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Colonial Recognition, a "New" Approach for Rapid Diagnostic Enteric Bacteriology

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The morphology, on MacConkey's agar, of colonies of the bacteria commonly found in enteric specimens is sufficiently characteristic and distinctive, when observed with transmitted oblique illumination and a low-power stereoscopic microscope, to enable identification at the generic (and occasionally the species) level with over 97% accuracy. Application of this technique resulted in great savings of time, manipulative steps, and media compared with the usual procedures of diagnostic enteric bacteriology, and there was an increase in sensitivity without sacrificing accuracy.

Bacteriologists often make "educated guesses" regarding the identity of colonies obtained from specimens submitted for diagnosis. In bacteriological studies of the upper-respiratory tract, observation of colonial morphology, usually on blood-agar, is perhaps the major, and certainly a first, consideration in the identification of the organisms present and in deciding whether they are pathogens or only normal residents. The bacteriologist is guided in these decisions by ancillary factors, such as hemolysis, and he confirms (or refutes) his preliminary estimates by additional tests, such as microscopic observations, serological testing, and biochemical reactions, as his experience dictates for different types of colonies. This experience is handed down, practically as "folk lore," from teacher to student; it is described to some extent in the textbooks of bacteriology, and it is acquired fairly rapidly by the practitioners of the art in the laboratory. In the area of enteric bacteriology, similar principles are applied, but the technician is supported by a wider array of selective and differential media and all but the most color blind can distinguish between the lactose-fermenters and the "potential pathogens."

In his examination of the gross appearance of colonies, the technician soon learns that with translucent media he can get the best differentiation if he holds the petri dish at an angle to a source of transmitted illumination. This principle was applied by Henry (5), who used transmitted oblique illumination for differentiating colonial variants of *Brucella*. The lighting effect was obtained by reflecting the beam from a microscope lamp upwards and at an angle through the petri dishes on the stage of a stereoscopic dissecting microscope by means of a mirror placed on the table. The lighting effect plus the magnification enable the observation of differences in colonial morphology, i.e., in texture, color, refractility, and structure, which are not apparent to the naked eye or by other means of illumination. The technique was later adopted by Braun (1) for the same purpose. Walters, Cooper, and Keller (9) demonstrated the value of this method in the rapid detection of *Shigella* in specimens submitted for diagnosis. They also described the colonial appearance of other organisms likely to be encountered. However, recognition of the potential value of the technique as a tool in enteric bacteriology in general has not been widespread, although it has been used for some specific purposes in some laboratories. Lankford (6) found the technique to be particularly useful in rapid identification of cholera vibrios. The colonial morphology of the cholera vibrios is practically unique, and Lankford's observations were soon confirmed and expanded by Feeley (2) and Finkelstein and Gomez (3). Lankford and Burrows (7) recently re-emphasized the general applicability of the method in diagnostic enteric bacteriology and demonstrated its usefulness in a *Salmonella* outbreak.

We were impressed with the potential of colonial recognition as a tool in general diagnostic enteric bacteriology. In the present study, we attempted to familiarize ourselves with the colonial morphology of the various bacteria likely to be encountered in enteric specimens submitted for

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bacteriological diagnosis and to determine whether, by application of this familiarity, it might be possible to increase the efficiency and speed of current conventional methods of determinative enteric bacteriology.

One of the variables in the colonial-recognition technique is the light source. The nature of the lamp itself and of the placement of the mirror, as used in previous investigations, is not standard, and both factors can have a profound bearing on the observable characteristics of colonies. Colonial appearances may vary in different laboratories and even in the same laboratory, from day to day, according to the positions of mirror and lamp. The mirror technique likewise does not provide even lighting throughout the microscopic field, and one is obliged to use only the central portion for observations. For these reasons, and to provide a more even distribution of light for photography, we found it expedient to design a special lighting arrangement which is described.

MATERIALS AND METHODS

Light source. The light source was basically a 30-w, circular, daylight fluorescent lamp [9 inches (22.9 cm) in diameter] mounted in a box with a circular hole in the top [5 inches (12.7 cm) in diameter] which was centered over the lamp (Fig. 1). The distance from the surface of the fluorescent tube to the petri dish, on the stage of the stereoscopic, low-power, dissecting microscope, markedly affected the appearance of the colonies. A vertical distance of about 5 inches (12.7 cm) provided maximal contrast. The inside of the box was painted dull black to reduce internal reflections. For observation and photography of colonies, we used the 7× magnification of an American Optical Cycloptic microscope, with the "Transilluminator Base" removed, centered above the light source. Other brands are undoubtedly equally suitable. The capability of shifting to higher magnification was useful on occasion for more detailed observation. For convenience, we installed a light source in a removable drawer in a laboratory table with a hole in the surface (Fig. 1). The hole can be filled with a circular glass (or other) plate when it is desirable.

