APPLIED MICROBIOLOGY, Dec. 1970, p. 880-883

Vol. 20, No. 6
Printed in U.S.A.

Identification of Members of the Family *Enterobacteriaceae* by the R-B System

WILLIAM JEFFERY MARTIN, ROBERT J. BIRK, PAULINE K. W. YU, AND JOHN A. WASHINGTON II

Mayo Clinic and Mayo Foundation, Department of Microbiology and Immunology, Rochester, Minnesota 55901

Received for publication 20 July 1970

The R-B system was evaluated in parallel with conventional bacteriological procedures for the identification of members of the family *Enterobacteriaceae* by using bacterial strains from a variety of clinical specimens and from stock cultures. The R-B tests found to be reliable were, in decreasing order, the reactions for phenylalanine deaminase, hydrogen sulfide, and indole, the production of gas from glucose, and the decarboxylation of ornithine. The reactions in the R-B system found to be unreliable were motility, the decarboxylation of lysine, and the fermentation of both glucose and lactose. In addition, the reactions of the R-B system were more difficult to read and interpret than those of the conventional system. On the basis of this evaluation, it was concluded that the R-B system is not an acceptable alternative to the conventional methods in the identification of the *Enterobacteriaceae*.

Recently, a two-tube composite test, designated R-B media (derived from the names of the coinventors, William Rollender and Orville Beckford), was made available for the identification of *Enterobacteriaceae*. This two-tube differential system was designed to permit the identification of the organism by the simultaneous study of eight biochemical reactions: production of gas from glucose, fermentation of lactose, decarboxylation of lysine, decarboxylation of ornithine, deamination of phenylalanine, motility, production of indole, and production of hydrogen sulfide. In previous reports, O'Donnell et al. (15) and Sellers, McCleskey, and Weddington (Bacteriol. Proc., p. 97, 1969) suggested that the R-B media method provides both a rapid and an acceptable alternative to conventional methods in the identification of *Enterobacteriaceae*. However, the 99% agreement found by O'Donnell et al. was with a "conventional system" made up of a relatively limited number of tests. Therefore, we compared the use of the R-B media with the conventional methods used in our laboratory; this report describes the results.

MATERIALS AND METHODS

With the exception of 47 stock cultures obtained from the Enteric Bacteriology Unit, Center for Disease Control, the specimens used for this study represent fresh clinical isolates that were selected at random (Tables 1 and 2). Methods for the isolation from clinical specimens of members of the family *Enterobacteriaceae* and other gram-negative bacteria have been described previously (3, 12).

The R-B media were purchased on the open market and used according to the directions of the manufacturer (Diagnostic Research, Inc., Roslyn, N.Y.). Tube 1, which provides the tests for phenylalanine deaminase, production of hydrogen sulfide, fermentation or gas production from glucose, fermentation of lactose, and lysine decarboxylase, was inoculated in the same manner as a triple sugar-iron (TSI) slant. This tube was incubated with the screw cap loosened for 18 to 24 hr at 37 C. Tube 2 provides the tests for the production of indole, motility, and ornithine decarboxylase; it was inoculated by a single-line stab directly after the inoculation of tube 1. The incubation of tube 2 was similar to that of tube 1 except that the screw cap was kept tightened.

The basic set of biochemical tests used routinely in this laboratory for each gram-negative colony consists of the following: TSI, lysine-iron agar (LIA), Christensen's urea agar, Simmons' citrate agar, peptone water, and ornithine decarboxylase (Moeller base) in 0.3% agar. Although some strains initially were identified with this basic set, additional biochemical tests, such as those described by Martin (12) and Washington, Yu, and Martin (18), were routinely incorporated in the identification process. For those strains that needed further study after inoculation in the R-B media, additional biochemical tests were also used. The decarboxylase tests were modified by the addition of 0.3% agar (8), which avoids the need for the oil overlay. The indole, methyl red, and Voges-Proskauer tests were modified by the method of Douglas and Washington (2). The nomenclature and biochemical reactions used in this

