A Rapid Method for the Characterization of Enteric Pathogen Using Paper Discs[1]

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The changing concept of warfare and civil defense during the last decade has stimulated a re-evaluation of diagnostic methods employed in medical bacteriology. The expeditious laboratory diagnosis of communicable diseases occurring epidemiologically has become a matter of great importance. For example, epidemics of dysentery encountered in the Armed Forces may be unprecedented in size; Hardy *et al.* (1952) following such an experience have emphasized the pressing need for acceleration in the diagnosis of enteric infections.

Investigations of epidemics or sporadic outbreaks of bacillary dysentery, infantile diarrhea, typhoid and paratyphoid fevers are complicated by the slowness of the usual bacteriologic procedures and the complexity of conventional identification systems. Indeed, newer knowledge regarding the biochemical and serologic characteristics of the enteric pathogens has served to lengthen the conventional diagnostic methods used to characterize them. In effect, the old and sometimes superfluous techniques continue to be used plus the newer systems which could have, partially at least, replaced them.

This report describes a rapid and economic procedure which combines the desirable features of a booster-screening system reported by Sanders (1953) with the practical utility of the paper disc techniques described by Soto (1949) and Snyder (1954). The method described will (a) reduce over-all laboratory time required for the definitive identification to less than 24 hr; (b) enhance the probability of detecting multiple bacterial serotypes; (c) provide a simple and economic procedure easily adapted to the working structure of all laboratories.

Materials and Methods

All of the experiments in this study were conducted using a basic agar medium seeded with various bacterial cultures onto the surfaces of which were placed paper bioassay discs each containing one of several carbohydrates. The ordinary Petri dish was used as a container, and all incubations were at 37 C.

Cultures. Both stock and lyophilized strains which included all serotypes and group types of the enteric pathogens were used in these investigations. The salmonellae had been lyophilized immediately after recovery from patients suffering from typhoid, paratyphoid and other enteric fevers. The shigellae included representative cultures of all stock strains maintained at the Walter Reed Army Institute of Research, and certain other lyophilized strains recovered from dysentery patients in the Far East. All Alkalescens-Dispar O Group cultures had been similarly collected. The pathogenic *Escherichia coli* group strains were recently isolated from children suffering with infantile diarrhea.

Preparation of paper discs containing carbohydrates. At the outset of this work preliminary experiments were conducted using paper discs ¼ in in diameter and 1 mm thick. It was immediately apparent that discs of such size could not absorb sufficient carbohydrate solution to preclude early reversal of the acid reactions. Accordingly, the bioassay discs of Schleicher and Schuell, No. 740E (½ in in diameter by 1 mm thickness) were employed in all subsequent experiments. Further preliminary surveys revealed that satisfactory results could be obtained with discs containing approximately 20 mg of substrate. Discs of this type were generously supplied by the Baltimore Biological Laboratory, Inc. No difficulties were encountered which could be directly attributed to the use of nonsterile discs; masked or otherwise obscure reactions never occurred. This observation suggests the potential utility of the method, especially under field or otherwise adverse laboratory conditions.

Media: lactose-sucrose booster broth. A booster-screening system was desired which would provide elimination of the lactose-sucrose fermenting intestinal organisms prior to the preparation paper disc-Petri dish cultures. A medium previously reported by Sanders and Okabe (1953) was used for this purpose, and prepared as follows:

[1] This material has been reviewed and there is no objection to publication by the Office of The Surgeon General, Department of the Army.

[2] From a thesis submitted to the Graduate School of the University of Maryland in partial fulfillment of the requirements for the degree of Master of Science.

Tryptose	20.0 g
Dibasic sodium phosphate	1.0 g
Sodium chloride	5.0 g
Bromcresol purple (1.6%)	0.5 ml
Water, distilled	1,000 ml

Autoclave the basic broth medium at 15 lb pressure for 15 min. Allow to cool and aseptically add:

	ml
Lactose (Difco concentrate, 10%)	10
Sucrose (Difco concentrate, 10%)	10

Dispense 2-ml aliquots into 13- x 100-mm test tubes and store in 4 C refrigerator.

