Identification of *Enterobacteriaceae* in the Clinical Microbiology Laboratory

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A total of 5,137 enterobacterial clinical isolates were examined with three series of biochemical tests. The data were analyzed with respect to the use of economical and practical procedures for the accurate identification of lactose-fermenting and -nonfermenting *Enterobacteriaceae* within 24 hr after isolation.

Numerous authors have established various biochemical tests and procedures for the identification of clinically significant isolates of *Enterobacteriaceae* (7, 9, 11, 12, 13, 15, 17–21, 27, 29, 33, 35, 36, 40, 41). It has been repeatedly stressed that mere colonial inspection or abbreviated series of tests entail considerable error (33, 36). On the other hand, some of the tests employed are known to require extended periods of incubation, thus delaying laboratory reports considerably. The present study served to determine the minimal number of biochemical tests necessary for accurate identification of lactose-fermenting (LF) and late- or non-lactose-fermenting (NLF) enterobacterial isolates within 24 hr after isolation.

MATERIALS AND METHODS

A total of 5,137 *Enterobacteriaceae* from various clinical sources were isolated on blood-agar (Casman's base, Difco; 5% added sheep blood), choloralyzed blood-agar, MacConkey agar (with crystal violet; Fisher Scientific Co.), or Salmonella-Shigella agar (Difco), during the period of January 1969 through January 1970.

The isolates were processed as follows. The top portion of a single, well-isolated colony was inoculated into tubes containing 2.5 ml of Trypticase Soy Broth (TSB; BBL). After incubation for 3 to 5 hr at 35 C, the growth was inoculated into sets of media and substrates (Table 1), including Kliger iron agar (Difco) for NLF organisms, which were incubated at 35 C overnight. Sectors of MacConkey agar without crystal violet (Difco; Y plates) were streaked for control purposes. Gelatin strips were added to the remainder of the original growth in TSB. Swarming *Proteae* were examined for motility, H2S, indole, and ornithine decarboxylase (Table 1). Enteropathogenic *Escherichia coli* isolates were subcultured to blood-agar after preliminary serological screening, tested for indole, and serotyped for confirmation. Stool NLF isolates were inoculated into Kliger iron agar and urea-agar, after which suspected enteric pathogens were screened serologically; if reactive, the organisms were examined with the complete battery of tests for NLF isolates. Isolates of *Salmonella*, *Shigella*, and *Alkalescens-Dispar* were serotyped; the identity of the former two was confirmed by the North Carolina State Board of Health, Raleigh. To exclude *Aeromonas* spp., the spot-oxidase test (23) was performed by rubbing a loopful of growth from the decarboxylase control tube into Whatman no. 1 filter paper soaked with the reagent (tetramethyl-p-phenylene-diamine HCl; Eastman Organic Chemicals). The isolates were reported as species of *Enterobacteriaceae* (11, 14), unless specified otherwise.

RESULTS

A total of 1,453 isolates of *E. coli*, including anaerobic biotypes (*Alkalescens-Dispar*), were encountered, of which 75% promptly fermented lactose (Table 2). Forty-three *E. coli* isolates (2.9%) were citrate-positive. Faulty inoculation of Simmons' citrate agar slants probably accounted for the majority of false-positive results. It is possible that some of these isolates were, in fact, atypical *Enterobacter cloacae* (Padlew ska) (38; V. M. Young, D. M. Kenton, and B. J. Hobbs, Bacteriol. Proc., p. 106, 1968) since 16 of these organisms were lysine decarboxylase-negative and urease-negative, a finding compatible with atypical *E. cloacae*. The number of *Shigella* isolates (Table 2) was too small to warrant any comment. Thirty strains of *Salmonella* (serogroups B, C1, and D) were isolated during the course of this study; their biochemical reaction patterns were typical (12, 13, 15, 16). A moderate number of *Citrobacter freundii* isolates were encountered; the majority of these were prompt lactose fermenters (58%). These isolates yielded positive ornithine decarboxylase tests roughly twice as often as expected (8, 12). Only 6 of 114 isolates (5.3%) were H2S-negative. Four
of these were motile, methyl red (MR)-positive, citrate-positive, and ornithine decarboxylase-negative lactose fermenters; the remaining two isolates were NLF organisms yielding typical reaction patterns. Upon re-examination with the NLF battery of tests, the four LF isolates of C. freundii again were H2S-negative, but otherwise typical.

