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Rapid Microbiochemical Method for Presumptive Identification of Gastroenteritis-Associated Members of the Family *Enterobacteriaceae*

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A method for rapid screening of isolates of pathogenic members of the family *Enterobacteriaceae* is described. Flow charts are used in conjunction with triple sugar iron agar, *o*-nitrophenyl-β-D-galactopyranoside–phenylalanine-motility sulfate screening media, oxidase test, and six rapid biochemical tests, namely, lysine decarboxylase, urease, indole, esculin hydrolysis, malonate, and xylose. This scheme is used to provide an inexpensive but rapid presumptive identification of *Salmonella*, *Shigella*, *Edwardsiella*, *Aeromonas*, *Plesiomonas*, *Vibrio*, and *Yersinia* isolates from stool cultures.

In clinical bacteriology, rapid identification of bacteria that cause gastroenteritis is essential for diagnostic, epidemiological, and public health reasons. When large numbers of fecal specimens are processed by a laboratory, there is a need for rapid screening of negative specimens and confirmation of positive specimens. A methodology that can reduce turn-around time by 1 day is therefore extremely useful.

In our laboratories, the search for enteric pathogens consists of initial screening by a two-tube method, triple sugar iron (TSI) agar and *o*-nitrophenyl-β-D-galactopyranoside–phenylalanine motility (ONPG-PA-M) sulfate agar (13), before confirmation by additional biochemical and serological testing.

A 4-h rapid microbiochemical (RM) method is described for presumptive identification of pathogenic enteric bacteria. This method, in which small amounts of media are used with large inocula of bacteria, is based on methods previously described for the identification of *Neisseria gonorrhoeae*, *Gardnerella vaginalis*, and corynebacteria (14, 16, 17).

We have also proposed a series of flow charts, based on key reactions, for using the RM tests to select appropriate further tests for each isolate. This approach is similar to that used by Shayegani et al. (11) for identifying nonfermentative gram-negative bacilli. The chart design is similar to that used by Ellner et al. (5) for identification of anaerobes.

This method is intended to screen for species of *Salmonella*, *Shigella*, *Edwardsiella*, *Aeromonas*, *Plesiomonas*, *Vibrio*, and *Yersinia* in fecal samples. It is not intended for complete identification, nor is it intended for identification of organisms from other types of specimens.

MATERIALS AND METHODS

Cultures. A total of 1,475 clinical isolates from stool samples were examined, including 626 *Salmonella*, 24 *Shigella*, 215 *Escherichia coli*, 73 *Enterobacter*, 62 *Proteus*, 35 *Providencia*, 320 *Citrobacter*, 36 *Klebsiella*, 23 *Serratia*, 1 *Edwardsiella tarda*, 1 *Vibrio cholerae*, 4 *Aeromonas hydrophila*, 2 *Plesiomonas shigelloides*, 46 *Yersinia*, and 7 *Arizona* (*Salmonella* subgenus III) isolates. In addition, 24 stock cultures of enteric organisms were obtained from the Central Public Health Laboratory, Ontario Ministry of Health, Toronto, Canada.

All organisms were initially isolated on one or more of the following enteric isolation media: modified salmonella-shigella agar (Oxoid Ltd.), MacConkey agar (GIBCO Diagnostics), cefsulodin-irgasan-novobiocin agar (10), thiosulfate citrate bile sucrose cholera medium (Oxoid), and bismuth sulfite agar (Oxoid).

Single-colony isolates from enteric isolation media were inoculated into slants of TSI agar (GIBCO) and tubes of ONPG-PA-M sulfate medium (13), followed by incubation at 36°C for 18 to 24 h. Each isolate was identified by using API 20E (Analytab Products). Growth from the TSI slant was used to inoculate the following RM and conventional substrates: lysine decarboxylase, indole, urea, esculin, malonate, and xylose. Oxidase tests were performed with filter paper impregnated with a freshly prepared 1% solution of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma Chemical Co.). Inoculum from TSI agar was rubbed on the filter paper with a sterile platinum loop.

