# A Vi VARIANT OF *SALMONELLA TYPHI* AND ITS APPLICATION TO THE SEROLOGY OF TYPHOID FEVER

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Since the description of the new typhoid Vi antigen and its corresponding antibody by Felix and his co-workers, various studies have appeared on this subject confirming their observations (Kauffmann, 1935*a*, *b*; Dyachenko, 1936; Horgan, 1936; Craigie & Brandon, 1936*a*, *b*; Robertson & Yu, 1936; Almon & Stovall, 1936; Bensted, 1937; and others).

The presence of Vi antigen in a typhoid strain can be inferred from its O inagglutinability and confirmed by agglutinating it against a pure Vi serum. The latter procedure, however, involves the preparation of a serum against a highly virulent strain of *S. typhi* and the complete absorption of O and H agglutinins from it.

The detection of Vi antibody, on the other hand, is a more laborious process, specially so, if the quantitative estimation of this antibody has to be made with a low-titre serum. Three different strains of *S. typhi*, sensitive to O, H and Vi agglutinins respectively, have to be employed and the Vi titre read in the simultaneous presence of O and H agglutination. This requires considerable practice. Where tentative conclusions have to be drawn from an investigation, the alternative method of completely absorbing the serum of its O and H agglutinins has to be resorted to.

Serological analyses during the past three years of sera from patients suspected of typhoid fever, suggested, to the senior author, that Vi agglutination may be a more reliable method of serological diagnosis of typhoid infection than the O and H types of agglutination. This, however, could only materialize if the detection of Vi antibody were made a practical proposition by discovering a strain of *S. typhi*, sensitive to Vi agglutinins in the same manner as the two well-known strains—"O 901" and "H 901", introduced by Felix to typhoid serology—are to their corresponding types of antibodies.

With this object in view, a complete antigenic analysis of a large number of typhoid strains was undertaken. 235 strains were collected from different parts of the world and were typed systematically against pure Vi, O and H sera. The present communication deals with one of these strains which behaves agglutinogenically as a pure Vi variant of *S. typhi*. It was isolated



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from the urine of a carrier and supplied to us through the courtesy of Dr F. Kauffmann, State Serum Institute, Copenhagen. This strain has been designated "Vi 1".

### Morphology, cultural and biochemical reactions

The strain "Vi 1" differs in certain respects from an ordinary typhoid bacillus. It is a non-motile, unusually small organism of the cocco-bacillary type. A single stained colony appears, under the microscope, as if the culture were contaminated. The bacilli are seen to lie in bunches and in twos and fours resembling a staphylococcal smear except that they are Gram-negative. Some of the organisms stain more deeply than others, the deeply stained bacteria having a lighter pink area round about them. A few are embedded in an oval homogeneous pink-stained matrix. The same pink material is seen to lie here and there in the field without any organisms being encased by it. The organisms appear as if they were possessed of a capsule.

In film preparations made with Indian ink, the great majority of bacteria of this strain were seen to be surrounded by a clear zone of considerable extent which reminded one of the gelatinous envelope, originally described by Rowland (1914) and later studied by Schütze (1932), which is seen around *B. pestis* when grown at 37° C.

Other Vi-containing strains of *S. typhi* had the appearance of an envelope specially when grown on glucose media (Hort, 1920) but this was never so well marked as with the strain "Vi 1".

This strain would be classified as a mucoid variant of *S. typhi* (see Bruce White, 1929). It grows freely on ordinary laboratory media but it emulsifies with difficulty in normal saline, though the suspension, once made, is quite stable. Two different kinds of smooth colonies were noticed on an agar plate: (*a*) opaque, and (*b*) transparent. The opaque colonies were more mucoid in character and the subcultures made from them were found to be more virulent than those from a transparent colony. The two types of colonies, however, did not breed true after a few subcultures.

The presence of an envelope and the mucoid nature of the growth of strain "Vi 1" would lend support to the suggestion of Kauffmann (1935*b*) that the Vi antigen is a sheath antigen enclosing the body of the bacillus in a kind of capsule. Successful experiments on the enhancement of the pathogenicity of bacteria, including the typhoid organism, by suspending them in mucin, carried out by a number of American workers (Rake, 1935), would further associate the high virulence of Vi-containing strains of *S. typhi* with the possession of a slimy cover, probably mucoid in character.

