SELECTION OF SALMONELLA AND SHIGELLA CULTURES FOR SEROLOGICAL CLASSIFICATION

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Among the microorganisms received for confirmation as *Salmonella* or *Shigella* types, a large number belong to neither group but are paracolon, coliform, or other enteric bacteria. For example, Ewing and Bruner (8) reported that 39 percent of the cultures submitted to them were *Proteus*, paracolons, etc., rather than *Salmonella* or *Shigella* types.

The aim of this paper is to outline procedures which should aid in the elimination of extraneous bacteria in the laboratory in which they are isolated and by which probable *Salmonella* and *Shigella* cultures may be differentiated.

The procedures outlined here are based upon the writers' experience gained during the bacteriological examination of fecal specimens, rectal swabs, urine sediments, and cultures derived from these sources. The subject will be presented first in a general way and then the differentiation of salmonellae and shigellae will be discussed separately.

There are several important points to be considered in the original treatment of the specimen. In bacillary dysentery and gastroenteritis or enteric fever caused by salmonellae, it is important to make cultures in the acute stage of the disease, since the number of causative microorganisms in the stool diminishes rapidly and late cultures are likely to be negative. Early in the disease, patients' stools are fecal in character and are formed. This stage is followed by a series of watery evacuations which contain shreds of mucous, and often, especially in bacillary dysentery, macroscopic blood. If the mucous shreds are washed in sterile physiological saline solution and then cultured directly by streaking over the surface of the plating medium, the inciting microorganism usually can be isolated. Further, it is very important that the specimen be cultured as quickly as possible after its collection. One of the best procedures is to deliver the stool in the pan directly to the laboratory, so that the bacteriologist can select suitable material for microscopic examination and for cultural work. Otherwise, some means for rapid delivery and prompt treatment of the specimen should be devised. If conditions are such that specimens cannot be cultured immediately, suitable portions, containing mucous shreds if present, are placed in a preserving medium until the specimen can be cultured. The buffered glycerol-saline preservative medium devised by Sachs (14) can be recommended. This medium should be tinted pink by the addition of phenol red, and it should not be used if it becomes acid. Other such media are reviewed by Bangxang and Eliot (2).

It is often a distinct advantage to collect and culture rectal swabs. An experienced team of two or three workers can collect and inoculate several hundred rectal swabs in a short space of time. The technic is valuable in the investigation of outbreaks, for examining food handlers, and for collecting specimens from dispensary or office patients.

In chronic cases where stool cultures may be negative, swabs collected directly from the lesions during proctoscopic examination often reveal the inciting microorganisms.

Plating media. The selective *Shigella-Salmonella* Agar (Difco) and Desoxycholate Citrate Agar (BBL) are good plating media and they have the advantage that a large inoculum may be used. Kauffmann (10) recommended the use of brilliant green agar for the isolation of salmonellae, and bismuth sulfite agar (Wilson-Blair) surpasses all others for the detection of typhoid bacilli (4). Fecal material or mucous on a swab may be inoculated over the entire surface of plates of these media since coliform bacteria are greatly inhibited. When fishing colonies from such media, care must be taken because coli-

form bacteria on the plate may be viable. This point is stressed because triple sugar iron agar (TSI) slants are received frequently which have two or three microorganisms present because of carelessness in fishing colonies. In routine work it is advisable to use a less inhibitory differential plating medium in addition to selective media. MacConkey agar or eosin methylene blue agar (enteric) is good for this purpose. Some knowledge of the general intestinal flora may be ascertained by this procedure and it allows for isolation of an occasional pathogen which may not grow well on the more selective media.

