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Efficiency of a Multitest System (Enterotube) for Rapid Identification of *Enterobacteriaceae*

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Enterotube, a multiple-test system which combines nine biochemical tests useful in the identification of members of the family *Enterobacteriaceae*, was tested and compared with the PathoTec test system in the identification of gram-negative bacilli isolated from clinical specimens. It was found that both the Enterotube and the PathoTec systems rapidly and accurately defined the position of the organisms in the major groups of the family *Enterobacteriaceae*. Discrepancies were noted between the results of citrate tests on Simmons' citrate-agar and in the Enterotube, as well as between Simmons' citrate-agar and the PathoTec citrate test. It was concluded that the Enterotube system provides a simple, reliable, and rapid method for the presumptive identification of *Enterobacteriaceae*. The major advantage of the Enterotube is that all tests are done simultaneously by inoculation from a single isolated colony.

Rapid identification of gram-negative bacilli of the family *Enterobacteriaceae* isolated from clinical specimens has long posed a problem for the clinical laboratory. "The group to which an organism belongs must be determined by a combination of biochemical tests, not by a single property" [e.g., lactose fermentation (4)]. Various schema based on selected biochemical tests have been advocated to simplify the identification of *Enterobacteriaceae* (4, 5, 7, 8). Edwards and Ewing (4) list eight biochemical reactions which serve to separate members of the family into four principle divisions: *Shigella-Escherichia; Salmonella-Arizona-Citrobacter* (*Escherichia freundii*); *Klebsiella-Aerobacter-Serratia;* and *Proteus-Providencia* (*Proteus inconstans*). Seventeen additional tests for further categorizing the organisms are listed by these authors. Isenberg and Berkman (7) recommend a battery of 12 biochemical reactions which characterize the broad subdivisions within the *Enterobacteriaceae*. It is evident that the preparation of 12 to 25 individual cultures is costly not only in terms of time but also in terms of media preparation and equipment.

Sanders et al. (11) devised an enrichment broth which permits rapid detection of lactose and sucrose fermentation, indole production, and motility, as well as a phenol-red-iron-agar medium which, when used with carbohydrate-impregnated paper discs, provides a simple system for the detection of carbohydrate fermentation and hydrogen sulfide formation.

The use of the PathoTec test reagent-impregnated paper strips (General Diagnostics Div., Warner-Chilcott Laboratories, Morris Plains, N.J.) for the determination of certain biochemical tests—cytochrome oxidase, phenylalanine deaminase, urease, and lysine decarboxylase activity, indole production, and acetylmethylcarbinol production (Voges-Proskauer test)—has been reported (1, 6, 10). The test-paper strips eliminate the need for special media and can be stored indefinitely. However, since a heavy growth of the organism is needed for the performance of the tests, a 24-hr pure culture, preferably on solid medium free of indicators, is required.

A method which would reduce the number of inoculations and the equipment needed to perform a series of biochemical tests for the identification of *Enterobacteriaceae* would be of great benefit to the diagnostic bacteriologist. Recently a multiple-test system, Enterotube, which combines in a single unit nine of the biochemical tests most useful for the identification of members of the family *Enterobacteriaceae*, has been developed. This paper describes a study carried out to test the efficiency and accuracy of the Enterotube system in comparison with tests routinely used in this laboratory.

MATERIALS AND METHODS

Strains tested. Nine laboratory strains of representative gram-negative bacilli and 57 gram-negative strains isolated from specimens submitted for diagnostic bacteriology by the special treatment unit of Newark City Hospital and the Hoffmann-La Roche dispensary were studied. The clinical strains comprise isolates from 29 urines, 4 sputa, and 2 throat swabs.

Broth cultures of the known laboratory strains were first streaked on Trypticase Soy (TS; BBL) and EMB (Difco) agar to obtain isolated colonies. Clinical specimens were plated directly on TS, goat blood, and EMB agar for primary isolation of the organisms.

