The Laboratory Identification of the V Form of B. Typhosus*

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RECENT investigation has shown that the form of B. typhosus which occurs in human infection is characterized by antigens which are distinct from the H and O antigens and which tend to be lost on repeated subculture on laboratory media. The V form of B. typhosus may be identified by means of two simple tests. These tests will be described here from the point of view of their possible use in the routine diagnostic laboratory either for the rapid provisional identification of B. typhosus or as additional aids in the identification of this organism.

Grinnell* compared a number of cultures of the Rawlings strain with freshly isolated strains of the typhoid bacillus and suggested that virulent smooth cultures should be substituted for the old Rawlings strain in the preparation of typhoid vaccine. Perry, Findlay and Bensted2 investigated the Rawlings strain further and found that its virulence and its protective qualities as a vaccine could be restored by mouse passage. Felix and Pitt3 demonstrated that virulent living stock strains of B. typhosus were characterized by a very marked resistance to agglutination by O serum. Shortly afterwards the same authors reported that virulence and resistance to O agglutination were related to the presence of a new antigen which they called Vi (virulence) antigen.4 More recent work by Felix and his associates5 would indicate that the Vi antigen is of a complex nature.

Vi antigen has the following antigenic properties: (a) it produces an agglutinin which specifically agglutinates the virulent O-resistant form of the organism, (b) it produces bactericidal antibody for the virulent form, (c) it produces specific phagocytosis-promoting qualities for the virulent form; and (d) it produces active immunity against infection with the virulent form and antibodies which will confer passive immunity.

Kauffmann6 made an extensive study of the typhoid bacillus primarily from the point of view of the agglutination reactions referable to the presence of the Vi antigen. He introduced the terms "V form" and "W form" to denote, respectively, the form of B. typhosus which develops the Vi antigen and the form which does not do so. The V form is inagglutinable in O serum but agglutinable in serum containing the Vi agglutinin. The W form, lacking Vi antigen, is agglutinated by O agglutinin but not by Vi agglutinin. Kauffmann noted that the majority of strains recently isolated from human cases of typhoid fever were V forms and this has been confirmed by Felix, Krikorian and Reitler,7 who found that Vi antigen was present in 88 out of 90 strains of B. typhosus isolated in Palestine. Craigie and Brandon8 examined cultures from 396 specimens submitted from 283 cases and carriers in the provinces of Ontario and Quebec. The primary object of this study was to determine the occurrence of the V form in infected individuals. It was found necessary to work out methods for the satisfactory identification of V and W forms and the differentiation of pure form and mixed form cultures. It was found that with the majority of pure V form cultures, age of the particular subculture used in the ag-

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glutination test had an important bearing on the behaviour of the organisms in pure V and pure O agglutinating sera. Four-hour living broth cultures of the V form invariably gave advanced agglutination in a pure V serum within two hours at 37°C, and were at the same time completely inagglutinable in pure O serum. Similarly, cultures of the W form were completely inagglutinable in the pure V serum but agglutinated rapidly in pure O serum. Older cultures of the majority of V form strains, however, lost their O-resistance and their V-agglutinability to some extent. It was concluded that only in the early logarithmic phase of growth were the V characters of *B. typhosus*, i.e., agglutinability by V agglutinin and resistance to O agglutination, at a maximum. The case and carrier cultures were therefore examined under the optimum conditions for the development of V form characters; i.e., living four-hour broth cultures were used in the agglutination tests. Examining the cultures in this way, it was found that the V form was obtained from 95.6 per cent of individuals submitting the specimens. Six individuals submitted one specimen each from which only the W form was obtained, but this, in view of the other findings and the age of the cultures before examination, does not exclude infection of these persons with the V form. Cultures from the other individuals from whom the V form was not obtained were found to contain a bacteriophage specific for the V form.