The reaction patterns obtained with Klebsiella are summarized in Table 3. Of 1,494 iso-

### Table 1. Tests performed for lactose-fermenting (LF), non-lactose-fermenting (NLF), and swarming Proteus enterobacterial isolates

| Test or substrate | Methods and references | Battery of tests employed for |
|-------------------|------------------------|-------------------------------|
|                   |                        | LF isolates | NLF isolates | Swarming protein |
| Gelatin liquefaction | Gelatin strips (Key Scientific Products Co., Los Angeles, Calif.) in Trypticase Soy Broth | x | x |
| H2S | Modified SIM medium (Difco; 33); Kovacs’ indole reagent added (22) | x | x | x |
| Motility | Same as for H2S | x | x | x |
| Indole | Same as for H2S | x | x | x |
| Methyl red | References 4, 33 | x | x |
| Voges-Proskauer | References 6, 33 | x | x |
| Citrate | Simmons’ citrate agar (Difco; 30) | x | x |
| Lysine decarboxylase | Reference 33 | x | x |
| Ornithine decarboxylase | Reference 33 | x | x | x |
| Decarboxylase control | Reference 33 | x | x | x |
| o-Nitrophenyl-β-D-galactoside | Reference 28 | x |
| Glucose | 1% carbohydrate/alcohol in Purple Broth base (Difco); Durham fermentation tube added | x |
| Arabinose | 1% carbohydrate/alcohol in Purple Broth base | x |
| Inositol | Same as for arabinose | x | x |
| Urease | Christensen’s urea agar (Difco; 3) | x |
| Phenylalanine deaminase | Phenylalanine agar (Difco); reagent added (11) | x |

### Table 2. Biochemical reactions of Escherichiae and Salmonelleae

| Test or substrate | E. coli (1,485 isolates: 1,082 LF, 371 NLF) | Shigella (9 isolates: all NLF) | Salmonella (30 isolates: all NLF) | C. freundii (114 isolates: 66 LF, 48 NLF) |
|-------------------|------------------------------------------|--------------------------------|---------------------------------|-----------------------------------|
|                   | No. | Per cent | No. | Per cent | No. | Per cent | No. | Per cent |
| Gelatin............. | 3   | 0.2      | 0   | 0        | 0   | 0        | 0   | 0        |
| H2S                | 0   | 0        | 0   | 0        | 30  | 100      | 108 | 94.7     |
| Motility............ | 1,104 | 76.1 | 0   | 0        | 30  | 100      | 109 | 94.7     |
| Indole.............. | 1,407 | 96.9 | 1   | 11.1     | 0   | 0        | 4   | 3.5      |
| Methyl red........... | 1,443 | 99.4 | 7   | 77.8     | 30  | 100      | 111 | 97.4     |
| Voges-Proskauer..... | 6   | 0.4      | 0   | 0        | 0   | 0        | 1   | 0.9      |
| Citrate.............. | 43  | 2.9      | 0   | 0        | 30  | 100      | 114 | 100      |
| Lysine decarboxylase | 1,104 | 76.1 | 0   | 0        | 30  | 100      | 109 | 94.7     |
| Ornithine decarboxylase | 1,040 | 71.1 | 6   | 66.7     | 28  | 93.3     | 32  | 28.1     |
| o-Nitrophenyl-β-D-galactoside | 358  | 96.5 | 6   | 66.7     | 0   | 0        | 37  | 77.1     |
| Glucose (acid + gas)a | 360  | 97.8 | 1   | 11.1     | 29  | 96.7     | 48  | 100      |
| Arabinosea........... | 367  | 99    | 7   | 77.8     | 29  | 96.7     | 47  | 97.9     |
| Inositol............. | 10   | 0.7    | 0   | 0        | 10  | 33.3     | 1   | 0.9      |
| Ureasea.............. | 30   | 0      | 0   | 0        | 0   | 0        | 42  | 87.5     |
| Phenylalanine deaminasea | 0   | 0      | 0   | 0        | 0   | 0        | 0   | 0        |