Salmonella serology was performed with omnivalent O + Vi and omnivalent H antisera obtained from the Enteric Reference Section, Central Public Health Laboratory, Toronto. The O + Vi antiserum contained O groups A to W as well as Vi antiserum. Slide agglutinations were performed in each antiserum and a phenolized saline control with growth from a TSI agar slant.

Preparation of RM media. Media for the RM biochemical tests were prepared as follows. Lysine decarboxylase broth contained 0.5 g of Moeller decarboxylase base (Difco Laboratories), 0.5 g of L-lysine hydrochloride (Sigma); and 25 ml of distilled water; pH was adjusted to 6.2 with 1 N NaOH. Decarboxylase control broth contained 0.5 g of Moeller decarboxylase base (Difco) and 25 ml of distilled water; pH was adjusted to 6.2 with 1 N NaOH. Tryptophane broth for indole determination contained 1 g of tryptose (Difco) and 25 ml of distilled water; pH was adjusted to 7.4 with 1 N NaOH. Urea broth contained 25 ml of "urea base" and 2.0 g of urea

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TABLE 1. Comparison of RM and conventional biochemical reactions of clinical isolates

Substrate	No. of specimens tested	No. (%) of reactions in agreement
Lysine	1,475	1,475 (100)
Urease	1,475	1,472 (99.8)
Indole	1,475	1,475 (100)
Esculin	1,122	1,122 (100)
Malonate	1,315	1,311 (99.7)
Xylose	1,122	1,116 (99.5)

(crystalline; Sigma); pH was adjusted to 7.0 with 1 N NaOH. (Urea base contained 0.01 g of yeast extract [Difco], 0.01 g of monopotassium phosphate, and 100 ml of distilled water.) Esculin broth contained 0.03 g of esculin hydrate crystalline (Sigma), 0.02 g of ferric citrate, 0.3 g of Bacto-Peptone (Difco), 0.17 g of sodium chloride, and 25 ml of distilled water; pH was adjusted to 7.2 with 1 N NaOH. Xylose broth contained 25 ml of RM base and 0.5 g of xylose (Sigma); pH was adjusted to 7.3 with 1 N sodium hydroxide (RM base contained 2.0 g of Casamino Acids [certified; Difco], 0.03 g of L-cysteine hydrochloride [Sigma], 0.03 g of sodium sulfate, 2.5 g of neopeptone [Difco], 0.1 g of phenol red, and 100 ml of distilled water). Malonate broth contained 0.2 g of malonate broth (Difco), 0.03 g of bromothymol blue, and 25 ml of distilled water.

All RM media were filter sterilized with a membrane filter (pore size, 0.2 μm; Nalgene small sterilization filter unit; Sybron/Nalge) and stored at 2 to 8°C in glass dropper bottles.

Preparation of conventional media. Lysine decarboxylase broth and decarboxylase control broth were made as described in the directions of the manufacturer with Moeller decarboxylase base (Difco) and L-lysine hydrochloride (Sigma). Tryptose broth for indole determination consisted of 10 g of tryptose (Difco), 5 g of sodium chloride, and 1,000 ml of distilled water. Urea agar slants consisted of urea agar base and 40% urea (Oxoid) and were made as described in the directions of the manufacturer. Esculin agar slants (15) consisted of 1 g of esculin (Sigma), 0.5 g of ferric citrate, 40 g of heart infusion agar (Difco), and 1,000 ml of distilled water. Xylose broth (15) consisted of 22.5 g of heart infusion broth (Difco), 10 g of xylose (Sigma), 0.02 g of bromocresol purple, and 1,000 ml of distilled water. Malonate broth (Difco) was made as described in the directions of the manufacturer.

