Numerical Diagnostic Key for the Identification of *Enterobacteriaceae*

HERMAN BAER AND LOUIS WASHINGTON

Department of Immunology and Medical Microbiology and Clinical Laboratories, University of Florida
College of Medicine, Gainesville, Florida 32601

Received for publication 2 June 1971

A numerical diagnostic key for enteric organisms is described which permits the identification of typical strains and of biochemical variants with high accuracy. Unknown strains are inoculated into a basic set of five media which permit the testing of eight biochemical reactions. The positive reactions are assigned points, and the score of a strain is added up, after which the identification of the strain is obtained from a table. In many instances, the final identification is obtained with this set of biochemical tests; and, in other instances, a small number of additional tests are required to distinguish between organisms giving the same score in the basic set of biochemical tests. The key permits an accurate, rapid, and economical differentiation of the typical and the more common atypical biotypes of enteric organisms in the clinical laboratory.

With the recently observed increase of hospital-acquired infections caused by enteric organisms, the need for a precise laboratory identification of these organisms is widely recognized. Biochemical differentiation of *Enterobacteriaceae* is usually achieved with the help of diagnostic tables or dichotomous keys (5). An interesting approach for the differentiation of enteric organisms and some other aerobic gram-negative bacteria was used by Fey (6), who designed a numerical diagnostic key, whereby the positive reactions of different biochemical tests are assigned points. The score of the strain tested is added up, and the diagnosis is obtained from a table. We have used Fey's diagnostic key in the Diagnostic Laboratory for several years with very satisfactory results. Extensive modifications of the design of the key became necessary, however, and this paper describes the key as it is presently used in our laboratory, together with an evaluation obtained by studying about 500 strains of enteric organisms both by this numerical key and according to the diagnostic tables of Edwards and Ewing (2) and Ewing (4).

**MATERIALS AND METHODS**

Most strains of gram-negative rods studied were isolated in the Clinical Microbiology Laboratories, Shands Teaching Hospital and Clinics, Gainesville, Fla., from a variety of clinical specimens. In addition, a number of stock strains from various sources were used. Single colonies from the eosine methylene blue (EMB) agar were used to perform the biochemical tests. Ewing's taxonomy and nomenclature for the *Enterobacteriaceae* were followed (3), and the biochemical tests were performed according to the procedures described by Edwards and Ewing (2), and Ewing (4). (Media were used obtained from Difco and BioQuest.) The method of Moeller was used for the degradation of amino acids, Christensen urea broth was used for the detection of urease activity, the fermentation of carbohydrates was tested in Phenol Red Broth, and SIM semisolid agar was used to test for motility and production of indole and hydrogen sulfide. Hydrogen sulfide production was verified on triple sugar-iron (TSI)-agar (2). Great care was used in the preparation of the KCN medium (2). The dispensed medium was stored no longer than 2 weeks, and the tubes were inoculated lightly.

**Description of the diagnostic key.** The key permits the identification of enteric organisms and some other gram-negative bacteria. Unknown strains are identified with the help of two sets of biochemical tests, called stage 1 and stage 2, respectively. The final identification of unknown strains is obtained either after incubation in the tests of stage 1 or after further testing in stage 2. Usually, this information is obtained within 24 or 48 hr.

**Stage 1.** The unknown strain is inoculated into five media: mannitol broth with a gas trap, lactose broth, SIM semisolid agar, urea broth, and KCN broth. This same set of biochemical reactions was used by Fey (6). Instead of recording the results of the reactions as positive or negative, the positive reactions, with the exception of H₂S production, are assigned points, as described by Fey (6) and shown in the footnote to Table 1. The points of a strain are added up, and the score is entered into Table 1. This table was originally constructed by Fey (6), and it
### Table 1. Diagnostic key for gram-negative rods: stage I