a Procedures used for NLF isolates.
Table 3. Biochemical reactions of Klebsiella

| Test or substrate          | K. pneumoniae (1,49) isolates: 1,149 LF | E. cloacae (249 isolates: 199 LF, 50 NL) | Atypical E. cloacae (44 isolates: 2 LF, 42 NL) | E. aerogenes (345 isolates: 108 LF, 237 NL) | E. liquifaciens (96 isolates: 21 LF, 71 NL) | E. hafniae (16 isolates: 11 NL) | S. marcescens (249 isolates: all NL) |
|----------------------------|---------------------------------------|------------------------------------------|-----------------------------------------------|--------------------------------------------|------------------------------------------|---------------------------------|----------------------------------|
| No. | Percent | No. | Percent | No. | Percent | No. | Percent | No. | Percent | No. | Percent | No. | Percent | No. | Percent |
| Gelatin                     | 18 1.2 | 0 0  | 0 0  | 3 0.9 | 96 100 | 0 0  | 248 99.6 |
| H2S                         | 0 0  | 0 0  | 0 0  | 0 0  | 0 0  | 0 0  | 0 0  |
| Motility                    | 0 0  | 241 96.7 | 43 97.7 | 325 94.2 | 94 97.9 | 15 93.7 | 247 99.2 |
| Indole                      | 147 9.8 | 0 0  | 44 100 | 0 0  | 0 0  | 0 0  | 3 1.2  |
| Methyl red                  | 66 4.2 | 4 1  | 44 100 | 0 0  | 0 0  | 0 0  | 2 12.5  |
| Voges-Proskauer             | 1,444 96.7 | 245 98.4 | 0 0  | 344 99.7 | 96 100 | 10 62.5 | 247 99.2 |
| Citrate                     | 1,490 99.7 | 249 100 | 44 100 | 344 99.7 | 96 100 | 12 75 | 249 100 |
| Lysine decarboxylase        | 1,410 94.4 | 0 0  | 0 0  | 345 100 | 12 12.5 | 16 100 | 249 100 |
| Ornithine decarboxylase     | 0 0  | 244 98 | 44 100 | 338 98 | 89 92.7 | 16 100 | 239 96 |
| a-Nitrophenyl-D-galactoside | 343 99.4 | 147 98 | 42 100 | 236 99.6 | 71 100 | 15 93.7 | 249 100 |
| Glucose (acid + gas)a       | 340 98.6 | 150 100 | 42 100 | 237 100 | 70 98.6 | 15 93.7 | 11 4.4 |
| Arabinosea                  | 342 99.1 | 146 97.3 | 42 100 | 235 99.2 | 70 98.6 | 16 100 | 0 0  |
| Inositol                    | 1,434 96 | 2 0.8 | 0 0  | 258 74.8 | 6 6.2 | 2 12.5 | 0 0  |
| Ureasea                     | 334 96.8 | 141 94 | 36 85.7 | 18 7.6 | 55 77.5 | 2 12.5 | 48.2  |
| Phenylalanine deaminasea    | 0 0  | 0 0  | 0 0  | 0 0  | 0 0  | 0 0  |

* Procedures used for NLF isolates.

