SEROLOGICAL IDENTIFICATION OF *SHIGELLA FLEXNERI* BY MEANS OF FLUORESCENT ANTIBODY

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A minimum of 24 to 72 hr is required for even well equipped laboratories to identify the etiological agents of enteric disease by present methods. The fluorescent antibody technique described by Coons and Kaplan (1950) has been applied in the rapid identification of microorganisms (Thomason *et al.*, 1956, 1958; Whitaker *et al.*, 1958; Moody *et al.*, 1958).

This communication gives the results of our studies in which we used the fluorescent antibody technique to serotype various strains of *Shigella flexneri*. The first portion compares the specificity of the staining procedure with that of the slide agglutination test while the latter part summarizes our preliminary attempts to identify *S. flexneri* in the feces of guinea pigs experimentally infected by the method of Formal *et al.*, 1958*a*, *b*.

MATERIALS AND METHODS

Most of the strains of Shigellae used in this study were from the culture collection of the Walter Reed Army Institute of Research; an additional few were isolated in 1954 and 1955 from cases of bacillary dysentery in Mexico and Japan.

Preparation of antisera. All anti-Shigella sera were prepared by intravenous immunization of rabbits with living cultures of the appropriate strains. Portions of the *S. flexneri* antisera were rendered type-specific by standard adsorption procedures. Sheep anti-rabbit globulin sera were obtained from Dr. Robert Feinberg.

Conjugation with fluorescein dyes. Globulin fractions were obtained from all sera except the adsorbed type-specific reagents by 50 per cent saturation with ammonium sulfate and subsequent dialysis. These immune preparations were conjugated with either fluorescein isocyanate employing the method of Coons and Kaplan (1950), or with fluorescein isothiocyanate as described by Riggs *et al.* (1958) and modified by Marshall *et al.* (1958). Generally, antisera labeled by the latter method were more satisfactory.

Preparation of smears and staining methods. All cultures were grown on nutrient agar (Difco) supplemented with 0.3 per cent beef extract (BBL) and hereafter designated meat extract agar. The stock cultures were streaked on meat extract agar plates and incubated for 16 to 18 hr at 37 C. Smooth translucent colonies were suspended in saline, smeared on individual slides, air-dried, and fixed. There were no differences in the staining quality of the cells whether fixed by methanol, ethanol, or heat, but in the case of fixation by heat, an increase in background fluorescence was noted in a number of instances probably due to extractable soluble antigenic material fixed to the slides. Absolute methanol was selected for routine use.

The fixed smears were layered with fluorescein-labeled antiserum, placed in a moist chamber for 20 min, and then washed in Coplin jars with several changes of buffered saline (0.01 M phosphate buffer, pH 7.0 to 7.2). Cover slips were mounted with buffered glycerol. Controls consisted of smears stained with fluorescein-labeled normal rabbit serum globulin and unstained preparations. The specificity of the labeled antisera was also established by demonstrating that staining reactivity was lost after the antiserum was adsorbed with the homologous Shigella serotype. Further, the specificity of all the antisera was tested by inhibition tests with unlabeled homologous antisera (Goldman, 1957).

The slides were viewed with a Leitz Ortholux microscope fitted with a darkfield condenser; the ultraviolet light source was a Phillips CS 150 W high pressure mercury arc lamp. The exciter filter used was a blue primary vitamin B_2 assay filter (Photovolt Corporation, New York, New York) and the barrier filter was the yellow secondary filter used in the vitamin B_2 assay. The degree of fluorescence was graded by using

neutral density (N.D.) filters (Corning) placed between the exciter filter and the object stage according to the following scale:

± = Visible but not through a 0.3 N.D. filter,
+ = visible through a 0.3 N.D. filter,
2+ = visible through a 0.6 N.D. filter,
3+ = visible through a 0.9 N.D. filter, and
4+ = visible through a 1.2 N.D. filter.

For photographic purposes a Corning no. 5113 blue filter (standard thickness) was substituted as the exciter filter. All photomicrographs were taken with 35 mm Tri-X black and white film with exposure times of 2 to 3 min.

RESULTS

Except where noted, each fluorescein-labeled antiserum or globulin fraction employed in this work was maximally diluted to the point where it still strongly stained its homologous antigen. This was done to reduce as much as possible the many undesirable cross reactions which were anticipated.

Initial efforts were designed to test the specificity of the staining reactions of labeled Shigella grouping sera with the various *S. flexneri* serotypes. The results, summarized in table 1, show that a high degree of specificity was achieved using this procedure, since significant staining of strains of *S. flexneri* was observed only with the group B serum. There was a good correlation between the results obtained by the staining technique and by the slide agglutination method.

