Comparison of Conventional Methods, the R/B System, and Modified R/B System as Guides to the Major Divisions of Enterobacteriaceae

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Primary grouping of more than 2,000 members of Enterobacteriaceae, freshly isolated from clinical specimens, was performed on the basis of eight conventional laboratory tests used routinely in this laboratory and by the R/B system. Results indicate that both systems perform well in this first step in the identification of various medically significant species of the family. Difficulties encountered with certain reactions of the R/B system were corrected with a physical modification of the tubes. An additional 100 representatives of Enterobacteriaceae were compared with conventional methods and the modified R/B system. Results indicate improvement in observing and interpreting findings with the R/B system.

Several factors account for the mounting interest accorded the findings of clinical microbiological analyses in recent years. The waning efficiency of antimicrobial agents has persuaded the clinician to insist on guidance for specific therapy (1). The selective pressures generated by nonmedical as well as medical uses of these same drugs in the community and medical facilities have resulted in perplexing shifts within the human microbiota, with emphasis on groups of bacteria hitherto not considered significant or totally unknown in the intimate biosphere of man (6). The demand for very rapid recognition of significant bacteria in clinical specimens has created difficulties with the accepted but slow modalities used in most laboratories, a situation complicated still further by the paucity of personnel experienced and trained in clinical microbiology at the technical as well as the professional level. As a result, several simplified combinations of media have appeared with the objective of easing the workload while increasing the opportunity of recognizing medically significant bacteria. These innovations still require primary isolation on enrichment or selective media and stress the significance of colonial morphology as the first step in delineating the identity of an organism. The R/B system combines eight reactions, helpful in categorizing members of the family Enterobacteriaceae, into two tubes (7–9). Results should permit the grouping of various representatives of Enterobacteriaceae into categories such as is achieved by a series of conventional tests used for this purpose. This report is such a comparison using more than 2,000 members of the family Enterobacteriaceae isolated from various clinical materials.

MATERIALS AND METHODS

Primary isolation. All bacteria were isolated from clinical specimens which were screened by methods reported earlier (5). The sources of the specimens were as follows: nasopharynx, 282; ear and skin lesions, 126; urine, 579; blood, 6; other body fluids, 197; sputum, 129; feces, 431; eye, 11; postoperative wounds, 98; autopsy tissues, 162; stock collection, 11.

Conventional media for approximating enterobacterial classification. Isolated bacterial colonies on any of the selective or enriched agar media, the colonial morphology and reactions of which suggested that they belonged to the family Enterobacteriaceae, were transferred to tubes containing 2.0 ml of Trypticase soy broth (BBL) and were incubated for 2 to 4 hr at 35 C. Thereafter, they were inoculated as drops via sterile Pasteur pipettes onto triple sugar-iron-agar slants (TSI; BBL), tryptone broth (Difco), methyl red, Voges-Proskauer broth, Simmons citrate agar, urea broth, KCN, and malonate broths (all were BBL media prepared by manufacturer’s specifications and stored at 4 C for no longer than 5 days). TSI slants were streaked and stabbed; the citrate agar slant was streaked after the delivery of the preincubated drop. All eight tubes were incubated at 35 C for 18 hr and recorded; organisms were identified presumptively if possible or subcultured on further test media.

R/B tubes. These were procured manufactured from Diagnostic Research, Inc. (Roslyn, N.Y.) as the two tube system. Tube 1 contained appropriate media for recognizing bacterial activities such as phenylalanine
deaminase, lactose fermentation, gas production from glucose, lysine decarboxylase, and \( \text{H}_2\text{S} \) production. The second tube was used to elicited reactions such as motility, indole production, and ornithine decarboxylase. The manufacturer's instructions and admonitions about inoculation, loose capping, and incubation were adhered to meticulously.

Different technical personnel of the routine clinical microbiology laboratory performed all of the various tests. One technician examined some specimens first by one modality followed by examination by the other method; at other times one technician examined the organisms by both approaches simultaneously. Some 500 specimens were examined by two different members of the staff, each using one set of media; 500 additional specimens were examined by two persons, each using both approaches. Findings were recorded and interpreted for each condition entirely separately. Whenever discrepant results were encountered, a member of the research laboratory reexamined the cultures by reisolating the organism from the TSI slant and R/B tube 1 and performing all tests by both modes. The research staff also collated all of the findings and performed further identification studies.