* Less than 10% gas in Durham tube.

lates of K. pneumoniae, 345 were NLF organisms (23%); their biochemical behavior was typical (11, 13, 20, 24, 41). Only 11 of 345 strains (3.2%) were urease-negative (19, 20), and 147 isolates were indole-positive (9.8%). The 249 isolates of E. cloacae were unremarkable; 8 strains (3.3%) proved nonmotile but were positive for ornithine decarboxylase (26). Similarly, 20 of 345 (5.8%) isolates of E. aerogenes were nonmotile and ornithine decarboxylase-positive. All three gelatin-positive isolates of E. aerogenes produced a large volume of gas in glucose broth and fermented arabinose and inositol, thus ruling out Serratia marcescens. Forty-four isolates, two of which were prompt lactose fermenters, were identified as atypical E. cloacae; the reaction patterns agreed with those stated in the literature. A total of 96 strains of E. liquefaciens were isolated. Only 16 strains of E. hafniae and 25 strains of Pectobacterium were encountered during the course of this study. No pectate liquefaction tests were performed; thus, the designation of these latter isolates as Pectobacterium was not definitive, and the isolates are not listed in Table 3. The 249 strains of S. marcescens yielded typical reaction patterns; only 21 isolates (8.4%) were pigmented (2, 5, 39).

Approximately 75% of all clinical isolates of Proteus proved to be P. mirabilis (18, 34), as shown in Table 4. All of 115 strains of P. mirabilis that failed to swarm or that had been isolated on MacConkey agar exclusively were positive for phenylalanine deaminase and urease. Twelve ornithine decarboxylase-negative isolates of P. mirabilis (1.7%) were indole-negative and susceptible to ampicillin and cephalothin; 21 of 22 indole-positive strains of P. mirabilis were ornithine decarboxylase-positive and susceptible to these two antibiotics (J. M. Matsen, Bacteriol. Proc., p. 96, 1970), whereas the remaining isolate was ornithine decarboxylase-negative, H2S-positive, motile, and susceptible to ampicillin and cephalothin. Next in frequency were 171 isolates of P. morganii which yielded typical reactions with the exception of one phenylalanine deaminase-negative isolate (18, 32). The sole indole-negative strain was negative for gelatin, citrate, and H2S, positive for ornithine decarboxylase, and susceptible to ampicillin and cephalothin, thus raising the question as to whether this particular isolate might have been an atypical strain of P. mirabilis. Four of 52 isolates of P. vulgaris proved indole-negative, and one isolate was negative for phenylalanine deaminase (31); all were resistant to ampicillin, and all but two were not inhibited by cephalothin. The 24 isolates of P. rettgeri were typical biochemically. Of 38 isolates of Providencia, 23 fermented inositol, 10 pro-
TABLE 4. Biochemical reaction patterns of Proteaceae