Modified basic agar medium. In all experiments, Phenol Red Agar Base (PRAB), a product of the Baltimore Biological Laboratory, Inc., was used. The principal constituent of this medium is a casein peptone which is free of fermentable carbohydrates, and contains the following ingredients:

	g
Trypticase	10.0
Sodium chloride	5.0
Agar	15.0
Phenol red	0.018

For use, 30 g of the powdered medium were added to 1,000 ml of distilled water and dissolved with the aid of heat, followed by the addition of:

	g
Sodium thiosulfate	0.6
Ammonium ferrous sulfate	0.4

It was then sterilized in the autoclave under 15 lb pressure for 15 min. The final pH was 7.4.

The modified PRAB medium provided a source of sulfur as well as a metallic salt which has resulted in a unique and apparently accurate method for the rapid detection of hydrogen sulfide. Kligler's iron agar was used as a control medium in conjunction with this modified PRAB medium throughout these experiments. It was selected because of its wide usage as a double sugar-iron substrate, and serves a purpose in conventional methodology similar to that of the modified PRAB medium in the paper disc-Petri dish technique reported here.

Scheme of identification employing paper disc-Petri dish technique. Initial isolation procedure was accomplished by streaking a loopful of fecal material which had been artificially seeded with various combinations of test cultures onto the surfaces of SS (*Salmonella-Shigella*) and MacConkey's agar plates. Following incubation for 15 hr at 37 C further identification was carried out according to the procedure outlined in figure 1. Three nonlactose-fermenting (colorless) colonies were picked with a sterile needle and transferred into 3 tubes each of lactose-sucrose booster broth which had been preheated to 37 C. Following inoculation, the booster broth cultures were replaced in a 37 C water bath for 2 hr, after which all tubes showi ng an acid reaction were discarded as non