Reference strains. Our initial familiarization with the appearance of the colonies of the various enteric genera and species was accomplished by study of stock cultures maintained in the lyophile state in this laboratory. It should be noted that the colonial morphology of strains maintained for some period in the laboratory may exhibit some heterogeneity and variation beyond what is likely to be encountered with fresh isolates. For photography, drops of appropriate dilutions of culture were "spotted" on the surface of MacConkey Agar (BBL) to avoid the distortion of colonial morphology which is sometimes associated with streaking.

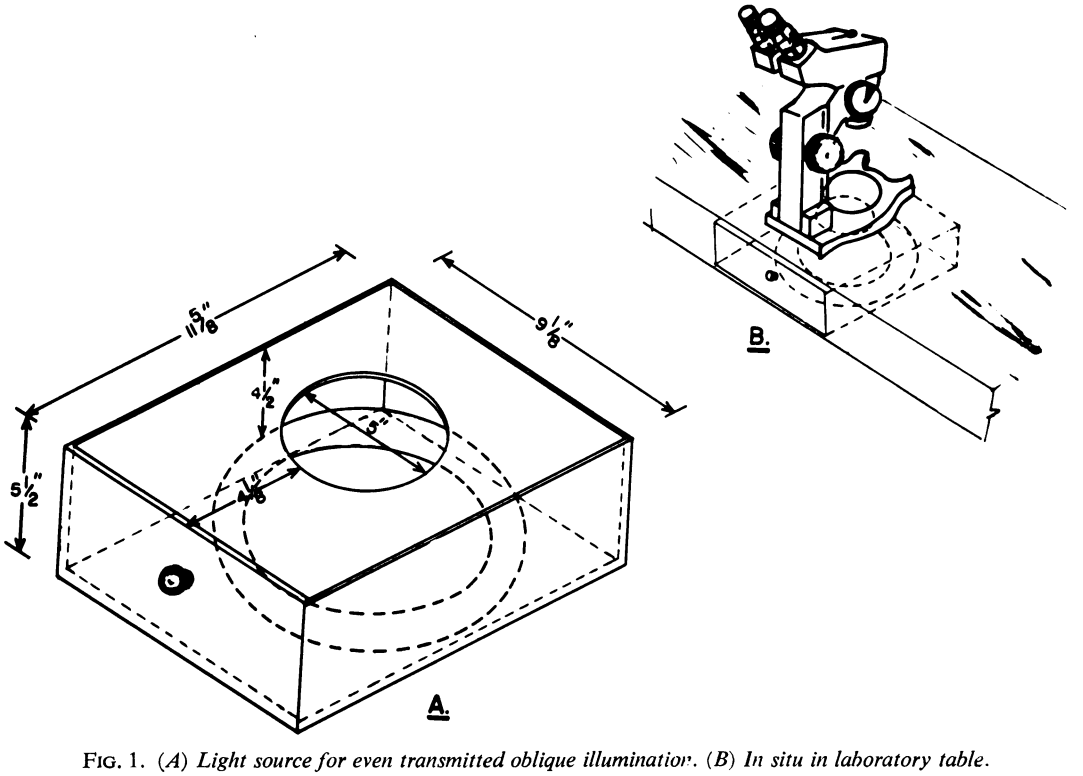

FIG. 1. *(A) Light source for even transmitted oblique illumination. (B) In situ in laboratory table.*

