SIMPLIFIED METHODS FOR THE SEROLOGICAL IDENTIFICATION OF SHIGELLA CULTURES

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A number of new *Shigella* types were added to the genus during recent years so that a relatively large number of microorganisms are now classified in the genus *Shigella* (6). The purpose of this paper is to outline serological methods by which those shigellae that are most common in the United States may be grouped and typed. The needs of many laboratories may be fulfilled by simply identifying a culture as a member of one of the groups, reporting it as such, and then forwarding the culture to a laboratory that is equipped to complete the typing. Other laboratories may desire to carry the identification beyond the grouping stage and to determine the type of the microorganisms. Methods designed to meet the requirements of both kinds of laboratories are given herein. The methods are based in part upon the work of Boyd (3,4), Wheeler (21,22), and upon the writer's studies.

A definition and the essential biochemical reactions of members of the genus *Shigella* are given by Ewing and Edwards (5) and need not be discussed here. However, it should be emphasized that cultures that are grouped or typed should be confirmed as members of the genus *Shigella* by the performance of biochemical tests.

Group A of the *Shigella* schema (fig. 1) is composed of those shigellae which characteristically do not form acid from mannitol. These are *Shigella dysenteriae* I (Shiga), *Shigella dysenteriae* II *(Shigella ambigua* or *schmitzii),* and the microorganisms described by Large and Sankaran in 1934 (14) *(Shigella dysenteriae,* types III to VII*).*

The microorganisms formerly called *Shigella paradysenteriae,* Flexner and now referred to as *Shigella flexneri,* types I to VI, make up group B. With a few exceptions, cultures belonging to this group ferment mannitol within 24 hours. Certain biotypes of *Shigella flexneri* VI do not utilize this substrate. Also, mannitol negative variants of *Shigella flexneri* IV may occur. Nelson (15) described a small outbreak caused by such a variant. The microorganisms described under the names of *Shigella rabaulensis* and of *Shigella rio* (20) appear to be varieties of *S. flexneri* IV that do not utilize mannitol.

The members of group C of the *Shigella* schema are similar to *S. flexneri* cultures as regards their cultural and biochemical characteristics but described types bear little serological relationship to members of group B. However, intragroup relationships among group C cultures are known (23,24).

Group D is composed of those shigellae which utilize lactose after continued incubation. These are *Shigella sonnei* and *Shigella dispar.*

The final group of the schema is made up of *Shigella alkalescens,* the commonly occuring forms of which were described by Andrewes (1) and by Stuart *et al.,* (19). Coliform and paracolon cultures that are serologically identical to *S. alkalescens* cultures were described by Stuart *et al.* (19) and Wheeler *et al.* (23).

S. alkalescens and *S. dispar* are retained in the genus *Shigella* for the purpose of the present discussion. However, it is probable that they will be removed from the genus and placed with the *Escherichia* because of biochemical and serological relationships to members of the coli group (7,9,12).

Cultures which appear to be shigellae are tested first in polyvalent or grouping serums to determine the group to which they belong. Six such antiserums are needed for grouping purposes. These are groups A, B, C, *S. sonnei* mixed, *S. dispar* mixed, and *S. alkalescens.*

The preparation and use of polyvalent *Shigella* antiserums is described by Ewing (8). Polyvalent A is prepared by the injection of two or more rabbits with a mixed vaccine made by pooling 20-hour broth cultures of each of the seven *S. dysenteriae* types. Polyvalent antiserums for group B and for group C are prepared in a similar manner, using mixed vaccines. All six of the *S. flexneri*

types are included in the pooled vaccine for group B because agglutinins for the specific antigen of each type should be present in the serum. For the preparation of a mixed *S. sonnei* antiserum broth cultures of form I (smooth) and form II ("rough") are pooled to make the vaccine. Mixed *S. dispar* serum is prepared in a similar way by mixing young broth cultures of types I and II. An antiserum for *S. alkalescens* is included with the grouping serums because of the high incidence of this type. Broth cultures to be used for the preparation of antiserums for *S. dispar* and *S. alkalescens* should

