 1 : 40 could be taken as diagnostic in this community if such infection occurred. It is doubtful if the present position in Victoria warrants the inclusion of a *Bact. paratyphosum* A suspension in routine tests.

Discussion of results

The results obtained for agglutinins for *Bact. paratyphosum* A call for little discussion, as they agree with those obtained by other workers in countries which are free from paratyphoid fever due to this organism. They emphasize the fact that normal agglutinins for this organism are very uncommon, and it seems reasonable to suppose that its antigenic components would be very rarely found in organisms or materials which could give rise to the non-specific stimulation of such normal agglutinins.

With regard to the H agglutinins for *Bact. typhosum* the incidence in normal sera agrees very well with that found by Smith *et al.* (1930) in a group of presumably uninoculated females in Manchester. At the titre of 1 : 80 the percentage of positive reactions was 0·6 % in Victoria and 0·7 % in Manchester. Similarly, with the O agglutinins for *Bact. typhosum* there is very good agreement with the English figures of Gardner & Stubington (1932). They obtained agglutination at dilutions of 1 : 50 and 1 : 100 with 6 and 2 % respectively of the normal sera tested, whereas our figures at 1 : 40 and 1 : 80 were 7·7 and 2·3 %. The pamphlet recently issued by the Medical Research Council from the Standards Laboratory of Oxford for the use of those working with Oxford Standard suspensions states that the standard titre of the average normal serum in the British Isles is well below 1 : 50 for *Bact. typhosum* O. It has already been mentioned that the average titre of O agglutination was raised in our tests on account of the unusual content of O agglutinins of sera from 200 pregnant women attending an antenatal clinic, but even in other members of the normal population we would consider that 1 : 50 is somewhat low for use as a diagnostic titre in this community.

It is especially worthy of note that the normal level of H agglutinins for *Bact. paratyphosum* B is as high as that for *Bact. typhosum*, and in neither case do we consider them to be the result of specific stimulation resulting from subclinical infection. Our results agree well with those of Smith *et al.* (1930) for normal sera from females in Manchester but are in marked contrast with

those obtained in Southern Rhodesia by Alves (1936) who failed to obtain any agglutination of this suspension with 530 sera at dilutions of 1 : 50. Again, we consider that the average normal titre of 1 : 25 for *Bact. paratyphosum* B specific H agglutinins suggested for the British Isles by the Oxford Standards Laboratory would be somewhat low in this community, and this is also our opinion concerning H agglutinins for *Bact. typhosum*. The importance of using a type suspension for the detection of H agglutinins for *Bact. paratyphosum* B was emphasized when we included in some of the tests a suspension of the monophasic *Bact. cholerae-suis* var. *kunzendorf*. Not infrequently a serum agglutinated this latter suspension but not the type suspension of *Bact. paratyphosum* B, thus indicating the presence of group agglutinins but the absence of specific agglutinins for the latter.

Such a result with the group H suspension indicates the probability that the presence of O agglutinins for *Bact. paratyphosum* B might have been due to infection with a *Salmonella* organism such as *Bact. typhi-murium* which contains the same O antigen. We do not think it likely that this would explain the majority of these reactions. A point of interest with regard to these O agglutinins is that in many normal sera they occur alone, and, in fact, in as many as 6·6 % of these sera at a dilution of 1 : 40 (see row 21, Table I). This suggests the presence of agglutinins for the somatic antigens IV and V and the absence of those for somatic antigen XII, which is present in *Bact. typhosum* and apparently in unusually large available amounts in the particular strain 901. Pure O serum prepared by inoculation of a rabbit with *Bact. abortus-ovis* (somatic antigen IV and XII) agglutinated our strain of *Bact. typhosum* 901 (somatic antigens IX and XII) and *Bact. paratyphosum* B (somatic antigens IV, V, XII) to the same titre. It is reasonable to suppose that if *Bact. paratyphosum* B was the specific stimulus producing agglutinins in these normal sera, then the O agglutinins for this organism would cause co-agglutination of *Bact. typhosum* 901. The absence of such co-agglutination of *Bact. typhosum* 901 by many sera showing O agglutinins for *Bact. paratyphosum* B (see row 21, Table I) leads us to the conclusion that the presence of normal O agglutinins for *Bact. paratyphosum* B are due to contact with antigens present in other organisms or in foreign protein. These findings provide a reasonable explanation of the presence of O agglutinins for an organism which, as far as is known, is not present as a cause of enteric fever in this State. Further, in view of the numbers of sera which showed a single O agglutinin for either *Bact. typhosum* or *Bact. paratyphosum* B (rows 11 and 21, Table I) it seems unlikely that the combination of both these agglutinins (row 13, Table I) is caused by co-agglutinins resulting from specific stimulation by either organism. It is more likely that in these sera with combined agglutinins the normal agglutinins for each organism have arisen from separate non-specific stimuli or that there is one compound non-specific antigen which gives rise to co-agglutinins. Such results further suggest to us that in cases of enteric fever where co-agglutination occurs, such as those described by

Wyllie (1937), the high titre attained for agglutination of the non-infecting organism may be largely the result of the stimulus of the common antigen of the infecting organism on a mechanism already operating, so that normal agglutinins for the non-infecting organism already present in a low titre, are quickly raised to a higher titre.

SUMMARY

1. Five hundred samples of sera sent for the Wassermann test were tested for the presence of H and O agglutinins for *Bact. typhosum*, *Bact. paratyphosum* A and *Bact. paratyphosum* B. Histories were obtained of nearly all those persons whose sera gave H agglutination at dilutions of 1 : 40 or higher, or O agglutination at 1 : 80 or higher. It was found that at least 20 people in this series had been inoculated or had suffered from enteric fever. The remaining 480 were considered to be normal. This assumption is likely to be correct in this community where typhoid is the only enteric fever which occurs, and that rarely, and where inoculation is not commonly practised.

2. The results are tabulated so as to show all the combinations of agglutinins which occurred at serum dilutions from 1 : 20 to 1 : 1280. Out of sixty-three possible combinations of agglutinins twenty-three were found to occur.

3. The interpretation of the results for single agglutinins and for certain combinations of agglutinins is discussed, and also the application of these results to the diagnosis of typhoid fever in this community and enteric fever elsewhere.

4. A comparison of the local "level" of normal agglutinins with that of other communities is made, and the origin of normal and co-agglutinins discussed.

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