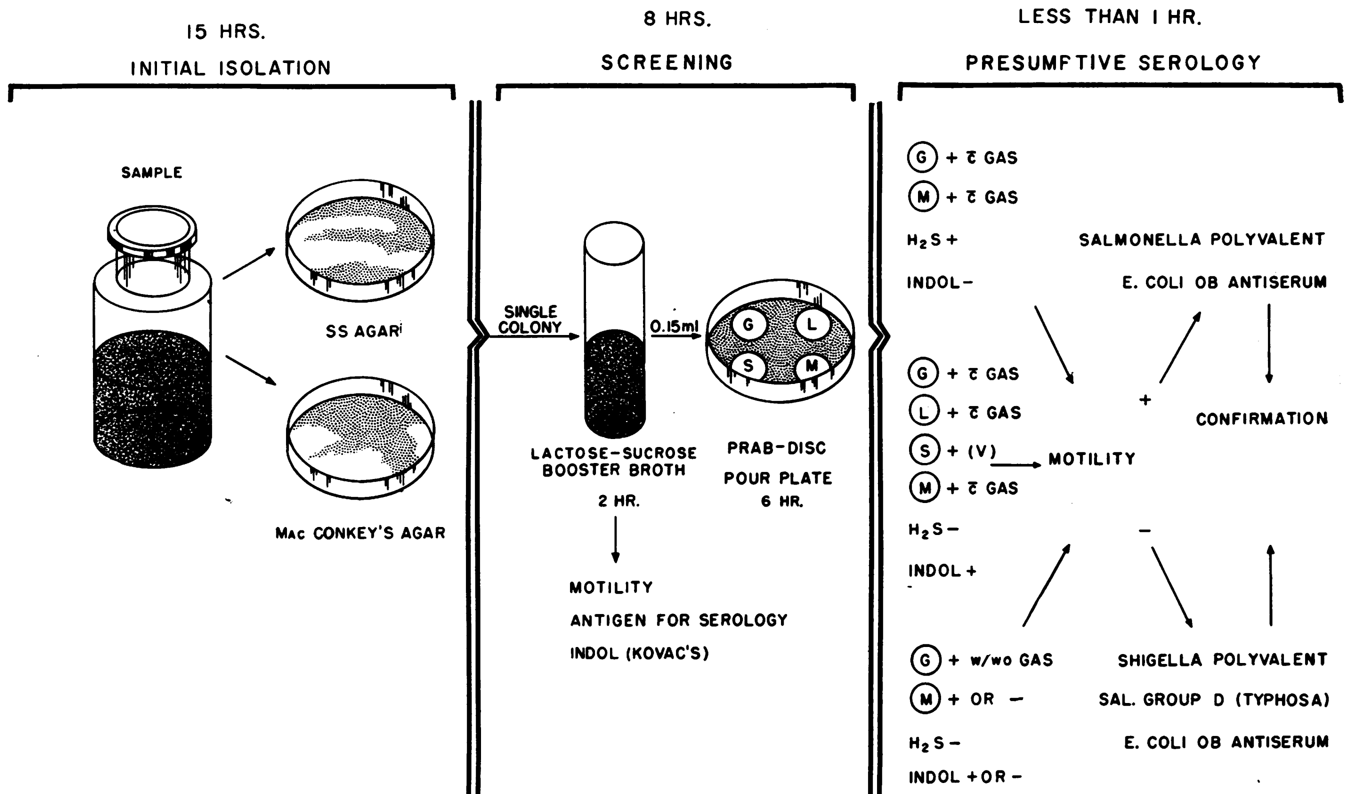

FIG. 1. Outline of simplified procedure for identification of enteric pathogens using paper discs

pathogens, that is, *Proteus*, *Aerobacter*, *Escherichia*, and so forth.

Pour plates were next prepared by inoculating 0.15 ml of each remaining alkaline booster broth into 25 ml of the melted modified basic medium (PRAB). After the seeded medium has hardened, four carbohydrate discs, each one containing a single sugar (G, glucose; L, lactose; S, sucrose; M, mannitol), were affixed one to each quadrant of the cultural surfaces. Reactions were recorded following 6 and 24 hr of incubation at 37 C.

Motility was determined by examining a drop of the alkaline booster broth cultures in a hanging drop preparation. At this time the booster broth cultures were also divided into two equal portions, one to be treated with Kovac's reagent for the indole reaction, while the other was retained for use as an antigen in the presumptive serologic tests.

Serologic screening was performed by placing a drop of appropriate polyvalent antiserum, diluted as indicated for the lot, on 1- x 3-in glass slides marked in approximately ½-in squares. Using a large loop, a single portion was transferred from the booster broth antigen and mixed directly into the diluted antiserum on the slide. For strongly positive reactions to occur rapidly, it was found necessary to prepare heavily turbid mixtures.

RESULTS

A total of 206 cultures representative of the *Enterobacteriaceae*, which included those pathogenic to man were used to check the reactions of this method. At least two serotypes of each serologic group within the salmonellae and shigellae were used. Three strains each of the several *E. coli* Group O cultures were also investigated; all were serotypes associated with diarrheal disease. Other cultures included members of the Alkalescens-Dispar O Group, Bethesda-Ballerup, *Paracolobactrum intermedium*, and *Escherichia freundii*. Cultures of *Alcaligenes faecalis*, *Proteus morganii*, and *Proteus vulgaris* were processed merely to check their reactions for comparison with those demonstrated by conventional methods. Reactions obtained employing these microorganisms, characteristic of their respective groups are shown in table 1. Excellent correlation was obtained with the control cultures on Kligler's iron agar in all tests.

Acid and acid and gas production. The fermentation of carbohydrates to produce acid and gas was readily apparent in the paper disc-Petri dish cultures after 6-hr incubation, and was manifested by development of a yellow zone(s) around and under the disc(s). The formation of gas, when present, was evidenced by the splitting of the medium within the acid or yellow zones (figure 2). Contrary to the experience of M. L.