| Score* | Organism | Common | Less common | Confirmed by |
|--------|----------|--------|-------------|--------------|
| 0      | Nonfermentative organisms |         |             |              |
| 5      | Shigella sonnei |         | S. boydii   | Serology     |
|        | S. flexneri |         | Salmonella typhi | Serology     |
| 15     | *Escherichia coli* |         | S. flexneri (type 6) | Serology     |
| 25     | *E. coli* |         |             |              |
| 40     | *Alcalessens-Dispar* biotype |         | S. boydii   | Serology     |
| 55     | *E. coli* |         |             |              |
| 65     | *Alcalessens-Dispar* biotype |         |             |              |
| 75     | *E. coli* |         |             |              |
| 115    | *Klebsiella pneumoniae* |         |             |              |
| 160    | Nonfermentative organisms |         |             |              |
| 165    | *S. typhi* |         | Salmonella spp. (anaerogenic) | Serology     |
| 175    | *Salmonella* spp. |         | E. coli     | Serology +   |
|        | *Arizona hinshawii* |         | Citrobacter freundii | Stage II     |
| 195    | *E. coli* |         | Salmonella spp. | Serology +   |
|        | *A. hinshawii* |         | C. freundii  | Stage II     |
| 200    | *Edwardsiella tarda* |         |             | H₂S +        |
| 215    | *E. coli* |         |             |              |
| 225    | E. coli |         |             |              |
| 235    | Nonfermentative organisms |         |             |              |
| 240    | Nonfermentative organisms |         |             |              |
| 325    | *Enterobacter hafniae* |         | Serratia marcescens | Stage II     |
| 335    | K. ozanae |         | E. cloacae  | Stage II     |
| 345    | K. ozanae |         | E. aerogenes | Stage II     |
| 355    | K. pneumoniae |         | K. ozanae   | Stage II     |
|        | E. cloacae |         | E. aerogenes | Stage II     |
| 360    | *Providencia alcalifaciens* |         |             | Stage II     |
| 365    | *P. stuartii* |         |             |              |
| 400    | *K. pneumoniae* |         | Proteus mirabilis | Stage II     |
| 415    |         |         |             |              |
| 425    | K. pneumoniae |         | K. pneumoniae |         |
| 435    |         |         |             |              |
| 440    | *P. vulgaris* |         |             | Stage II     |
| 445    | P. morganii |         |             |              |
| 455    | P. rettgeri |         |             |              |
| 465    | P. rettgeri |         |             |              |
| 475    | K. pneumoniae |         |             | Stage II     |
| 480    | Nonfermentative organisms |         | P. mirabilis | Stage II     |
| 485    | S. marcescens |         | E. hafniae  | Stage II     |
| 495    | E. cloacae |         | Aeromonas    | Serology +   |
|        | E. aerogenes |         | A. hinshawii | Stage II     |
|        | C. freundii |         | E. liquefaciens |         |
| 505    | S. marcescens |         | E. hafniae  | Stage II     |
| 515    | E. cloacae |         | Aeromonas    | Serology +   |
|        | E. aerogenes |         | A. hinshawii | Stage II     |
|        | C. freundii |         | E. liquefaciens |         |
| 520    | *P. alcalifaciens* |         | P. vulgaris | Stage II     |
|        | *P. stuartii* |         |             |              |

* Scores of biochemical reactions of stage I: mannitol acid produced, 5; mannitol gas produced, 10; lactose acid produced, 20; indole produced, 40; urea hydrolyzed, 80; motility present, 160; KCN resistant, 320; H₂S produced, +.
has been extensively modified and updated by us on the basis of newer data (4). If only one organism is listed under a score in the table, the identification is final. For instance, a score of 235 is obtained only with a typical Escherichia coli. E. coli is also listed under the scores of 225 (gasless), 215 (lactose-negative), 195 (indole-negative), 75 (nonmotile), etc. The first column of the key lists the more commonly isolated biotypes; the second column lists the more unusual variants. Since in about 20% of the strains of Klebsiella pneumoniae, the fermentation of lactose or the hydrolysis of urea, or both, are positive only after 48 hr of incubation, the lactose or urea broth (or both) of strains giving scores of 335, 345, 355, 375, 395, 405, and 415, after overnight incubation are always incubated for another day.

**Stage 2.** If several organisms are listed under the same score in Table 1, additional biochemical tests are required. These tests of stage 2 are listed in Table 2. The data used in the construction of Table 2 were obtained from Edwards and Ewing (2) and Ewing (4).