Method of inoculation of RM substrates. Just before use, approximately 0.05 ml (1 drop) of each sterile medium was dispensed into sterilized microtubes (disposable borosilicate glass, 6 by 50 mm; Kimble). The tubes were placed in microtiter V plates (Dynatech Laboratories, Inc.), and the media were allowed to warm to room temperature (22°C)

TABLE 3. Guide for chart selection

TEST	Test results[a] indicating use of the following chart:					
	A	B	C	D	E	F
H_2S[b]	+	+	−	−	−	−
TSI						
Slant			A	A	K	K
Butt			A	A	A	A
Gas			−	+	+	−
PA	−	−	−	−	−	−
ONPG	−	+				

[a] Test results: A, acid; K, alkaline; +, positive; −, negative.
[b] H_2S production may be determined from TSI agar or ONPG-PA-M sulfate medium.

TABLE 2. Presumptive identification of 24 stock cultures by using the RM and flow chart method

Organism	Reactions in RM tests[a]						Flow chart result
	LYS	URE	IND	ESC	MAL	XYL	
Salmonella typhi	+	−	−	−	−	+	Possible *Salmonella* (*S. typhi*)
Salmonella typhimurium	+	−	−	−	−	+	Possible *Salmonella* sp.
Salmonella newport	+	−	−	−	−	+	Possible *Salmonella* sp.
Salmonella paratyphi A	−	−	−	−	−	−	Posssible *Salmonella paratyphi A*
Salmonella paratyphi B	+	−	−	−	−	−	Possible *Salmonella* sp.
Arizona sp.	+	−	−	−	+	+	Possible *Arizona* sp.
Shigella flexneri	−	−	+	−	−	−	Possible *Shigella* sp.
Shigella sonnei	−	−	−	−	−	−	Possible *Shigella* sp.
Yersinia enterocolitica	−	+	−	−	−	−	Possible *Yersinia* sp.
Yersinia intermedia	−	+	+	+	−	+	Possible *Yersinia* sp.
Yersinia frederiksenii	−	+	+	+	−	+	Possible *Yersinia* sp.
Yersinia kristensenii	−	+	−	−	−	+	Possible *Yersinia* sp.
Yersinia pseudotuberculosis	−	+	−	−	−	+	Possible *Yersinia* sp.
Escherichia coli	+	−	+	−	−	+	Discard
Proteus mirabilis	−	+	−	+	−	+	Discard
Providencia rettgeri	−	+	+	+	−	−	Discard
Providencia stuartii	−	+	+	+	−	−	Discard
Citrobacter freundii	−	−	−	−	+	+	Discard
Edwardsiella tarda	+	−	+	+	−	−	Possible *Edwardsiella* sp.
Enterobacter cloacae	−	−	−	+	+	+	Discard
Klebsiella pneumoniae	+	+	−	+	+	+	Discard
Serratia marcescens	+	−	−	+	−	−	Discard
Vibrio cholerae	+	−	+	−	−	−	Possible *Vibrio* sp.
Aeromonas hydrophila	−	−	+	+	−	−	Possible *Aeromonas* sp.

[a] Abbreviations: LYS, lysine decarboxylase; URE, urease; IND, indole; ESC, esculin; MAL, malonate; XYL, xylose; +, positive result; −, negative result.

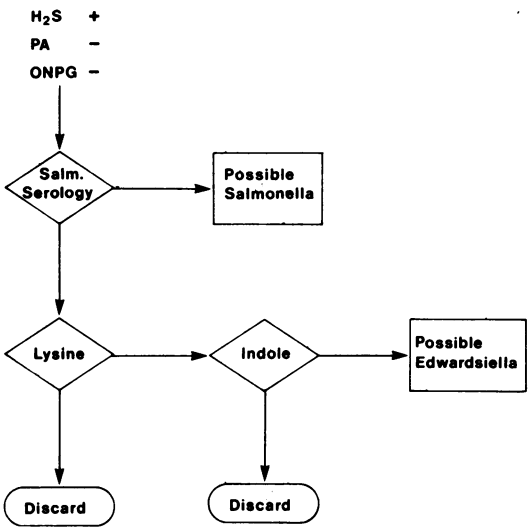

FIG. 1. Chart A. Scheme for organisms that are H_2S positive, PA negative, and ONPG negative. Symbols: →, positive result; ↓, negative result; ◇, test (as indicated); (Discard), no further identification necessary (nonpathogenic organism); ▭, further identification necessary.

before inoculation. One-third of a loopful (loop diameter, 3 mm) of pure growth of each isolate (grown for 18 to 24 h on TSI agar slants) was inoculated into each medium. Thorough mixing of inoculum and substrate was necessary to quicken the reaction time. After inoculation, the media in the urea and decarboxylase tubes were overlaid with 2 drops of sterile mineral oil. The inoculated tubes were incubated in a shallow water bath at 36°C.