Enrichment media. Tetrathionate broth is preferred for isolation of salmonellae. Galton and Quan (9) found a 164 percent increase in *Salmonella* isolation attributable to the efficacy of a combination of tetrationate enrichment (Kauffmann modification) and brilliant green plating agar. Unfortunately, tetrathionate broth is not a favorable medium for shigellae or for *Salmonella typhi*. Thus, selenite broth is probably the enrichment medium of choice for routine work, since salmonellae (including *S. typhi*) and shigellae usually can be isolated from it. Primary plating media are inoculated with mucous or fecal material on cotton swabs. Then the swabs, containing 0.1 to 1.0 gram of inoculum, are placed in tubes of enrichment media. The specimen must be emulsified in the enrichment broth. Large inocula can be used with enrichment media because coliform bacteria are inhibited while enteric pathogens may increase in number. Enrichment media are incubated for 16 to 18 hours and if primary plates are negative, a large loopful of enrichment broth culture is streaked on an additional (secondary) plate.

The primary plates usually are positive in acute cases of both salmonellosis and shigellosis so that enrichment media are of greatest value with specimens collected after the acute period of disease is passed, in chronic cases, and in carrier studies. Enrichment media may be used to advantage to culture blood, urine, or gall bladder drainage specimens in those cases in which a *Salmonella* type may be the etiological agent.

Differential media. If plates are found which contain colonies of lactose negative microorganisms several such colonies are picked and inoculated into TSI agar slants. After 18 to 20 hours incubation at 37° C., the TSI agar slants are examined and all those showing acid and gas throughout the medium are discarded. Occasionally slants may be observed that are acid throughout, without evidence of gas formation. These are usually anaerogenic coliform bacteria. Fecal streptococci produce a similar reaction. Cultures on TSI agar slants that exhibit a typical acid butt, with or without gas, and an alkaline slant are inoculated onto Christensen's (3) urea medium for the detection of *Proteus* cultures. The latter medium is incubated from 2 to 4 hours at 37° C., then a preliminary reading is made. Specimens that are negative at this reading then are tested in *Salmonella* and *Shigella* antiserum by the slide agglutination method. The urea agar cultures are reincubated after the preliminary reading. Positive reactions are characterized by complete alkalinization of the medium within 20 hours' incubation and may be recorded and discarded if desired. Negative urea agar slants should be retained for 48 hours since some paracolon bacteria yield a delayed reaction which is doubtful or weakly positive. Such a reaction aids in the differentiation of these cultures. Christensen's medium remains unchanged or becomes slightly acid when inoculated with *Shigella* or *Salmonella* cultures. For details of the use of Christensen's medium see Ewing (6). If preferred, the rapid urease test devised by Stuart, Van Stratum, and Rustigian (17) may be substituted for Christensen's medium.