TABLE 1. *Source of isolates*

Source	No.
Sputum	21
Urine	50
Throat	13
Stool	7
Blood	4[a]
Bile	4
Abscess	4
Nose	3[a]
Gallbladder	2
Thumb	2
Thigh wound	2
Abdominal wound	2
Lung	2[a]
Thigh	1
Wound	1
Abdominal fluid	1
Abdominal wall	1
Vagina	1
Anal ulcer	1
Ischioanal region	1
Peritoneum	1
Incision	1
Peritracheal node	1
Left orbit	1
Epididymis	1
Chest wall	1
Brain	1[a]
Duodenal stoma	1
Animal source	1
Stock cultures[b]	47
Total	179

[a] One at autopsy.
[b] Center for Disease Control, Atlanta.

TABLE 2. *Summary of isolates used*

Isolate	No.
Escherichia coli	40
Shigella flexneri	4
S. sonnei	3
Edwardsiella tarda	2
Salmonella typhi	2
S. choleraesuis	1
S. enteritidis[a]	6[b]
Arizona hinshawii	4
Citrobacter freundii	11
Klebsiella pneumoniae	36
K. rhinoscleromatis	1
Enterobacter cloacae	11
E. aerogenes	6
E. hafniae	2
E. liquefaciens	2
Serratia marcescens	11
Pectobacterium carotovorum	1
Proteus vulgaris	3
P. mirabilis	7
P. morganii	10
P. rettgeri	3
Providencia alcalifaciens	5
P. stuartii	1
"Atypical" *E. cloacae*	2
Aeromonas hydrophilia	4
A. shigelloides	1
Total	179

[a] Nomenclature based on the three-species concept (13).
[b] One was bioserotype Paratyphi A, and five were other serotypes.

study were based on the taxonomic system of Ewing (5).

RESULTS

In our hands, agreement between tests was good with the hydrogen sulfide, indole, gas from glucose, and phenylalanine deaminase reactions (Table 3). Moderate agreement was noted with the ornithine decarboxylase test. Agreement was poor in the reactions for lactose and glucose fermentation, motility, and the decarboxylation of lysine.

Eighty-one of the strains (45%) were correctly identified by the R-B method. Of the remaining 98 strains (55%), 20 (11%) were identified incorrectly by this method. For example, 10 indole-positive *Klebsiella pneumoniae*, the 2 atypical *Enterobacter cloacae*, 1 *Aeromonas hydrophilia*, and the *Pectobacterium carotovorum* were all identified as *Escherichia coli* on R-B media. Two anaerogenic, nonmotile *E. coli* and one *K. rhinoscleromatis* were identified as shigellae. One indole-negative *E. coli* was identified as an *Enterobacter* species, whereas two strains of *E. liquefaciens* were identified on R-B media as *Klebsiella* and *E. cloacae*, respectively. Because of the lack of clear-cut reactions and because some of the strains were atypical in their biochemical reactions, 78 strains (44% of the total) could not be identified on R-B media with any degree of accuracy, 39 (22%) because of atypical strains or because test results on R-B media did not correlate with the conventional methods of testing and 39 (22%) because glucose was not fermented and subsequently had an effect on the other R-B tests.

DISCUSSION

Because of the demand for adequate bacteriological examination of clinical specimens for identification of members of the family *Enterobacteriaceae*, there is an apparent need for a simplified procedure that not only is rapid but also is as accurate and reliable as the conventional method. A number of test systems have

TABLE 3. *Comparison of conventional and R-B media test systems*

Test	No. of strains positive		No. of strains negative		No. of strains weak		No. of strains delayed positive		No. of strains questionable		Other	
	Conventional	R-B	Conventional	R-B	Conventional	R-B	Conventional	R-B	Conventional	R-B	Conventional	R-B
Lactose fermentation[a]	93	73	86	78								28[b]
Glucose												
Acid	179	139	0	37						3		
Gas[c]	69G	83G	48	53								
	62g	43g										
Hydrogen sulfide	27	27	151	152			1					
Ornithine motility	85	63	81	104	11	8			2	4		
Indole	71	65	103	108	5	4						2[d]
Lysine	95	47	78	109		5	6			17		1[e]
Ornithine	88	76	84	97			7			6		
Phenylalanine deaminase	28	28	149	151	2							