$S \rightarrow R$ variation was observed to take place readily with this strain. The organism was grown on agar prepared from Lab Lemco, the usual medium used in the military laboratories of India. Although daily subculture and weekly colony selection was practised throughout, rough variants were seen

to appear readily if the strain were seeded on Lemco agar which had been steamed more than once or where the *p*H of the medium was less than 7·6. Attention had also to be paid to normal saline. Where its reaction happened to be less than 7·4 granularity was observed in the suspensions prepared with it.

Stock cultures of this strain, as well as of the other Vi-containing strains of *S. typhi*, were found to maintain their full quota of Vi antigen on a simple egg medium (recommended to us by Kauffmann) consisting of three parts of whole egg and one part of pure sodium chloride, heated at 85° C. for 2 hr. and then sloped. The tubes must, however, be hermetically sealed. Similarly culture stabs prepared with Lemco agar, as practised by Felix (personal communication) can be relied upon to preserve the Vi content of these strains. A strain of *S. typhi*—T 58—stocked by drying *in vacuo*[1] was found on sub-culturing to possess the Vi antigen of the same order as that of the well-known strains "Ty 2", "Watson" and "Giglioli".

The biochemical reactions of strain "Vi 1" differed in no essential respect from those of other typhoid bacilli.

## THE ANTIGENIC ANALYSIS OF STRAIN "Vi 1"

In order to determine its content of the three typhoid antigens the strain "Vi 1" was agglutinated against pure Vi, O and H sera. In addition, immune sera were prepared against it in rabbits.

The results obtained in agglutination are summarized in Table I. Strains "O 901" and "H 901", employed extensively in typhoid serology for the determination of O and H antibodies respectively, were also included in the experiment, as well as a highly virulent Vi-containing strain—T 58—of the type designated "inagglutinable" by Felix and his co-workers and "V" forms by Kauffmann (1935*a*). The production of antibodies by "Vi 1" is exemplified in Table II.

The contrast between the agglutinability of strain "Vi 1" by the three different types of sera is striking. While complete agglutination is shown against the pure Vi serum, there is no agglutination whatever by the H serum and only a trace in 25 with the O serum. As this organism had already been observed to be non-motile, the absence of flagellar agglutination was not unexpected. Its complete failure to produce H antibody in the serum prepared against it (see Table II) confirmed the earlier observations.

Although permanently non-motile variants of Vi-free strains of *S. typhi* have been described—"O 901" of Felix is an example—we have not read, in the small literature on Vi antigen, of a non-motile strain belonging to this group. In the antigenic analysis of as many as 235 strains this was the only organism found to be completely devoid of H antigen. We have experimented

[1] Received through the courtesy of Colonel J. F. Siler, M.C., U.S. Medical Corps, from the laboratories of the Army Medical School, Washington D.C., this strain is being used in the preparation of T.A.B. vaccine for the United States Army.

Table I. *Agglutination of different types of strains of* Salmonella typhi *by pure* Vi, O *and* H *sera*

| Typhoid serum | | Strains of *S. typhi* | | | |
|---|---|---|---|---|---|
| Type | Dilution | Vi 1 | T 58 | O 901 | H 901 |
| Pure Vi serum | 25 | + + + | + + + | − | − |
| (1 : 2000) | 50 | + + + | + + ± | − | − |
| | 100 | + + ± | + + ± | − | − |
| | 200 | + + ± | + + | − | − |
| | 500 | + + | + ± | − | − |
| | 1000 | + ± | ± | − | − |
| Pure O serum | 25 | Trace | + + + | + + + | + + + |
| (1 : 20,000) | 50 | − | + + + | + + + | + + + |
| | 100 | − | + + | + + + | + + + |
| | 200 | − | + | + + + | + + + |
| | 500 | − | ± | + + + | + + + |
| | 1000 | − | Trace | + + + | + + + |
| Pure H serum | 25 | − | + + ± | − | + + ± |
| (1 : 5000) | 50 | − | + + ± | − | + + ± |
| | 100 | − | + + ± | − | + + ± |
| | 200 | − | + + | − | + + ± |
| | 500 | − | + + | − | + + ± |
| | 1000 | − | + + | − | + + ± |

*Note:* (*a*) The figures in brackets indicate the standard titres of these sera.

