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J. Clin. Microbiol. **1991, 29(10):2300.**

JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1991, p. 2300–2304
0095-1137/91/102300-05$02.00/0

Vol. 29, No. 10

Enterosistem 18-R: Description and Comparative Evaluation with Conventional Methods for Identification of Members of the Family *Enterobacteriaceae*

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Received 1 May 1991/Accepted 23 July 1991

The efficiency and accuracy of Enterosistem 18-R (Liofilchem s.r.l., Roseto degli Abruzzi, Teramo, Italy) were compared with those of conventional biochemical methods to identify 360 members (38 species) of the family *Enterobacteriaceae*. Overall, 329 strains (91.3%) were correctly identified (percentage of identification, ≥90.0), with 37 (11.2%) requiring additional tests for complete identification. For 11 isolates (3.1%), Enterosistem 18-R gave only genus identifications, and for 14 (3.9%), the strains did not correspond to any key in the codebook and could not be identified by the manufacturer's computer service. Only six isolates (1.7%) were misidentified. The new system accurately identified common and several newly described isolates of the family *Enterobacteriaceae*, such as *Enterobacter gergoviae*, *Providencia rustigianii*, *Serratia odorifera*, and *Serratia rubidaea*. The system is highly reproducible, simple to perform, easy to handle, and inexpensive. With adjustments in supplementary code numbers for some strains, Enterosistem 18-R is a suitable alternative for identification of members of the *Enterobacteriaceae* in clinical laboratories.

Identification of members of the family *Enterobacteriaceae* is a major feature of clinical bacteriology laboratories since these bacteria, alone, are the etiological agents of more than 50% of hospital infections (17). With increased government attention to health costs (2), today the clinical microbiologist is more interested than ever in rapid reporting and reductions in laboratory costs (1). Therefore, it is essential to develop new, simple, and economic systems for rapid and accurate identification of this bacterial group, and many commercial multitest systems are now available for this purpose (4, 10, 16).

Computer-assisted identification systems are already available in miniaturized test kits such as Micro-ID, Minitek, API 20E, and Enterotube (11, 12, 15, 18). Enterosistem 18-R (Liofilchem s.r.l., Roseto degli Abruzzi, Teramo, Italy) is a new system designed to identify members of the *Enterobacteriaceae* to the genus and species levels in 18 h. The system, at present available only in Europe, consists of a disposable tray with 18 wells containing the dehydrated biochemical substrates. With inoculation of a bacterial suspension in each well, a six-digit octal number can be generated from 18 different biochemical reactions. From this octal number, an identification is derived from a codebook furnished to laboratories.

To evaluate the accuracy and utility of Enterosistem 18-R, we have compared this system with conventional biochemical methods in identifying 360 isolates of members of the family *Enterobacteriaceae*.

MATERIALS AND METHODS

Bacterial strains. A total of 360 strains were tested. Of these, 213 were fresh clinical isolates from our clinical bacteriology laboratory, 109 were stock cultures from our collection that have been kept frozen (−80°C) in 20% glycerol, and 38 were reference strains from different international culture collections.

Before the experiment, the 109 stock cultures and the 38 reference strains were subcultured three times into sheep blood agar (Liofilchem s.r.l.) to raise their levels of enzymatic activity. Before testing, all of the 360 isolates were grown in brain heart infusion broth (Oxoid Italiana S.p.A., Garbagnate Milanese, Milan, Italy) and then subcultured in a sheep blood agar to ensure purity and viability. To mask the identity of all microorganisms throughout the experiment, we adopted the use of a progressive numbering system for each microorganism (from 1 to 360). Two study groups were cross-employed in this work. The first identified all isolates and revealed the results to the second group only at the end of the study. The second group, employed as a control for the first group, conducted the work on 72 randomized isolates and used the 38 reference strains as a quality control. Furthermore, to evaluate the possible effect of the growth medium on Enterosistem 18-R, the same reference strains were grown on sheep blood and MacConkey agar plates (Oxoid Italiana S.p.A.). To ascertain the reproducibility of results from the system, growth from both media was employed as an inoculum on three separate occasions, each time by a different study group.