TABLE 1. *A comparison of biochemical reactions obtained by paper disc-Petri dish technique with conventional Kligler's iron agar reactions*

Organism	Paper Disc-Petri Dish Tests					Kligler/Iron Agar Control Tests
	Glucose	Lactose	Sucrose	Mannitol	H_2S	
Salmonella						
typhimurium	AG/AG*	−/−	−/−	AG/AG	+/+	$-/\frac{AG}{+}$
typhosa	A/A	−/−	−/−	A/A	+/+	$-/\frac{A}{+}$
Shigella						
dysenteriae	A/A	−/−	−/−	−/−	−/−	$-/\frac{A}{-}$
flexneri	A/A	−/−	−/−	A/A	−/−	$-/\frac{A}{-}$
flexneri 6	AG/AG	−/−	−/−	AG/AG	−/−	$A/\frac{AG}{-}$
Escherichia coli 055	AG/AG	AG/AG	AG/AG	AG/AG	−/−	$A/\frac{AG}{-}$
Alkalescens-Dispar O1	A/A	−/−	−/−	A/A	−/−	$-/\frac{A}{-}$
Proteus						
morganii	A/A	−/−	−/−	−/−	−/−	$-/\frac{A}{-}$
vulgaris	AG/AG	−/−	AG/AG	−/−	+/+	$+/\frac{AG}{+}$
Alcaligenes faecalis	−/−	−/−	−/−	−/−	−/−	−/−
Pseudomonas aeruginosa	−/−	−/−	−/−	−/−	−/−	−/−

* AG/AG: Acid and gas reactions recorded at 6 and 24 hr, respectively. A/AG: Acid reaction recorded at 6 hr; acid and gas, H_2S positive at 24 hr.

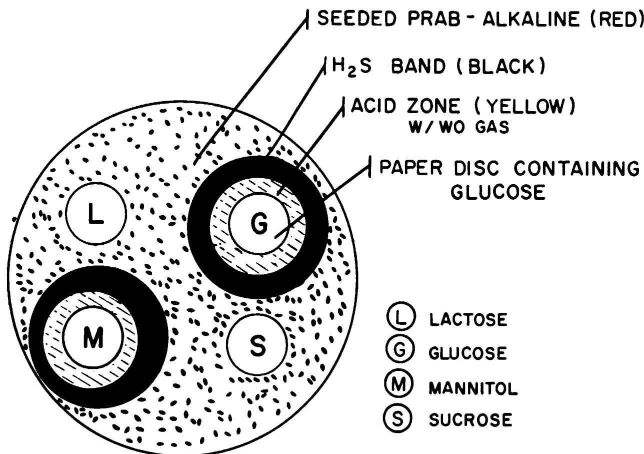

FIG. 2. Configuration of paper disc-Petri dish culture reactions.

Snyder (personal communication), no difficulty was encountered in the detection of gas, and no failures were recorded with the numerous aerogenic strains tested. Similarly, among the anaerogenic shigellae, a strain of *Shigella flexneri* 6, the single gas-producing member, was detected with ease after 6-hr incubation at 37 C.

Complete reversal of the acid zones never occurred before 36 hr, although it took place rapidly thereafter. However, such reversal of reaction did not prevent detection of delayed acid formation by the late lactose fermenting strains of *E. coli*, which sometimes did not occur before 5 days. Plates held as long as 7 days displayed no evidence of gross contamination or marked dehydration of the medium.

Kligler's iron agar was used as the control medium in conjunction with the experimental PRAB, and again excellent correlation was obtained after 24-hr incubation. However, only the PRAB medium was capable of demonstrating reliable results after 6-hr incubation.

Hydrogen sulfide production. The striking results obtained with known H_2S producing cultures were unexpected. It had been assumed that active H_2S producers would cause a blackening of the entire basic medium because of the fairly even distribution of bacteria throughout the poured plates. Conversely, the metallic sulfide indicative of H_2S production occurred only at the periphery of the acid zone(s) around the carbohydrate disc(s) which were fermented (figure 2). In no instance did it ever occur in the absence of acid formation.

The experimental PRAB medium used in the paper disc-Petri dish method was superior to Kligler's iron agar slants for H_2S detection. The remarkable difference between the two mediums was the ability of the PRAB medium to elicit the reaction in 6 hr. Of interest also was the observation that several strains of *Salmonella typhosa* failed to produce H_2S gas in the presence of glucose, although they actively formed it in the presence of mannitol.