(*b*) The agglutination tubes incubated for 2 hr. at 37° C. and the readings taken after a further 20–22 hr. at room temperature.

Table II. *The titration of antibodies produced by the strain* "Vi 1" (*on rabbit immunization*)

| | | Strains of *S. typhi* | | |
|---|---|---|---|---|
| Serum | Dilution | Vi 1 | O 901 | H 901* |
| Vi 1 | 10 | + + + | + + + | − |
| | 25 | + + + | + + ± | − |
| | 50 | + + + | + + | − |
| | 100 | + + + | + ± | − |
| | 200 | + + ± | + | − |
| | 500 | + + ± | − | − |
| | 1000 | + + | − | − |
| | 2000 | + ± | − | − |
| | 5000 | ± | − | − |
| | 10,000 | − | − | − |

* The agglutination tests against strain "H 901" were carried out after O antibody had been removed from the serum by absorption with strain "O 901".

with it constantly for the last 4 months. At no time has the strain "Vi 1" given any evidence of the presence of H antigen.

It was, however, more surprising to come across an organism which, while showing perfectly smooth colonies and maintaining its stability in normal physiological saline, should at the same time exhibit such a very low degree of O agglutinability (a trace in dilution 1 : 25).

Although the term "inagglutinable" had been applied, in connexion with their O agglutination, to certain Vi-containing strains, it was understood to have been used in a comparative sense, since fairly good agglutination was always noticeable with them against an O serum within the range of dilutions

employed by us (1 : 25 to 1 : 1000). Strain "T 58" whose reactions in agglutination are included in Table I will serve as an example. The explanation for the hypoagglutinability of such strains by O antisera is to be found in the presence of Vi antigen which in some unknown way prevented an interaction between their O antigen and the corresponding O antibody. That they contained a fairly large quota of O antigen was never in doubt, since by the employment of such agents as heat and alcohol, which destroyed their Vi antigen, they could be made O agglutinable easily. Besides, they gave rise to a considerable amount of O antibody on animal immunization.

A reference to Table IV will show that neither heat nor alcohol extraction has the same effect on strain "Vi 1" as on the other Vi-containing strains. Its O inagglutinability is unaltered by treatment with these reagents. When this observation is taken in conjunction with the production of a very low titre of O antibody on animal immunization, as is evident from Table II, it may be concluded that the O inagglutinability of "Vi 1", or, to be more precise, its very low O agglutinability, is directly related to its very low content of O antigen, small enough to let this strain behave as a pure Vi variant of *S. typhi* but, at the same time, large enough to maintain it in a perfectly smooth state under optimal conditions of growth and environment.

It can also be seen from Table II that a considerable amount of Vi antibody is produced when strain "Vi 1" is inoculated into rabbits. The animals were immunized with living saline suspensions and by the intravenous route. The initial dose was 100 million bacilli increased at 2 days' interval to 200, 400, 600, 800, 1000, 1200, 1400 and 1600 million organisms. No difficulty was experienced till a dose of 800 million bacilli was reached. The interval between inoculations had then to be increased as animals lost weight, refused food and became out of condition. Great help in this connexion was obtained if the animal were previously prepared by feeding it with egg and cod-liver oil mixture and with plenty of greens and carrots.

This procedure was only necessary where a high Vi titre was desired against a smooth organism. Higher Vi titres, on the other hand, were obtained with greater ease against rough variants of "Vi 1". As Felix & Pitt (1935) have already shown that the Vi antigen does not necessarily disappear, *pari passu* with the O antigen, when $S \rightarrow R$ variation takes place in the Vi-containing strains of *S. typhi*, this seemed to be the explanation for the retention of Vi agglutinogenic activity by strain "Vi 1" when in a rough state.