Tests were then carried out to determine the degree of cross-staining reactions between various *S. flexneri* serotypes and their labeled unadsorbed antisera. As noted above, these sera were used at a dilution where they still strongly stained their homologous strain. At the same time that the staining was carried out, slide agglutination tests were also performed. The results of these studies are summarized in table 2. It can be seen that a large number of cross-staining reactions occurred even though the sera were diluted. In general the cross-staining reactions appeared to be more intense when group factor antigens 3, 4, or 6 were involved than when factors 7, 8, or 9 were the common reactants. It was noted in a number of instances that a given antiserum agglutinated a particular culture but did not stain it. This apparent difference in sensitivity between the two procedures was borne out by further tests which showed that tube cross-agglutination titers

TABLE 1
Fluorescent staining and slide agglutination reactions of Shigella flexneri serotypes with Shigella grouping sera

S. flexneri		Labeled Antisera against Shigella Groups*									
Serotype	Strain	A		B		C		D		NSG†	
		Agg	Stain	Agg	Stain	Agg	Stain	Agg	Stain	Agg	Stain
1a	1-3	−	−	++	3	−	−	−	−	−	−
1b	1-9	−	−	+	4	−	−	−	−	−	−
2a	2457T	+	−	++	3	−	−	−	±	−	−
2b	16B	−	−	++	2	−	−	−	±	−	−
3	3-5	−	−	++	3	−	−	−	−	−	−
4a	4-10	−	−	++	3	−	−	−	−	−	−
4b	G-2	−	−	++	3	−	−	−	−	−	−
5	M90A	−	−	++	3	−	−	−	−	−	−
6	6-1	−	−	++	4	−	−	−	−	−	−
"X" variant	D-1	−	−	++	3	−	−	−	−	−	−
"Y" variant	F-2	−	−	++	4	−	−	−	−	−	−

* Fluorescein-labeled globulin fractions of polyvalent grouping sera; it should be noted that sera against groups A, C, and D yielded strong staining reactions with their various homologous antigens.
++ = Complete agglutination—large floccules; + = partial agglutination, ± = little agglutination usually appearing after 4 to 5 min, − = no visible agglutination.
† NSG = Fluorescein-labeled normal rabbit serum globulin; Agg = agglutination.

TABLE 2

Fluorescent staining and slide agglutination reactions of some Shigella flexneri serotypes with unadsorbed labeled S. flexneri antisera

S. flexneri			Fluorescein-Labeled *S. flexneri* Antisera															
			1b		2a		2b		3		4a		5		6		NSG*	
Serotype	Strain	Antigenic Schema	Agg	Stain	Agg	Stain	Agg	Stain	Agg	Stain	Agg	Stain	Agg	Stain	Agg	Stain	Agg	Stain
1a	1-3	I-4, 9	+†	3‡	++	1	++	–	++	–	++	2	–	–	+	–	–	–
1b	1-9	I-4, 6, 9	+	4	++	2	–	–	++	2	±	1	±	–	±	–	–	–
2a	2457T	II-3, 4	–	–	+	4	++	2	–	–	–	1	–	–	±	±	–	–
2b	16B	II-7, 8, 9	–	–	+	3	+	3	–	–	–	±	–	–	–	–	–	–
3	3–5	III-6, 7, 8, 9	++	2	+	–	±	–	++	3	+	–	+	–	–	–	–	–
4a	4-10	IV-4	–	–	++	2	–	–	+	–	++	4	+	1	–	–	–	–
4b	G-2	IV-6	++	3	–	–	+	–	++	2	+	3	–	–	–	–	±	–
5	M90A	V-7, 8, 9	±	–	+	–	+	–	±	–	++	2	++	3	–	–	–	–
6	6-1	VI--	–	–	–	–	–	–	–	–	–	–	–	–	+	3	–	–
"X" variant	D-1	--7, 8, 9	++	±	++	1	++	2	++	1	+	1	+	1	+	–	–	–
"Y" variant	F-2	--4	++	1	++	3	+	1	++	1	++	2	++	2	++	±	±	–

* NSG = Fluorescein-labeled normal rabbit serum globulin; Agg = agglutination.

† See table 1.

‡ See Materials and Methods.

of at least 1:160 were required to produce good cross-staining reactions. These latter results are similar to those of Thomason *et al.* (1957).

Studies were then conducted to determine whether the cross-staining reactions noted above could be removed by the usual agglutinin adsorption procedures which render the sera type-specific. For this work, undiluted antisera were employed. The results of these experiments using sera which had been adsorbed to render them type-specific (by slide agglutination tests) are given in table 3. As can be seen, the staining reactions were also type-specific; however, they were not as intense as those noted above where unadsorbed sera were used.