Figure 1.
THE GENUS SHIGELLA
Dysentery and Related Bacteria

Proposed Designation	Other Designations					
Group A. *Shigella dysenteriae,*	I	*Shigella dysenteriae* (Shiga-Kruse bacillus) *Bacterium shigae.*				
	II	*Shigella schmitzii, Schigella ambigua, Bacterium ambiguus,* etc.				
	III	Q 771 of Large-Sachs group, Type 8524 Gober, *et al. Shigella arabinotarda A.* Christensen and Gowan.				
	IV	Q 1167 of Large-Sachs group; *Shigella arabinotarda B.*				
	V	Q 1030				
	VI	Q 454				
	VII	Q 902				
Group B. *Shigella flexneri,*		**Kauffmann & Ferguson**	**Boyd-Wheeler**	**Andrewes & Inman**	**Weil**	**Other**
	I	1a	F.I	V	F.I	
	I	1b	F.I	VZ	F.I.III	
	II	2a	F.IIa	W	F.II	
	II	2b	F.IIb	WX	F.II,VII	
				X	F.VII	
				Y	F.VIII	
	III	3	F.III	Z	F.III	
	IV	4a	F.IV		F.IV	Boyd 103
	IV	4b	F.IV		F.III,IV	
	V	5	F.V		F.V.	Boyd P.119
	VI	6	F.VI		F.VI	Boyd 88, Newcastle and Manchester bacilli, *Shigella newcastle*
Group C. *Shigella boydii,*	I		B.I		F.IX	Boyd 170
	II		B.II		F.X	Boyd P.288
	III		B.III		F.XI	Boyd D.1
	IV		B.IV		F.XIV	Boyd P.274
	V		B.V		F.XIII	Boyd P.143
	VI		B.VI		F.XII	Boyd D.19
	VII					"Lavington," Type T, *Shigella etousae*
Group D. *Shigella sonnei* *Shigella dispar*		Sonne - Duval bacillus, Kruse - E - Ruhr, *Shigella ceylonensis A*				
	I	Serotype I, Carpenter & Stuart; *Shigella madampensis* (Castellani)				
	II	Serotype II, Carpenter & Stuart; *Shigella ceylonensis B* (Castellani)				
Group E. *Shigella alkalescens*		Andrewes, Stuart *et al.*, Type I, DeAssis.				

B. - Boyd; F. - Flexner

From Ewing, Bact., 57: 633-638 (modified) (1949).

be heated at 100° C. for 2½ hours to destroy L antigens which may cause cross reactions with coliform cultures that contain related antigens (10,12). All newly prepared serums should be tested with a living culture of a bacterium known to contain alpha antigen (17,11). If alpha agglutinins are present, the antiserum should be absorbed with the alpha antigen culture.

While it is possible to use the grouping serums in the unabsorbed state if cognizance is taken only of rapid and complete agglutination, it usually is desirable to free them of intergroup reactions by absorption with appropriate microorganisms. *S. alkalescens* cultures may react in A antiserum because of a relationship of this type to *S. dysenteriae* I. Also, *S. alkalescens* reacts in B and C antiserums. The agglutination in B serum is brought about by minor relationships to certain *S. flexneri* types and the reaction in polyvalent C is caused by the relationship of *S. alkalescens* to *S. boydii* I and IV. Further, *S. dispar* I and II cultures are agglutinated by B and C grouping serums. Certain *S. flexneri* types, particularly *S. flexneri* IV and the "X" and "Y" varieties, react slightly in C antiserum. *S. sonnei* II cultures are agglutinated by antiserum C because of the relationship of that type to *S. boydii* VI. See table 1 for method of absorption of polyvalent serums.

Mixed *S. sonnei*, mixed *S. dispar*, and *S. alkalescens* antiserums are employed in the unabsorbed condition.