In our experience with the rough variants of "Ty 441", a strain recommended by Felix & Pitt (1935) for the immunization of horses in the preparation of therapeutic antityphoid sera, we have not been able to produce such a high titre of Vi antibody by the use of this strain in rabbits as with the strain "Vi 1". It is, therefore, suggested that the strain may prove to be more useful in this connexion than the strain employed at present.

### Average lethal dose and protective power

The average lethal dose of strain "Vi 1" was found to be approximately $200 \times 10^6$ organisms. Its virulence, however, was of the same order as that of the other "inagglutinable" members of the Vi group.

In experiments on passive immunization, a Vi-containing strain with a lethal dose of about 100 million organisms, such as the strain "Ty 2", is usually employed. In none of our experiments have we been able to reduce this dose to the region of 100 million bacilli with any of the "inagglutinable" types including "Ty 2". The explanation would appear to be the unsuitability of Lab Lemco and other meat extracts as nutrient agents for the routine growth of the organisms of this group. The deleterious effect of such artificial media on the antigenic complex of the highly virulent strains of typhoid-paratyphoid group has already been commented on by various observers (Horgan, 1936; Bhatnagar *et al.* 1937*b*; Bensted, 1937).

The protective power of Vi antibody produced against strain "Vi 1" was of the same order as attributed to this antibody in general. It is, however, of interest to note that no difference was noticed, in passive immunization experiments on mice, between the protective power of antibody elaborated against smooth forms of this bacillus and that against its rough variants.

### The application of strain "Vi 1" to typhoid serology

The behaviour of strain "Vi 1" as a pure Vi variant of *S. typhi* in agglutination reactions naturally suggested its ideal suitability as an agglutinating agent for the detection of Vi antibody. Before this could be contended, it was, however, essential to determine its comparative sensitivity in Vi agglutination in relation to the other strains, specially "Ty 2" and "Watson", commonly employed for this purpose.

Three different Vi sera were, therefore, titrated against live saline suspensions of strains "Ty 2", "Watson" and "Vi 1". A sample experiment is reproduced in Table III. The serum marked "Lister" was very kindly supplied by Dr Felix. The other two sera—"T 58 " and "Vi 1"—were prepared by us. They are labelled after the organisms employed in their production.

It is seen from Table III that the strain "Vi 1" is much more agglutinable by the Vi antibody of all the three sera included in the experiment than either of the other two strains. As an example, taking one plus (+) as the standard titre of these sera, while a reading of 1 : 200 is given by the strain "Ty 2" for the Lister serum, a five times higher figure (1 : 1000) is recorded by the strain "Vi 1". Differences of similar magnitude are observed with the other two sera as well. Again when comparisons are made between this strain and the strain "Watson" a distinct superiority is noticeable in favour of strain "Vi 1" with all the three sera.

Table III. *Comparative titration of* Vi *antibody against different* Vi *strains of* S. typhi

| Pure Vi sera | Dilution | Vi strains | | |
|---|---|---|---|---|
| | | Ty 2 | Watson | Vi 1 |
| Lister | 50 | + + ± | + + + | + + + |
| | 100 | + + | + + ± | + + + |
| | 200 | + | + + | + + ± |
| | 500 | – | + ± | + + |
| | 1000 | – | – | + |
| | 2000 | – | – | – |
| | 5000 | – | – | – |
| T 58 | 50 | + + ± | + + + | + + + |
| | 100 | + ± | + + ± | + + + |
| | 200 | + | + ± | + + |
| | 500 | – | + | + ± |
| | 1000 | – | – | ± |
| | 2000 | – | – | – |
| | 5000 | – | – | – |
| Vi 1 | 50 | + + + | + + + | + + + |
| | 100 | + + ± | + + + | + + + |
| | 200 | + + ± | + + ± | + + + |
| | 500 | + ± | + + | + + ± |
| | 1000 | ± | + ± | + + |
| | 2000 | – | + | + ± |
| | 5000 | – | – | ± |

From the experiments described, it is evident that the Vi antibody content of a serum can be estimated easily with the help of strain "Vi 1". Greater accuracy is ensured since the uncertainty involved in the interpretation of Vi titre in the simultaneous presence of O and H agglutinations is avoided. The complete exhaustion of O and H antibodies, which had frequently to be resorted to, is rendered unnecessary. At the same time a more sensitive result than is possible by the employment of any other Vi-containing strain experimented on so far, is obtained. The strain is thus seen to occupy the same position with regard to Vi agglutination as the strain "O 901" does for the O type of agglutination.