Enterosistem 18-R identification method. The Enterosistem 18-R identification method consists of a plastic tray containing 18 different reaction wells covered with a transparent plastic cover (Fig. 1). The 18 biochemical tests included in the system are listed in the legend to Fig. 1.

The tray was inoculated according to the manufacturer's instructions with some modifications, such as the inoculum conditions and the incubation time. One to three well-isolated colonies were emulsified in 4.5 ml of physiological sterile solution to reach an opacity equal to 0.5 MacFarland standard. The reaction wells were inoculated with 200 μl of the bacterial suspension by using a multichannel pipette (Titertek; Flow Laboratories, Milan, Italy). Wells for lysine

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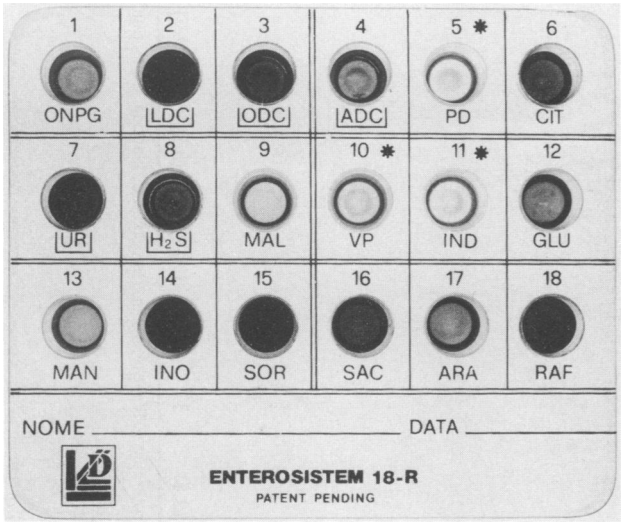

FIG. 1. Enterosistem 18-R. Test wells: 1, ONPG; 2, lysine decarboxylase; 3, ornithine decarboxylase; 4, arginine dihydrolase; 5, phenylalanine deamination; 6, citrate; 7, urea hydrolysis; 8, H_2S production; 9, malonate utilization; 10, VP; 11, indole production; 12 through 18, fermentation of glucose, mannitol, inositol, sorbitol, sucrose, arabinose, and raffinose, respectively.

decarboxylase, ornithine decarboxylase, arginine dihydrolase, urease, and H_2S tests were covered with sterile mineral oil. The tray was closed with the plastic cover and then incubated for 18 h at 37°C in an aerobic atmosphere.

After incubation, 2 drops of a 10% ferric chloride solution were added to the phenylalanine deaminase well and an immediate green reaction was evident. At the same time, 3 drops of α-naphthol plus 1 drop of 40% NaOH solution (Voges-Proskauer [VP] reagents) and 2 drops of Kovács reagent were added, respectively, to the VP test and indole production wells. The VP reaction was evident before 12 to 15 min, while an immediate red-ring appearance in the indole production well demonstrated a positive tryptophan metabolism.

All of the reactions were read by a color chart provided with the kit. The biochemical reactions were recorded on data sheets provided with the kit, and a six-digit octal number was generated for each microorganism, which was then identified as a single species or as one of several possible species by using the Enterosistem 18-R codebook index.

Unlisted profiles were interpreted by referring to the manufacturer's computer.

In the Enterosistem 18-R codebook, identifications are classified according to the percentage of identification (% ID) (19) as follows: excellent (% ID, ≥99.9), good (% ID, ≥90.0), acceptable (% ID, ≥80.0), and low confidence (% ID, <80.0, but with the % ID sum of the first two or three taxa greater than or equal to 80).

In this paper we have considered a correct identification as % ID ≥ 90.0.

Conventional biochemical tests. The 360 *Enterobacteriaceae* isolates employed in this study were identified according to procedures described by Ewing (13). A few strains which could not be accurately identified with this procedure were fully characterized by using the following additional tests as reported by Farmer et al. (14): growth in KCN and tyrosine clearing (*Citrobacter amalonaticus*); fermentation of L-arabinose (*Edwardsiella tarda*); fermentation of α-methyl-D-glucoside and D-arabitol (*Enterobacter gergoviae*); salicin fermentation, esculin hydrolysis, and chloramphenicol susceptibility (*Proteus penneri*); fermentation of D-galactose (*Providencia rustigianii*); and fermentation of mucate and gelatin hydrolysis by rapid film method at 36°C (*Salmonella arizonae*). *Enterobacter hormaechei* (enteric group 75) was identified according to the scheme proposed by O'Hara et al. (20). *Yersinia* spp. were characterized as reported by several authors (3, 5, 6, 8, 21).