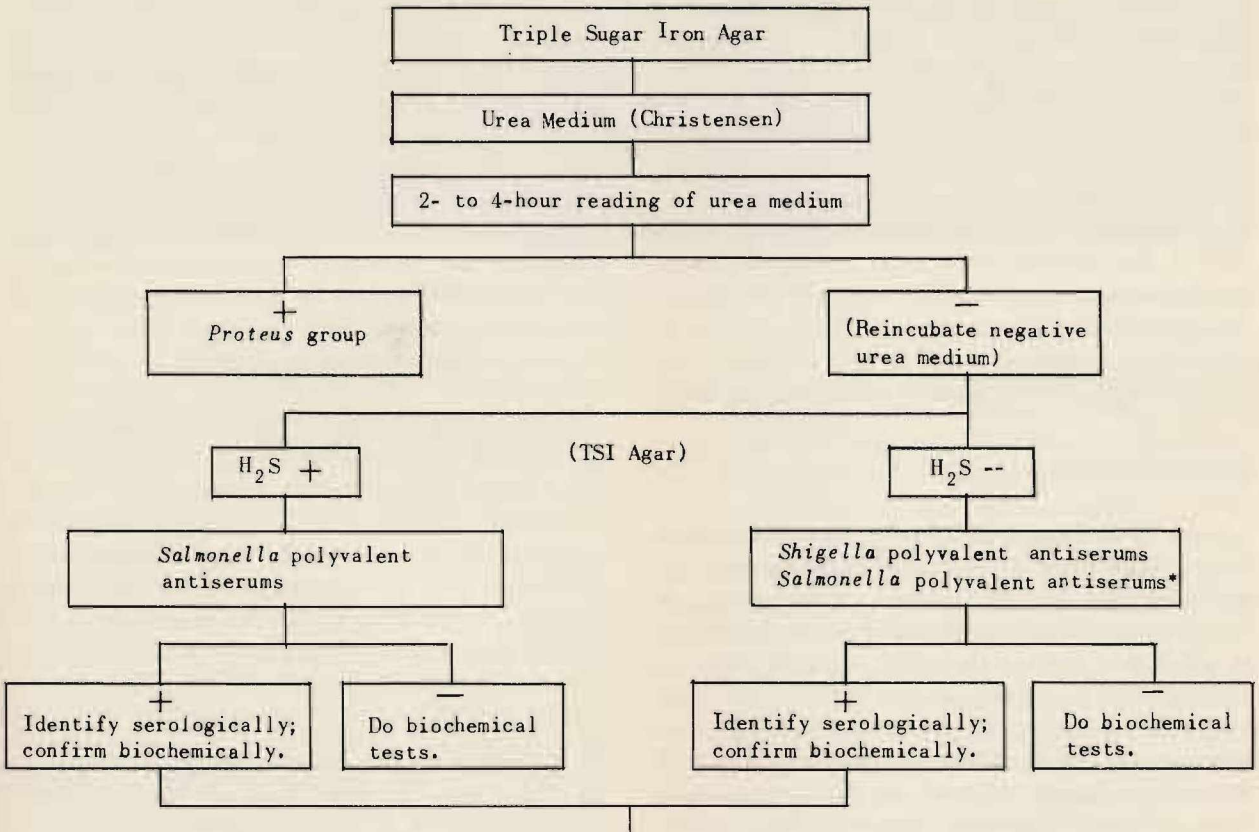
Biochemical reactions. A study of their biochemical reactions is the only method available at present for the final elimination of most paracolon bacteria. Any culture thought to be a member of either the *Salmonella* or the *Shigella* genus should be confirmed as such by the application of a few biochemical tests before it is forwarded to a central laboratory for serologic examination. The essential reactions are listed in table 1.

For hydrogen sulfide and indol tests a medium composed of 2 percent Bacto peptone and 0.5 percent sodium chloride is used. After inoculation, a strip of oxalic acid paper (Gnezda's test for indol) and a strip of lead acetate paper are inserted into a tube and held in place by the cotton stopper.*

* Oxalic acid papers may be made by soaking strips of filter paper (about 3 by ¼ inches) in a warm saturated solution of oxalic acid. The lead acetate papers are made by impregnating similar strips in a warm, saturated solution of neutral lead acetate. After drying, the paper strips may be stored in petri dishes or screw-capped bottles; they do not require further sterilization.

Table 1

OUTLINE OF PROCEDURE FOR IDENTIFICATION OF SALMONELLA AND SHIGELLA CULTURES



	Salmonella	*Shigella*
Glucose	AG	A
Lactose	-	V (positives delayed)
Sucrose	-	V (positives delayed)
Mannitol	not needed	V
Salicin	-	-
Adonitol	-	-
Citrate (Simmons')	V	-
M R	+	+
V P	-	-
H_2S (paper)	+ (usually)	V
Indol	-	V
Motility	V	-

*Occasional *Salmonella* cultures may fail to produce hydrogen sulfide in TSI agar. Also certain salmonellae and shigellae cross agglutinate (see text). *Salmonella typhi* and *Salmonella gallinarum* are anaerogenic. Rarely anaerogenic cultures of other types appear.

The papers must not come into contact with the medium. It is advisable to test for indol with Kovac's or Pringsheim's reagent after the papers are observed 48 to 72 hours. This procedure affords confirmation of negative findings by Gnezda's method.

Motility of microorganisms belonging to the family Enterobacteriaceae is determined by the use of a semisolid medium. This is a far more accurate method than direct microscopic examination. Edwards and Bruner (5) described a modification of the motility medium of Jordan, Caldwell, and Reiter that is an excellent one both for motility determination and for separation of phases. As an alternative, a medium of peptone or tryptone water to which 0.25 percent agar is added, may be used.

