SIMPLIFIED SEROLOGIC IDENTIFICATION OF SALMONELLA CULTURES

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The growing multiplicity of *Salmonella* types and the numerous diagnostic serums required for the recognition of all of them make impractical complete serologic typing of salmonellae in the average laboratory. However, this situation should not deter the small laboratory from making accurate diagnoses of *Salmonella* infections or from exact recognition of those *Salmonella* types which are of greatest importance in the epidemiology of salmonelloses of man. To persons not familiar with the typing of *Salmonella* cultures, the Kauffmann-White schema of the antigenic types thus far recognized seems very complicated and the fast growing numbers of types add to the confusion of the average technician. Actually, the pioneer work of White and the meticulous systematic studies of Kauffmann provide the only sure basis for recognition of types encountered frequently in diagnostic laboratories. By taking advantage of the knowledge of the genus *Salmonella* which has been gained through the studies of many workers over a period of years which has culminated in the Kauffmann-White system of classification, it is possible to evolve a simplified method of diagnosis which can be carried on even in the small diagnostic laboratory.

Simplified *Salmonella* diagnosis is by no means new. As early as 1930 Kauffmann (1) proposed a set of 12 OH serums which permitted recognition of the principal types in the genus. Bornstein (2) made a similar proposal in 1942. Kauffmann and Edwards (3) in 1947 described a simple method for the recognition of the most important *Salmonella* types. Other workers have made similar suggestions.

The method described here, with a few exceptions, is similar to that of Kauffmann and Edwards. It is based on the necessity for the exact identification of those *Salmonella* types particularly adapted to man, which are disseminated from man to man, which produce severe enteric fevers, and which tend to become endemic in the population since numerous permanent carriers result from the infections. Among these types are *S. typhi*, *S. paratyphi A*, *S. paratyphi B*, *S. paratyphi C*, and *S. sendai*. It is true that *S. paratyphi A* and *S. paratyphi C* occur only infrequently in the United States and that *S. sendai* has been found only in the Orient. Nevertheless, these types are important human pathogens in various parts of the world, and it is desirable that they be recognized so that they may be dealt with promptly and efficiently when they appear. The importation of *S. paratyphi C* from the Pacific area is already a matter of record. In addition to the types particularly adapted to man, *S. cholerae-suis* should be identified exactly since it so often produces septicemia and localizations in man; and *S. typhi-murium* should be recognized since it occurs more frequently in man than does any other of the numerous types which produce acute gastroenteritis.

Fortunately the great majority of *Salmonella* cultures isolated from man, including the types mentioned above, are members of the first five somatic groups of the Kauffmann-White classification. Edwards and Bruner (4) found that 98.3 percent of 3,019 cultures from man and animals were members of these five groups, and Seligmann, Saphra, and Wassermann (5) obtained an identical figure in 2,916 cultures from man. Bruner and Joyce (6) classified 99.5 percent of 1,007 cultures isolated from man in Italy in these groups. Thus, it is necessary for the average laboratory to have O serums only for groups A through E of the genus.

In the light of these facts the following diagnostic scheme for the average routine laboratory is proposed. A polyvalent serum which at least contains agglutinins for antigens I, II, III, IV, V, VI, VII, VIII, IX, X, XII, XV, XIX and Vi should be available. Such a combination is a minimum requirement for a polyvalent serum. The serum may be prepared from smooth cultures of *S. para-*

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typhi A, S. paratyphi B, S. thompson, S. virginia, S. gallinarum, S. anatum, S. newington, and *S. senftenberg,* and a culture of the Ballerup type which is in the Vi form. Antigens of the eight first-mentioned cultures are prepared by the method described below for the production of O serums and are mixed in equal amounts. Usually six injections of the mixture in amounts of 0.5, 1.0, 2.0, 4.0, 5.0 and 5.0 ml are given to rabbits at intervals of 4 days. To the last four injections, dried organisms of the Vi form of the Ballerup strain should be added just before injection. (The method of preparation of the Vi organisms and amounts administered are described in a subsequent paragraph.) After four injections a trial bleeding may be taken. If it is found that agglutinins for a particular component are lagging the amount of that particular antigen in the mixture may be doubled. The rabbits are bled on the 6th day following the last injection and the serum preserved with 0.25 percent tricresol. The serum is then standardized against all the antigens injected as described below under standardization of O serums. It is used in slide tests in the highest dilution in which it readily will agglutinate all the injected antigens. It also should be tested against a living Vi culture of *S. typhi* to assure the presence of Vi agglutinins.