Specimens and procedures. Specimens submitted for enteric bacteriological examination were used. In the routine procedure, the specimens (rectal swabs or stool) were streaked (initial streak) on MacConkey Agar and S S Agar (Difco) and were placed in Selenite-F (BBL) broth for enrichment. The next day, suspicious colonies were transferred to Kligler Iron Agar (KIA, from BBL), and the Selenite-F enrichment broth cultures were streaked on S S Agar and on Desoxycholate Citrate Agar (Difco; second streak). On the 3rd day, biochemical tests were initiated based upon the reactions observed in KIA of the colonies picked from the initial streak, and colonies were transferred to KIA from the second streak. The 4th day, serological testing was performed on isolates (from the initial streak) which produced biochemical reactions similar to those of pathogens, and differential media were inoculated with strains isolated from the second streak based upon their KIA reactions. Serological tests for identity were performed on the 5th day on the latter strains. Occasionally, serological tests were performed with the growth on KIA prior to biochemical tests. In the modified technique, only the MacConkey Agar plates from the initial streaking were used. After colonies had been selected by the routine enteric section of our laboratory, plates were examined by the oblique-light technique at 18 to 24 hr. When the colonial morphology revealed a possible pathogen (*Salmonella* or *Shigella*), slide agglutination tests were performed with appropriate antisera, or, if insufficient colonial material was available for testing, broth was inoculated to build up sufficient antigen. A record was kept of the presumptive identification based on colony morphology of all different kinds of colonies selected. Each of the different colony types was transferred to KIA for further identification by routine procedures to confirm or refute the original presumption.

RESULTS

Colonial morphology of some common enteric bacteria: Escherichia coli (Fig. 2). Little need be said about the familiar and characteristic morphology and color of colonies of *E. coli*. Frequently, however, one may observe several colonial types of *E. coli* in a single specimen. The differences, primarily in color and refractility, are difficult to describe. The presence of multiple colonial forms was most easily recognized when they happened to occur in contiguous colonies which manifested a sharp refractile interface. Colonies identified as *E. freundii* (*Citrobacter*) differed from the usual *E. coli* colony type by having a more irregular edge, but we found some *E. coli* strains which could not be differentiated by colonial morphology from *E. freundii*. We were not able to differentiate enteropathogenic serotypes of *E. coli*, although application of this technique may aid in the selection of different colonies for further serological tests.

Aerobacter aerogenes (Fig. 3). The appearance of *Aerobacter* colonies is likewise well known and they are easily identified. The group appeared to be fairly homogeneous insofar as colonial morphology is concerned.

Paracolons (Fig. 4 and 5). For practical purposes, we kept the designation *Paracolon* for those organisms which resembled *Escherichia* or *Aerobacter* (or intermediate forms) biochemically, but which gave a delayed reaction in lactose. In addition, some isolates which appeared to resemble *Shigella* biochemically but which failed to be agglutinated in *Shigella* antisera were included in this heterogeneous group, as were some organisms which resembled H_2S-negative *Salmonella* but which could not be typed. As might be expected from the heterogeneous nature of the group, the colonial morphology of different strains also exhibited a rather wide range of variation. Two rather diverse forms are illustrated in Fig. 4 and 5. Forms resembling those in Fig. 4 were most common. There was a superficial morphological resemblance between this colonial form and that seen with the *Salmonellae* (Fig. 6–10). However, under actual observation, the differences were far greater than these photographs suggest, and there was little reason for confusion of the two groups. One obvious distinction was that the *Paracolon* colonies were more refractile and appeared more opaque. The colonial type illustrated in Fig. 5 superficially resembled colonies of *Shigella boydii* (Fig. 11), but, again, under actual observation the distinction was clear. The *Paracolon* colonies were larger and more opaque. Some difficulty was experienced in differentiating the *Paracolon* type pictured in Fig. 5 from colonies of the *Providencia* group (Fig. 12). Again, the *Paracolon* colonies had a more mucoid appearance and were more refractile, but the distinction could not invariably be made. Some colonies which were considered to be *Paracolon* were subsequently identified as *Aerobacter*.

Salmonella (Fig. 6–10). Interestingly, all of the *Salmonella* colonies encountered exhibited a strong familial resemblance which was characteristic for the group. There was some strain-to-strain variation and occasionally variation within a strain (Fig. 6), but it was rather easy to conclude, with reasonable accuracy, that colonies of this general kind were indicative of the presence of *Salmonella*. It was not feasible, however, to speciate or type the salmonellae on the basis of colonial morphology. The colonies had irregular edges and rather flat surfaces. Generally, they were quite translucent and exhibited a greenish coloration, particularly in the central area. Colonies of bacteria subsequently identified as *Bethesda* species could not be differentiated from