Salmonella

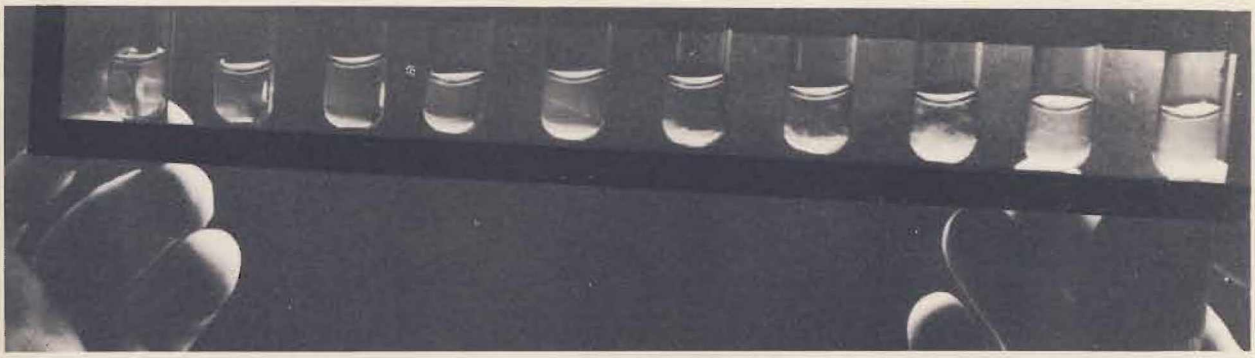
Most of the organisms isolated from feces which bear a superficial resemblance to *Salmonella*, but which actually are not members of the genus, can be eliminated from consideration in the laboratory in which they are isolated. The currently accepted definition of the genus is as follows: "A large genus of serologically related, Gram-negative and non-sporing bacilli; 0.4 – 0.6 microns by 1 – 3 microns 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. Barely 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." (15).

The above definition admits certain cultures which ferment lactose or sucrose or which produce indol to the genus, and it is true that *Salmonella* cultures possessing these characteristics have been found. However, they are extremely unusual forms and for practical purposes it may be said that ANY CULTURE WHICH FERMENTS LACTOSE, SUCROSE, SALICIN, OR ADONITOL, OR WHICH PRODUCES INDOL IN AMOUNTS DETECTABLE IN THE USUAL TESTS IMMEDIATELY CAN BE EXCLUDED FROM THE GENUS. The only exceptions to this statement are cultures which, in spite of possessing aberrant biochemical characters, possess both O and H antigens characteristic of *Salmonella* types and thus can be oriented in the Kauffmann-White scheme. In addition, *Salmonella* cultures are methyl red positive and Voges-Proskauer negative. Further, in the case of *Salmonella*, the urea agar of Christensen exhibits absolutely no evidence of urease activity after 24 or 48 hours' incubation. Certain paracolon organisms which show no urease activity after the short incubation period used to detect *Proteus* cultures will produce a distinct alkalinity in the medium after 24 to 48 hours' incubation. Many such cultures belong to the Bethesda or Ballerup groups which otherwise are extremely difficult to distinguish from *Salmonella* except by prolonged incubation of fermentation tests. Such cultures often have a pronounced putrefactive odor which is absent in *Salmonella*.

Fermentation of lactose, sucrose, or salicin may be delayed, and tests should be incubated for 30 days before being discarded. The tubes may be stoppered with corks soaked in hot paraffin to decrease the time necessary for the production of detectable acid in fermentation tubes. By corking the tubes, cultures which ferment lactose or sucrose slowly often can be eliminated in one-half the time necessary if uncorked tubes are incubated.

The majority of *Salmonella* strains other than *S. typhi* produce gas and abundant hydrogen sulfide in TSI slants. In addition to being anaerogenic, certain cultures of *S. typhi* produce insufficient hydrogen sulfide to blacken TSI agar. Also, certain other types of *Salmonella* infrequently encountered in the United States fail to produce hydrogen sulfide. Among these are *S. choleraoe-suis* (diphasic), *S. paratyphi A.*, *S. berta* and certain strains of *S. senftenberg*. Further, anaerogenic strains of any *Salmonella* type may appear. When gas production and hydrogen sulfide formation are apparent in TSI medium it is logical to suspect the presence of a *Salmonella* type other than *S. typhi*. Such cultures may be tested at once with polyvalent *Salmonella* serum.