The following technic of agglutinin absorption may be used for the preparation of absorbed polyvalent antiserums and for absorption of specific antiserums. Smooth cultures of the microorganisms to be employed for absorption are inoculated into infusion broth. After incubation for 15 to 18 hours at 37° C., the broth cultures are used to seed infusion agar plates. Standard 90 mm petri dishes containing 20-25 ml of infusion agar are employed. Each plate is seeded with 0.3 to 0.4 ml of broth culture and the inoculum is spread over the entire surface of the agar. Such plates are incubated in an upright position for 18 to 20 hours at 37° C. The growth is removed with formalinized (0.3 percent of 40 percent formaldehyde solution) physiological saline solution. If the suspension is to be boiled, plain physiological salt solution should be used. The microorganisms are sedimented by centrifugation and the supernatant fluid discarded. Diluted antiserum is added to the packed bacteria and the cells are resuspended. Antiserums to be absorbed are diluted 1:5 or 1:10 and added to an absorptive dose calculated to be in excess of that required. The mixtures are incubated at 37° C. for 6 hours and placed in an icebox (about 4° C.) overnight. They are then centrifuged and the antiserum removed. In most cases, the number of plates indicated in the tables effectively removes the heterologous agglutinins from 1.0 of antiserum. There are exceptions, of course, in which antiserums must be reabsorbed by additional bacteria to remove all of the heterologous agglutinins.

If a culture suspected of being a *Shigella* type is not agglutinated by any of the six grouping serums in slide tests, a suspension made with plain saline solution should be heated for ½ hour, cooled, and retested. Some shigellae, particularly *S. alkalescens* cultures, contain thermolabile antigens that inhibit O agglutination (2,12,16).

The use of the grouping antiserums described above and the biochemical criteria given by Ewing and Edwards (5) will enable laboratory personnel to determine the group to which an unknown *Shigella* type belongs and to confirm it as a member of the genus *Shigella*. The needs of many laboratories will be fulfilled by this procedure. Such cultures, together with information as to source, clinical diagnosis, age and sex, should be forwarded to a laboratory equipped for typing of

Reaction of group B *Shigella* cultures in slide agglutination tests with polyvalent serums.

shigellae, *e.g.*, a State health department laboratory.

Those laboratories which desire to make specific identification of cultures belonging to the various groups should have type specific antiserums for this purpose. Such antiserums may be prepared in the same way as that mentioned for grouping serums except that single types are employed as vaccines.

The incidence in the United States of *Shigella* types belonging to group A is not high. In most cases antiserums for *S. dysenteriae* I, II, and III are all that are needed for this group. Newly prepared antiserum for *S. dysenteriae* I should be tested with a boiled suspension of *S. alkalescens* to determine the amount of cross reaction. If the titer for *S. alkalescens* is in the magnitude of 1:320 or higher, the antiserum should be absorbed to remove the heterologous agglutinin. Antiserums for *S. dysenteriae* II and III are usually specific and may be employed in the unabsorbed state in slide tests.

The shigellae of group B are common in the United States and if typing is to be done, it is advisable to have antiserums for all of the six types. Of the six, *S. flexneri* II, III and VI appear to occur most frequently. Since there are extensive intragroup relationships among *S. flexneri* cultures it is necessary to use absorbed type specific serums to identify them. The one exception of this is in the case of *S. flexneri* VI. By using a freshly isolated culture of this type it is sometimes possible to obtain an antiserum which may be used in a dilution of 1:10 or 1:20 without absorption.

Each *S. flexneri* type contains a specific or major antigen and a number of common group or minor factors. It is possible to absorb antiserums in such a way as to remove all of the group agglutinins and thus render the serum specific. The manner in which *S. flexneri* serums may be treated to render them specific is shown in table 2.

At present, group C of the *Shigella* schema includes seven types (fig. 1). With the exception of *Shigella boydii* VII, these were isolated originally in India by Boyd (3,4) and his collaborators. *S. boydii* types since have been recognized in various parts of the world and most of the types have been found in the United States. *S. boydii* VII was isolated originally in North Africa in 1943 by Stock *et al.* (18). This serotype was found later in Italy, England, and France. As yet it is unreported in the United States.

