THE DIAGNOSIS OF SALMONELLA TYPES

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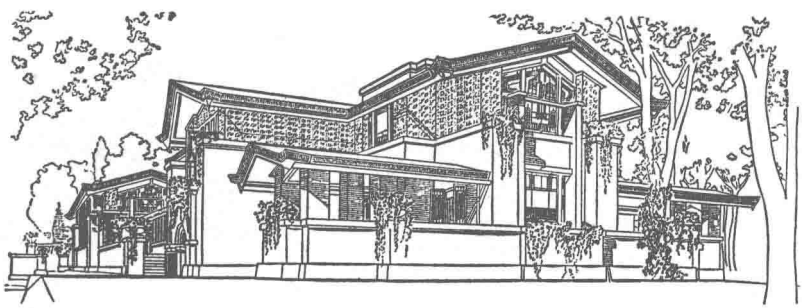

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Preface

THE SCOPE of this work is limited to a discussion of the natural phenomena and methods used in the practical classification of the Salmonella group by serologic and biochemical methods. The classification, which has been widely accepted and generally used, is based upon the antigenic analysis of cultures with due regard to the several forms of variation known to affect antigenic structure.

In order to classify the organisms successfully and, in fact, to prepare the reagents necessary for serologic study, it is imperative that the worker understand the variational changes which occur in the group. These include the O-H variation of Weil and Felix, the S-R variation of Arkwright, the V-W variation, and form variation of Kauffmann, and the phase variation of Andrewes. If these phenomena are not understood and their effects not kept constantly in mind, confusion and failure to arrive at a correct diagnosis can only result.

If the above mentioned changes are recognized and if the recommended methods are strictly adhered to, little difficulty should be experienced in the classification of *Salmonella* cultures.

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Serologic Diagnosis

No EXACT definition of the Salmonella group is possible, because there are no sharply delimited groups in nature. The Salmonella group comprises various types of Gram-negative bacteria which are characterized by certain biochemical and serologic properties. In 1947 the Salmonella Sub-Committee modified Bruce White's definition as follows:

"A large genus of serologically related Gram-negative and non-sporing bacilli, 0.4–0.6$\mu \times 1$–3μ in usual dimensions, but occasionally forming short filaments; showing, with certain exceptions, a motile peritrichous phase in which they normally occur; in fact adhering to the pattern of *S. typhi* in staining properties and morphology. Rarely fermenting lactose or sucrose, liquefying gelatin or producing indole, they regularly attack glucose with, but occasionally without gas production. All the known species are pathogenic for man, animals, or both."

Thus in the arbitrary delimitation of the Salmonella group from other, related groups, consideration is given to both biochemical and serologic characteristics; but in subdividing the Salmonella group itself use is made chiefly of serologic methods, by means of which it is possible to set up a large number of sero-types. These sero-types are then further divided into biochemical types by biochemical methods; in other words, the serologic division is primary.

Serologic type diagnosis is made by means of agglutination and one must have regard both to the somatic (O and Vi) and the flagellar (H) antigens of the Sal-

monella bacteria. Moreover, factors of considerable importance are serologic variation, e.g., the "form variation," among the somatic antigens and the "phase variation" among the flagellar antigens. The indispensable prerequisites of type diagnosis are good media and suitable immune sera. In every agglutination reading within the Salmonella group a distinction must be made between the granular (somatic) and the floccular (flagellar) agglutinations.

General Technic

Preparation of Antigens

O Antigen

For the preparation of O immune sera, which must be free of H agglutinins, the culture is heated for 2½ hours at 100° C. when the bacteria are of the flagellated type. The purpose of heating is to remove the antigenic effect of the flagella. When working with non-flagellated bacteria—the so-called O forms—the organisms may be killed by any method; immunization may also proceed with living bacteria. The culture may be either broth culture or agar culture suspended in saline. The killed culture may be stored in the refrigerator during the period of immunization. Formalin, 0.5%, is added as a preservative; this is the commercial 40% formaldehyde, which is added in 0.5 ml. amount to 100 ml. broth. If living culture is employed for immunization, it must always be freshly prepared.