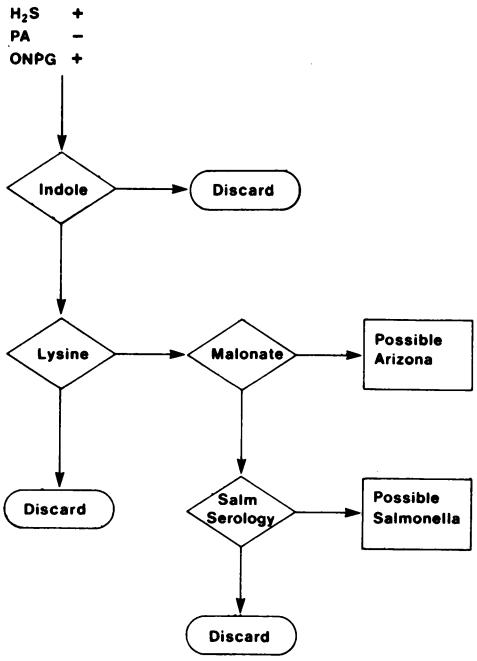

FIG. 2. Chart B. Scheme for organisms that are H_2S positive, PA negative, and ONPG positive. Symbols are described in the legend to Fig. 1.

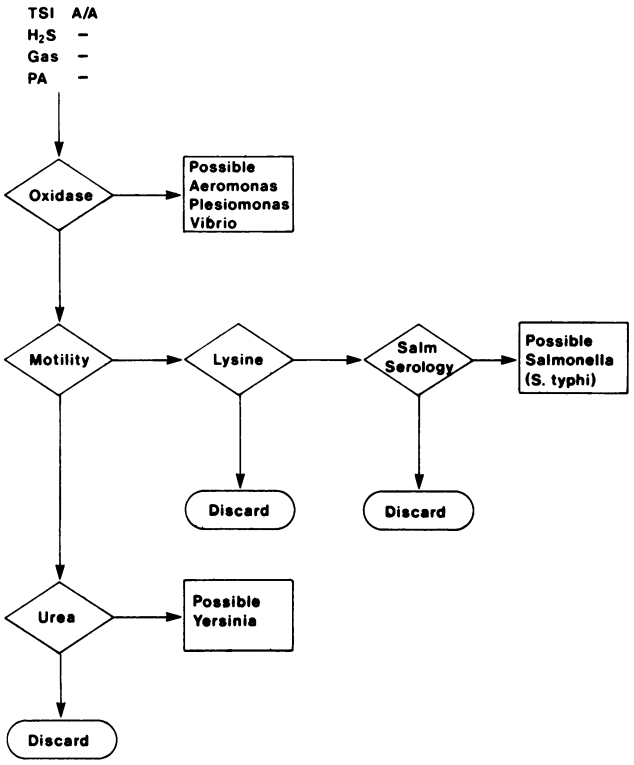

FIG. 3. Chart C. Scheme for organisms with an acid slant, an acid butt without gas in TSI agar, and H_2S-negative and PA-negative results. Symbols are described in the legend to Fig. 1.

Lysine decarboxylase results were read after 2 h of incubation; a positive test result was indicated by a purple color, provided that the decarboxylase control tube was yellow. The indole result was read in 4 h. After the addition of one drop of Kovac reagent, a positive test produced a red color. All other test results were observed after 2 h, but tubes showing negative reactions were incubated for a total of 4 h. A positive urease test was indicated by a pink color. A positive malonate test was blue, and a negative test was green. In the esculin test, a positive result was indicated by development of a black color. A positive xylose test was yellow and a negative test was red.