To obtain the results summarized in tables 1 and 3, it was necessary to have 4 fluorescein-labeled Shigella grouping sera and 6 similarly tagged but adsorbed type-specific *S. flexneri* antisera. It would seem possible to achieve these same results with only a single fluorescein-labeled antiserum by employing the indirect technique of staining described by Weller and Coons (1954). However, our efforts in this direction were not successful because the two fluorescein-labeled sheep anti-rabbit globulin sera available for this work contained "normal" antibodies against various Shigellae. Thus, when these reagents were

TABLE 3

Reactions of some Shigella flexneri serotypes with type-specific fluorescein-labeled S. flexneri antisera

S. flexneri		Fluorescein-Labeled Type-Specific *S. flexneri* Antisera						
Serotype	Strain	1	2	3	4	5	6	NSG*
1a	1-3	2†	–	–	–	–	–	–
1b	1-9	2	–	–	–	–	–	–
2a	2457T	–	2w‡	–	–	–	–	–
2b	16B	–	2w	–	–	–	–	–
3	3-5	–	–	2	–	–	–	–
4a	4-10	–	–	–	2	–	–	–
4b	G-2	–	–	–	3	–	–	–
5	M90A	–	–	–	–	3	–	–
6	6-1	–	–	–	–	–	4	–
"X" variant	D-1	–	–	–	–	–	–	–
"X" variant	D-3	–	–	–	–	–	–	–
"Y" variant	F-2	–	–	–	–	–	–	–

* NSG = Fluorescein-labeled normal rabbit serum globulin.

† See Materials and Methods.

‡ w = weak 2+ reaction.

employed, "nonspecific" staining was the result. Presumably these antibodies could be removed by adsorption procedures, but since these sera also stained organisms in the feces of normal guinea pigs (see below) the procedure of indirect staining was not pursued further.

The data presented in tables 1 and 3 show that it is possible serologically to group and type strains of *S. flexneri* in pure culture by means of the fluorescent antibody technique. Attempts were then made to apply this procedure to the identification of *S. flexneri* in the feces of experimentally infected guinea pigs (Formal *et al.*, 1958*a*, *b*). In general, the technique employed was to suspend the specimens, obtained either by rectal swab or from fecal material, in saline and centrifuge the suspension at low speed to sediment the debris. Smears of the supernatant were made on several clean slides, air-dried, and fixed in methanol. The slides were stained with either fluorescein-labeled polyvalent Shigella grouping serum, labeled *S. flexneri* antiserum, labeled *S. flexneri* typing serum, or with labeled normal rabbit serum globulin. All sera had previously been adsorbed twice with guinea pig tissue powder to decrease nonspecific staining as much as possible. Bacteriological examination of the fecal samples was concomitantly carried out employing standard procedures.

The results of this study are not encouraging. Again difficulty was experienced with nonspecific staining on the part of certain reagents. Labeled grouping sera, especially those against Shigella groups A and C and the two sheep anti-rabbit globulin sera, stained occasional organisms in the feces of uninfected animals with some degree of regularity. Attempts to isolate these organisms have not been successful. It is true that only a few organisms in these normal feces were stained (figure 1) but by the same token on relatively rare occasions similarly few dysentery organisms may be present and be stained in the feces of experimentally infected animals (figure 2). Usually, however, the feces of infected animals, severely ill from an experimental infection, contain a large number of *S. flexneri* bacilli (figure 3) which may be present in almost pure culture.