In cases where O antigens only are to be used for agglutination, flagellar bacteria may be heated for 30 minutes at 100° C., whereas nonflagellated bacteria may be killed with 0.5% formalin. The cultures may be either broth cultures or agar plate cultures suspended in saline. After being killed, these antigens are kept in the refrigerator.

Long years of experience have shown that alcohol suspensions are excellent for O agglutination. It should be observed, however, that the alcohol treatment simply abolishes the H agglutinability of the bacteria, and not their antigenic property. For this reason alcohol treatment should not be used in preparing immunizing antigens for O sera.

For the preparation of alcohol suspensions the method is to make a dense suspension in saline from a 20-hour agar plate culture, add the same quantity of 96% alcohol and incubate the mixture for 20 hours at 37° C. in the incubator. Then place the suspension in the refrigerator, where it will keep for years. For agglutination purposes use this dense alcohol suspension for preparing a suitable dilution in saline.

It is particularly important when preparing O antigens to employ only smooth forms. With this in view the culture should be streaked out on agar plates in such a manner as to produce single colonies, so one can exclude any rough forms that may be present. One should use only bright, domed colonies which are stable in saline and which agglutinate well in the homologous O serum.

Vi Antigen

In the Vi antigen we have a special, somatic antigen, belonging to Kauffmann and Vahlne's group of K antigens (1) and with a toxic action in animal experiments. The assumption of Felix and Pitt (2) that this was a "virulence" antigen was refuted by Ørskov and Kauffmann (3) in respect to *S. typhi*, and by Kauffmann (4) as regards *S. paratyphi C*. Therefore we use the term "Vi" solely as a symbol of a certain somatic antigen, and do not call it a virulence antigen.

For the preparation of Vi serum the author generally employs living bacteria from a 20-hour agar plate culture suspended in saline. One may also work with a killed culture and sterilize with formalin, alcohol, or Hg preparations. However, it is usual to employ the relatively innocuous Ballerup bacteria in living culture rather than *S. typhi*.

On the other hand, for Vi agglutination, for instance in

the Vi Widal reaction, one may employ a freshly prepared saline suspension of *S. typhi* killed by the addition of 0.5% formalin which has been permitted to stand for an hour or two at 37° C.

H Antigen

As an H antigen it is usual to employ formalin-broth culture prepared in the following manner:

To a six-hour broth culture (taken from a 20-hour agar plate), which must be tested for motility, add 0.5% formalin and then leave the culture overnight in the incubator at 37° C.; thereafter it should be stored in the refrigerator. Should broth cultures of certain strains not be sufficiently motile (all individuals must be actively motile when viewed by microscope), pass the culture once or twice through semi-fluid agar in a U-tube and start the broth culture from there. One may also transfer the culture to soft agar plates (swarm-agar) and from these suspend the culture in formalin-saline.

Quite exceptionally one encounters non-motile cultures which, despite their immobility, are possessed of flagella and produce good H sera.

For the preparation of phase sera it is necessary always to start cultures intended for immunization from tested single colonies; otherwise, pure, or preponderantly pure phase sera will not be obtained. However, the best method is to use nothing but cultures from swarm-agar plates according to Gard's method (5), i.e., swarm-agar plates to which immune serum has been added. If it is the intention, for instance, to make a pure culture of phase 1 of *S. paratyphi B*, add to the swarm-agar an H serum of phase 2, which contains the H antibodies 1, 2. This prevents phase 2 of *S. paratyphi B* from swarming, whereas phase 1 (b) grows over the plate without hin-

drance. In selecting the H serum, care must be taken that it contains no O agglutinins or H agglutinins for phase 1. In order to obtain the purest possible H antigens, two swarm plates must be started, one after the other, plate 2 being inoculated from the extreme outer edge of plate 1. Inoculation is made with a needle in the center of the plate by a single spot culture. On suspending organisms from the swarm plates in formalin-saline, we get antigens of the required phase, suitable for agglutination and for preparing sera.