Polyvalent *Salmonella* serum may be prepared in several ways. Probably the simplest serum that is at all serviceable is a pure O serum which contains agglutinins for *Salmonella* O groups A through E_3. Experience has shown that 98 to 99 percent of the *Salmonella* types isolated from man belong to these groups. If desired, a serum which contains agglutinins for selected H antigens in addition to the above mentioned O antigens may be made. Finally, it is possible to produce a polyvalent serum for all the O and H antigens of the genus. Such a serum



Positive and negative H agglutination tests.

is difficult to prepare and has a greater tendency to react with paracolon cultures than does a serum containing O agglutinins for only groups A through E_3.

Agglutination tests with polyvalent serum can be performed by suspending a small amount of growth from a TSI agar slant in a droplet of physiological saline solution on a slide, adding a small drop of properly diluted serum, and tilting the slide back and forth a few times. If *Salmonella* is present agglutination quickly should become apparent. If agglutination occurs, and if the necessary serums are available, the next step is the grouping or typing. If these procedures are not carried out in the laboratory, the culture should be confirmed by biochemical study and sent to a laboratory in which *Salmonella* typing is done. Biochemical study is necessary since a certain number of *Shigella* and paracolon strains are agglutinated by polyvalent serum. However, if typical agglutination is obtained and preliminary biochemical results are characteristic, it is justifiable to report, "*Salmonella*, type undetermined."

If the organism fails to agglutinate in polyvalent serum, it should be subjected to the biochemical tests outlined above and listed in table 1. If the organism cannot be eliminated from the genus *Salmonella* by these tests it should be sent to a laboratory specializing in the study of enteric bacteria. In the absence of agglutination in polyvalent serum it is justifiable to report that probably no *Salmonella* type was found.

Shigella

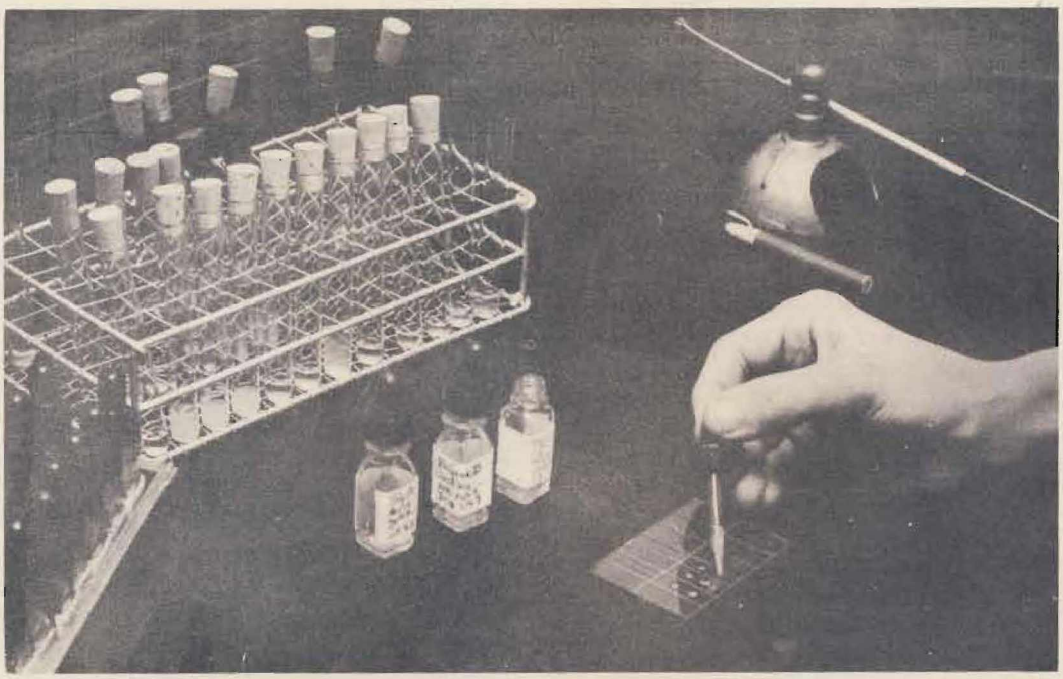
The following definition of the genus *Shigella* (7) provides a working basis for a discussion of the differentiation of shigellae: "gram-negative bacteria that are aerobic, nonsporulating, nonmotile, and, with a few exceptions, nonproductive of gas from fermentable substances. They do not utilize salicin, adonitol, or citrate, or hydrolyze urea, liquefy gelatin, or form acetylmethylcarbinol. Lactose is utilized by only two recognized species (*Shigella sonnei* and *Shigella dispar*), and by these only upon prolonged incubation."