The Beckford modification of the R/B tubes system became available for screening toward the close of the investigation and was substituted for the original R/B system in the comparison with conventional methods. This modification is physical, consisting of a constriction in the base of each of the tubes. This permits separation of the lysine medium in the first tube while permitting a decrease in the agar concentration of the medium in tube 2.

**RESULTS**

The conventional eight-tube system, employed routinely in this laboratory as the primary approximation of the medically significant genera and certain species of the family *Enterobacteriaceae*, yielded the results summarized in Table 1. The table lists, in percentages, the expected findings and the reactions obtained. The results reported are those found after 18 hr of incubation at the first reading and do not include corrections obtained after reexamination.

Generally, the findings were in agreement with Ewing's compilation (2), especially since results were recorded after 18 to 24 hr of incubation, eliminating the reported delayed positive reactions from consideration at the first attempt at categorizing these microorganisms. The greatest divergence from expected results in this conventional approach occurred with the cyanide findings. Agreement was only 50% with the four *Arizona* strains and 85.7% with the seven shigellae. Although *Arizona* somatic serogroup 21 tolerates KCN and Ewing reports 8.7% of this genus capable of growth, the two arizonae were KCN-intolerant on reexamination. The disagreement with the shigellae vis-a-vis KCN was resolved also when the bacterium yielding this false reaction was reexamined and found KCN-intolerant. As is customary in this laboratory, each examination is quality controlled daily with established positive and negative bacteria for each reaction. The control reactions for KCN were quite correct on the occasion of the first examination, and the erroneous results remain unexplained although they are quite probably an inoculum error. Another departure worthy of note is the urease reaction. This test is performed in liquid medium intentionally. This choice singles out the genus *Proteus*. Those members of the tribe Klebsiellae which elborate urease at a slower rate usually do not yield positive results in this broth medium during 18 hr of incubation. The response of *Enterobacter aerogenes* in urease broth as well as the *Enterobacter hafniae* indole reactions are findings which were confirmed several times on repeat examination. The only explanation one can proffer for these minimal disagreements with expected reaction patterns is that bacteria isolated from clinical specimens and examined for a few pertinent responses do not fall into categories as easily as do the strains which have been domesticated in laboratories for many years.

These eight conventional substrates measuring 11 bacterial reactions are obviously insufficient for speciating all of the medically significant members of the family *Enterobacteriaceae*. Results were read according to the scheme as shown in Fig. 1. These reactions can help to recognize some species and provide very presumptive evidence for others. However, in many instances additional reactions must be elicited before recognition of species or genus on the basis of biochemical and physiological characteristics can be achieved; additional work is influenced by considering the source of the specimen and the history of the patient (3).

Table 2 summarizes the findings achieved with the same bacteria tested with the original R/B tubes. Agreement between the reactions observed and expected was very acceptable. Indole-positive strains of *Citrobacter* were somewhat greater (12%) than the 6.7% reported by Ewing (2). The reaction obtained with the conventional indole procedure was within those limits (Table 1). Retesting of the indole-positive *Citrobacter* representatives by conventional methods confirmed this observation. Repeat examination of these bacteria with tube 2 of the R/B system indicated a very low level of indole production, suggesting greater sensitivity of the R/B system reaction although not ruling out the remote possibility that related pyrrole derivatives may account for this positive result (4). The negative findings with the indole reaction observed in the
**Table 1. Results obtained with conventional methods**