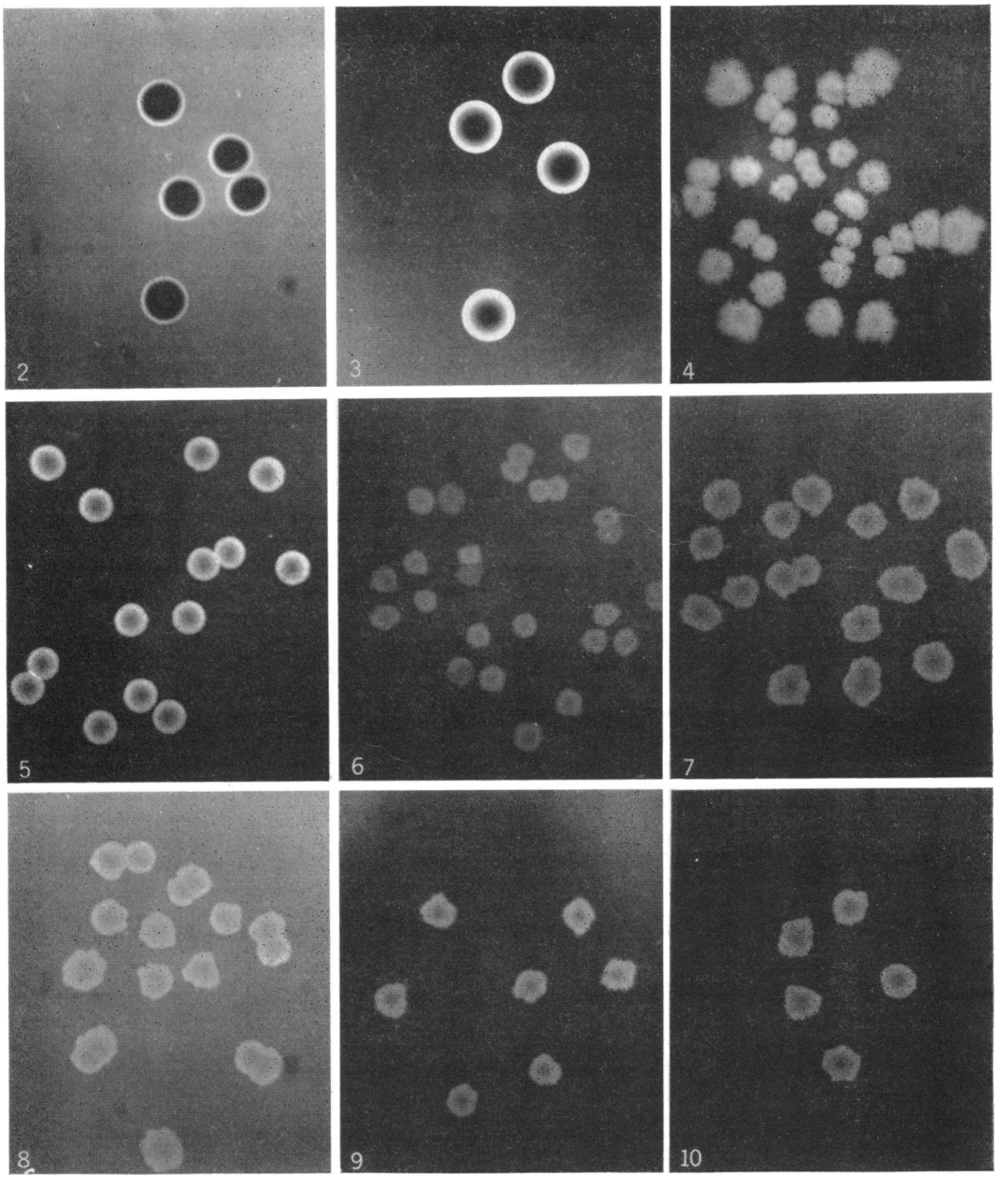

FIG. 2–10. *Colonies of (2) Escherichia coli, (3) Aerobacter aerogenes, (4, 5) Paracolon, (6) Salmonella paratyphi A (group A), (7) S. paratyphi B (group B), (8) S. montevideo (group C), (9) S. typhi (group D), (10) S. anatum (group E), on MacConkey Agar, at 18 hr. Oblique transmitted illumination; 7× original magnification.*

the salmonellae on the basis of colonial morphology.