Enterotube. This is a prepared test system contained in a half-round, molded plastic tube which is divided into eight compartments, each containing a slant of a standard biochemical test medium. The flat side of the tube is sealed with a plastic film. A single wire, which serves as an inoculating needle, extends lengthwise through the center of the tube and projects from either end. The ends are covered by plastic screwcaps. Small air holes in the side of three compartments (citrate, lysine, lactose) are sealed by a removable "activator" strip which is peeled off at the time of inoculation to provide aerobic conditions during incubation.

To inoculate the Enterotube, the plastic caps are removed and the inoculating wire is touched to the center of an isolated colony of the organism to be tested. The wire is then withdrawn from the tube, thereby inoculating each of the media in the compartments. The wire may then be used to inoculate other test media such as broth or motility test medium. After the wire has been withdrawn from the Enterotube, the plastic caps are replaced and the activator strip removed. The test reactions are read after incubation for 18 to 24 hr at 37 C. After the reactions have been recorded, the tube may be reincubated to detect possible delayed reactions.

Media in the compartments and the reaction of each are listed below in the order in which they are contained in the tube starting from the first compartment inoculated. (i) Citrate agar: a positive reaction is shown by a change of the indicator, bromothymol blue, from green to blue. (ii) Modified lysine-lactose medium: a positive reaction is shown by the change of the indicator, phenol red, from yellow to pink. (iii) Lactose fermentation: the fermentation of lactose changes the indicator, phenol red, from pink to yellow. (iv) Dulcitol fermentation: the fermentation of dulcitol changes the indicator, phenol red, from pink to yellow. (v) Urea agar: the production of ammonia from urea is shown by a change of the indicator, phenol red, from yellow to pink. (vi) Phenylalanine deaminase: to test for phenylalanine deamination, approximately 0.3 ml of 10% ferric chloride solution is injected into the compartment through the plastic film by means of a syringe. This test must be performed after incubation for 18 to 24 hr. A positive reaction is indicated by the presence of a dark green color on the surface of the slant. (vii) Hydrogen sulfide-indole agar: hydrogen sulfide production is demonstrated by the formation of a black precipitate along the line of inoculation.

To detect the production of indole, approximately 0.3 ml of Kovac's reagent (9) is injected through the plastic film into the compartment by means of a syringe. In the presence of indole a cherry-red color develops. (The reagent used was prepared with *p*-dimethylaminobenzaldehyde, USP, obtained from Industrial Chemicals Division of Allied Chemical, Morristown, N.J.) (viii) Dextrose fermentation: the fermentation of dextrose changes the indicator, phenol red, from pink to yellow. All *Enterobacteriaceae* ferment dextrose promptly. Therefore, the failure of an organism to give a positive reaction in this test indicates either that the inoculum was not carried through all the compartments or that the organism is not a member of the family *Enterobacteriaceae*. The question of sufficient inoculum may be easily resolved if the inoculating wire is used to inoculate a broth or motility test medium when it is withdrawn from the Enterotube.

Paper strip tests (PathoTec). The growth of the organisms on TS-agar plates (18 to 24 hr) was used in the performance of the PathoTec tests according to the manufacturers directions.

RESULTS

Laboratory strains. The results of the tests with known strains of representative gram-negative bacilli are listed in Table 1. From the table, it may be seen that both the Enterotube tests and the PathoTec tests serve well in determining the position of the organisms studied in the major groups of the family *Enterobacteriaceae*. The classification of *Escherichia*, *Salmonella*, and *Proteus* groups by either method is clear cut. The general lack of biochemical activity of the indole-negative, dulcitol-negative *Shigella* strain tested is reflected in both test systems. In either case, motility studies would be necessary to classify the organism. A study of fermentation reactions, especially of dextrose and lactose, would also be necessary with the PathoTec system. *Klebsiella* are more readily classified by the Enterotube system than by the PathoTec system with which ancillary tests for motility and carbohydrate fermentation would be required to determine to which group the organism belonged. Nonpigmented strains of *Serratia*, although definitely falling into the *Klebsiella-Aerobacter-Serratia* division when tested by either method, would probably be classed as *Aerobacter* C by the Enterotube system. Motility, fermentation, and gelatin liquefaction studies would be required for a more definitive classification.