| Bacteria                  | No. of strains | TSI | Citrate | Urease | MR | VP | Indole | KCN | Malonate |
|---------------------------|----------------|-----|---------|--------|----|----|--------|-----|----------|
| *Escherichia coli*        | 430            | A/AG| -       | -      | +  | -  | -      | -   | -        |
|                           | 99.5           | 99.3| 100     | 99.8   | 99.8| 99.8| 97.0   | 99.5| 96.5     |
| *Shigella sp.*            | 7              | B/A | -       | -      | +  | -  | -      | -   | -        |
|                           | 100            | 100 | 100     | 100    | 100 | 100 | 85.7   | 100 | 100      |
| *Edwardsiella sp.*        | 3              | H2S | -       | -      | +  | -  | -      | -   | -        |
|                           | 100            | 100 | 100     | 100    | 100 | 100 | -      | -   | -        |
| *Salmonella sp.*          | 51             | d/H2S| +       | -      | -  | -  | -      | -   | -        |
|                           | 98.0           | 98.0| 100     | 100    | 100 | 98.0| 98.0   | 100 | 100      |
| *Arizona sp.*             | 4              | d/H2S| +       | -      | -  | -  | -      | -   | +        |
|                           | 100            | 100 | 100     | 100    | 100 | 100 | 50.00  | 100 | 100      |
| *Citrobacter sp.*         | 50             | H2S | +       | -      | -  | -  | -      | +   | -        |
|                           | 100            | 100 | 100     | 100    | 100 | 98.0| 98.0   | 100 | 100      |
| *Klebsiella pneumoniae*   | 326            | A/AG| -       | -      | +  | -  | -      | +   | -        |
|                           | 100            | 99.7| 94.5    | 99.4   | 99.7| 99.1| 99.7   | 97.2| 94.1     |
| *Enterobacter cloacae*    | 136            | A/AG| +       | -      | -  | +  | -      | +   | -        |
|                           | 100            | 99.3| 94.1    | 96.3   | 97.8| 97.8| 97.0   | 94.1| 97.0     |
| *E. aerogenes*            | 114            | A/AG| +       | -      | +  | -  | +      | -   | -        |
|                           | 100            | 99.1| 86.8    | 99.1   | 96.5| 99.1| 96.5   | 100 | 100      |
| *E. liquefaciens*         | 20             | A/AG| +       | -      | -  | +  | +      | -   | -        |
|                           | 95.00          | 95.00| 95.00  | 95.00  | 95.00| 95.00| 95.00  | 100 | 100      |
| *E. hafniae*              | 6              | A/AG| +       | -      | -  | +  | +      | -   | -        |
|                           | 100            | 100 | 100     | 100    | 100 | 83.3| 100    | 100 | 100      |
| *Serratia marcescens*     | 81             | A/AG| +       | -      | +  | -  | +      | -   | -        |
|                           | 100            | 98.8| 95.0    | 97.5   | 98.8| 100 | 95.0   | 100 | 100      |
| *Proteus mirabilis*       | 549            | H2S | +       | +      | -  | +  | -      | +   | -        |
|                           | 99.6           | 99.6| 100     | 99.8   | 100 | 99.8| 100    | 100 | 100      |
| *P. vulgaris*             | 21             | H2S | d       | +      | +  | -  | +      | -   | -        |
|                           | 100            | 100 | 100     | 100    | 100 | 100 | 95.2   | -   | -        |
| *P. morganii*             | 170            | B/AG| +       | -      | +  | -  | +      | -   | -        |
|                           | 96.5           | 99.4| 100     | 100    | 100 | 95.9| 100    | 98.8| 98.8     |
| *P. rettgeri*             | 8              | B/AG| +       | -      | +  | -  | +      | -   | -        |
|                           | 100            | 87.5| 87.5    | 100    | 100 | 100 | 100    | 100 | 100      |
| *Providencia sp.*         | 10             | B/AG| +       | -      | +  | -  | +      | -   | -        |
|                           | 100            | 100 | 100     | 90     | 90  | 90  | 100    | 100 | 100      |

*a* Abbreviations: A, acid; G, gas; B, alkaline or no change; d, variable; TSI, triple sugar-iron-agar; MR, methyl red; VP, Voges-Proskauer.

R/B system with *Proteus morganii* and *Providencia* were reexamined with conventional and R/B determinations. These repeat examinations were positive in both modalities. The original negative findings were in error, quite probably as a result of inadequate inoculation discussed below. This inadequate inoculation of tube 2 of the R/B system also accounts for the discrepancies observed in the ornithine decarboxylase reactions of *Enterobacter liquefaciens* and *E. hafniae*, since these tests were positive when repeated. Lack of motility found in the case of *E. aerogenes* and *E. liquefaciens* was confirmed by repeat examinations with the R/B system but was not substantiated by conventional means. The explanation of this observation is best coupled with the omission of the eighth test of the R/B system from Table 2, namely lactose fermentation. Evaluation of this indicator is not of great consequence in categorizing representatives of the family *Enterobacteriaceae* by the R/B system as seen in Fig. 2, which demonstrates the sequence of checking microbial reactions. Consideration of lactose fermentation is consequential only as a guide to *Enterobacter* classification. Nevertheless, reactions with lactose contrary to expectations upset the acceptance of any scheme for categorizing *Enterobacteriaceae*.