Providencia (*Fig. 12*). As mentioned above, colonies of this group closely resembled and were most difficult to distinguish from some *Paracolons*. This could be expected, since the *Providencia* group was formerly included among the *Paracolon* bacteria. The colonies were round with entire edges, generally slightly convex, and of an intermediate degree of opacity, somewhat less than that of the related *Paracolon* colonies. A dark (translucent) central area was rather char-

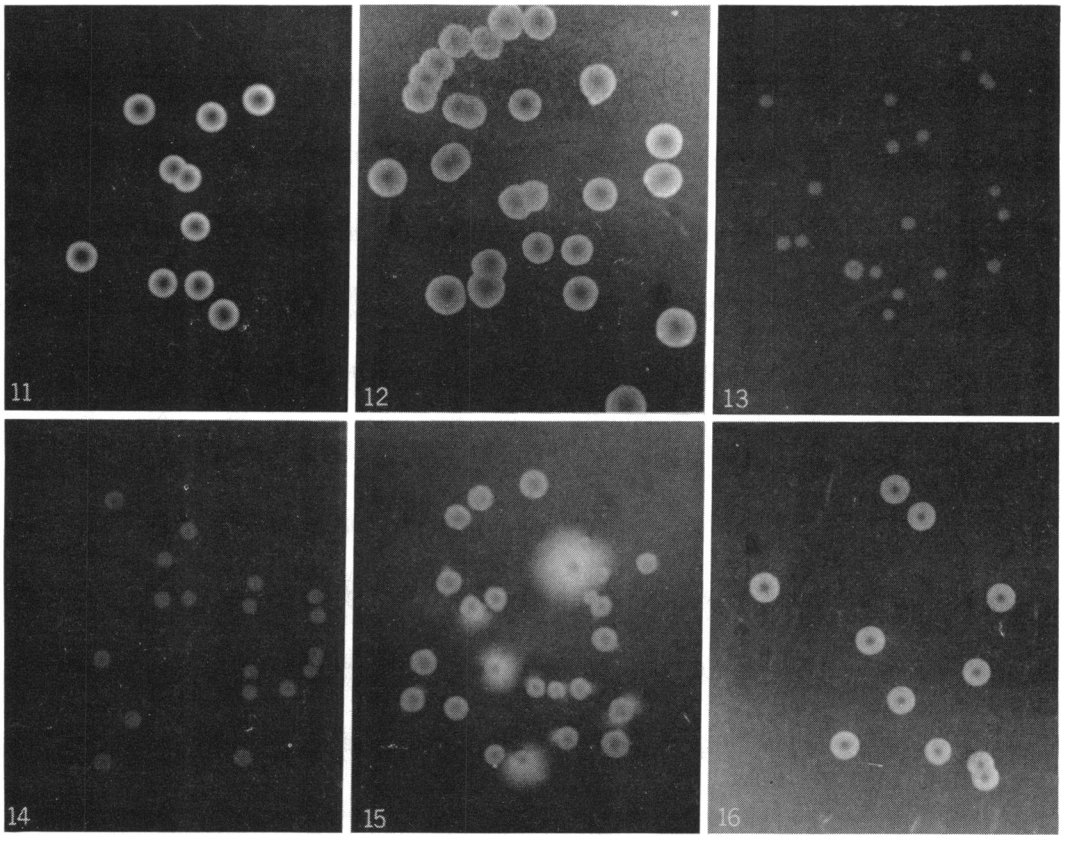

FIG. 11–16. *Colonies of (11) Shigella boydii (subgroup C), (12) Providencia, (13) S. dysenteriae (subgroup A), (14) S. flexneri (subgroup B), (15) S. sonnei, (16) Proteus sp. (H_2S-negative), on MacConkey Agar, at 18 hr. Oblique transmitted illumination; 7× original magnification.*

acteristic and served to differentiate the *Providencia* group from the other *Paracolons*.

Shigella (*Fig. 11, 13–15*). The colonial morphology of the *Shigella* group was quite distinctive and easily recognized. There was some variation within the group which, in some instances, permitted a presumption as to species. The colonies were generally small, poorly developed, and of an intermediate degree of opacity. *S. boydii* colonies (Fig. 11) were somewhat larger, granular, and more opaque. A presumption as to the presence of *S. sonnei* could also be made, with considerable accuracy, from the colonial morphology (Fig. 15). The colonies were larger, coarser, more granular, and somewhat more opaque than the other *Shigella* species. As was reported by Walters et al. (9), *S. sonnei* colonies also had a fuzzy appearance. In addition, *S. sonnei* was frequently present in two colonial forms, the second being a rough-appearing variant (Fig. 15) which was sometimes found as an outgrowth of a smoother colonial type. The simultaneous appearance of both colonial forms may be regarded as practically pathognomonic.