[a] Determined on TSI agar only in conventional system.
[b] Red slants due to deaminase activity.
[c] G, gas production >50%; g, gas production <50%.
[d] Both were weakly positive initially; on repeat test both were negative.
[e] No change; R-B lysine, as directed, not kept beyond 24 hr.

been designed in recent years with the goal of providing rapid and accurate identification of these bacteria with a minimum of time and personnel (1, 7, 14; W. J. Martin, S. F. Bartes, and M. M. Ball, Amer. J. Med. Technol., *in press*).

In attempts to achieve a simplified biochemical method for preliminary differentiation of members of the family *Enterobacteriaceae*, the use of composite media testing two or more biochemical or other characteristics in one culture tube has been advocated by several investigators. Russell (16) introduced the double-sugar medium for determination of fermentation reactions of glucose and lactose in the butt and on the slant of the medium, respectively. Many modifications of this medium have since been introduced, such as the addition of 1% sucrose (11) to exclude the so-called intermediates which have a greater preference for sucrose than for lactose when grown under aerobic conditions. Kligler (9) reported that the incorporation of lead acetate in Russell's double-sugar medium provided a method of differentiating *Salmonella typhi* and species of *Shigella*. The familiar TSI medium had its origin when Sulkin and Willett (17) described a triple sugar-ferrous sulfate medium for use in the identification of enteric organisms. Other composite media that have been used with varying degrees of success include the two-tube test originally described by Kohn (10) and later evaluated extensively by Gillies (6) and the LIA of Edwards and Fife (4).

The unreliable reactions in the R-B media yielded a large number of false-negative results. Such results were particularly noteworthy with the motility and lysine decarboxylase reactions. With the increasingly large number of strains, isolated from nosocomial infections, which belong to one of the four genera of the tribe *Klebsielleae*, it is of paramount importance that these bacteria be identified adequately, and the tests that are considered most useful in the identification of members belonging to the genus *Klebsiella* are motility and the decarboxylation of lysine and ornithine. O'Donnell et al. (15) also found that the motility reaction was unreliable in the R-B system and that the lysine decarboxylase reaction was difficult to read, especially with the anaerogenic bacteria; however, the latter reaction ceased to be a problem in their studies once they became used to the appearance of positive tubes. Generally, we could not become used to the appearance of a positive reaction on R-B media. Often we interpreted such reactions as negative. In certain instances, we encountered the same difficulty with the R-B ornithine decarboxylase reactions. Therefore, since experienced bacteriologists have trouble reading such reactions in the R-B system, lesser accuracy can be expected in those laboratories lacking trained personnel.

It was further noted by O'Donnell et al. (15) that the false-negative motility reactions in the R-B media did not interfere with the correct identification of members of the tribe *Klebsielleae* because other criteria were available. For exam-

ple, the ornithine decarboxylase reaction provides an additional test that is helpful in differentiating *Klebsiella* from *Enterobacter*. However, the marginal acceptability of this test in the R-B media (at least in this study) limits its usefulness in making this differentiation.

One aspect of this study that deserves particular attention is the large number of cultures (44%) that could not be categorized because they were either atypical in character or they did not ferment glucose. It could be argued that several of the strains shown in Table 2 were selected because of their atypical reactions or are isolated infrequently and therefore are not of much concern in the clinical laboratory. All but one (*P. carotovorum*) of the 20 strains that were identified incorrectly by the R-B method, however, were isolates of clinical significance, and the same was true for the 78 that were categorized as not fitting into any biochemical pattern.