On the other hand, adsorbed labeled type-

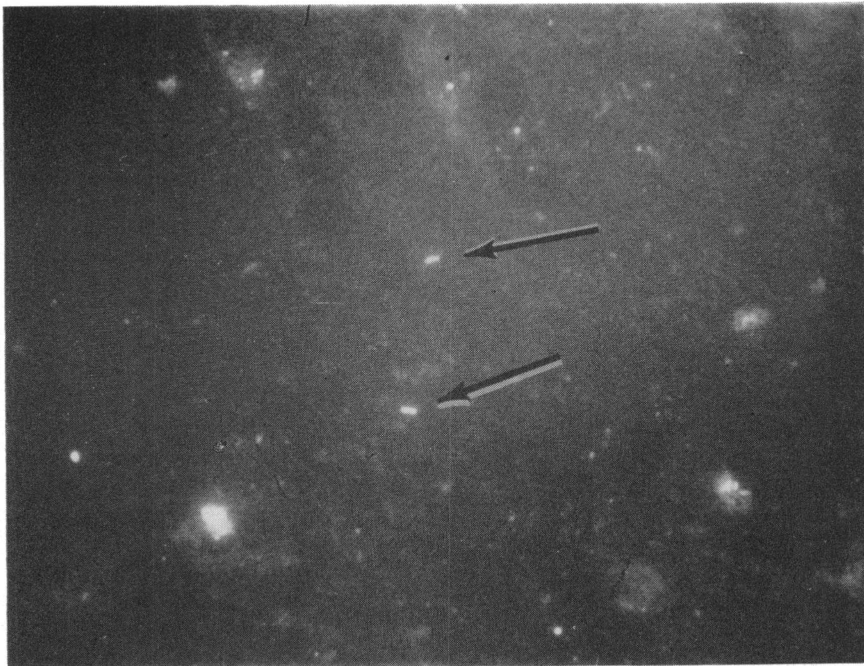

Figure 1. Fecal smear from a noninfected guinea pig stained with labeled Shigella group A antiserum. Note the 2 fluorescent bacilli in the center of the photograph. Attempts to isolate the responsible organisms were not successful. Only a few such organisms morphologically similar to Shigella were found on the slide (original magnification, 420×).

Figure 2. Smear made from fecal specimen from a guinea pig infected with *Shigella flexneri* strain 2a and stained with labeled Shigella group B antiserum. Only a few organisms were present in the specimen. However, positive identification was made by conventional techniques (original magnification, 420×).

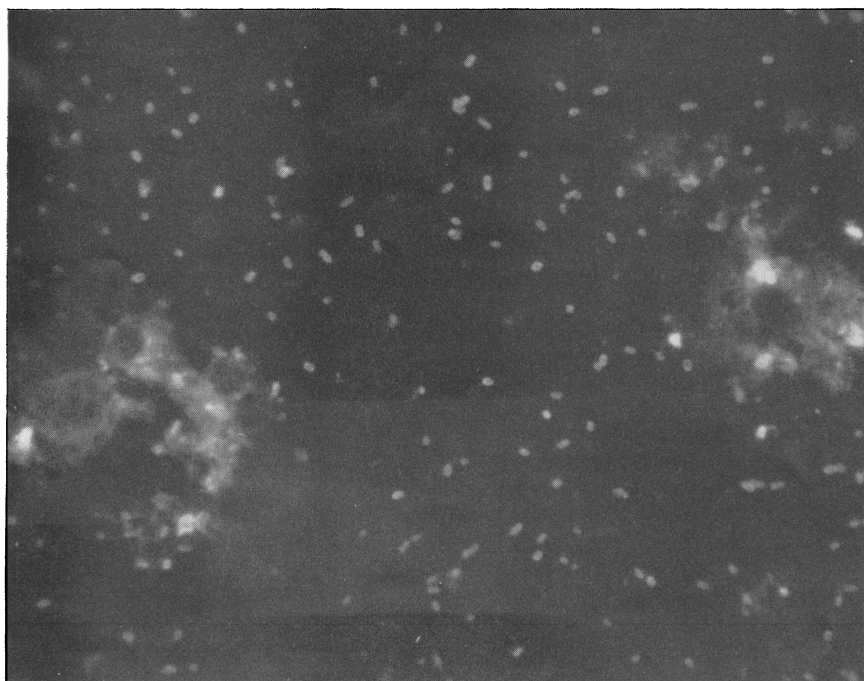

Figure 3. Fecal smear made from a moribund guinea pig experimentally infected with *Shigella flexneri* strain 2a and stained with Shigella group B antiserum. Large numbers of Shigella were noted in every field (original magnification, 320×).

specific *S. flexneri* sera were quite satisfactory. Staining of organisms in the feces of normal guinea pigs was not observed and even the relatively weak reacting type 2 reagent (table 3) stained *S. flexneri* 2a in the feces of experimentally infected animals with sufficient intensity to render these organisms readily visible.

Further, fecal specimens obtained from 8 normal human volunteers were found to contain many organisms which were stained with the various labeled grouping sera. Although many of the organisms viewed were obviously not Shigellae purely on their morphological appearance, there were microorganisms present which were morphologically indistinguishable from Shigella. All specimens were plated on eosin-methylene-blue agar and no lactose negative colonies were found. Nonspecific staining of organisms in the feces of normal individuals was minor or not observed when *S. flexneri* typing sera were employed; only one fecal specimen contained organisms exhibiting a very weak staining reaction. This occurred when labeled *S. flexneri* 1 and 3 typing sera were used.