Six O serums for groups A to E, respectively, should be prepared as follows:

A	I, II, XII	*S. paratyphi A*
B	IV, V, XII	*S. paratyphi B*
C_1	VI, VII	*S. thompson*
C_2	VIII	*S. virginia*
D	IX, XII	*S. gallinarum*
E	III, X, XV	*S. anatum* and *S. newington*

These serums may be prepared from smooth cultures of the indicated types. Twenty-four-hour infusion broth cultures should be heated at 100° C. for 2 hours. After cooling, 0.3 percent formalin is added. These formolized antigens are injected into rabbits in amounts of 0.5, 1.0, 2.0, and 4.0 ml at intervals of 4 days. The rabbits are bled 6 or 7 days following the last injection. If desired a trial bleeding may be taken before the rabbits are exsanguinated. If a sufficient titer has not been obtained an additional injection of 4.0 ml may be given. Experience has shown that additional injections are without effect. Although not necessary, it is desirable to inject two rabbits with each antigen since certain animals fail to develop a satisfactory titer. The antigens should be injected as soon after preparation as convenient and should be refrigerated during the period of immunization. Antigens which have been held for long periods before injection are not as effective as freshly prepared suspensions.

The serums should be tested with homologous antigens by slide agglutination. A convenient method of preparing antigens for slide agglutination is to scrape the organisms from the surface of a slant and suspend them in 1.0 ml of absolute alcohol. After heating the alcoholic suspensions at 60° C. for 1 hour, the tubes are centrifuged, the alcohol decanted, and the organisms suspended in 0.5 ml of carbolized saline. Such antigens, when kept in the refrigerator, can be used for several weeks. The serums to be tested are diluted 1 to 5, 1 to 10, and 1 to 20. A small drop of each dilution is placed on a slide and mixed with a drop of antigen of equal size. The slide is then tilted back and forth and the reactions observed. A satisfactory serum when diluted 1 to 10 should produce pronounced agglutination of alcohol-treated organisms within 30 seconds to 1 minute.

After the rabbits are bled and the serum collected it should be preserved by addition of an equal amount of glycerol. Preservation with glycerol has two advantages: it produces no precipitate in the serum, and it prevents rapid drying of slide tests. Serums refrigerated at 4° C. retain their potency for several years.

After the serums have been preserved they should be standardized. This is accomplished by preparing different dilutions of each serum and determining the highest dilution in which it will give pronounced reactions with the homologous antigen within a given time limit, usually about 30 seconds after tilting of the slide is begun. It should be remembered that alcohol-treated organisms are more resistant to agglutination than are living cultures. Therefore, the serums should be standardized against living antigens if living organisms are to be used in routine tests.

As a rule, cross reactions between the various O groups are not pronounced but certain minimal cross reactions should be expected. If one is careful to observe with which serum an antigen first reacts it is not difficult to determine to what O group an organism belongs. Representative reactions of antigens with the grouping serums are illustrated in table 1.