Amino acid decarboxylase tests are read after overnight incubation. Clear cut positive or negative results are reported, whereas inconclusive tests are reincubated. Samples of the methyl red (MR) and Voges-Proskauer (VP) media are tested after overnight incubation, and all tests with indeterminate or negative results are reincubated. With most strains, conclusive results are obtained after overnight incubation, however.

Standard serological procedures are used if the biochemical tests indicate a Salmonella or Shigella (2, 4).

Most scores of 0, 160, 320, and 480 in stage 1 are obtained with nonfermentative organisms that belong to the groups Pseudomonas or Acinetobacter, among others. These strains are differentiated with the help of diagnostic tables (1, 7).

**RESULTS**

The accuracy and reproducibility of the results obtained with this diagnostic key were evaluated in two ways.

**Confirmation of strains presumptively identified in stage 1 and stage 2 by additional tests.** A total of 461 consecutive isolates from our clinical laboratories, presumptively identified with the numerical key, were tested further by using the tests described by Edwards and Ewing (2) and Ewing (4). There were 102 strains presumptively identified as lactose-positive E. coli, 30 as lactose-negative E. coli, 2 as anaerogenic E. coli (Alcaligenes-Dispar biotype), 4 as Citrobacter freundii, 1 as Edwardsiella tarda, 107 as K. pneumoniae, 6 as K. ozaenae, 38 as Enterobacter cloacae, 20 as E. aerogenes, 7 as E. hafniae, 1/ as Serratia marcescens, 76 as Proteus mirabilis, 14 as P. morganii, 14 as P. vulgaris, 4 as P. rettgeri, 15 as Providencia stuartii, and 9 as P. alcalifaciens. K. rhinoscleromatis and E. liquefaciens were not observed during the period of time for collection of these strains. Salmonella and Shigella strains were not included because the initial screening of stool cultures for these enteric pathogens is done on TSI agar and in urea broth.

The results of the presumptive identification of 456 strains were confirmed by the additional biochemical tests. One strain designated as E. cloacae on the basis of the presumptive results in the numerical key turned out to be an intermediary strain and could not be assigned to any species of Enterobacter or other group of enteric organisms with certainty. Four strains presumptively identified as P. mirabilis were found to belong to other species of Proteus.

**Identification of stock strains in stage 1 and stage 2.** It is possible that the evaluation described above was biased to a certain degree because the laboratory personnel doing the identification usually knew the results of the antibiotic sensitivity determinations which often provided a clue to the identity of the organisms. In cases in which the antibiotic sensitivity pattern showed a deviation from the one usually observed in a given species, the technologists may have repeated biochemical and sensitivity tests and thus eliminated a cer-
tain number of errors. This may have been notably the case in the *Klebsiella-Serratia-Enterobacter* group (8).

To eliminate this bias in the evaluation of the results obtained with this diagnostic key, 83 strains were coded and identified with the help of stage 1 and stage 2. Most of these strains were old stock strains, but some strains had been isolated in our laboratory, and their biochemical reactions had been studied extensively. The results of the antibiotic sensitivity tests were not known to the person who did the identification.

The following strains were used: *Shigella* group B and D (4); *E. coli* (12); anaerogenic *E. coli* (Alcalescens-Dispar biotype) (1); *Salmonella* groups A, B, C, D, and E (15); C. freundii (4); *K. pneumoniae* (6); *E. cloacae* (6); *E. aerogenes* (4); *E. hafniae* (2); *S. marcescens* (7); *P. mirabilis* (6); *P. vulgaris* (3); *P. morganii* (4); *P. rettgeri* (3); *P. alcalificiens* (3); *P. stuartii* (3).

Eighty-one strains were identified correctly by using stage 1 and stage 2. Two strains of
Klebsiella pneumoniae were misdiagnosed as Enterobacter aerogenes, because the motility test in the SIM media was interpreted incorrectly. When the motility test was repeated, the strains were clearly nonmotile. Both strains were ampicillin-resistant and cephalothin-sensitive so the error would have been detected, in all probability. This error could also be avoided by performing the ornithine decarboxylase test on all Klebsiella-Enterobacter strains, but, at the present time, we do not think that the number of possible errors would justify the delay in reporting the results on all Klebsiella strains for at least 24 hr.