RESULTS

Table 1 shows 99.8% agreement between test results obtained with conventional and RM methods in 7,984 individual tests. All RM reactions were complete within 4 h, although with most substrates, positive reactions were apparent within 2 h of inoculation. Urease, malonate, and xylose tests yielded more positive reactions in the conventional tests than in the RM tests, with three, four, and six discrepancies, respectively. Three *Klebsiella* isolates showed false-negative urease results; three *Escherichia coli*, two *Providencia*, and one *Serratia* isolate showed false-negative xylose results; and two *Klebsiella* and two *Enterobacter* isolates showed false-negative malonate results with RM media. Tests which were negative by the RM method but positive with conventional substrates were considered to be false-negative.

Table 2 shows the reactions of 24 stock cultures tested by the RM method. All RM results agreed with those of

conventional tests and with published results for each species (3, 4, 6).

All *Salmonella*, *Shigella*, *Edwardsiella tarda*, *Vibrio cholerae*, *Aeromonas hydrophila* group, *Plesiomonas shigelloides*, and *Yersinia enterocolitica* isolates were detected by this system. This included one lactose-positive stock culture of *Salmonella newport* that was H_2S negative in TSI agar.

DISCUSSION

Good correlation (99.8%) was demonstrated between the RM and conventional biochemical tests. There were discrepancies in 13 individual tests, with the RM tests appearing to be undersensitive in each instance. None of the discrepancies occurred in a test critical to the identification of the organism involved. Other papers have described rapid tests for screening of enteric pathogens (2, 9, 12), esculin tests (8), and identification schemes (7). Burns et al. (1) have proposed an extensive series of flow charts to aid in the identification of both fermentative and nonfermentative gram-negative bacteria. The proposed method facilitates the discarding of isolates which have no clinical relevance without extensive identification. In this regard, an important screening step is the use of a positive phenylalanine deaminase (PA) reaction to screen for *Proteus*, *Providencia*, and *Morganella* spp., which are discarded as normal enteric flora without further biochemical testing.

Table 3 directs the investigation into one of the flow charts based on reactions obtained in the preliminary screening media, TSI and ONPG-PA-M sulfate.

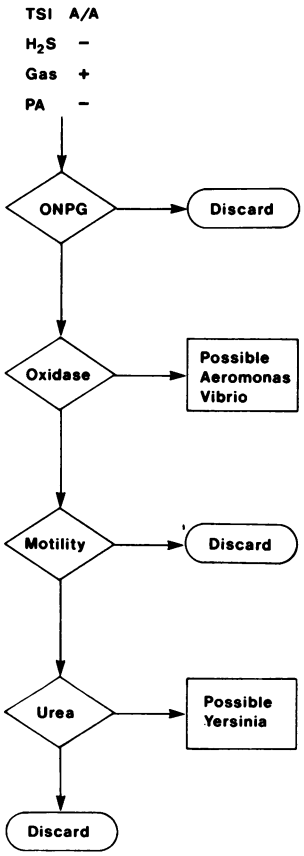

FIG. 4. Chart D. Scheme for organisms that have an acid slant, an acid butt with gas in TSI agar, and H_2S-negative and PA-negative results. Symbols are described in the legend to Fig. 1.