Certain differences, important from the practical point of view, were noticed between pure Vi agglutination and pure O agglutination. While both types of reactions were essentially granular in character, the clumped bacilli of Vi strains were found to give rise to finer particles than those observed with O- agglutinated bacteria. When readings were taken according to the method described by Felix, it was noticed that the Vi agglutinated bacilli did not, in the great majority of cases, settle down to the bottom of the tube, as happens in the O type of agglutination, but distributed themselves uniformly round about the circumference of the lower quarter of an inch of the agglutination tube. What then appeared to the naked eye was not a definite deposit of agglutinated bacteria but a general haze in this area, which with the help of a 10× lens was detected to be a big mass of somatic agglutination. The standard reading, which was *one plus* (+) in every case, had, therefore, to be taken with a lens. When the tubes were shaken, the agglutinated bacilli were easily seen, floating in the saline as very fine particles.

We would strongly recommend the use of wide tubes of uniform internal diameter (1·25 cm.) with a flat bottom in preference to Dreyer's tubes for this type of agglutination. The differences between agglutinated bacteria and those that have simply settled down to the bottom of the tube can be made out by shaking the former type of tube. Moreover, the interpretation of results is much easier and far more satisfactory.

### The preservation of Vi antigen in killed suspensions of strain "Vi 1"

In view of the objection to the use of live organisms in agglutination, the activity of suspensions of this strain, rendered sterile by heat and chemicals, was investigated. The results obtained are exemplified in Table IV.

Table IV. *The effect of heat and chemicals on the agglutinability of the strain* "Vi 1"

| | | "Vi 1" treated differently | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Serum | Dilution | Live | Heated 100° C. 10 min. | Phenolized | Alcoholized | Acetonized | Formolized | Mercolized ($HgCl_2$) |
| Pure Vi | 50 | + + + | Trace | + + | + + | + + | + + + | + + + |
| | 100 | + + + | − | + | + ± | + + | + + ± | + + + |
| | 200 | + + ± | − | ± | + | + ± | + + | + + ± |
| | 500 | + + | − | Trace | ± | + | + + | + + |
| | 1000 | + ± | − | − | Trace | Trace | + ± | + ± |
| | 2000 | + | − | − | − | − | ± | + |
| Pure O | 25 | Trace | ± | Trace | ± | ± | Trace | Trace |
| | 50 | − | − | − | − | − | − | − |
| | 100 | − | − | − | − | − | − | − |
| | 200 | − | − | − | − | − | − | − |
| | 500 | − | − | − | − | − | − | − |
| | 1000 | − | − | − | − | − | − | − |

It is seen from this table that strain "Vi 1" conforms in general to what is already known of the susceptibility of Vi antigen to the action of heat and chemical reagents. Its exposure to a temperature of 100° C. for as short a period as 10 min. destroys its agglutinating power almost completely. The deleterious effect of phenol, pointed out by Felix & Bhatnagar (1935), is also evident. The phenolized suspension employed in this experiment was 4 weeks old. Its sensitivity to Vi agglutination was quite satisfactory when prepared fresh. The organisms, however, became less and less agglutinable with the lapse of time. While alcohol-extracted suspensions have proved their efficacy in the O type of agglutination, it is clear from Table IV that neither alcohol nor acetone extraction can be recommended for Vi antigen.