In addition to the O grouping serums, a Vi serum

should be prepared. Freshly isolated cultures of *S. typhi* do not agglutinate readily with O serum since the reaction is masked by Vi antigen (7). A potent Vi serum can be prepared from the organism described by Kauffmann and Moeller (8) as *S. ballerup*. Vi colonies of this organism are decidedly more opaque than O colonies. Vi colonies should be selected repeatedly until no O colonies appear upon plates. After a pure Vi form is obtained, the are administered. This procedure rarely fails to yield a potent Vi serum which will agglutinate freshly isolated typhoid bacilli in a dilution of 1 to 20 or 1 to 40 in slide tests. Such a serum has a distinct advantage over serums prepared with *S. typhi* since it contains no O or H agglutinins for typhoid bacilli and may be used in the unabsorbed state. It does contain O and H agglutinins for the Ballerup strain but such antigens occur very rarely

Table 1

REACTIONS OF REPRESENTATIVE ANTIGENS WITH O GROUPING SERUMS

Antigens	Serums					
	Group A I, II, XII	Group B IV, V, XII	Group C_1 VI, VII	Group C_2 VIII	Group D IX, XII	Group E III, X, XV
I, II, XII	++	-	-	-	±	-
I, IV, XII	+	++	-	-	±	-
I, IV, V, XII	+	++	-	-	±	-
IV, V, XII	-	++	-	-	±	-
VI, VII	-	-	++	-	-	-
VI, VIII	-	±	±	++	-	-
VIII	-	±	-	++	-	-
VIII, XX	-	±	-	++	-	-
IX, XII	-	±	-	-	++	-
I, IX, XII	+	±	-	-	++	-
III, X	-	-	-	-	-	++
III, XV	-	-	-	-	-	++
I, III, XIX	+	-	-	-	-	++

± to ++ indicates different degrees of agglutination.

growth from the plates is suspended in absolute alcohol, centrifuged, and the alcohol decanted and replaced with fresh alcohol. The culture is suspended in the alcohol, again centrifuged, and the fluid poured off. The organisms are dried in a vacuum desiccator, ground to a fine powder, and stored until needed. The bacteria are suspended in physiological saline just before injection. The amounts administered are the same as those used for the production of O serums. The first injection should be of a density comparable to a 24-hour broth culture; but in succeeding injections the suspension may be increased rapidly in density as well as in amount so that toward the end of the period of immunization quite dense suspensions in enteric organisms. The use of the serum is explained below.

In addition to the polyvalent, O, and Vi serums, it is necessary to prepare six H serums as follows:

a – *S. paratyphi A*
b – *S. paratyphi B*, phase 1 or *S. minnesota*, phase 1
c – *S. cholerae-suis*, phase 1
d – *S. typhi*
i – *S. typhi-murium*, phase 1
1, 2, 3, 5 – *S thompson*, phase 2, and *S. newport*, phase 2

All the above-named organisms occur in monophasic form so that one need not isolate the desired

phases. In order to prepare the serums the organisms are passed through two or three successive tubes of semisolid agar to insure maximum motility. They are then inoculated into infusion broth, incubated overnight, and an equal amount of physiological saline solution containing 0.6 percent formalin is added to the cultures. The formalinized suspensions are injected into rabbits in the same manner and amounts as are the antigens used to produce O serums. The rabbits are bled on the 6th day following the last injection and the serums are preserved with glycerine. It is unnecessary to take trial bleedings because satisfactory titers are almost invariably obtained. The serums should be titrated against the homologous antigens. A titer of 5,000 or more is adequate.

EXAMINATION OF CULTURES

It is usually convenient to examine cultures which give reactions typical of *Salmonella* on triple-sugar-iron-agar (TSI) slants and which produce no alkalinization of Christensen's urea agar. The growth from the TSI slants may be used to make a dense saline suspension. This suspension should be tested on a slide with polyvalent serum. In performing slide agglutination tests it is advisable to use amounts of antigen and serum approximately equal to a 3 mm loopful. If larger drops are used nonspecific reactions are more likely to appear. Also, in the use of the O grouping serums, the use of large drops emphasizes cross reactions between groups. Suspensions which are agglutinated by polyvalent serum should then be tested with the O grouping serums.

Any culture which is agglutinated in a typical manner by polyvalent serum and by one or more of the O grouping serums may be reported as a probable *Salmonella* provided preliminary biochemical examination indicates that it is a member of the genus. Results should be confirmed by extended biochemical tests since many paracolon bacteria contain antigens related to the O antigens of *Salmonella*.