Proteus (*Fig. 16 and 17*). Because we examined fairly young cultures on MacConkey Agar, we did not encounter any of the swarming which is supposed to be characteristic of *Proteus*. Instead, the colonies were discrete, circular, and somewhat raised. Colonies of *Proteus* strains which were subsequently found not to produce H_2S were generally smaller and more convex, and they had a more translucent (dark) central area (Fig. 16). The H_2S-positive strains usually had yellowish centers and were flatter (Fig. 17). The latter type could be confused with *Salmonella* by an inexperienced observer, particularly when the colonies were distorted by streaking. Furthermore, some of these *Proteus* strains agglutinated in our *Salmonella* polyvalent serum (from a commercial source) but were nonreactive in grouping sera (from the Walter Reed Army Institute of Research).

Pseudomonas (*Fig. 18*). Colonies of *Pseudo-*

monas were relatively easily differentiated. The colonies were generally rather large, flat, granular, and translucent. They usually exhibited alternative concentric zones of opacity and translucency and some bluish-green tint. Some strains, on primary isolation, formed only very small colonies which were difficult to see.

Cholera vibrios (*Fig. 19*). Although we do not recommend MacConkey Agar for the isolation of cholera vibrios, they may occasionally be encountered on this medium in laboratories in endemic or epidemic regions. As with other media, the colonies of cholera vibrios, of both classical and El Tor varieties, are practically unique in their translucency. On MacConkey Agar, they do not develop as well as on other media, and their small size and translucency make it possible for the inexperienced observer to overlook them since there is so little contrast with the background.

"Nonagglutinable" (NAG) or noncholera vibrios (NCV) (*Fig. 20*). These constitute a large, heterogeneous, and inadequately studied group which are frequently identified on the basis of their growth on selective media designed for the isolation of cholera vibrios, and their nonreactivity in diagnostic cholera sera. The colonies were well developed, quite opaque, and finely granular with a slightly yellowish tint. In previous experience with a nonselective medium (3; Finkelstein, *unpublished data*), it was extremely rare to find a colony which resembled that of a cholera vibrio but which could not be confirmed with cholera antiserum. Generally, colonies of NCV were markedly more opaque; in those instances when the colonies were translucent, there was usually a brownish tint which enabled differentiation from the grayish cholera vibrio colonies. Some NCV have been associated with diarrheal conditions (8).

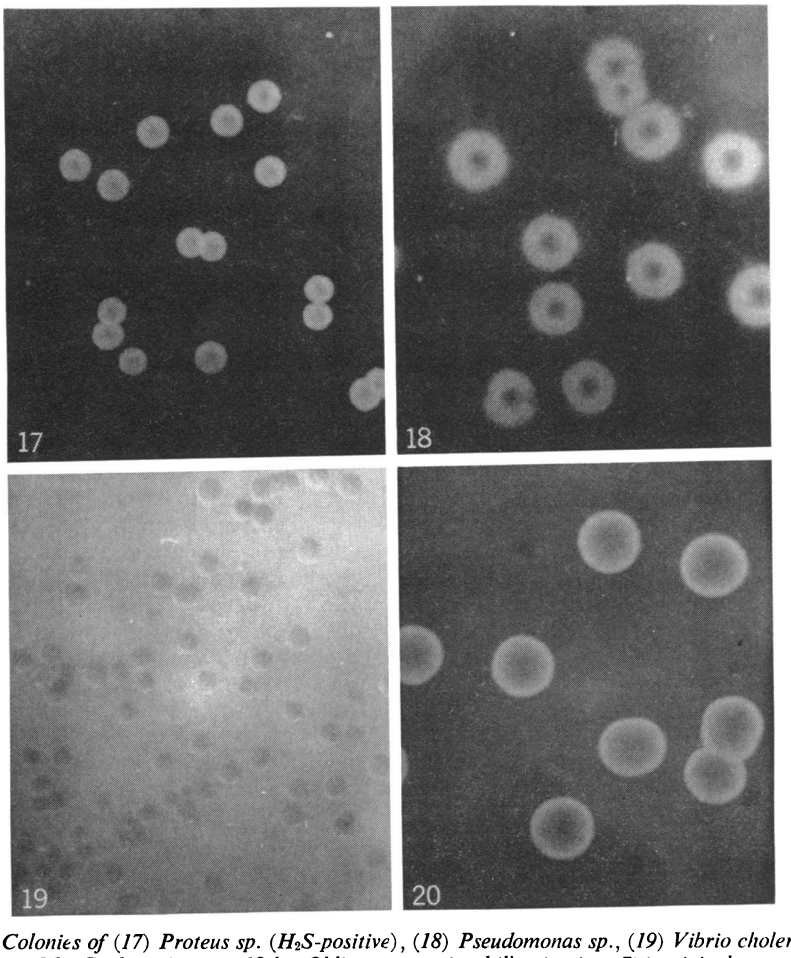

FIG. 17–20. *Colonies of (17) Proteus sp. (H_2S-positive), (18) Pseudomonas sp., (19) Vibrio cholerae, (20) noncholera vibrios, on MacConkey Agar, at 18 hr. Oblique transmitted illumination; 7× original magnification.*