The recognition of these difficulties, especially the interaction between lysine decarboxylase and lactose fermentation end products and the occasional inability of the R/B system to demonstrate...
motility, led to the Beckford modification of the R/B tubes. This is a physical modification of the glass tubes carrying the media. It consists of a constriction at the lower end of tube 1 of the R/B system which isolates the lysine decarboxylase medium effectively from the other reactions occurring in this tube. The diffusion of end products is prevented completely. A similar constriction in tube 2 permits a reduction in the agar concentration which facilitates the macroscopic demonstration of motility.

This modified R/B system was used to screen an additional 100 bacteria, which on the basis of their colonial morphology belonged to the Enterobacteriaceae, as well as representatives from the collection used earlier in the trial. The conventional tests were performed simultaneously. The findings with these routine examinations did not differ from the findings described in Table 1. Results with the modified R/B system are summarized in Table 3 and indicate clearly that the modification has resulted in correcting the short-comings of the original approach. Especially noteworthy are the lactose reaction and motility readings, which conform with reactions based on Ewing's authoritative tables (2). The Escherichia coli studied contained anaerogenic and slow lactose-fermenting representatives. A Klebsiella pneumoniae strain also fermented lactose slowly. All other reactions did not deviate in the slightest from those expected. Other bacteria were also included primarily because they may be found in specimens harboring representatives of Enterobacteriaceae, grow on most

*At 37°C and 25°C

Fig. 1. Guide to the groups of Enterobacteriaceae with conventional procedures.
Enterobacteriaceae

Aeromonas sp. represented bacter of not certain members of the Enterobacteriaceae in if

Providencia sp., Pseudomonas sp., and certain members of the Enterobacteriaceae

Serratia, Proteus, Providencia, and Enterobacter are important members of the family Enterobacteriaceae in clinical laboratories. They are not representative of all members of the family Enterobacteriaceae.

**DISCUSSION**

The findings obtained in this study indicate that the R/B system, especially in its recently modified form, represents a real alternative for the first approach to the classification of the family Enterobacteriaceae in the clinical laboratory. It is of great importance to stress that this system does not differ in intent or achievement from the primary approximation of identity afforded by more established methods. The R/B system can guide the clinical microbiologist to the tribes, genera, and species of the family Enterobacteriaceae. This system, like all others, fails if it is applied to those representatives of the family which do not react typically vis-a-vis one or more of the eight reactions which constitute its spectrum. Like the conventional methods, tailored to the preference or conviction of the individual clinical microbiologist, the R/B system requires that certain bacteria be studied further. This is an accepted condition in all clinical microbiology laboratories which must balance the source of specimen, the past history of the patient, and the presumptive identity of the bacterium with the need to continue the work toward

**TABLE 2. Results obtained with the R/B system**

| Bacteria                        | No. of strains | Agreement found with expected responses (per cent) |
|--------------------------------|---------------|---------------------------------------------------|
|                                 | PAD | H₂S | Indole | Motility | LDC | ODC | Gas |
| Escherichia coli                | 430 | -   | +     | d        | d   | d   | +   |
| Shigella sp.                    | 7   | -   | -     | d        | d   | d   | -   |
| Edwardsiella sp.                | 3   | -   | +     | +        | +   | +   | +   |
| Salmonella sp.                  | 51  | 98.04 | 98.04 | 96.08 | 100 | 100 | 100 |
| Arizona sp.                     | 4   | 100  | 100   | 100     | 100 | 100 | 100 |
| Citrobacter sp.                 | 50  | 100  | 100   | 100     | 100 | 100 | 100 |
| Klebsiella sp.                  | 326 | 100  | 100   | 99.38   | 95.70 | 98.46 | 99.38 |
| Enterobacter cloacae            | 136 | 100  | 100   | 97.79   | 96.32 | 100 | 100 |
| Enterobacter aerogenes          | 114 | 100  | 100   | 98.24   | 84.21 | 100 | 72.81 |
| Enterobacter liquefaciens       | 20  | 100  | 100   | 90.00   | 75.00 | 100 | 60.00 |
| Enterobacter hafniae            | 6   | 100  | 100   | 100     | 100 | 83.33 | 100 |
| Serratia marcescens             | 81  | 100  | 100   | 100     | 100 | 98.76 | 100 |
| Proteus mirabilis               | 549 | +    | +     | -       | +   | +   | +   |
| Proteus vulgaris                | 21  | 99.81 | 99.63 | 100     | 99.45 | 99.27 | 100 |
| Proteus morganii                | 170 | +    | +     | -       | +   | +   | +   |
| Proteus rettgeri                | 8   | 100  | 100   | 100     | 100 | 85.71 | 100 |
| Providencia sp.                 | 10  | 100  | 100   | 100     | 100 | 100 | 100 |