If it is one's intention to demonstrate H antigen with living culture, by means of slide agglutination, the agar plates must be soft (1.8–2.0%), moist and thick. Hard, too dry, and thin plates are unsuitable for the optimal development of H antigen, a fact which cannot too often be emphasized.

Another point to be observed is that all strains are not equally suitable for producing formalin-broth cultures. Strains inclined towards spontaneous agglutination must be excluded; this is done by microscopic examination of the broth culture, polar agglutination being an expression of spontaneous agglutinability.

In preparing antigens and sera, it is usual to employ only smooth forms of the culture, and not rough forms. Furthermore, to ensure a normal antigen development the use of suitable media is unconditionally necessary.

Strains frequently used are kept best on sloped egg medium. If long storage is desired, dried or stab culture on meat extract agar are recommended (see *media*).

Preparation of Immune Sera

Rabbits are employed in the preparation of diagnostic immune sera. The animals are injected several times with living or, most frequently, killed bacteria at certain in-

tervals of days. In the case of formalin-broth cultures or boiled broth cultures the animals are injected intravenously with 0.25 ml., 0.5 ml., 1.0 ml., 1.5 ml., and 2. ml. amounts, respectively, at intervals of five days (i.e., with four days between). Eight to ten days after the last injection the blood is taken by sterile means from the carotid. If suspensions are used as antigens, their density must be adjusted to the density of the broth culture.

The blood is collected in sterile tubes; after coagulation the coagulate is divided and loosened from the wall of the tube with a glass rod and allowed to stand at room temperature. The serum fluid is poured off and centrifuged until clear. Glycerin, equal parts, or 0.5% phenol is used as a preservative, or merthiolate 0.01%, adding 1 ml. of a sterile 1% stock solution in distilled water for every 100 ml. serum. The finished sera are stored in the refrigerator at +5° C.

Preparation of O Sera

When using OH forms, the culture must be heated for 2½ hours at 100° C. in order to destroy the H antigen. With O forms (non-flagellated) one can immunize with living bacteria or with formalin-broth culture. For the preparation of immune sera possessing a high V titer (O antigen No. 5), the culture must be a formalin-broth culture of the O form, recently described by F. Kauffmann (6).

Preparation of Vi Sera

For diagnostic purposes it is recommended that sera be prepared with living Ballerup bacteria, though care must be taken to use only V forms (*i.e.* colonies with Vi antigen). For this purpose the culture must each time before use be streaked out on agar plates to produce

single colonies. The V forms, recognizable by their opaque appearance and giving positive agglutination in a typhoid Vi serum, are suspended in saline.

Preparation of H Sera

For preparation of H sera one uses either formalin-broth culture for immunization, or, better—where diphasic cultures are concerned—suspension from swarm-plates according to Gard (see under Preparation of Antigens).

All immune sera must be tested before use for the presence of α antigen; this may be done by means of slide agglutination with a strain containing α antigen (for details see F. Kauffmann (7)).

Agglutination

Slide Agglutination

Slide agglutination is not merely an orienting agglutination but also a method which, with the proper technic, gives such definitive results that in most cases test-tube agglutination is superfluous. Here, as in all other forms of agglutination, consideration must be given separately to reactions with the O, H and Vi antigens of the bacteria.

On an ordinary glass slide (7.5 cm. by 2.5 cm.), lying on a black background, place side by side two or three drops of various diluted immune sera, whose working or test dilutions have been determined. With a loop or inoculating needle stir into each drop sufficient agar culture to produce a distinct, homogeneous turbidity. For control purposes mix the same culture into a drop of 0.9% saline in order to test its stability. After the culture has been stirred well, tilt the slide back and forth for thirty seconds and observe against a light for ag-