If a culture reacts with polyvalent serum but not with the O grouping serums, it should be tested with Vi serum since most freshly isolated strains of *S. typhi* are not agglutinated by O serum for group D, to which they belong. If the culture reacts with Vi serum a portion of the suspension should be heated in a boiling water bath for 10 minutes, and after cooling, again should be tested with the O serums and with Vi serum. After boiling, *S. typhi* cultures should react with group D serum; and *S. paratyphi C* cultures, which also may contain Vi antigen, should react with group C_1 serum. From a practical standpoint, cultures which react with Vi serum, and which, after heating, react with group D serum may be reported as *S. typhi*, although in order to complete their identification the H antigens should be identified and biochemical tests performed. Rarely rough cultures of *S. typhi* are isolated which, after boiling, agglutinate neither in Vi or O serums. Such cultures also may be nonmotile. They are identifiable only by their content of Vi antigen and by their biochemical characteristics.

Cultures which continue to agglutinate in Vi serum after heating and which are not agglutinated by the O grouping serums may be presumed to contain O antigens related to those of the Ballerup strain. Such cultures now are classified as intermediate paracolon bacteria.

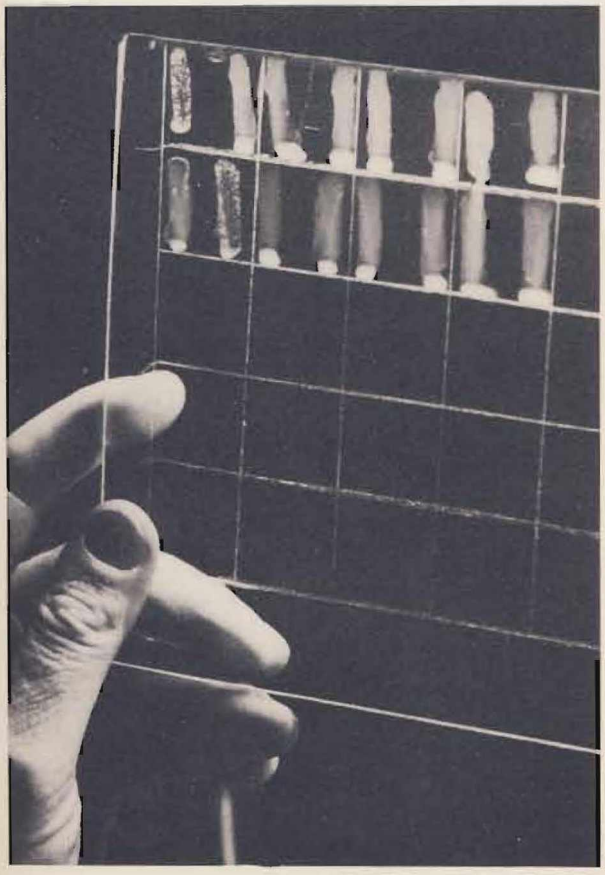
After the O group of the culture is determined its H antigens should be examined. This may be done by testing growth from an agar slant on a slide with H sera which are diluted 1 to 50 or 1 to 100. An alternate method is that recommended in 1942 by Edwards and Bruner (10), in which an infusion broth culture is diluted with an equal amount of saline containing 0.6 percent formalin and tested with appropriate sera at 50° C. Only one dilution (1 to 1,000) is used and results are read after incubation for 1 hour in a water bath.

The group A cultures should be tested with serum a to assure that they are *S. paratyphi A*, the only known type in that group. In addition, they should be subjected to differential biochemical tests such as nonfermentation of xylose, and inability to utilize tartrates and citrate. The majority of *S. paratyphi A* strains produce little or no H_2S.