Observations on this V bacteriophage have been reported elsewhere. The available evidence indicates that this bacteriophage is as specific in its action as the V agglutinin and that the bacterial substances present on the surface of the organism which are responsible for the adsorption of V agglutinin and V bacteriophage are very closely related, if not identical. Only the V form of *B. typhosus* which develops V agglutinin is sensitive to this bacteriophage, its sensitivity being demonstrable in two ways, either by the inhibitory effect of the V phage on growth when the phage is applied to an agar plate culture or by manifest lysis of the V forms in broth culture. When a pure V form culture is lysed by this phage the secondary growth which ultimately appears consists of the W form. The W form, either phage-produced or naturally occurring, is resistant to V bacteriophage and does not adsorb it. The presence of V bacteriophage in a stool culture may result in the rapid elimination of the V form, a pure W culture thus being obtained. Apart from manifest phage action, the V form may degrade, on subculture, to the W form, the capacity to elaborate V agglutinogen being lost. Strains of the typhoid bacillus vary very greatly in their tendency to show W degradation on subculture on laboratory media. Some may show the W form after a few subcultures in broth. Others, like the strain Ty 2, are outstanding in the stability of the V form on repeated subculture. It is to be noted V → W degradation occurs independently of S-R and H-O variation. The characters of V and W forms are summarized in table I.

**TABLE I**

<table>
<thead>
<tr>
<th>Form</th>
<th>Agglutinogens present</th>
<th>Agglutination*</th>
<th>Sensitivity to V Phage</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>V+O</td>
<td>++++</td>
<td>Sensitive</td>
</tr>
<tr>
<td>W</td>
<td>O</td>
<td>-</td>
<td>Resistant</td>
</tr>
</tbody>
</table>

*Four-hour, living broth culture, 2 hours at 37°C.

Both V and O agglutinogens are somatic (body) antigens. Loss of O agglutinogen may occur without loss of V agglutinogen, thus giving a rough V form. V forms may be flagellate (H form) or non-flagellate.
METHODS FOR THE IDENTIFICATION OF THE V FORM

In view of the finding that a very high percentage of individuals infected with *B. typhosus* are infected with the V form, it is justifiable to employ methods for the identification of the V agglutininogen as methods for the identification of *B. typhosus*. Neither the H nor the O antigens alone are sufficient for the serological identification of *B. typhosus*. The typhoid bacillus shares the O antigen with *S. enteritidis* (Gaertner) and six other members of the *Salmonella* group. It shares its specific H antigen with two other *Salmonella* species (*S. stanley* and *S. muenchen*). As far as is known, the V agglutininogen of the typhoid bacillus is more specific than H antigen and as yet has been identified only in one strain of one other *Salmonella* species, the Witts strain of *S. paratyphi-C*.8 9,11

Craigie and Brandon8 tested an extensive series of micro-organisms with typhoid V bacteriophage, including a series of species representative of the *Salmonella* group, and found that only the typhoid bacillus showed sensitivity to this bacteriophage. This bacteriophage differs from other phages for *Salmonella* species which have been studied in that it is a phage for heat-labile somatic antigen. The *Salmonella* phages extensively investigated by Burnet9 and Levine and Frisch10 showed specificity related to the heat-stable somatic O antigens which are widely shared in this genus. Typhoid V bacteriophage, on the other hand, shows a degree of specificity which would permit of its use as a diagnostic reagent not merely for *B. typhosus* but for the identification of the V agglutininogen of this micro-organism. The test for sensitivity to V bacteriophage, being simpler than the agglutination test, will be described first. It should be stated that all freshly isolated cultures of *B. typhosus* which we have examined have shown sensitivity to this bacteriophage.

PHAGE PLATE TEST

A colony suspected of being a colony of *B. typhosus* is tested for sensitivity to V bacteriophage in the following way. A portion of the colony is rubbed over a sector of a dry but not corrugated, nutrient agar plate with a smooth spreader. A loopful of potent V phage filtrate, the preparation of which is described below, is applied to the centre of the inoculated area and the plate is transferred to a 37° C. incubator. If the inoculum of organisms has been sufficiently heavy, growth will be apparent in six hours, and if the culture is the V form of *B. typhosus* an area of inhibition of growth where the phage was applied will be readily apparent. This inhibition of growth indicates

![Figure 1](image)

**Figure 1**

Pure V form, nos. 1 to 4; mixed V and W form, no. 5; pure W form, no. 6.

sensitivity of the culture to the bacteriophage. On further incubation overnight the effect produced by the phage will be more apparent (fig. 1). If the culture is a pure V culture the phage-inoculated area will be absolutely devoid of growth. If a few colonies appear, they will either be colonies of the W form of *B. typhosus* which is resistant to the phage or colonies of some contaminating micro-organism. Very rarely, in about one
to two per cent of cultures of the V form, some growth with subsequent lysis may occur, with the result that a number of nibbled colonies or a confluent growth showing a glassy degeneration will be found in the phage-inoculated area. Even late lysis such as this is readily distinguished from the unrestrained growth of the W form of *B. typhosus* or of other *Salmonella* species.