DISCUSSION

As the situation now stands, the identification of Shigellae is based both on biochemical and serological tests. A culture with the identical antigenic structure of one of the Flexner serotypes but which ferments lactose would not be considered as a member of the genus *Shigella.* It would take much time, work, communication, and conversation to establish that an isolate represents a new dysentery serotype.

The advantages of rapid diagnosis are obvious, but it necessarily follows that the fluorescent antibody technique, being a serological procedure, offers only a part of the data necessary for an accurate identification of Enterobacteriaceae. Thus the problem revolves around the question of the incidence of nonspecific reactions and this present study emphasizes the fact that specificity is a major problem which one encounters when he attempts to identify by means of this procedure microorganisms in material taken from highly contaminated areas.

Nonspecific staining may occur under at least two sets of circumstances. First of all a labeled serum may contain "normal antibody." We met this problem while still working with pure cultures for when attempts were made to use the indirect technique it was found that both of two labeled sheep anti-rabbit globulin sera which were available reacted with various Shigella serotypes and also with organisms in the feces of normal guinea pigs. The same results probably would have occurred if labeled horse anti-rabbit globulin had been employed, for in preliminary experiments in other work (Formal and Baker, 1953), all normal sera from over 50 horses had agglutinins for *S. flexneri* serotypes. It seems likely that this is not an isolated instance and that similar situations may be encountered with other microorganisms.

While the problem of normal antibodies is a thorny one, incorrect diagnosis can at least be limited by the use of proper controls. However, it does not seem possible at this time to eliminate the false positive results which may be obtained when the material under study contains organisms possessing antigens in common with the pathogen which is being sought. Obviously there will be a greater incidence of these cross reactions when polyvalent grouping sera are employed than when adsorbed type-specific sera are used. This was the case in this study. Polyvalent grouping sera stained organisms in the normal guinea pig and human feces, whereas only minor difficulty was encountered with the type-specific reagents.

From a practical viewpoint, nonspecific staining was troublesome in only a very small percentage of the samples used in this study. In the guinea pig the experimental fatal enteric infection with *S. flexneri* is usually an overwhelming one and the organisms are present in the feces in large numbers. Thus even though occasional organisms might be stained by groups A and C sera, so many more were positive with group B serum, that it was difficult to doubt the diagnosis. Identification could then be accomplished with confidence by using type-specific reagents which up to this time have not been shown to stain organisms in the feces of the normal guinea pig.

We have not yet had an opportunity to study stool specimens from humans with diarrheal disease. For at least two reasons we anticipate greater difficulty in making accurate diagnoses with material from man than with guinea pig stools. First of all there is a large percentage of human dysentery cases passing stools in which dysentery bacilli are present in relatively small numbers (as indicated by standard isolation pro-

cedures). Secondly, the normal human enteric flora, unlike that of the guinea pig, contains large numbers of *E. coli* and other members of the family Enterobacteriaceae in which case a larger number of cross-staining reactions could be expected. Consequently, the presence of relatively low numbers of dysentery bacilli in a significant proportion of stool specimens combined with the possibility of even an increased incidence of cross-staining reactions of non-Shigella organisms may render grouping sera unusable.

Unless these anticipated difficulties can be resolved, it does not seem likely that the fluorescent antibody technique can be practically employed as a routine procedure to diagnose the occasional case of diarrheal disease. On the other hand, the fact that no difficulty has yet been encountered with the type-specific reagents indicates that this procedure may be employed to advantage in epidemics or even in areas where relatively few serological types of diarrheal agents predominate.

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SUMMARY

The fluorescent antibody technique has been employed to group and type *Shigella flexneri* in pure culture. When the direct staining procedure was employed the staining reactions were of the same specificity as those of the slide agglutination method. *S. flexneri* was stained by Shigella group B antiserum but not by antisera against Shigella groups A, C, or D. Similarly, by using labeled, adsorbed, type-specific *S. flexneri* sera it was possible to type *S. flexneri* cultures with this staining procedure. The indirect staining method was not satisfactory because the available sheep anti-rabbit globulin contained normal antibody against various strains of Shigella.

Attempts to identify *S. flexneri* in fecal specimens obtained from experimentally infected guinea pigs were only partly successful. When labeled Shigella grouping sera were employed, diagnosis could not be made with any degree of accuracy when the numbers of Shigella organisms present was small since, on occasion, group A and C antisera stained significant numbers of morphologically similar organisms in the feces of normal animals. Fecal smears from normal, healthy human volunteers were found to contain microorganisms which were stained by the various grouping sera. When labeled *S. flexneri* typing sera were employed, nonspecific staining was not observed.

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