Final identification was determined according to the table from the work of Ewing (13) and Farmer et al. (14). All fresh clinical isolates of *Salmonella* or *Shigella* species were confirmed by serological tests.

RESULTS

The results obtained with Enterosistem 18-R and conventional methods in identifying the 360 strains belonging to 38 different species of *Enterobacteriaceae* are shown in Table 1.

Enterosistem 18-R agreed with the conventional methods in the identification of the 329 of 360 isolates (91.3%) at the species level (% ID, ≥90.0). Among these 329 strains, the system provided an excellent identification (% ID, ≥99.9) for most species, in particular those often isolated in bacteriological laboratories, such as *Citrobacter diversus* (8 of 9 strains tested), *Citrobacter freundii* (9 of 12), *Edwardsiella tarda* (3 of 3), *Enterobacter aerogenes* (8 of 10), *Enterobacter cloacae* (24 of 27), *Escherichia coli* (59 of 66), *Klebsiella oxytoca* (12 of 13), *Klebsiella pneumoniae* (25 of 33), *Morganella morganii* (8 of 11), *Proteus mirabilis* (41 of 45), *P. penneri* (8 of 10), and *Providencia alcalifaciens* (2 of 2). All the *Salmonella* and *Shigella* species were correctly identified (% ID, ≥90.0) to the genus and species level. However, serological confirmation was made throughout the evaluation of the system. Most of the uncommon or newly described microorganisms, such as *E. gergoviae* (two of two strains tested), *Providencia rustigianii* (three of three), one strain of *Serratia odorifera*, and one isolate of *Serratia rubidaea*, were correctly identified (% ID, ≥99.9) by the system.

In 11 cases (3.1%), Enterosistem 18-R provided only genus identification.

Of the above-mentioned 329 strains, 54 were not directly identified: 37 needed additional tests, and 17 were identified by the manufacturer's computer service.

Fourteen microorganisms (3.9%), including the newly described strains, such as *E. hormaechei* and *Yersinia frederiksenii*, could not be identified because the generated six-digit octal numbers were included neither in the codebook which is furnished to laboratories nor in the data base available in the manufacturer's computer. However, these 14 isolates produced biochemical reaction patterns identical to those obtained with the conventional methods. The majority (10 of 14 strains tested) of these isolates were from the *Klebsiella* spp. (4 of 60), *Proteus vulgaris* (2 of 17), *Providencia* spp. (2 of 17), and *Yersinia pseudotuberculosis* (2 of 3).

Enterosistem 18-R disagreed with conventional methods for only 6 of the 360 isolates (1.7%) at both genus and species levels (complete disagreement). One *C. diversus* strain was identified as lysine-negative *E. coli*; one atypical strain of *E. coli* (*o*-nitrophenyl-β-D-galactopyranoside [ONPG] negative, H_2S positive, and sorbitol negative) was called *Edwardsiella tarda*; one *Enterobacter agglomerans* strain was identified

TABLE 1. Comparison of Enterosistem 18-R system with conventional methods for identification of members of the *Enterobacteriaceae*