| Test or substrate | P. vulgaris<sup>a</sup> (52 isolates, 33 swarming) | P. mirabilis<sup>a</sup> (726 isolates, 611 swarming) | P. morgani<sup>a</sup> (117 isolates) | P. retigeri<sup>a</sup> (24 isolates) | Providencia<sup>a</sup> (38 isolates) |
|------------------|--------------------------------------------------|--------------------------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|
|                  | No. | Per cent | No. | Per cent | No. | Per cent | No. | Per cent | No. | Per cent |
| Gelatin          | 10  | 58.8     | 96  | 83.4     | 0   | 0        | 0   | 0        | 0   | 0        |
| H<sub>2</sub>S<sup>b</sup> | 43  | 82.7     | 635 | 87.5     | 0   | 0        | 0   | 0        | 0   | 0        |
| Motility<sup>b</sup> | 50  | 96.2     | 709 | 97.7     | 168 | 98.2     | 23  | 95.8     | 16  | 42.1     |
| Indole           | 48  | 92.3     | 22  | 3        | 170 | 98.8     | 24  | 100      | 19  | 50       |
| Methyl red       | 13  | 76.4     | 43  | 37.4     | 161 | 94.2     | 23  | 95.8     | 22  | 57.9     |
| Voges-Proskauer  | 0   | 0        | 38  | 33       | 1   | 0.6      | 0   | 0        | 3   | 7.9      |
| Citrate          | 2   | 11.8     | 68  | 59.1     | 0   | 0        | 24  | 100      | 29  | 76.3     |
| Lysine decarboxylase | 0   | 0        | 1   | 0.9      | 2   | 1.2      | 0   | 0        | 0   | 0        |
| Ornithine decarboxylase<sup>b</sup> | 1   | 2.1      | 714 | 98.3     | 164 | 95.9     | 0   | 0        | 3   | 7.9      |
| o-Nitrophenyl-β-D-galactoside | 0   | 0        | 0   | 0        | 0   | 0        | 0   | 0        | 2   | 5.3      |
| Glucose (acid + gas) | 14  | 82.3     | 113 | 98.3     | 168 | 98.2     | 2   | 8.5      | 10  | 26.3     |
| Arabinose        | 2   | 11.8     | 5   | 4.3      | 2   | 1.2      | 1   | 4.2      | 8   | 21.1     |
| Inositol         | 1   | 5.9      | 1   | 0.9      | 2   | 1.2      | 7   | 29.2     | 23  | 60.5     |
| Urease           | 17  | 100      | 115 | 100      | 171 | 100      | 24  | 100      | 0   | 0        |
| Phenylalanine deaminase | 16  | 94.1     | 115 | 100      | 170 | 99.4     | 24  | 100      | 38  | 100      |

<sup>a</sup> Seventeen of 52 P. vulgaris and 115 of 726 P. mirabilis isolates did not swarm on primary isolation media.

<sup>b</sup> Procedures used for swarming Proteus.

duced gas in glucose, 2 were o-nitrophenyl-β-D-galactoside (ONPG)-positive, and 19 proved indole-positive; all were phenylalanine deaminase-positive and urease-negative. This permitted one to designate 23 and 10 isolates, respectively, as P. stuartii and P. alcalifaciens; the remaining 5 isolates were reported as Providencia.

One strain of K. ozaenae and one strain of Edwardsiella tarda were isolated during the 1-year study period.

DISCUSSION

The major aim of this study was to analyze the data obtained with different batteries of biochemical tests to determine the reliability and accuracy of the procedures with regard to the identification of enterobacterial isolates within 24 hr after isolation. An attempt was made to ascertain the minimal numbers of tests necessary to assure accurate identification of the isolates.

The data indicated that the tests employed for NLF isolates were adequate for the majority of isolated Enterobacteriaceae, with the exception of Pectobacterium. It is planned to substitute rhamnose for arabinose, in an effort further to facilitate the differentiation between E. cloacae and E. liquefaciens.