In order to obtain complete inhibition of the V form a sufficiently potent phage filtrate must be employed and care must also be taken that heavy inoculation of the plate with organisms is not carried to too great an extreme. When the plate is inoculated there should not be any visible area of opacity where the colony was spread. Potent V phage filtrates may be obtained in the following way. One 1/10 to 1/4 cc. of active phage filtrate is added to 5 cc. of broth, pH 7.6 to 7.8, and a sufficient amount of an 18-hour V form broth culture is added so as to yield a slight opacity. The tube is incubated at 37°C. While waiting for lysis to occur in this tube, the 18-hour culture is also inoculated rather heavily into fresh broth in order to provide a supply of young actively growing V culture. The broth tube containing phage is inspected at intervals and as soon as definite clearing sets in more young V form culture is added. When lysis occurs for the second time a still larger quantity of V form inoculum is added. Lysis with immediate addition of further V form culture is allowed to proceed for at least six to seven hours, and when lysis occurs again the broth culture is filtered through a Seltz EK filter which has been washed out with a buffer solution, pH 7.0, to neutralize its normal alkalinity. This method can be modified in order to yield larger quantities of phage filtrate. If 50 to 100 cc. are to be prepared, it is best to inoculate the broth with phage and a six-hour culture of the V form the previous evening so as to have available a moderately high concentration of phage the following morning. The disadvantage of commencing propagation of the phage the previous day is that without some experience as a guide to the proportion of phage and organisms to be inoculated, an undue amount of secondary growth of the W form may occur overnight. We have found the Rawlings V form a very suitable strain for the propagation of V bacteriophage. Before a culture is employed for the propagation of phage, it is plated out and phage-tested in order to ensure that it is a pure V form culture and that the W degradation has not occurred. The potency of the phage filtrate is readily estimated by making a series of tenfold dilutions of the filtrate in broth and seeding the broth very lightly with the V form of the Rawlings strain. The tubes showing clearing in twenty-four hours and several of the dilutions beyond this, are plated and phage applied to the plate culture in order to determine accurately the end-point of the phage.

Using the method of propagation which has been described, filtrates active in dilutions from 10^-9 to 10^-11 may be obtained. For the phage plate test, filtrates active in a dilution of at least 10^-9 should be used.

**Identification of the V Form by the Agglutination Test**

The V agglutinability of many V strains decreases as the culture ages, being usually much less marked in 18-24 hour agar slope cultures than it is in four-hour broth cultures. Four-hour broth cultures have an added advantage in that their use permits the carrying out of the agglutination test on the same day as isolation of the colony. If the purpose of the agglutination test is to determine whether the culture is a pure V form or a pure W form or a mixed V and W form culture, then pure O and pure V agglutinating sera must be used. In this case only a three tube test is required, since the test is a purely qualitative one (Craigie and Brandon). O and V agglutination being
mutually exclusive in the case of pure form cultures and a range of serum dilutions therefore offers no advantages. A pure V serum, however, is troublesome to prepare. When the living or formalin-killed V form is injected into a rabbit, H and O agglutinins are formed in addition to V agglutinin. The H and O agglutinins are present in considerably higher titre than the V agglutinin and it is necessary to adsorb the H and O agglutinins completely in order to obtain a pure V serum. This requires massive and frequently repeated adsorption with a W culture such as H 901 and subsequently rigorous testing of the serum for absence of H and O agglutinin. A V serum, however, does not require adsorption, provided that four-hour broth cultures are employed, if the object in its use is merely the identification of *B. typhosus*. The degree of flagellar development in four-hour broth cultures is usually very slight, and even so, the degree of H agglutination with such a culture in two hours at 37°C is rarely perceptible. As has been stated above, nearly all freshly isolated cultures of *B. typhosus* are V form cultures and thus in four-hour broth culture are resistant to O agglutination. Any agglutination which will occur, therefore, with a four-hour broth culture of a freshly isolated strain will be predominantly V agglutination and the V agglutination of *B. typhosus* is at least as specific as is its H agglutination.