Some *S. boydii* types may be identified by the use of unabsorbed serums in slide tests. When an antiserum for *S. boydii* I is prepared, it should be tested with a suspension of *S. alkalescens* and if the titer of the latter is 1:320 or more, the serum should be absorbed with *S. alkalescens*. Unabsorbed *Shigella boydii* II and III serums may be employed in slide tests if trials indicate little or no cross reactions with other shigellae. It usually is necessary to absorb *S. boydii* IV antiserum with a culture of *S. alkalescens* because these microorganisms cross agglutinate to considerable degree. The reciprocal of this absorption is necessary also, that is, *S. alkalescens* serum should be absorbed by *S. boydii* IV.

Of the members of group C, *S. boydii* I, II, and IV appear to be more common in the United States. It is probable that most laboratories will find that antiserums for these three types will meet their requirements. The manner in which *S. boydii* and *S. alkalescens* antiserums should be absorbed in order to render them specific is given in table 3. If *S. boydii* V and VI serums are employed, they should be absorbed as indicated in table 3.

S. sonnei is one of the most common *Shigella* types isolated in the United States, and in other

Table 1

ABSORPTION OF POLYVALENT ANTISERUMS

Serum	Absorbing Cultures	Number of Plates
Polyvalent A (*S. dysenteriae* I-VII) 1.0 ml	*S. alkalescens*	Growth from 10 plates
Polyvalent B (*S. flexneri* I-VI) 1.0 ml	*S. alkalescens* *S. dispar* I *S. dispar* II	Growth from 10 plates do do
Polyvalent C (*S. boydii* I-VII)	*S. alkalescens* *S. dispar* I *S. dispar* II *S. sonnei* II	Growth from 10 plates do do do

Table 2

PREPARATION OF SPECIFIC S. FLEXNERI ANTISERUMS

Antiserum 1.0 ml.	Absorbing culture(s)	Number of plates	Specific factor
I	III	10	
	IV	10	I
	V	5	
	VI	10	
II	I	10	II
	"X" variant	10	
	"Y" variant	10	
III	I	10	III
	II	10	
	IV	5	
	V	5	
IV	I	10	IV
	II	10	
	III	10	
V	I	10	V
	"X" variant	5	
	III	10	
VI	I	10	VI

parts of the world, as well. This type exists in two "phases," I and II (24). The phases are sometimes referred to as smooth and rough but this terminology is inaccurate and confusing, for in addition to forms I and II there exists a true rough variant. We prefer to designate these variants as form I, form II, and R(ough) rather than as phases. The term phase should be reserved for variation in the flagellar antigens of Enterobacteriaceae. Transitional forms between the three variants often are encountered and it is not uncommon to find cultures which are mixtures of forms I and II. Such cultures react in antiserum for form I, form II, and *S. boydii* VI. Pure form I and form II cultures cross-react very little or not at all, but pure form I and form II antiserums are difficult to obtain so that it usually is necessary to cross absorb the antiserums if one wishes to separate the two forms by means of slide tests. As indicated in table 3, *S. boydii* VI antiserum should be absorbed with a form II *S. sonnei* culture before it is utilized for diagnostic purposes, because the two types cross-react to considerable degree. Such an absorption leaves the specific agglutinins in *S. boydii* VI antiserum but the absorption of form II *S. sonnei* antiserum by *S. boydii* VI results in the removal of all agglutinins from the antiserum. Thus, it appears that form II *S. sonnei* contains the group antigens of *S. boydii* VI.

S. dispar I and *S. dispar* II antiserums should be cross-absorbed if specific typing is desired. While these microorganisms occur relatively frequently, it is likely that a mixed antiserum which agglutinates both I and II will meet the needs of many laboratories. Serotype II of *S. dispar* appears to be more common than type I.

If laboratories, such as those of the level of State health department laboratories, have specific antiserums for the first three members of group A (fig. 1), the six members of group B, *S. boydii* I, II and IV (of group C), *S. sonnei*, *S. dispar*, and *S. alkalescens*, most *Shigella* types found in the

Table 3

PREPARATION OF SPECIFIC S. BOYDII AND S. ALKALESCENS ANTISERUMS

Antiserum 1.0 ml.	Absorbing culture	Number of plates per ml
S. boydii I	*S. alkalescens*	5
IV	*S. alkalescens*	10
V	*S. dispar* I and II	5 (of each)
VI	*S. sonnei*, Form II	10
S. alkalescens	*S. boydii* IV	10

United States may be identified. Cultures belonging to other types and microorganisms that appear to conform to the description of the genus *Shigella* but fail to type, may be forwarded to a laboratory especially equipped to identify them.