Group B cultures should be tested with H serums for antigens b, i, and 1,2,3,5. In the event that agglutination occurs in b or i serum, the culture is almost certainly *S. paratyphi B* or *S. typhi-murium*, respectively. Diphasic cultures often occur only in one phase when recently isolated. If phase 2 only is present, it is necessary that phase 1 be obtained to identify the culture. Thus, if the culture is agglutinated only by 1,2,3,5 serum, it should be planted on a Gard plate (9) with 1,2,3,5 serum or placed in a tube containing 2 or 3 ml of semisolid medium to which a loopful of that serum has been added. Phase 1 will migrate through medium and can be isolated from the spreading growth.

Useful modifications of the Gard technique have been described by Edwards and Bruner (10), Hajna (11), and Juenker (12). After phase 1 is obtained it can be determined whether the culture is *S. paratyphi B*, *S. typhi-murium*, or some other type within group B.

Group C cultures should be tested with c and 1, 2, 3, 5 serums. If agglutination occurs in c serum, the culture may be identified as *S. paratyphi C* or *S. cholerae-suis* depending upon its biochemical reactions. *S. paratyphi C* usually ferments arabinose and always ferments trehalose promptly, whereas *S. cholerae-suis* attacks neither of these sugars. *S. paratyphi C* ferments dulcitol promptly while *S. cholerae-suis* gives delayed or negative reactions in dulcitol mediums. If phase 2 only is present, phase 1 should be isolated as previously described and tested with c serum. It should be remembered that the majority of cultures of *S. cholerae-suis* belong to the Kunzendorf variety in which phase 1 is more or less completely suppressed; so any group C culture which agglutinates in 1, 2, 3, 5 serum, fails to ferment arabinose and trehalose, and gives a negative or delayed result in dulcitol may be reported as *S. cholerae-suis*.



Reactions of group A and group B *Salmonella* cultures with grouping serums.

Group D cultures should be tested in a, d, and 1, 2, 3, 5 serums. Cultures which agglutinate only in d serum, which are anaerogenic, and which possess the usual biochemical attributes of *S. typhi* may be reported immediately. The organism will have been examined already for the presence of Vi antigen so that its identity is fairly well established. Some recently isolated cultures of *S. typhi* are very poorly motile and may fail to flocculate in d serum. Such cultures can be typed as *S. typhi* provided they possess IX, XII, and Vi antigens and the biochemical properties of *S. typhi*.

Identification of *S. sendai* depends upon the recognition of both phases and biochemical examination. It is necessary that the organism agglutinate both in a and 1, 2, 3, 5 serums. If only one phase is present the second must be isolated as previously described. Further, this type is H_2S and citrate negative and gives a negative result in Stern's glycerol-fuchsin broth. Arabinose is fermented immediately but xylose and sorbitol give delayed reactions. Cultures with similar antigens but different biochemical reactions belong to another type. *S. sendai* occurs frequently only in the Orient.

Cultures which belong to group E are not examined further but are reported simply as group E *Salmonella*.

By adoption of the system outlined above, or a similar one, the diagnosis of *Salmonella* infections in the routine laboratory should be greatly improved. The small laboratory should not attempt the exact typing of all *Salmonella* cultures but should identify only the more important types. The remaining cultures should be designated "*Salmonella*, type undetermined" and sent to a center for identification. The adoption of such a system permits the rapid diagnosis of 98 to 99 percent of *Salmonella* infections. At present many laboratories continue to rely on ordinary agglutination tests in which O and H reactions are not distinguished and upon biochemical reactions. Reports on enteric cultures therefore are often long delayed and inaccurate.

The system proposed above is flexible and may be modified to suit the needs of the individual laboratory. Further, as workers gain familiarity with the method it is likely that they will wish to extend the number of types which they identify. This may be done by increasing gradually the

number of H serums to the point that all the types which occur frequently may be recognized. Any proposal short of complete typing must be a compromise and each laboratory must decide to what length they wish to go in the identification of *Salmonella* cultures. The methods proposed here are based upon experience, with a view toward the employment of a minimal number of serums.

SUMMARY

A simplified method for the serological grouping of *Salmonella* is described. Methods for the exact identification of the principal human pathogens of the genus are included. Methods for the preparation of the necessary serums, for the preparation of antigens, and for the performance of the tests are given in some detail.

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