Abbreviations: PAD, phenylalanine deaminase; LDC, lysine decarboxylase; ODC, ornithine decarboxylase.

if not all of the selective media used to separate this family, and in some instances approach certain members of the Enterobacteriaceae in colonial appearance. The bacteria included represented several Pseudomonas and Acinetobacter species, Yersinia enterocolitica, and Aeromonas sp. They were recognized as nonmembers of Enterobacteriaceae by both systems.
Identification (3). Like other methods, the R/B system depends on selective and enrichment media to separate the gram-negative rods on colonial morphology and interactions with media indicators to select suitable subjects for study, and on the skill of technical personnel to remove an adequate, uncontaminated portion of an isolated colony for the inoculation of its two tubes, an end achieved by using an inoculation needle bent back together on itself at its very end. Similarly, the instructions issued concerning access of air (by keeping the screw cap loosely closed after inoculation) must be heeded. The R/B system can achieve a first intimation of identity with but two tubes encompassing eight tests and bring this modality to clinical labora-
tories unable or unwilling to identify Enterobacteriaceae even to this extent by conventional methods. Undoubtedly, clinical microbiologists who have been speciating the medically significant representatives of Enterobacteriaceae shall continue to do so with biochemical, physiological, immunological, and even viral reagents. However, the growing awareness of the inadequacy of lactose-fermenting versus non-lactose-fermenting categories and the recognition of the role played in human disease by members of the family other than salmonellae and shigellae make the R/B system a valuable tool for every clinical laboratory charged with the responsibility of microbial diagnosis. The admonitions of O'Donnell et al. (8), that careful technique and judicious interpretation of results are required, cannot be emphasized often enough. Our findings fully support the results obtained by these investigators (8). One might add that control bacteria with known, stable reactions should be included often to insure proper technique and interpretation. The objections to the R/B system voiced by Martin et al. (7) must be considered in light of the bacteria studied, the tests performed with other systems, and the interpretation of R/B results. These investigators used indole-positive K. pneumoniae and Enterobacter cloacae, Aeromonas hydrophilia, and Pectobacterium carotovorum and identified these organisms as E. coli by using the R/B system. Certainly, bacteria which react in the manner of this collection present a challenge to any screening system. Their recognition requires many additional tests as well as a diagnostic acumen encountered with regrettable infrequency in the clinical laboratories. One might have expected a degree of suspicion on part of these investigators when considering the motility of Klebsiella and the lysine reactions of E. cloacae and Pectobacterium,
in addition to the latter's ornithine decarboxylase. These reactions might also have served to infer the possibility of the aeromonad, often distinguishable by its white pigmentation on isolation media. These investigators found also that two anaerogenic, nonmotile E. coli and one Klebsiella rhinoscleromatis were identified as shigellae.

No doubt the reactions of these bacteria suggest quite strongly the possible diagnosis of Shigella with any system. No initial screening system can be used to differentiate these bacteria, only to alert and guide additional testing including serological grouping. In all fairness, it must be stated that the literature originally supplied with the R/B system implied that the user could recognize various genera and species of Enterobacteriaceae and did not emphasize the preliminary nature of the R/B approximation. Also omitted were the various cautionary explanations concerning loose caps during incubation, glucose fermentation as a basic requirement almost unique to Enterobacteriaceae, and the lysine decarboxylase-lactose fermentation interplay as well as motility. The corrections in the instructions in the use of the R/B system as well as the recent modification in physical design of the tubes might have tempered the judgment of the Mayo group (7).