A V agglutinating serum for *B. typhosus* is prepared in the following way. An 18-hour agar slope culture of a pure V form (Rawlings) is washed off with saline and killed with 0.25 per cent formalin. A series of intravenous injections of 500 million and 2,500 million organisms is given to a rabbit at four to five day intervals for five injections. A serum sample is taken and if of satisfactory titre the animal is bled out on the fifth and sixth day after the last injection. The V titre of the serum is tested in the following way. A series of twofold dilutions from 1:40 to 1:2,560 in 0.4 cc. of saline is set out and 0.1 cc. of a four-hour broth culture of a pure V form is added to each tube. The tubes are incubated for two hours at 37°C and then examined. If any H agglutination should occur, it is readily distinguished by its flocculent character and the fact that no concomitant decrease in opacity of the tube occurs. O agglutination will not occur since the test culture used was a young pure V form culture. Any granular agglutination which takes place will therefore be due to the presence of V agglutinin.

When a portion of a colony is transferred to an agar plate for the phage-plate test, another portion may be transferred to a tube containing 1 cc. of nutrient broth. Sufficient growth will occur in three to four hours to permit of the V agglutination test being carried out. It should be pointed out that in this agglutination test living, actively growing cultures are used and that since no preservative is added to the serum employed, growth occurs during the period of two hours' incubation at 37°C. This continued growth during the period of incubation greatly enhances the clear-cut nature of the coarse granular V agglutination, which is extremely rapid with very young broth cultures. With V serum in a concentration eight to ten times its titre, many V form cultures will show macroscopically visible agglutination within half an hour, and all will show definite agglutination in two hours at 37°C. In addition to the tube containing the V serum and a saline-suspension control tube, a tube containing O serum 18 to 20 times its titre may be included. As pointed out in a previous paper (Craigie and Brandon), O agglutination is extremely rapid when a living four-hour broth culture is employed. The O serum tube will, however, be found to be negative with nearly all cultures and its inclusion, apart from providing an additional control, has no advantage except for the recognition of an occasional W form culture. A W
form culture direct from the primary isolation plate generally indicates the presence of V bacteriophage in the culture or specimen of origin.

In a study of over 400 cultures a complete correlation was found between agglutinability in pure V agglutinating serum and sensitivity to V bacteriophage. While the agglutination test employs an orthodox reagent, i.e., V agglutinin, for the detection of an antigen, the phage test has the following features to recommend it:

1. It can be much more rapidly applied than the agglutination test.
2. With a little experience in its use, inhibition of growth of the V form of B. typhosus can be demonstrated within six hours from the selection of a suspected colony.
3. It will give a positive result with a very high percentage of freshly isolated strains of B. typhosus. Indeed, resistance of a freshly isolated strain of this organism to V phage probably indicates that V phage was present in the specimen from which the culture was derived.

Extensive trial of V bacteriophage by laboratories engaged in routine diagnostic work will, of course, be necessary before its value under such conditions can be assessed, but the study which has already been made of this phage indicates that it is worthy of such a trial. Only by very extensive application of the V phage test to freshly isolated Salmonella cultures can it be determined whether sensitivity to V bacteriophage is specifically confined to B. typhosus. No B. paratyphosus B strains sensitive to this phage have been encountered and this is in agreement with the finding of Felix and Pitt that the Vi antigen of this isolated Salmonella is serologically distinct from that of B. typhosus. The reader is referred to another paper for a discussion of the work of Burnet and Levine and their coworkers which has shown that sensitivity to strains of bacteriophage is in most cases specifically related to the presence of heat-stable antigens in the bacterial cell. The present position with regard to Vi phage may be summed up in the statement that the same antigen appears to be responsible for the adsorption of Vi agglutinogen and for the adsorption of V phage. It is obvious, therefore, that any Vi phage sensitive strains of Salmonella species other than B. typhosus, or any Vi phage resistant virulent strains of B. typhosus which future investigation may reveal, will be of more than passing interest. Apart from the significance of such strains in the future investigation of the nature and specificity of bacteriophage, strains of B. typhosus atypical in their reaction to V phage may prove of value in the further immunological investigation of the antigenic qualities included under the term Vi (or virulence) antigen.

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(b) J. Immunol., 1936, 38:63.