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Evaluation of the Titertek-NF System for Identification of Gram-Negative Nonfermentative and Oxidase-Positive Fermentative Bacteria

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The Titertek-NF (TT-NF) system (Flow Laboratories GmbH, Meckenheim, Federal Republic of Germany) was evaluated for the identification of 1,289 strains of gram-negative, nonfermentative bacteria and some gram-negative, oxidase-positive bacteria. The oxidase test was also performed. Identifications were classified as correct, not identified (two or more taxa possible and identification score of <80%; supplementary tests for furthering the identication were not performed), and incorrect. Correct identification results were further subdivided by the correct level of species or biotype identification as ≥98% (category a), 90 to 97% (category b), and 80 to 89% (category c). When compared with conventional identification results, the TT-NF system correctly identified 90.3% of strains (1,164 of 1,289 strains), with 72.5% (935 strains) belonging to category a, 14.7% of strains (189 strains) belonging to category b, and 3.1% of strains (40 strains) belonging to category c. Among the remaining strains, 104 (8.1%) were not identified and 14 (1.1%) were misidentified, and the system failed to generate identification results for 7 strains (0.5%). Reactions within the TT-NF system were reproducible, with an estimated probability of erroneous test results of 0.2%.

Although gram-negative, nonfermentative bacteria increasingly are becoming implicated in human disease, identification of these organisms has often been neglected in clinical microbiology laboratories, especially when antibiotic susceptibility data are available (2). A battery of conventional biochemical tests is needed for their precise identification of the taxonomically heterogeneous group of gram-negative, nonfermentative bacteria (13–15, 27). In recent years, several commercial kits have been developed, some of which are for the identification of members of the family *Enterobacteriaceae* and commonly encountered nonfermenters and others of which are for the specific identification of gram-negative nonfermentative bacteria (8).

During a 3-year study, a screening was performed in order to find miniaturized biochemical tests that would make possible an extensive species differentiation within these bacterial genera (20, 21). As a result, in cooperation with Flow Laboratories GmbH (Meckenheim, Federal Republic of Germany), the Titertek-NF (TT-NF) system was developed; it provides for the computer-aided identification of 57 different species. This study was undertaken to determine the accuracy, suitability, and reproducibility of the TT-NF system for use in clinical microbiology laboratories.

MATERIALS AND METHODS

Bacteria. A total of 1,289 strains of nonfermentative, gram-negative bacteria and fermentative, oxidase-positive organisms were tested. Of these, 621 were obtained from the National Collection of Type Cultures, Central Public Health Laboratory (Colindale, London, United Kingdom) (123 reference strains and 498 field strains). They were identified by the methods of the National Collection of Type Cultures (18). An additional 668 strains were stock cultures obtained from the Institute of Medical Microbiology, University of Heidelberg, Heidelberg, Federal Republic of Germany; Centre Hospitalier St. Nicolas, Sarrebourg, France; and the Culture Collection of the University Göteborg, Göteborg, Sweden, and were identified by conventional methods (13) or the API 20NE system (Analytab Products, Plainview, N.Y.) (12). Before testing, all isolates were grown on sheep blood agar (Oxoid, Ltd., Hampshire, United Kingdom) for 24 h at 30°C (strains of the genera *Moraxella* and *Pasteurella* and *Bordetella bronchiseptica* isolates were grown for 48 h at 30°C). Oxidase testing was carried out by using oxidase test strips (E. Merck AG, Darmstadt, Federal Republic of Germany). To assess purity, cultures were checked throughout the study by colony morphology.

TT-NF system. The TT-NF system consists of 32 dehydrated substrates in a standard microplate format (28 test and 4 control media). The system includes tests for indole production; esculin hydrolysis; urease; arginine dihydrolase; ornithine decarboxylase; fermentation of glucose and sucrose; assimilation of D-glucose, mannose, maltose, *N*-acetyl-D-glucosamine, mannitol, gluconate, β-hydroxybutyrate, DL-lactate, adipate, suberate, malate, phenylacetate, and histidine; and hydrolysis tests for *o*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenylphosphorylcholine, 2-deoxythymidine-5′-*p*-nitrophenylphosphate, L-proline-*p*-nitroanilide, *p*-nitrophenyl-α-D-maltoside, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, and bis-*p*-nitrophenylphosphate. Each microplate contains three test sets for the identification of three different isolates. Inoculation of the test kit was as follows. Each well was filled with 100 μl of a bacterial suspension in a defined minimal basal medium with a density equivalent to a 0.1 McFarland standard. Test wells and control wells of esculin, esculin control, arginine dihydrolase, ornithine decarboxylase, urease, glucose and sucrose fermentation, and the fermentation control were overlaid with 50 μl of sterile mineral oil. After inoculation, the microplate was sealed with a sterile plastic sealer. Following incubation for 24 h at 30°C, 50 μl of the dimethylaminocinnamaldehyde reagent was added to the indole test wells, and then the test plates were read with a photometer (Multiscan MCC 340; Flow Laboratories) connected to a personal computer (Desk-