Examination of the data obtained for LF enterobacterial isolates permits the conclusion that inositol is unnecessary, as are the MR and Voges-Proskauer (VP) tests. One further purpose of this study was to speciate all isolates of Klebsiellaeae and to determine their disc susceptibility antibiograms (to be reported elsewhere). All species of Enterobacter yielded essentially identical antibiograms with the exception of atypical E. cloacae (Padlewskia); the antibiogram of this organism corresponded to that of K. pneumoniae (38). It was decided to discontinue the speciation of Enterobacter isolates and to delete gelatin, MR, VP, and lysine decarboxylase from the battery of tests for enterobacterial lactose fermenters. Thus, our present series of tests for lactose fermenters consists of the following: H<sub>2</sub>S, motility, indole, citrate, and ornithine decarboxylase. Those LF isolates that are H<sub>2</sub>S-negative, but positive for motility, indole, citrate, and ornithine decarboxylase are regarded as possible atypical E. cloacae, false citrate-positive E. coli, or atypical C. freundii, and are subsequently examined with the NLF battery of tests. The "HOC" scheme of Wolfe and Amsterdam (40) is sound; however, the three tests (H<sub>2</sub>S, ornithine decarboxylase, and citrate) employed would fail to differentiate among occasional H<sub>2</sub>S-negative, ornithine decarboxylase-positive C. freundii, false citrate-positive E. coli, Enterobacter, and atypical E. cloacae. Thus, the inclusion of tests for indole production and motility would reduce the number of "atypical" isolates. All strains of Arizona and...
more than 90% of *Salmonella* isolates are lysine decarboxylase-positive, whereas *C. freundii* invariably is lysine decarboxylase-negative (12). Thus, the exclusion of the lysine decarboxylase test from the LF battery of tests renders differentiation of H$_2$S-producing, ornithine decarboxylase-positive LF *C. freundii* from LF *Arizona* and extremely rare LF *Salmonella* isolates virtually impossible. Likewise, the "HOC" scheme would fail to differentiate among these organisms. On the other hand, not a single strain of *Arizona* or LF *Salmonella* was encountered among the enterobacterial isolates during the course of this study; this is possibly an indication of the relative scarcity of these organisms in the greater Winston-Salem area.

An additional 1,233 LF enterobacterial isolates were examined with the revised series of tests ("HOCIM" system); the results obtained were highly satisfactory (Table 5). Tests for citrate utilization were rigidly controlled, and all 650 isolates of *E. coli* were citrate-negative.

Currently, the so-called "MIO" medium (motility, indole, ornithine decarboxylase) is being evaluated with regard to its suitability for the routine identification of *Enterobacteriaceae* (10). Preliminary results appear promising.

The results obtained with swarming *Proteae* indicate that tests for indole are less reliable than tests for ornithine decarboxylase (Matsen, Bacteriol. Proc., p. 96, 1970); nevertheless, the battery of tests for "swarming Proteus" was discontinued for reasons of economy. Instead, swarming *Proteae* presently are spot-indole tested (37), with the use of the reagent *p*-dimethylaminocinnamaldehyde (25), and disc susceptibility antibiograms are performed. Any isolate that is spot-indole negative and resistant to ampicillin and cephalothin, or spot-indole positive and susceptible to the two antibiotics, is examined with the NLF battery of tests. Divergent results have been encountered only twice among 252 isolates of swarming *Proteus*; one isolate yielded a negative spot-indole but a positive tube-indole test, whereas the other isolate was tube-indole negative, yet spot-indole positive after subculture to blood-agar. Both isolates were susceptible to ampicillin and cephalothin. It appears that the use of Casman's base in blood-and chocolate-agar and this particular spot-indole reagent minimized the number of false-negative results. However, if a significant number of discrepant results should occur in the future (27), the original series of tests (H$_2$S, motility, indole and ornithine decarboxylase) could be reinstituted.

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**Table 5. Biochemical reaction patterns obtained with 1,233 additional LF enterobacterial isolates, by use of the "HOCIM" series of tests**

| Test or substrate                  | *E. coli* (650 isolates) | *K. pneumoniae* (46 isolates) | *Enterobacter spp.* (142 isolates) | *C. freundii* (25 isolates) |
|-----------------------------------|--------------------------|-------------------------------|-----------------------------------|-----------------------------|
|                                   | No. | Per cent | No. | Per cent | No. | Per cent | No. | Per cent |
| H$_2$S                            | 0   | 0        | 0   | 0        | 0   | 0        | 25  | 100      |
| Motility                          | 523 | 80.5     | 0   | 0        | 137 | 96.5     | 25  | 100      |
| Indole                            | 647 | 99.5     | 41  | 10.0     | 0   | 0        | 0   | 0        |
| Citrate                           | 0   | 0        | 416 | 100      | 142 | 100      | 24  | 96.0     |
| Ornithine decarboxylase           | 508 | 78.2     | 0   | 0        | 142 | 100      | 4   | 16.0     |
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