Because ambiguity of reactions was noted on R-B media on several occasions, it was difficult to determine which additional tests to use with this system. In most cases, tubes of R-B media that yielded weak or ambiguous reactions had to be incubated for an additional 18 to 24 hr, even though this is contrary to the directions of the manufacturer. Therefore, we think that valuable time was lost in interpreting the results, whether additional biochemical tests were or were not needed for identification.

In our studies, the conventional system proved more versatile in providing preliminary information and thereby permitting changes to be made to complete the identification process. The shortcomings of the R-B system outweigh the advantages of its convenience.

LITERATURE CITED

1. Borchardt, K. A. 1968. Simplified method for identification of enteric and other gram-negative bacteria using reagent-impregnated strips. Amer. J. Clin. Pathol. 49:748–750.
2. Douglas, G. W., and J. A. Washington II. 1969. Identification of *Enterobacteriaceae* in the clinical laboratory. National Communicable Disease Center, Atlanta.
3. Edwards, P. R., and W. H. Ewing. 1962. Identification of *Enterobacteriaceae*, 2nd ed. Burgess Publishing Co., Minneapolis.
4. Edwards, P. R., and M. A. Fife. 1961. Lysine-iron agar in the detection of Arizona cultures. Appl. Microbiol. 9:478–480.
5. Ewing, W. H. 1968. Differentiation of Enterobacteriaceae by biochemical reactions. National Communicable Disease Center, Atlanta.
6. Gillies, R. R. 1956. An evaluation of two composite media for preliminary identification of *Shigella* and *Salmonella*. J. Clin. Pathol. 9:368–371.
7. Grunberg, E., E. Titsworth, G. Beskid, R. Cleeland, Jr., and W. F. Delorenzo. 1969. Efficiency of a multitest system (Enterotube) for rapid identification of *Enterobacteriaceae*. Appl. Microbiol. 18:207–213.
8. Johnson, J. G., L. J. Kunz, W. Barron, and W. H. Ewing. 1966. Biochemical differentiation of the *Enterobacteriaceae* with the aid of lysine-iron-agar. Appl. Microbiol. 14:212–217.
9. Kligler, I. J. 1918. Modifications of culture media used in the isolation and differentiation of typhoid, dysentery, and allied bacilli. J. Exp. Med. 28:319–322.
10. Kohn, J. 1954. A two-tube technique for the identification of organisms of the *Enterobacteriaceae* group. J. Pathol. Bacteriol. 67:286–288.
11. Krumwiede, C., Jr., and L. A. Kohn. 1917. A triple-sugar modification of the Russell double-sugar medium. J. Med. Res. 37:225–227.
12. Martin, W. J. 1970. *Enterobacteriaceae*, p. 151–174. *In* J. E. Blair, E. H. Lennette, and J. P. Truant (ed.), Manual of clinical microbiology. American Society for Microbiology, Bethesda, Md.
13. Martin, W. J., and W. H. Ewing. 1969. Prevalence of serotypes of *Salmonella*. Appl. Microbiol. 17:111–117.
14. Matsen, J. M., and J. C. Sherris. 1969. Comparative study of the efficacy of seven paper-reagent strips and conventional biochemical tests in identifying gram-negative organisms. Appl. Microbiol. 18:452–457.
15. O'Donnell, E. D., F. J. Kaufman, E. D. Longo, and P. D. Ellner. 1970. Evaluation of the R-B system for the identification of *Enterobacteriaceae*. Amer. J. Clin. Pathol. 53:145–148.
16. Russell, F. F. 1911. The isolation of typhoid bacilli from urine and feces with the description of a new double sugar tube medium. J. Med. Res. 25:217–229.
17. Sulkin, S. E., and J. C. Willett. 1940. A triple sugar-ferrous sulfate medium for use in identification of enteric organisms. J. Lab. Clin. Med. 25:649–653.
18. Washington, J. A., II, P. Yu, and W. J. Martin. 1969. Biochemical and clinical characteristics and antibiotic susceptibility of atypical *Enterobacter cloacae*. Appl. Microbiol. 17:843–846.