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pro286; Compaq, Munich, Federal Republic of Germany). Different tests were read at different wavelengths, and the resulting adsorbance values were coded in binary data. The resulting test profiles were compared with a probability matrix, according to the methods of numerical identification (39). This probability matrix was constructed by the methods given in the literature (17, 24), but with upper and lower limits of 0.999 and 0.001, respectively. The evaluation results were classified by the different ranges of likelihoods (identification scores), which are given as percentages throughout this report.

The reproducibility of the TT-NF system was determined by testing 50 randomly chosen isolates on 3 different days. The resulting test profiles were compared, and an estimate of the probability of an erroneous test result was calculated by methods described elsewhere (19).

RESULTS

The results of the evaluation of the TT-NF system are summarized in Table 1. Of 1,289 strains tested, 935 (72.5%) were correctly identified with an identification score of ≥98%. For 189 strains (14.2%), the correct identification scores were between 90 and 97%, while for 40 strains (3.1%), the correct identification scores were between 80 and 89%. For a total of 104 strains (8.1%), the identification score was below 80%, and hence, these strains were not identified correctly. To further the identification of 79 strains, one to four additional tests were necessary; these appeared in the form of percent positive test results on the computer screen in cases of low discrimination (additional tests recommended by Flow Laboratories for the TT-NF system were nitrogen formation from nitrate, nitrite formation from nitrate, motility, growth on salmonella-shigella agar, growth in the presence of cetrimide, growth on MacConkey agar, and growth at 42°C).

Incorrect identifications were obtained for 14 strains (1.1%) (Table 2). For seven strains (0.5%) no identification could be obtained. Investigations on the reproducibility of the TT-NF system provided the following results. A total of 50 strains were chosen at random, and 28 tests were performed on each strain, in triplicate, for a total of 4,200 test results. Of these 4,200 test results, there were 7 errors (a single negative result in one of the triplicate tests was classified as an error). The corrected error rate was calculated as follows:

$$C = 1/2\ (1 - \sqrt{1 - 4E/N})$$

where E is the number of errors observed, and N is the number of test results, as described previously (19). The corrected error rate for the TT-NF system was calculated to be 0.2%, with a 95% confidence interval of 0.7 to 1.4%. The reproducibility of individual tests is summarized in Table 3.

DISCUSSION

The TT-NF system was easy to set up and posed no problems of interpretation. Indole tests were sometimes difficult to read photometrically, because some strains of the *Alcaligenes faecalis-A. odorans* group and also some *Pseudomonas acidovorans* strains produced a cherry red color after the wells were filled with the indole reagent (4-dimethylaminocinnamaldehyde). This caused higher adsorbance values and, hence, false-positive results. These nonspecific reactions were considered in the probability values of *Alcaligenes faecalis-A. odorans* and *Pseudomonas alcaligenes* in the data base.

The reproducibility of the kit tests was excellent, with an estimated probability of erroneous results of 0.2%. This value is low when compared with probabilities of 2 to 4% found in other studies (19), probably because the tests that were performed are well established in our laboratory.

Seventeen species of the genus *Pseudomonas* were included in our study. Most strains were identified correctly with an identification score of ≥90%. For the 5 of 18 *Pseudomonas pseudoalcaligenes* strains and the 8 of 24 *Pseudomonas testosteroni* strains tested, only identification scores of <80% could be obtained. The differentiation among inactive strains of the species *Pseudomonas alcaligenes*, *Pseudomonas pseudoalcaligenes*, and *Pseudomonas testosteroni* was not satisfactory, as has already been described for other test systems, especially the API 20NE system (2, 23, 26).

Strains of *Chryseomonas luteola* (CDC group Ve-1) were correctly identified, whereas for two of the five strains of *Flavimonas oryzihabitans* (CDC group Ve-2), higher identification scores of ≥80% were prevented.

Within the genus *Alcaligenes*, *Alcaligenes faecalis* and *Alcaligenes odorans* were grouped together (*Alcaligenes odorans* is a later synonym for *Alcaligenes faecalis* [22]).