After cultures which appear suspicious on TSI agar slants are tested for urease activity, differentiation of shigellae may be begun. Those cultures which are anaerogenic, hydrogen sulfide negative (TSI) and urease negative are selected for examination as possible shigellae. A small amount of gas along the line of stab or at the bottom of a tube of TSI agar should not lead one to eliminate the culture from examination if there is no evidence of hydrogen sulfide production. Certain biochemical variants of *Shigella flexneri VI* (Newcastle, Manchester) produce gas from fermentable substances and sometimes enough gas is evolved to become apparent in TSI agar. *Shigella* cultures are not known to produce blackening of TSI agar or Kligler's iron agar in the ordinary 20- to 24-hour incubation period. However, many *Shigella* types do produce various degrees of discoloration of lead acetate papers suspended over 2 percent peptone water, and such reactions do not eliminate microorganisms from the genus *Shigella;* the lead acetate paper test is simply a more sensitive method.

Suspected shigellae then should be tested in polyvalent or grouping antiserums by the spot plate technic. Thick suspensions of the bacteria are prepared by suspending them in formalinized normal physiological saline solution. Triple sugar iron agar slants may be utilized for this purpose, or infusion agar slants may be inoculated and the growth examined following 6 hours' incubation or more.

Polyvalent antiserums are prepared against members of group A, B, and C in the *Shigella* schema (7). To prepare an antiserum for members of group A, for example, smooth cultures of each of the seven members of the group are cultivated and the growth pooled into a mixed vaccine. Then a single rabbit, or group of rabbits, is injected with the mixed vaccine. It is advisable to test-bleed the animals near the end of the immunization period to determine whether or not the agglutinin content of the antiserum is satisfactory for each constituent of the mixed vaccine. If antibody for any one of the microorganisms is unsatisfactory, the rabbit is injected again with a vaccine made from that particular microorganism. Such grouping antisera are used in slide tests and should be employed in a dilution that permits prompt and complete agglutination of microorganisms belonging to the group. Delayed, incomplete cross reactions are seen between certain members of the three groups but cognizance is taken only of those agglutination reactions that are prompt and complete. When such reactions occur, the suspension then is tested in specific antiserums prepared against each member of the group. In addition to the polyvalent antiserums prepared against members of groups A, B, and C, three other antiserums are employed for preliminary grouping of shigellae. If a suspension does not react in polyvalent A, B, or C antiserum, or is weakly agglutinated by one of them, the suspension then is tested with *Shigella alkalescens*, *Shigella sonnei* (mixed form I and II), and *Shigella dispar* (mixed I and II) antiserums.

Use of the above-mentioned six antiserums permits preliminary grouping of all except a few of the rarer *Shigella* types. Cultures which appear to be shigellae but which do not react in the antiserums should be suspended in plain saline and heated (100° C., ½ hour) and then retested. Certain *Shigella* may contain heat labile antigens, some of which belong to the class of antigens designated K by Kauffmann (11,12). One member of this group of antigens is designated L, and it is this type that is found in some microorganisms included in the *Shigella* group. The heat labile L antigen is found in *S. alkalescens* cultures (13). When present, L antigen markedly or completely inhibits O agglutination. For other references to these inhibitory antigens see Archer (1), Stuart *et al.*, (16), and Schuetze (19).