REFERENCES

(1) Andrewes, F. W. Dysentery bacilli: the differentiation of the true dysentery bacilli from allied species. Lancet I. 560-563 (1918).

(2) Archer, G. T. L. Factor inhibiting agglutination of *Bact. alkalescens* by homologous serum. J. Roy. Army M. Corps. 79: 109-117 (1942).

(3) Boyd, J. S. K. The antigenic structure of the mannitol fermenting group of dysentery bacilli. J. Hyg. 38: 477-499. (1938).

(4) Boyd, J. S. K. The laboratory diagnosis of bacillary dysentery. Tr. Roy. Soc. Trop. Med. & Hyg. 33: 553-571. (1940).

(5) Ewing, W. H. and Edwards, P. R. Selection of *Salmonella* and *Shigella* cultures for serological classification. CDC Bulletin IX: (5) 1-8 (1950).

(6) Ewing, W. H. *Shigella* nomenclature. J. Bact. 57: 633-638 (1949).

(7) Ewing, W. H. The relationship of *Shigella dispar* to certain coliform bacteria. J. Bact. 58: 497-500 (1949).

(8) Ewing, W. H. *Shigella* grouping antiserums. In press (1950).

(9) Frantzen, E. Biochemical and serological studies on *alkalescens* and *dispar* strains. Acta Path. et microbiol. Scandinav., in press (1950).

(10) Kauffmann, F. The serology of the coli group. J. Immunol. 57: 71-100 (1947).

(11) Kauffmann, F. Studies on the alpha antigen of Stamp and Stone. Acta path. et microbiol. Scandinav. 24: 582-587 (1947).

(12) Kauffmann, F. On the classification of the Enterobacteriaceae. Acta path. et microbiol. Scandinav. 26: 879-881 (1949).

(13) Kauffmann, F. and Ferguson, W. W. Memorandum on the Flexner group. Acta path. et microbiol. Scandinav. 25: 620-622 (1948).

(14) Large, D. T. M. and Sankaran, O. K. Dysentery among troops at Quetta. The non-mannite fermenting group of organisms. J. Roy. Army M. Corps. 63: 231-237 (1934).

(15) Nelson, M. G. Non-mannitol-fermenting *Shigella flexneri*, type 103. J. Path. & Bact. 59: 316-318 (1947).

(16) Schuetze, H. The agglutinability of *Bact. shigae*. J. Path. & Bact. 51: 317-325 (1944).

(17) Stamp, Lord and Stone, D. M. An agglutinogen common to certain strains of lactose and non-lactose-fermenting coliform bacilli. J. Hyg. 43: 266-272 (1944).

(18) Stock, A. H., Eisenstadt, I., Triplett, G. W., and Catto, A. Field studies in bacillary dysentery in U. S. military personnel and civilians in North Africa and Italy. J. Infect. Dis. 81: 56-75 (1947).

(19) Stuart, C. A., Rustigian, R., Zimmerman, A., and Corrigan, F. V. Pathogenicity, antigenic relationships, and evolutionary trends of *Shigella alkalescens*. J. Immunol. 47: 425-437 (1943).

(20) Weil, A. J. and Slafkovsky, H. *Shigella rio*, a new type of *Shigella*. J. Immunol. 58: 23-26 (1948).

(21) Wheeler, K. M. Antigenic relationships of *Shigella paradysenteriae*. J. Immunol. 48: 87-101 (1944).

(22) Wheeler, K. M. Serological identification of dysentery bacilli. Am. J. Pub. Health. 34: 621-629 (1944).

(23) Wheeler, K. M., Stuart, C. A., and Ewing, W. H. The antigenic complex of *Shigella paradysenteriae*, Boyd type P. 274. J. Bact. 51: 169-176 (1946).

(24) Wheeler, K. M. and Mickle, F. L. Antigens of *Shigella sonnei*. J. Immunol. 51: 257-267 (1946).