Five taxa of the genus *Moraxella* were tested. The two species *Moraxella nonliquefaciens* and *Moraxella lacunata* are not easily separated on routine biochemical tests and were therefore combined into a single taxon. Of the 14 strains of these two species, 13 achieved identification scores of only 68.7%. For 8 strains of the 12 *Moraxella phenylpyruvica* strains, a score of <80% was obtained. For the 11 strains of *Oligella urethralis* (*Moraxella urethralis*), 10 identification scores were only at a level of 77.1%. Within the genera *Flavobacterium*, *Pasteurella*, and *Vibrio*, as for the species *Aeromonas hydrophila*, *Plesiomonas shigelloides*, and *Chromobacterium violaceum*, identification in most cases were correct, with scores of >90%. Strains identified with a lower accuracy (scores from 80 to 90%) in most cases showed atypical test patterns. For *Vibrio vulnificus*, percentages should be verified, with the inclusion of more reference organisms in the data base needed.

Previous investigations on the accuracy of commercially available systems for the identification of nonfermenters produced various results (2, 8, 23, 26). Identification of gram-negative fermenting and nonfermenting bacteria by probabilistic methods has been described in the literature (6, 16, 18), and most commercially available test kits for the identification of various bacteria are based on these methods (40). Most evaluation results of commercially available kits reported difficulties in identifying weak oxidative or inactive strains. The combination of carbon substrate assimilation tests with enzyme tests in the API 20NE system has offered the highest accuracy so far for identification to the species or biogroup level (2). Carbon source assimilation tests and carbon substrate alkalinization tests have been used earlier for the differentiation of representatives of the families *Pseudomonadaceae* (3, 4, 25, 28, 30, 31, 33–35, 37, 38) and *Alcaligenaceae* (29, 32) and the genus *Flavobacterium* (7). Miniaturization of these methods (9–11, 20, 21) has made their application possible in commercially available test kits. The application of qualitative enzyme tests with chromogenic substrates improved the accuracy of differentiation of glucose-nonfermenting, gram-negative, and oxidase-positive, glucose-fermenting bacteria (1, 5).

The TT-NF system is a combination of miniaturized classical biochemical tests, carbon substrate assimilation tests, and growth-dependent qualitative enzyme tests with

TABLE 1. Results of the evaluation of the TT-NF system

Species	No. of strains tested	No. of strains correctly identified with an identification score of:			No. of strains with an identification score of <80%[a]	No. of strains incorrectly identified[b]	No. of strains not identified[c]
		>98%	90–97%	80–89%			
Pseudomonas aeruginosa	113	106	6	1			
Pseudomonas fluorescens	72	58	6	5	3		
Pseudomonas putida	61	42	6	5	8		
Pseudomonas pseudomallei	29	27	1		1		
Pseudomonas cepacia	42	39	3				
Pseudomonas pickettii	19	15		2	2		
Pseudomonas stutzeri	22	20		1	1		
Pseudomonas mendocina	22		20		2		
Pseudomonas alcaligenes	8		7		1		
Pseudomonas pseudoalcaligenes	18	4	9		5		
Pseudomonas acidovorans	44	38	3	1	2		
Pseudomonas testosteroni	24		15	1	8		
Pseudomonas diminuta	24	21	1	1		1	
Pseudomonas vesicularis	25	20	3		1	1	
Pseudomonas maltophilia	57	47	7	1	2		
Pseudomonas paucimobilis	20	16	1	1	1	1	
Shewanella putrefaciens	17	17					
Chryseomonas luteola (CDC group Ve-1)	6	6					
Flavimonas oryzihabitans (CDC group Ve-2)	5	3			2		
Alcaligenes faecalis-A. ordorans	54	50			2	2	
Alcaligenes xylosoxidans subsp. *denitrificans*	13	2	5	2	4		
Alicaligens xylosoxidans subsp. *xylosoxidans*	11	10			1		
Ochrobactrum anthropi (*Achromobacter* group V-d)	23	19	4				
Bordetella bronchiseptica	28	25	1	2			
Agrobacterium tumefaciens	30	26		1	1	1	1
Moraxella nonliquefaciens-M. lacunata	14				13	1	
Moraxella osloensis	10		9		1		
Moraxella phenylpyruvica	12	4			8		
Moraxella atlantae	5		4		1		
Oligella urethralis (*Moraxella urethralis*)	11				10	1	
Acinetobacter calcoaceticus	26	12	11	2	1		
Acinetobacter lwoffii	25	20	3		2		
Flavobacterium meningosepticum	25	25					
Flavobacterium odoratum	21	15	3		2		1
Flavobacterium breve	18	9	7		1	1	
Flavobacterium multivorum	42	11	22	6	3		
Flavobacterium thalpophilum	7		5		2		
Flavobacterium spiritivorum	31	29		1	1		
Flavobacterium species group IIb	28	22	1	1	3	1	
Weeksella zoohelcum (CDC group IIj)	14	14					
Pasteurella aerogenes	1		1				
Pasteurella multocida	34	32	2				
Pasteurella haemolytica type A	2	2					
Pasteurella haemolytica type T	2		2				
Pasteurella pneumotropica	3		2	1			
Pasteurella ureae	4		4				
Aeromonas hydrophila	28	25	1		1	1	
Plesiomonas shigelloides	23	17	1	1	1	1	2
Vibrio alginolyticus	22	20	2				
Vibrio parahaemolyticus	18	14	2		1		1
Vibrio vulnificus	10	2	2	1	4	1	
Vibrio cholerae	23	19	3	1			
Vibrio mimicus	5	2		2			1
Vibrio metschnikovii	12	12					
Vibrio furnissii	10	7	2		1		
Vibrio fluvialis	6	3	2		1		
Chromobacterium violaceum	10	8				1	1
Total (percent)	1,289 (100)	935 (72.5)	189 (14.7)	40 (3.1)	104 (8.1)	14 (1.1)	7 (0.5)