Method of preparing slide agglutination tests with *Shigella* polyvalent serums.

If a laboratory cannot prepare grouping antiserums, serological examination of suspected cultures may be omitted and primary differentiation made by the use of a few essential biochemical reactions

(table 1). Those cultures which conform to the foregoing description of the *Shigella* group may then be selected for serological examination either in the laboratory where they are isolated or in other laboratories equipped for such work. Further, it is to be emphasized that cultures that appear to be shigellae according to their reactions in polyvalent or grouping antiserums must be subjected also to the same biochemical tests in order to confirm them as members of the genus *Shigella*.

Shigella cultures are nonmotile, and if a simple test in semisolid medium is made, many extraneous microorganisms can be eliminated from consideration as possible shigellae by this test alone. All members of the genus produce acid from glucose. Microorganisms of the *Alcaligenes* group are sometimes found in feces, and cultures of *Alcaligenes fecalis* are sometimes submitted for typing as shigellae. *A. fecalis* does not ferment glucose, but it sometimes is confused with shigellae because it produces increased alkalinity on the slant of TSI agar. This gives the impression that acid is present in the butt. If such slants are compared with an uninoculated tube of TSI it is observed that there is no change of reaction in the butt. Likewise, *Pseudomonas aeruginosa* produces increased alkalinity on the slant, and in this case there is a purplish cast to the medium caused by diffusion of pigment. *P. aeruginosa* cultures produce a characteristic aromatic odor.

Mannitol is included in the list of biochemical test substances because it aids in the subdivision of the genus *Shigella* into groups. Members of group A do not utilize this substrate, while with a few exceptions, members of group B, C, D, and E ferment it. Mannitol negative cultures of *S. flexneri* IV and *S. flexneri* VI sometimes occur.

The fact that *S. sonnei* and *S. dispar* serotypes ferment lactose and occasionally attack sucrose after 48 hours or more serves to distinguish these microorganisms from other shigellae. The average time required for *S. sonnei* and *S. dispar* cultures to ferment lactose is 6 or 7 days. If lactose and sucrose tubes are plugged (see above, under *Salmonella*) fermentation is generally accelerated. It should be mentioned that if fermentation tubes are to be plugged, an indicator which is not an oxidation-reduction indicator must be used. Early fermentation of lactose or sucrose, i.e., within 24 hours, means that the culture is not a *Shigella* type. Ancillary evidence to this is afforded when gas is also present. Most paracolon cultures produce relatively large amounts of gas but anaerogenic forms are not uncommon (16,21). Regardless of their action toward lactose and sucrose or their gas producing propensities, most paracolon cultures can be differentiated from shigellae on the basis of other biochemical tests, motility, and serology.

Members of the genus *Shigella* do not utilize salicin or adonitol, and cultures which produce acid from these test substances may be eliminated from consideration as possible shigellae. None of the described *Shigella* types grow on Simmons' citrate agar. For this reason Simmons' citrate medium is useful in separating paracolons that are able to utilize citrate and ammonium salts. However, most *Salmonella* cultures utilize citrate, *S. typhi* being among the exceptions.

Use of the tests listed in table 1 should allow laboratory personnel to confirm microorganisms that react in antiserums as shigellae, to confirm cultures which do not agglutinate in antiserums for the more common shigellae as possible *Shigella* types, and to eliminate most paracolon cultures from consideration as members of the genus *Shigella*. Microorganisms that conform to the definition as regards their biochemical reactions but which cannot be typed serologically can be sent to laboratories equipped for complete serological studies.

Summary

Serological and biochemical tests are outlined which will guide workers in their identification of *Shigella* and *Salmonella* types. Use of these notes will aid in the elimination of many paracolon and coliform bacteria in the laboratory where they are isolated, and should decrease the percentage of error in cultures sent for typing as salmonellae and shigellae.

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