Kauffmann (1935*b*) has already established the suitability of formolized suspensions for the demonstration of Vi agglutination. While fully supporting this we found that perchloride of mercury, recommended as a general sterilizing agent for bacterial suspensions by Bridges (1936), proved to be a better preservative for Vi antigen than formalin. When agar slopes were washed

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*with normal saline to which to this reagent had been added in the strength* of 1 : 1000, the organisms died almost immediately, the suspension kept well and was found to be more sensitive to Vi agglutination in comparison to that prepared with formalin.

It must, however, be added that in our experience the preserved suspensions of strain "Vi 1" have not proved to be as stable as those of the O and H variants of *S. typhi*. We have not yet succeeded in preparing a suspension which has maintained its original degree of agglutinability for more than 10 weeks.

### THE IMPORTANCE OF Vi AGGLUTINATION IN THE DETECTION OF TYPHOID INFECTION PAST AND PRESENT

From personal experience and from the study of a large number of records of cases of typhoid fever in an inoculated population such as the army in India, it was observed that the estimation of O and H agglutinins, practised so extensively in this type of infection, did not justify the amount of trouble taken in carrying them out. The rise in the titre of these agglutinins, judged *from a series of agglutination reactions at intervals of a few days throughout the* course of infection, did not, in a large majority of cases, offer the evidence adequate for diagnosis. Reliance had, therefore, to be placed on the isolation of the infecting organism from the blood, urine or faeces. If one failed to isolate the bacillus from these sources, one was commonly confronted with a positive clinical picture and negative serological findings.

Since evidence was available pointing to the absence of Vi antibody, either as a natural agglutinin or as an immune body elaborated in response to T.A.B. inoculation with a vaccine in which a Vi-containing strain of *S. typhi*, preserved in phenol, was incorporated (see Bhatnagar *et al.* 1937*a*), sera from patients suspected to be suffering from an enteric type of infection were, as a routine, titrated for their antibody content against four different strains of *S. typhi*—"Ty 2", "Watson", "O 901", and "H 901"—and absorbed where it was considered desirable. A large volume of evidence had thus accumulated which would have justified us in stating some time ago that the detection of Vi antibody in the serum of a patient had been found to be of help in the detection of typhoid infection. In view of the technical difficulties involved in carrying out this procedure, which have already been enumerated, this observation, if made earlier, would have remained of theoretical interest only. The identification of strain "Vi 1" has, however, made it a practical possibility. Experiments since carried out have fully confirmed our earlier observations. As they raise many side issues, they are being made the subject of a separate communication. It is, however, confidently hoped that the practice of Vi agglutination with the help of a strain which is sensitive only to this type of antibody would, in the light of experience gained, be eventually successful in ousting H and O agglutinations from the important place they occupy at present in the serological diagnosis of typhoid fever.

A definite correlationship between the typhoid carrier condition and the presence of Vi antibody in the serum has been noticed. Thousands of persons are examined in the military laboratories in India, in connexion with their employment in the handling of food and drinks. Evidence of past enteric or dysenteric infection is looked for in their stools and urine. All the typhoid carriers detected by us were excreting highly virulent Vi-containing typhoid organisms. At the same time the sera of all of them were found to contain the Vi antibody of a fairly high titre that was easily detectable by the old technique. Since the identification of strain "Vi 1", the practice of agglutinating the sera of individuals presenting for carrier examination has been adopted as a routine. The detection of Vi antibody is taken to be an indication for a very thorough search for typhoid bacilli in their faeces and urine. We have not yet found an individual who, while excreting typhoid bacilli, failed to produce evidence for the presence of Vi antibody in his serum. In this connexion a statement of Felix *et al.* (1935) on the possible utility of Vi agglutination as a reliable serological method for the diagnosis of typhoid carrier condition is of interest.

SUMMARY

1. The description of a strain which behaves as a pure Vi variant of *S. typhi* is given. Complete absence of H antigen from it is demonstrated. The non-interference in its Vi agglutinating activity by the very small amount of O antigen contained in it is shown. This strain has been designated "Vi 1".

2. With the help of this strain, the practical applicability of Vi agglutination as a reliable method for the routine serological diagnosis of typhoid infection and for the detection of typhoid carrier condition, in preference to O and H types of agglutination, is stressed.

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