[a] Low discrimination results (identification results of strains grouped in this category showed two or more taxa possible [among them the correct taxon]), but they failed to reach the identification level (scores of ≥80%); a correct identification can be made by performing the suggested supplementary tests to differentiate between the most likely taxa suggested.

[b] Strains reached the identification level (scores of ≥80%), but to the incorrect taxon.

[c] No identification result is shown (comparison of test profiles with the data base generated identification scores that were too small).

TABLE 2. Misidentifications with the TT-NF system

Conventional identification (no.)	Identification with the TT-NF system
Pseudomonas diminuta (1)	*Moraxella atlantae*
Pseudomonas vesicularis (1)	*Flavobacterium meningosepticum*
Pseudomonas paucimobilis (1)	*Pseudomonas diminuta*
Agrobacterium tumefaciens (1)	*Flavobacterium multivorum*
Moraxella nonliquefaciens-M. lacunata (1)	*Pasteurella haemolytica* type A
Oligella urethralis (1)	*Moraxella phenylpyruvica*
Flavobacterium breve (1)	*Moraxella nonliquefaciens-M. lacunata*
Flavobacterium species group IIb (1)	*Flavobacterium meningosepticum*
Aeromonas hydrophila (1)	*Vibrio furnissii*
Plesiomonas shigelloides (1)	*Pasteurella multocida*
Vibrio vulnificus (1)	*Flavobacterium* species group IIb
Chromobacterium violaceum (1)	*Flavobacterium multivorum*
Alcaligenes faecalis-A. odorans (2)	*Alcaligenes xylosoxidans* subsp. *denitrificans* and *Moraxella phenylpyruvica*

chromogenic substrates. Hence, this system is designed to offer a relatively specific level of identification compared with that offered by other methods.

The TT-NF system allows, in most cases, the correct identification (identification scores of >90%) of commonly encountered nonfermenting bacteria and oxidase-positive, fermenting bacteria; and also, those groups of bacteria that are rarely encountered in the clinical microbiology laboratory were well separable. Disadvantages of the system included the lack of adequate resolution within the genus *Moraxella*, the lack of adequate differentiation among nonreactive *Pseudomonas testosteroni*, *Pseudomonas alcaligenes*, and *Pseudomonas pseudoalcaligenes* species and *Alcaligenes xylosoxidans* subsp. *denitrificans*. Combining these rarer species or genera into groups and identifying them on the genus or the group level only could improve the percent identification values, as has been suggested for the PASCO MIC-ID system (36). For the majority of laboratories, collective groupings as *Moraxella* sp. and *Pseudomonas alcaligenes*, *Pseudomonas pseudoalcaligenes*, and *Pseudomonas testosteroni* would be adequate. *Vibrio vulnificus* isolates also posed problems for the TT-NF system. Certain biotypes are not represented in the data base.

TABLE 3. Reproducibility of individual tests

Test[a]	No. of errors[b]	Corrected error rate (%)[c]
Glucose fermentation	2	1.4 (0–2.8)[d]
Gluconate assimilation	1	
Histidine assimilation	1	
Mannose assimilation	1	0.7 (0–2.0)
Phenylacetate assimilation	1	
Urease	1	

[a] Other tests gave 0 errors in the 150 tests that were performed.

[b] A single negative result in one of the triplicate tests was classified as an error.

[c] The corrected error rate is given by $C = 1/2\,(1 - \sqrt{1 - 4E/N})$, as described in the text.

[d] The 95% confidence intervals are given in parentheses.

Despite these problems, the TT-NF system provides a highly differentiating approach for the identification of gram-negative, nonfermenting and gram-negative, oxidase-positive, fermenting bacteria, and thus offers a practical alternative to already existing identification systems for this heterogeneous group of organisms.

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