The pseudomonads were tested because they are clinically important gram-negative bacilli which might be confused with the *Enterobacteriaceae*. Although *Pseudomonas aeruginosa*

TABLE 1. *Comparison of Enterotube and PathoTec systems for the identification of gram-negative bacilli: representative laboratory strains*[a]

System	Biochemical test	*Shigella-Escherichia* division		*Salmonella-Arizona-Citrobacter* division[b]		*Klebsiella-Aerobacter-Serratia* division		*Proteus-Providencia* division[c]	*Pseudomonas*	
		Shigella flexneri	*Escherichia coli*	*Salmonella typhosa*	*Salmonella schotmuelleri*	*Klebsiella pneumoniae*	*Serratia marcescens*	*Proteus mirabilis*	*Pseudomonas aeruginosa*	*Pseudomonas pseudomallei*
Enterotube	Dextrose	+	+	+	+	+	+	+	?	±
	Hydrogen sulfide	−	−	+	+	−	−	−	−	−
	Indole	−	+	−	−	−	−	−	−	−
	Phenylalanine	−	−	−	−	−	−	+	−	−
	Urea	−	−	−	−	±	−	+	−	−
	Dulcitol	−	−	−	+	+	−	−	−	−
	Lactose	−	+	−	−	+	−	−	−	(+)
	Lysine	−	−	(+)	+	(±)	−	−	+	−
	Citrate	−	−	−	+	+	+	±	+	−
	Identification	*Shigella* or *Aerobacter* C	*Escherichia*	*Salmonella*	*Salmonella*	*Klebsiella*	*Aerobacter* C or *Serratia*	*P. mirabilis*	Non-*Enterobacteriaceae*	Non-*Enterobacteriaceae*
PathoTec	Cytochrome oxidase	−	−	−	−	−	−	−	+	+
	Indole	−	+	−	−	−	−	−	−	−
	Phenylalanine	−	−	−	−	−	−	+	−	−
	Urea	−	−	−	−	−	−	+	−	−
	Voges-Proskauer	−	−	−	−	+	+	−	−	−
	Lysine	−	−	+	+	−	+	−	−	−
	Citrate	−	−	−	+	+	+	+	+	−
	Identification	*Shigella* or *Aerobacter* C	*Escherichia*	*Salmonella*	*Salmonella*	*Klebsiella-Aerobacter-Serratia*	*Klebsiella-Aerobacter-Serratia*	*P. mirabilis*	*Pseudomonas*	*Pseudomonas*

[a] Symbols: +, positive; (+), delayed positive; ±, weakly positive; (±), delayed weakly positive; −, negative.
[b] *Escherichia freundii*.
[c] *Proteus inconstans*.

usually produces a characteristic pigment, nonpigmented strains are known to exist (12). *P. pseudomallei* may become slightly pigmented after several days of incubation, but the colonies of many strains are colorless on TS agar for the first 24 to 48 hr and may be colorless on EMB as well. The two pseudomonas strains tested gave equivocal results by the Enterotube system since the fermentation of dextrose was weak or questionable. The PathoTec CO test, on the other hand, immediately demonstrated that the organisms were pseudomonads.

Identification of clinical isolates. A total of 57 colonies of gram-negative bacilli suspected of being *Enterobacteriaceae* were isolated from clinical specimens and tested by the Enterotube system. The same isolates were tested by the PathoTec system after isolation in Sanders' Enrichment Medium (Difco) and plating on TS and EMB agar. Many strains were also tested on Kligler's Iron Agar (Difco) and Simmons' Citrate Agar (Difco) (Table 2). Employing the schema of Edwards and Ewing (4), 30 strains were classified as members of the *Shigella-*

TABLE 2. *Comparison of results with Enterotube and PathoTec tests in the identification of clinical isolates*[a]