Species as determined by conventional methods	No. and origin of strains				No. of strains					
					Correctly identified by:					
	Total	Fresh clinical isolates	Stock cultures	International collection[a]	Species	Genus	Other tests[b]	Computer[c]	Not identified[d]	Misidentified
Citrobacter amalonaticus	2		1	ATCC 24405	2		1	1		
Citrobacter diversus	9	4	4	ATCC 27156	8					1
Citrobacter freundii	12	6	5	ISM 80/67	11			1		1
Edwardsiella tarda	3	1	1	ATCC 15947	3					
Enterobacter aerogenes	10	5	4	NCTC 10006	10		1	1		
Enterobacter agglomerans	6	3	2	NCTC 9381	3	1	1	1	1	1
Enterobacter cloacae	27	18	8	CIP 60-85	26	1	1			
Enterobacter gergoviae	2		1	ATCC 33028	2					
Enterobacter hormaechei (enteric group 75)	1			ATCC 49162					1	
Enterobacter sakazakii	3	2		ATCC 29544	2	1				
Escherichia coli	66	51	14	ATCC 25922	64		3		1	1
Hafnia alvei[e]	10	5	4	NCTC 8106	10		10			
Klebsiella oxytoca	13	7	5	CIP 66-6	13		1			
Klebsiella ozaenae	10	5	4	ATCC 11297	6	1	1	1	3	
Klebsiella pneumoniae	33	21	11	ATCC 13883	31	2	2	2		
Klebsiella rhinoscleromatis	4	1	2	NCTC 5046	3		1	1	1	
Morganella morganii	11	7	3	ATCC 25830	11		1	2		
Proteus mirabilis	45	37	7	ATCC 29906	45		3	1		
Proteus penneri	10	2	7	ATCC 33519	10		2			
Proteus vulgaris	17	14	2	ATCC 13315	13	2		1	2	
Providencia alcalifaciens	2		1	ATCC 9886	2					
Providencia rettgeri	7	4	2	ATCC 29944	6		1	1	1	
Providencia rustigianii	3	1	1	ATCC 33673	3					
Providencia stuartii	5	2	2	ATCC 29914	4		1		1	
Salmonella arizonae	3	2		ATCC 12323	3					
Salmonella paratyphi A	4	1	2	ISM 75/33	4					
Salmonella typhi	3	1	1	NCTC 8385	3					
Serratia marcescens	10	5	4	CIP 67-55	8	2	3	1		
Serratia liquefaciens	8	3	4	ISM 76/99	7	1	2	1		
Serratia odorifera	1			NCTC 11214	1					
Serratia rubidaea	2		1	ATCC 27593	2					
Shigella boydii	2		1	ISM 80/60	2					
Shigella flexneri	3	1	1	ATCC 11836	3					
Shigella sonnei	2		1	ATCC 11060	2					
Yersinia enterocolitica	5	3	1	NCTC 10598	4		1	1		1
Yersinia frederiksenii	1			CIP 80-29					1	
Yersinia intermedia	2		1	ATCC 29909	2		1	1		
Yersinia pseudotuberculosis	3	1	1	ATCC 29833					2	1
Total (%)[f]	360 (100)	213 (59.2)	109 (30.2)	38 (10.6)	329 (91.3)	11 (3.1)	37	17 (10.3)	14 (3.9)	6 (1.7)

[a] ATCC, American Type Culture Collection, Rockville, Md.; CIP, Collection de l'Institut Pasteur, Paris, France; ISM, Collezione dell'Istituto Sieroterapico Milanese, Milan, Italy; NCTC, National Collection of Type Cultures, London, England.

[b] Isolates correctly identified by additional tests.

[c] Isolates correctly identified with the aid of the manufacturer's computer service.

[d] The profile did not correspond to any key in the codebook furnished to laboratories or in the manufacturer's computer data base.

[e] This species is registered as *Enterobacter alvei* in the codebook index.

[f] All percentages reflect a total number of strains of 360, except that values in the "Other tests" and "Computer" columns reflect a total number of 329.

as *S. rubidaea*; one *Yersinia enterocolitica* strain that showed unusual features, such as ornithine, urease, and sorbitol negativity, was identified as *Enterobacter agglomerans*; finally, one ONPG-negative and H_2S-, arginine-, and ornithine-positive *C. freundii* strain was misidentified by Enterosistem 18-R as *Salmonella* spp., and one *Y. pseudotuberculosis* was identified as ONPG-positive and indole- and sorbitol-negative *Shigella* spp.

Instructions for Enterosistem 18-R suggest the use of the sheep blood agar as the primary medium plate from which to prepare the tray inoculum. In consideration of the relatively high cost and storage of this medium, we compared the effects of the growth media (sheep blood versus MacConkey agar) on the identification. With the exception of the mucoid strains, such as *Klebsiella rhinoscleromatis* (insufficient growth), we found that MacConkey agar can be used as a substitute for sheep blood agar for the preparation of the inoculum.