TABLE 1. *Comparison between colonial recognition and the routine method, as applied to 207 enteric specimens*

Organism	Colonial recognition		Routine	
	No. of isolates[a]	Errors[b]	No. of isolates from MacConkey Agar	Total isolates[c]
Aerobacter aerogenes	33		13	30
Bethesda	—		1	6
Escherichia coli	77	+ 4 *E. freundii*	94	115
E. freundii	—		3	10
Paracolon	136	+ 3 *Aerobacter* + 1 *Proteus* (H_2S+)	98	106
Proteus H_2S+	44		14	49
Proteus H_2S−	28		16	36
Providencia	11		1	3
Pseudomonas	33		19	44
Salmonella	25	+ 3 *Bethesda*	6	13
Shigella	14		8	11
Total[d]	401/440		273/327	423/891

[a] Excluding duplications in the same specimen.
[b] See text.
[c] From all media excluding duplications in the same specimen.
[d] Isolations/total picked, including duplications in the same specimen.

TABLE 2. *Enteric pathogens isolated by both procedures*

Organism	No. of strains isolated by	
	Colonial recognition	Routine method
Salmonella anatum	2	1
S. derby	2	2
S. newport	2	2
S. paratyphi B	17	6
S. typhi	1	1
S. saintpaul	1	
S. enteritidis		1
Shigella dysenteriae 1	1	1
S. flexneri 1	1	1
S. flexneri 3	5	5
S. flexneri 4	4	2
S. sonnei I	2	1
S. sonnei II	1	1

Comparison between colonial recognition and the routine method of enteric bacteriological diagnosis. To evaluate the colonial-recognition technique, a series of 207 enteric specimens was processed, in parallel, by both methods. The results are summarized in Table 1.

Perhaps the most significant observation was the identification of 39 enteric pathogens (25 *Salmonella* and 15 *Shigella*) by the colonial-recognition technique as opposed to 24 (13 *Salmonella* and 11 *Shigella*) by routine methodology. Of the 13 *Salmonella* strains recovered by the routine procedure, 2 were missed by the colonial-recognition method. One of these was isolated from the initial streak on S S Agar (but not MacConkey Agar), and the other was recovered only after enrichment (from the Desoxycholate Citrate Agar plate). Only 11 "mistakes" in identification based solely on colonial recognition were made in a total of 440 colonies picked from the 207 plates used. Thus, the accuracy of identification, based only on colonial morphology, was over 97%. The errors consisted of four strains which were considered to be *E. coli* and were subsequently identified as *E. freundii;* four strains judged to be *Paracolon*, of which three were identified as *Aerobacter* and one as *Proteus*; and three strains considered to be *Salmonella* which were later identified as *Bethesda*.

In the routine procedure, 891 colonies were picked from the 828 plates of media used. Of these, 327 were picked from the MacConkey Agar plate. Picking in the routine manner from the MacConkey plate resulted in the isolation of only 14 pathogens (6 *Salmonella* and 8 *Shigella*).

A summary of the enteric pathogens isolated by both techniques is given in Table 2.

DISCUSSION

Colonies of the various bacterial genera present in normal and pathological specimens submitted for enteric bacteriology, when observed by the

oblique-light technique, exhibited strong familial resemblances not recognizable by the naked eye. Their characteristics enable the experienced technician to make an early and reasonable guess regarding the identity of the colonies. This may then be confirmed by immediate application of appropriate biochemical or serological tests, or both. In addition to the advantages of being able to provide a diagnosis before the patient has either died or recovered, the method offers possibilities for the conservation of media and manipulative steps with an increase in sensitivity and without sacrifice of accuracy.