Classification by Enterotube	PathoTec results	Other tests
30 *Shigella-Escherichia* division		
27 *Escherichia:* I +, PA −, Lact +	13 I +, PA −, C −, VP −	8 Simmons' −, 1 ±
15 C−	1 I +, PA −, C +, VP −	1 Simmons' −
	1 I +, PA −, C −, VP +	1 Simmons' −
4 C +, 8 C (+)	7 I +, PA −, C −, VP −	7 Simmons' −
	1 I −, PA −, C +, VP −	1 Simmons' +
	3 I +, PA −, C +, VP +	3 Simmons' +
	1 I +	1 Simmons' −
3 "Atypical" *Escherichia:* I +, PA −, Lact −, C −, Lys (+)	3 I +, PA−, C−, Lys +, VP −	3 Simmons' −, KIA Lact −, 2 EMB Lact +, 1 motile 1 EMB Lact −, motile
17 *Klebsiella-Aerobacter-Serratia* division		
3 *Klebsiella:* I −, PA −, Lact +, C +, Lys +, H_2S − [2 U +, 1 Dulcitol +]	1 I − PA −, C +, Lys −, VP +	1 Simmons' +, not motile
	1 C +, VP +	1 Simmons' +, not motile
	1 I +, C +, VP +	
4 *Aerobacter* A (*A. cloacae*): I −, PA −, Lact +, C +, Lys −, U +, H_2S −, Dulcitol −	3 I −, PA −, C +, Lys −, VP +, U ±	3 Simmons' +,
	1 I−, PA −, C +, Lys −, VP +, U −	1 Simmons' +
1 *Aerobacter* B (*A. aerogenes*): I −, PA −, Lact +, C +, Lys (±) U −, H_2S −, Dulcitol −	1 I −, PA −, C −, Lys −, VP +	1 Simmons' +, motile
9 *Aerobacter* C (*A. liquefaciens*): I −, PA −, U −, H_2S −, Dulcitol −		
[1 Lact −, Lys (+), C (+)]	1 I −, VP −, Lys +, C +	1 Simmons' +, motile
[1 Lact −, Lys −, C +]	1 I−, PA −, VP −, Lys +, C −, U−, CO −	1 Simmons' +, motile
[1 Lact (±), Lys (+), C +]	1 I −, PA −, VP +, Lys +, C +, U ±, CO −	1 Motile
[2 Lact +, Lys −, C −]	1 I −, PA −, VP −, Lys −, C−, U −, CO −	1 Simmons' (±)
	1 I −, VP +, C +, CO −	
[4 Lact −, Lys + or (±), C (+) or (±)]	4 CO +	
8 *Proteus-Providencia* division		
7 *Proteus mirabilis:* I −, H_2S +, PA +, U + Lact −, C + or (+)	2 I −, PA +, U +, C +	
	1 I −, PA +, U +, C−	
	3 I −, PA +	1 Simmons' ±, 1 −
	1 I −	
1 *P. rettgeri:* I +, H_2S ±, PA +, U +, C +	1 I −, PA −, U −, C +, CO +	
2 Indeterminant: dextrose +		
[1 Lact (+), all other tests −]	1 CO +	Mixed culture
[1 I +, H_2S +, PA +, Lact +, Lys −, C ±]	1 Lys +	Mixed culture

[a] Abbreviations: I, indole; PA, phenylalanine deaminase; U, urease; Lact, Lactose; Lys, lysine decarboxylase; C. citrate; VP, Voges-Proskauer; CO, cytochrome oxidase; KIA, Kliger's Iron Agar. Symbols-+, positive; (+), delayed positive; ±, weakly positive; (±), delayed weakly positive; −, negative

Escherichia division since they were indole-positive and phenylalanine deaminase-negative when tested by Enterotube. Of these strains, 27 were found to be *Escherichia* since they also fermented lactose in the *Enterotube*. This was confirmed by the demonstration of lactose fermentation on EMB agar. Of the 27 strains, 15 were citrate-negative in the Enterotube.

TABLE 3. *Correlation of the Enterotube and PathoTec tests*[a]

Test	Entero-tube positive	Entero-tube negative	Total agreeing
Indole	29/31	18/19	47/50
Phenylalanine deaminase	6/7	38/38	44/45
Urease	6/8	19/21	25/29
Lysine decarboxylase	7/10	22/26	29/36
Citrate	16/26	18/20	34/46

[a] Values are expressed as the number of PathoTec test in agreement/number tested.