Reproducibility of the tests with the quality control strains was 100% successful.

DISCUSSION

Our results demonstrate that the Enterosistem 18-R identification kit produced a good level of identification accuracy (% ID, ≥90.0) for members of the *Enterobacteriaceae* as compared with conventional methods.

Of the 329 correctly identified isolates that were recognized at species level, 54 needed further tests or the aid of the manufacturer's computer service. Among these strains were of all the *Hafnia alvei* strains. This might be due to the incubation temperature (37°C) that we used, which is not the optimum growth temperature for these species (7). Similar problems were reported by Izard et al. with the API 20E system (16). At present, this is not mentioned in the user's instructions for Enterosistem 18-R.

Although Enterosistem 18-R correctly identified 91.3% of the 360 isolates tested, the profiles of 14 isolates, including the newly described strains (*E. hormaechei* and *Y. frederiksenii*), did not correspond to any key in the computer data base or the codebook furnished to laboratories. *Klebsiella* spp., *P. vulgaris*, *Providencia* spp., and *Y. pseudotuberculosis* are the most difficult species to identify for Enterosistem 18-R. If these 14 unlisted strains and respective code numbers had been incorporated in the codebook and in the manufacturer's data base, the percentage of microorganisms correctly identified by the system would have risen from 91.3% to 95.3%.

Only six strains were really misidentified by the Enterosistem 18-R method because they were atypical in a certain character or they gave one or two negative results with Enterosistem 18-R and positive results when tested conventionally (especially by ONPG, sorbitol fermentation, and ornithine decarboxylase tests).

One strain of *C. freundii* and one strain of *Y. pseudotuberculosis* were incorrectly identified as *Salmonella* and *Shigella* species, respectively. This is not a real difficulty because in any case, identification of *Salmonella* and *Shigella* species must include serological confirmation; in this case, then, it is unlikely that the two misidentified species will be confused.

Although it was misidentified, the *Y. enterocolitica* strain was differentiated from *E. agglomerans* by motility, apart from the additional tests. Since no relative indication was given by the manufacturer, we assumed a temperature of 35°C, at which the *Y. enterocolitica* is nonmotile, whereas both of the species should be motile at 18 to 22°C.

It has been reported previously (11) that stock cultures would be less suitable to evaluate the performance of a bacterial identification system than fresh clinical isolates. After multiple in vitro passages or storage, bacteria may undergo genotypic and phenotypic variations and somehow lose one or more biochemical characteristics. Because Enterosistem 18-R reveals constitutive bacterial enzymes, it is possible that the storage of *Enterobacteriaceae* strains changes enzyme systems, resulting in negative or weak reactions, while fresh isolates may produce a positive reaction. We did not find any difference with fresh clinical isolates or frozen cultures (including those from the international culture collections). In fact, of the 213 fresh clinical isolates, 92% (196 strains) were correctly identified by Enterosistem 18-R, a figure which is comparable to the 91.8% (135 of 147) of frozen cultures and reference strains correctly identified.

With the lack of instructions relative to the preparation of the tray inoculum, we suggest that the turbidity of the bacterial suspension be equivalent to 0.5 MacFarland standard. This is especially important because for some strains with an inoculum density not carefully controlled, their identification by Enterosistem 18-R is difficult because of the appearance of false-positive or false-negative biochemical reactions.

Moreover, the requirement for 4.5 ml of a bacterial suspension equivalent to 0.5 MacFarland standard might preclude the use of this system for many primary culture isolates after an overnight incubation. However, we discovered that if insufficient microorganisms are available, it is possible to use the suspension of a 6-h culture in brain heart infusion broth.

In conclusion, we have evidence that Enterosistem 18-R, in its present form, accurately identifies common and several uncommon and newly described members of the family *Enterobacteriaceae*. From a practical point of view, the system was simple to use, easy to handle, and inexpensive. With adjustments in supplementary code numbers for some strains, the accuracy and utility of Enterosistem 18-R would be comparable to the qualities of other commercial systems.

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