In our comparison of the colonial-recognition procedure with routine methods in current use in this laboratory, the proposed method enabled identification of a significantly greater number of pathogens in less time and with greater economy of effort and materials. In the routine procedure, a total of 891 colonies was picked from the 828 plates of media used in the examination of 207 specimens. In many instances, there was duplication since two or more colonies of the same organism were picked and subjected to all the ensuing biochemical and serological tests. These duplicate colonies were frequently picked from the same plate because the technician was unable to distinguish between colonies which appeared similar to the naked eye. If we consider all of the subcultures of a given species isolated from the same individual to be identical, then the picking resulted in 423 distinct isolates and 468 duplications. In contrast, with the colonial-recognition procedure, there were only 28 duplications among 440 pickings from the 207 plates. Actually, if we had relied on our judgment only, and had not followed through on the identification of the various saprophytes present (which were identified for the sake of evaluating that judgment), it would have been possible to identify all the *Salmonella* and *Shigella* species isolated on primary streaking of 207 specimens by picking only 48 colonies with 6 duplications and 3 "false-positive" errors (colonies which looked like *Salmonella* but which were subsequently identified as *Bethesda*). Since multiple additional tests are performed for each colony picked, the unnecessary duplication involved in routine procedures results in considerable expense of time, effort, and media.

The errors which were made in colonial recognition were relatively minor, consisting only of four strains considered to be *E. coli* and subsequently identified as *Citrobacter*, four misdiagnosed *Paracolons*, and the three Bethesda strains mentioned above, of the total of 440 colonies picked. Only two isolates of pathogens were "missed" by use of the colonial-recognition technique, and these were recovered by the conventional method only on a more selective medium (which takes a heavier inoculum) or after selective enrichment. For this reason, it might prove beneficial to enrich all specimens and then restreak those which are negative at the 18-hr reading of the primary streak.

Although we elected to use MacConkey Agar, as a rather "broad-spectrum" enteric medium, the technique may be performed with any other translucent medium. Colonial characteristics will vary with the medium used, so it is necessary to become familiar with the range of morphology on the medium selected. Colonies on opaque media are best observed with the stereoscope by use of reflected oblique illumination obtained by directing the light of a microscope lamp downwards, at an angle of about 40°, at the colonies on the petri dish.

It must be emphasized that we do not recommend that diagnosis be based solely on colonial recognition. Rather, this should be used as an adjunct to conventional methods and as a means of guiding the technician to appropriate definitive tests. For example, if a colony looks like a *Salmonella* colony and there is sufficient colonial material for testing, direct slide agglutination tests in polyvalent and grouping sera could enable an immediate provisional diagnosis which could then be confirmed, if necessary, by appropriate biochemical tests. If there is not enough colonial material for testing, a small amount of broth could be inoculated, and a few hours later the indicated serological tests could be performed. Colonies should be transferred while the technician is actually looking at them through the dissecting microscope. In this way, there will be fewer errors and fewer mixed cultures since the surface of the colony can be touched, and the possibility of including dormant contaminants, inhibited but not killed by the medium, will be minimized.

There are serological cross-reactions, especially among the enteric bacteria, which, in conventional diagnostic bacteriology, are sufficiently common to make direct serological testing a dangerous procedure. However, by combining the two parameters of colonial appearance and serological reactivity, a new dimension of specificity is introduced. Quick biochemical tests may also be used to rule in or out a particular identification which is suggested by colonial morphology. There is no limit to the combinations of tests which can be used in various laboratories, and we would not begin to suggest a standard operating procedure at this time. The approach is only recommended to aid in providing an early practical diagnosis at the generic (or in some cases, the species)

level. For more sophisticated results and definitive serotyping, more appropriate methods are necessary.

In this technique, there is no substitute for experience: a colony cannot be described so that another individual can invariably recognize it. However, once an individual has observed a colony, it is much easier for him to recognize it again, and with further practice this ability becomes appreciably more developed. For this reason, it is possible, and even likely, that, if this study were to be repeated in other laboratories, the results in terms of comparative yields of pathogens might vary to some degree depending on the skill of the technicians and their diligence in picking colonies by the conventional "eyeball" technique. However, we believe that the advantages of transmitted oblique illumination with low-power microscopic observation as an aid in the selection of colonies are incontrovertible in practically the same manner as the use of a telescope is an advantage to the astronomer. It is conceivable that in the future the procedure may be instrumented and automated to remove the variable of human judgment. A significant step in that direction has already been made by the development of a computer-controlled, flying-spot scanner which recognizes the differences among bacterial colonies and provides reproducible optical-density profiles (4).

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