The PathoTec test results with these 15 strains were in agreement with the Enterotube results except for one strain which was citrate-positive by the PathoTec test. Another strain, although PathoTec citrate-negative was Voges-Proskauer (VP)-positive. One of 11 strains tested grew on Simmons' citrate agar, although it was citrate-negative by both the Enterotube and PathoTec citrate tests.

The remaining 12 indole-positive, lactose-fermenting strains were found to be citrate-positive in the Enterotube. These characteristics indicated that the organisms were *E. intermedia* (2). However, seven of these strains were citrate-negative and VP-negative in PathoTec tests and failed to grow on Simmons' Citrate Agar. One strain was PathoTec and Simmons' citrate-positive but indole- and VP-negative by the PathoTec test. Three strains which were citrate- and VP-positive in the PathoTec tests grew on Simmons' Citrate Agar. The twelfth strain did not grow on Simmons' Citrate Agar. It was indole-positive in the PathoTec test but was not otherwise tested in this system.

Three indole-positive, phenylalanine deaminase-negative strains did not ferment lactose in the Enterotube or in Kligler's Iron agar. These strains were lysine decarboxylase-positive both in the Enterotube and the PathoTec test, indicating that the organisms were not *Shigella*. Lactose fermentation or motility was demonstrated by conventional methods and the organisms were classed as "atypical" *Escherichia*.

Seventeen isolates were placed in the *Klebsiella-Aerobacter-Serratia* division since they were indole-, phenylalanine deaminase-, and hydrogen sulfide-negative by the Enterotube tests. Eight strains which were lactose- and citrate-positive were classified on the basis of their reactions with lysine, dulcitol, and urease as *Klebsiella* (three strains), *Aerobacter* A (*A. cloacae*, four strains), or *Aerobacter* B (*A. aerogenes*, one strain). The PathoTec tests were in agreement except for one citrate test which was negative, although the strain was positive in the Enterotube and on Simmons' citrate agar. The remaining nine strains were classified as *Aerobacter* C (*A. liquefaciens*) since they displayed negative or delayed reactions with lactose, lysine, and urea or were citrate-negative. However, the PathoTec CO test demonstrated that four of the isolates were cytochrome oxidase-positive and thus were pseudomonads. The PathoTec test results with the remaining five strains identified as *Aerobacter* C by Enterotube also indicated that the organisms were *Aerobacter* C, although the citrate tests with two strains disagreed with the citrate tests in the Enterotube.

Eight isolates were placed in the *Proteus-Providencia* division since in the Enterotube they were phenylalanine deaminase- and urease-positive. Seven were identified as *Proteus mirabilis*, being indole-negative and hydrogen sulfide-positive. The PathoTec tests also identified these organisms as *P. mirabilis*. One isolate which appeared to be *P. rettgeri* from the Enterotube tests was found to be a *Pseudomonas* species when tested for oxidase activity with the PathoTec CO strip.

Two colonies which gave confusing, therefore indeterminant, results in Enterotube were found to be mixed cultures upon examination of TS agar plates streaked from the broths inoculated with the Enterotube wires.

When the results with individual tests represented in both systems were compared (Table 3), few discrepancies were noted in the indole, phenylalanine deaminase, and urease tests. In 3 of 50 indole tests, 1 of 45 phenylalanine, and 4 of 29 of the urease tests, the PathoTec results were contrary to those obtained with the Enterotube. Mann and Gandelman (10) reported a number of discrepancies between the PathoTec indole test and the conventional indole test with strains of *Proteus vulgaris* and *P. rettgeri*. The discrepancies noted here occurred with one strain each of *Escherichia*, *Klebsiella*, and a pseudomonad. The organism which was phenylalanine-positive by Enterotube but -negative by PathoTec test was one of the pseudomonads identified by the PathoTec CO test. This pseudomonad was also urease-positive in the Enterotube but -negative by PathoTec test. The two organisms that gave positive urease reactions by PathoTec but were negative in the Enterotube were *Aerobacter* C strains, organisms which are generally variable in urease activity. Of 10 strains which were lysine decarboxylase-positive in the Enterotube, seven were also positive by the PathoTec test. The PathoTec lysine decarboxylase-negative organisms were one indole-positive, citrate-positive strain classified as *E. intermedia*, a *Klebsiella*,