DISCUSSION

The unique feature of this diagnostic key is the use of numerical values for the positive biochemical tests in the first stage of the identification. One advantage of this numerical system is that it saves time compared to the use of a diagnostic table, for instance. More important to us, however, seems to be the elimination of a considerable amount of uncertainty and ambiguity inherent in all diagnostic keys that list certain biochemical properties as variable. In this numerical key, biochemical variants are simply listed as different scores of stage 1. In general, biotypes of common organisms that are reported in the literature to occur with a frequency of about 3 to 5% were included; some variations that are rare according to the literature, but were found repeatedly among our isolates under our conditions of testing, i.e., nonmotile S. marcescens or nonmotile P. mirabilis, were added. It is easy to include other organisms or biotypes, if necessary. For instance, S. dysenteriae or the recently reported indole-positive E. cloacae (9) were not included, because we have no indication that these organisms do occur in our material, at the present time. On the other hand, when an unusual organism is isolated or when a score is obtained which is not listed in the key, additional biochemical reactions are done by using the standard tables (1, 2, 7).

In a formal evaluation of the accuracy of the results obtained with the key on over 500 strains, the generic identification was correct in all but two instances and the species was determined correctly in about 99% of the cases. It was found that all biochemical tests of stage 1 showed very good reproducibility with the exception of the KCN broth, in which a small number of false-positive and false-negative results was observed, even though the necessary precautions in the preparation, storage, and inoculation of the medium were strictly adhered to (2). To avoid diagnostic errors due to failures of the KCN test, these anticipated wrong results have been considered in the design of the key. For instance, a false-positive KCN may lead to the misdiagnosis of E. coli as Aeromonas liquefaciens (score 535), but the confirmation is done by performing the oxidase reaction. On the other hand, a false-negative KCN may cause an Enterobacter (score 495) to be misdiagnosed as a lactose-negative and indole-negative E. coli (score 175). It is, therefore, of paramount importance that all the tests of stage 2 are performed as required, and the results must be exactly as listed in Table 2. If deviations are observed, the strain is first checked for purity and then retested. If the results fail again, more extensive biochemical testing according to the standard tables mentioned above must be performed. This was found to be a rare event, however. Extensive testing is also performed if scores are observed in stage 1 which are not listed in Table 1.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service General Research Support Grant FR-05362-09 and by Training Grant STAI1-0128 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Cowan, S. T., and K. J. Steel. 1965. Manual for the identification of medical bacteria. Cambridge University Press, England.
2. Edwards, P. R., and W. H. Ewing. 1962. Identification of Enterobacteriaceae. Burgess Publishing Co., Minneapolis.
3. Ewing, W. H. 1966. Enterobacteriaceae: taxonomy and nomenclature. Communicable Disease Center, Atlanta.
4. Ewing, W. H. 1966. Lecture outline series. I. Isolation and identification of Enterobacteriaceae: principles and practice. II. Differential reactions of Enterobacteriaceae. III. Preliminary serologic examination of Salmonella and Shigella cultures. Communicable Disease Center, Atlanta.
5. Ewing, W. H., J. N. Willert, L. J. Kunz, M. Dumoff, and H. D. Isenberg. 1969. Roundtable: How far to go with Enterobacteriaceae? J. Infec. Dis. 119:217-215.
6. Fey, H. 1959. Differenzierungsschema fuer grammegative, aerobe Staebchen. Schweiz. Z. Pathol. Bakteriol. 22: 641-652.
7. King, E. O. 1967. Roundtable: current trends in diagnostic microbiology. The identification of unusual pathogenic gram-negative bacteria. Communicable Disease Center, Atlanta.
8. Ramirez, M. J. 1968. Differentiation of Klebsiella-Enterobacter (Aerobacter)-Serratia by biochemical tests and antibiotic susceptibility. Appl. Microbiol. 16:1548-1550.
9. Washington, J. A., III, P. Yu, and W. J. Martin. 1969. Biochemical and clinical characteristics and antibiotic susceptibility of atypical Enterobacter cloacae. Appl. Microbiol. 17:843-846.