The original R/B system has been evaluated also by Smith and co-workers (9) at the Center for Disease Control. Using pure cultures submitted for identification to the Enteric Bacteriology Laboratory of the Microbiology Branch of CDC from all parts of the United States and from human, animal, and food sources, they tested the eight component R/B tubes exactly as prescribed supplementing the system when indicated with 10 additional tests suggested by the manufacturer. As comparison, 18 tests conventionally used at CDC, complemented by additional tests when indicated, were employed. Good correlation was obtained except with parameters such as gas from glucose, motility, and lysine decarboxylase. The diagnostic correlation in identifying 327 representatives of Enterobacteriaceae on biochemical grounds averaged 89% for the R/B system and 96.4% by the more extensive system conventional at CDC. The modified R/B system resulting in improved performance of lysine decarboxylase and motility tests as well as the ability to use lactose fermentation as part of the system, omitted from evaluation by Smith and co-workers as well as by us, ought to lead to an even better correlation. Certainly, the objectives of this governmental agency charged with the evaluation of a system are not identical to those of the clinical laboratories as the authors clearly indicate. The nature of their test material is also not identical to that received by a clinical laboratory.

Smith and his co-workers conclude that the greatest usefulness of the R/B system is as a screening device. The results obtained in this study have been predicated on the use of the system for precisely this purpose. The system can perform this function well. The improved system and more realistic instructions now provided make it possible for clinical laboratory personnel to place members of Enterobacteriaceae into proper general divisions, to test important organisms further biochemically or, with proper training, immunologically, and, if unable to carry out further testing, refer such cultures to the proper governmental agencies.

Experience with the more than 2,000 representatives of Enterobacteriaceae from clinical specimens indicates that the original R/B system performs comparably to the conventional system presently in use. Thus, it could be used as a guide or screening device for the major categories of medically significant members of the family. The improvement effected by the recent physical modification permits the recognition of several members of Enterobacteriaceae at the species level. Diagnostic speciation of those not categorized by eight biochemical reactions of the modified R/B system can be accomplished with but a few additional reactions. Thus, anaerogenic, nonmotile E. coli can be separated from the shigellae by the use of acetate. The genus Providencia may be distinguished from Proteus rettgeri by the absence of urease and may be differentiated into its species by the fermentation of inositol. Malonate or dulcitol can be used to separate arizonae from salmonellae. It is obvious from Fig. 2 that E. aerogenes, E. liquefaciens, E. hafniae, Serratia marcescens, and H3S-negative species of Salmonella require further testing for recognition at the species level. The guidance provided by the modified R/B system makes this a readily performed examination involving but four reactions, namely deoxyribonuclease production as well as sorbitol, raffinose, and rhamnose fermentation.

The R/B system resembles all of the other biochemical or physiological approaches to identification by its inability to accommodate those bacteria which do not react typically. Organisms with aberrant reaction patterns tend to confuse even the expert taxonomists. They are of great interest because they are misleading and difficult to recognize unless clinical microbiologists are made aware of their peculiarities. Usually they are isolated very infrequently. Their odd
reaction patterns can be detected and should alert personnel to perform whatever tests are required for identification if the source of specimen, the patient's history, or repeated recovery from the same institutional area warrant such an effort.

The R/B system is an alternative approach to grouping representatives of *Enterobacteriaceae* which requires a considerable effort on the part of those clinical microbiologists who, like ourselves, have accustomed themselves to tests such as those described as routine in this laboratory. Those among our technical staff not habituated to TSI, IMViC (indole, methyl red, Voges-Proskauer, citrate), KCN, etc., reactions accepted the R/B system much more readily, quite often because the latter constituted a considerable saving in time and effort in a busy laboratory and required fewer quality control measures. There was no difficulty encountered in training totally inexperienced college students or recent graduates in the performance of the tests and the interpretation of results. This latter observation deserves special emphasis in view of the growing demand for improved clinical microbiological analyses in laboratories and institutions lacking such services now. It is especially in these areas that the R/B system and similar kits can improve the rendering of microbiological analyses for the benefit of the patient, the clinician, the institution, and the community.

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