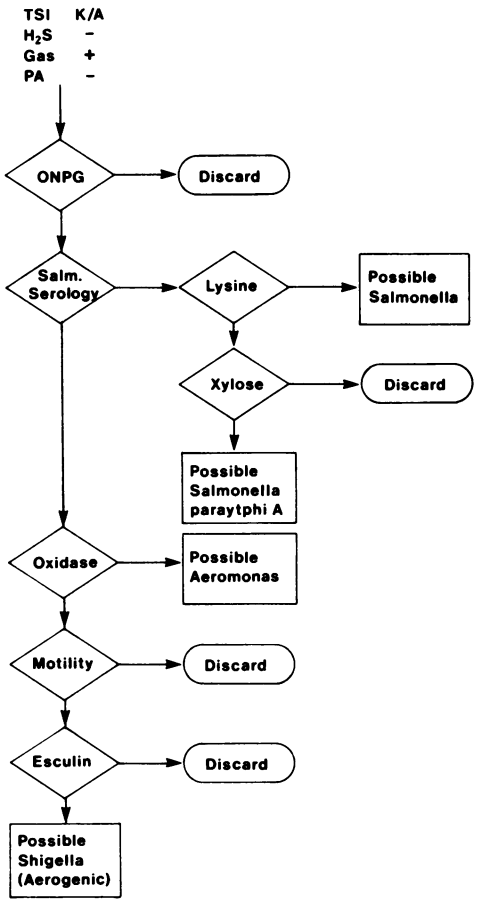

FIG. 5. Chart E. Scheme for organisms with an alkaline slant, an acid butt with gas in TSI agar, and H_2S-negative and PA-negative results. Symbols are described in the legend to Fig. 1.

Figure 1 is the simplest chart and would probably be the most extensively used in a laboratory processing a large number of *Salmonella* spp. isolates. The key is the same for all six flow charts. Each symbol gives processing directions. For each substrate all arrows from the right-hand apex represent positive reactions. The ovoid discard symbol means that the organism has been screened as a nonpathogen and no further identification is necessary. The rectangular identify symbol means that further identification is necessary to confirm the presumptive identification or to differentiate between several possibilities. Identification was performed by using API 20E during this project, but other systems or conventional testing could be used.

For these flow charts, the TSI reactions, PA, ONPG, and motility were all derived from the two media used for preliminary screening, TSI slants and ONPG-PA-M sulfate agar. Often, only these reactions would be necessary to produce a preliminary identification. When the charts indicated that more tests were necessary, the remaining substrates were inoculated simultaneously. The flow charts then provided an order in which to interpret test results.

Figures 2 through 6 present schemes of various complexities, but the directions for use remain the same.

Since the initial study, this scheme has been used routinely in one of our laboratories for 22 months. During this

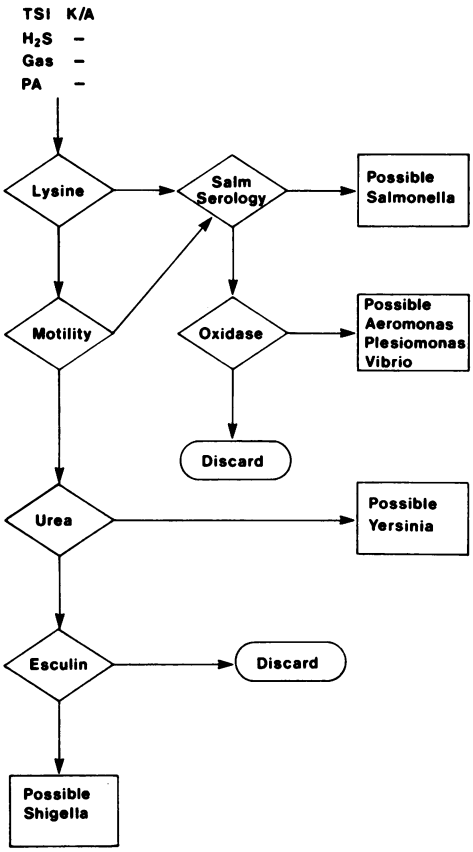

FIG. 6. Chart F. Scheme for organisms with an alkaline slant, an acid butt with no gas in TSI agar, H_2S-negative, and PA-negative results. Symbols are described in the legend to Fig. 1.

period, ca. 21,000 stool cultures were processed, resulting in the detection of the following pathogens: *Salmonella* spp. (1,712 isolates), *Shigella* spp. (19 isolates), *Yersinia enterocolitica* (63 isolates), and *Vibrio cholerae* (4 isolates). Sixty-seven isolates of nonpathogens which were not screened out by the flow chart scheme but required further identification by API 20E included *Citrobacter* spp. (28 isolates), anaerogenic *Escherichia coli* (38 isolates), and *Morganella morganii* (1 isolate).

These RM tests, when used with the flow chart scheme, provide a practical and economical diagnostic tool for dealing with routine enteric bacteriology.

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