Motility and indole reaction. The booster-screening broth proved to be a satisfactory vehicle for the motility and indole determinations. Motility was quickly determined by examining a drop of the broth culture in a hanging drop preparation following the initial 2-hr incubation period. At this time 1 ml of the remaining alkaline broth cultures was treated with 0.5 ml of Kovac's reagent, and the indole reactions were promptly detected, with no interference caused by the carbohydrates present in the booster-screening medium. In these studies, except among the shigellae, there appeared to be an inverse relationship between indole formation and H_2S production. Since a relationship of this kind is apparent when conventional methods are employed, it was regarded as a point of confirmation of the rapid technique employed here.

DISCUSSION

The paper disc-Petri dish method used in conjunction with a booster-screening system as described in this paper offers a practical method for the cultural differentiation of enteric pathogens in a short period of time. The basic medium (PRAB) containing sodium thiosulfate and ammonium ferrous sulfate used in conjunction with a set of four carbohydrate discs constitutes a simple and expeditious diagnostic system. It is one that could be utilized under the most adverse laboratory conditions without concern for problems such as preparation of fresh materials, and the exacting task of carbohydrate solution preparation and sterilization. If necessary, the paper disc-Petri dish method can be used independently of the lactose-sucrose booster broth without sacrificing speed or accuracy. The use of a single basic medium differs from the usual multiple sugar media in two respects: (a) It is a more simple matter to detect organisms of doubtful pathogenicity from information derived by using plates than from multiple sugar tubes, and to observe whether such organisms are occurring in a series of epidemiologically related cases; and (b) streaking for purity required in descriptive bacteriology can be eliminated by careful colony selection because impurity is more readily discernible on agar surfaces than in tubes of liquid media.

Paper discs proved to be an excellent vehicle for carbohydrates when used on the surfaces of seeded agar plates, although it was found important that they contain an adequate amount of sugar. When carbohydrates are placed in paper discs and used in this manner, each covering a small section of an agar plate, diffusion becomes an important factor. The detail, sharpness and size of the acid zone depends on a

balance determined by the amount of fermentable substrate, the rate of utilization, and the alkalinity produced by further growth of the culture. For reasons such as these, it was found necessary to use paper discs sufficiently large to contain a reservoir of sugar, thus preventing reversal of the acid reactions for as long as 36 hr.

The method outlined in figure 1 was found to be well suited for the practical application of the paper disc-Petri dish technique. As shown, the isolation-identification procedure is divided into three phases, namely, initial plating, screening, and presumptive serology. The time required for initial plating (18–24 hr) when conventional methods are used remains excessively long, and it is in this phase of diagnostic procedure where additional time can be saved. There is no contradictory information available and, indeed, Bicknell *et al.* (1952) have shown that nonlactose fermenting colonies may be picked from a selective medium in 10 to 12 hr following inoculation. In the course of the artificial seeding experiments conducted in this work, all initial isolations were performed after 15 hr of incubation, although growth was sufficiently heavy to have allowed colony picking earlier. Careful selection and picking of colonies was considered essential, and such practice insured the detection of multiple serotypes present in a single fecal sample. This has been shown to be of paramount importance in the effective management of epidemic enteric disease. Using conventional bacteriologic methods, such a practice would impose an almost impossible workload on well-staffed and -equipped laboratories.

The initial plating and screening phases of the method described have been successfully used in extensive field trials by A. V. Hardy (personal communication) and O. Felsenfeld (personal communication), but experience has shown a requirement for a biochemical screening system more rapid and accurate than those which are dependent on Kligler's iron agar. The results obtained in this study would indicate that the modified PRAB medium employed with a set of four carbohydrate discs admirably satisfies this need from both the standpoint of rapidity and accuracy.

Summary

A modified phenol red agar base medium and a new method for the rapid characterization of enteric pathogens which combines the desirable features of a booster-screening system with the demonstrated utility of a paper disc-Petri dish technique are described.

A new method for the rapid detection of hydrogen sulfide has been demonstrated.

The reactions of this medium and method were checked with 206 cultures, including selected pathogenic members of the *Enterobacteriaceae*. Excellent correlation was demonstrated between the reactions obtained by the experimental method and the control Kligler's iron agar medium.

Use of this paper disc-Petri dish technique provided for *complete* isolation and identification of enteric pathogens in less than 24 hr after receipt of the fecal sample.

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