TABLE 4. *Comparison of the Enterotube and PathoTec tests for citrate utilization with tests on Simmons' citrate agar*[a]

Test	Citrate	
	Positive	Negative
Enterotube	14/23	13/15
PathoTec	11/12	19/23

[a] Values are expressed as the number of Simmons' citrate in agreement to the number tested.

and an *Aerobacter* strain. There was agreement between the two test systems with 22 of the 26 strains which were lysine decarboxylase-negative in the Enterotube. Two *Escherichia*, one *P. mirabilis*, and one *Aerobacter* C strain were positive in the PathoTec test. Thus, a total of 29 of the 36 tests were in agreement. Gandelman and Mann (6) reported that 16 strains of *P. mirabilis* which were lysine decarboxylase-positive in the PathoTec test were -negative upon testing with lysine decarboxylase broth (Difco). No *Escherichia* or *Aerobacter* strains were tested in their study. The PathoTec tests for citrate utilization were in agreement with the Enterotube with 34 of the 46 strains compared. One *Aerobacter* C and one *Escherichia* which were Enterotube citrate-negative were -positive by the PathoTec test, whereas seven *Escherichia*, the *Aerobacter* B, one *Aerobacter* C, and one *P. mirabilis* strain were positive in the Enterotube but negative in the PathoTec test.

When the results of the citrate tests with the two systems were compared with the results of tests on Simmons' Citrate Agar (Table 4), it was found that 27 of 38 (71%) of the Enterotube tests and 30 of 35 (86%) of the PathoTec tests were in agreement with the Simmons' citrate tests. Thus, it appears that discrepancies in citrate utilization may be expected with either the Enterotube or the PathoTec system when compared to Simmons' Citrate Agar. The results with the PathoTec citrate tests reported here differ somewhat from those reported by Borchardt (1), who found no false positives and a low percentage of false negatives with the PathoTec test when compared with Simmons' citrate. However, it was pointed out that false negatives would occur if the inoculum for the test was not sufficiently heavy. Eight of the Simmons' citrate-negative strains which were citrate-positive in the Enterotube were considered to be *Escherichia* intermedia. The citrate reactions of these strains were weak or delayed in the Enterotube.

DISCUSSION

It was the purpose of this investigation to assess the rapidity and accuracy with which gram-negative bacilli of the family *Enterobacteriaceae* could be presumptively identified by the use of the Enterotube in comparison with the use of the reagent-impregnated paper strip tests of the PathoTec system. Previous experience had shown that the PathoTec tests, when used in conjunction with conventional fermentation tests, were of great help in providing information about the biochemical characteristics of isolates of gram-negative bacilli. The tests for cytochrome oxidase and phenylalanine deaminase activity and indole production are easily and quickly performed and they quickly detect pseudomonads and members of the Proteus-Providence groups, especially when the organisms are present in pure culture in the original specimen. The preparation of the remaining tests for urease and lysine decarboxylase activity, citrate utilization, and acetylmethylcarbinol production (Voges-Proskauer test) are simple and require relatively short incubation periods. Only one reagent, 40% KOH for the VP test, is required. However, since a large inoculum is necessary for the performance of the tests, the organism, unless present originally in pure culture, must be isolated and subcultured on solid medium. On the other hand, although overnight incubation is required before the tests can be read, the Enterotube may be inoculated directly from the suspect colony on the primary isolation plate. After overnight incubation, two reagents are added and the results of all the tests can be read immediately. Since a series of inoculations are required with the PathoTec tests, the number depending on the organisms being studied, whereas a single inoculation suffices for the Enterotube, the latter takes considerably less time to perform.

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