Rapid Identification of Nonfermentative Gram-Negative Rods by the Corning N/F System

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A total of 1,298 nonfermentative gram-negative rods were used to evaluate the performance of the individual biochemical tests in the N/F System and to determine whether the observed results included sufficient key reactions for rapid identification. The system included an oxidase test, two screening tubes providing 5 test reactions designed primarily to identify pigment-producing strains of *Pseudomonas aeruginosa*, and a plate providing 12 test reactions designed to identify other nonfermenters. With both the tubes and plates, most results were consistent with expected conventional test reactions. Use of the tubes permitted the identification of 90% of the strains of *P. aeruginosa* in 24 h and 97% in 48 h. Use of the plates permitted the identification of 95% of the other oxidative nonfermenters within 24 h and 96% within 48 h. Only 26% of weak and nonoxidative nonfermenters were identified because of the nonreactivity of these organisms in this system. There were no misidentifications based on misleading test results.

The N/F (nonfermenter) System (Corning Medical Products, Roslyn, N.Y.) is a new system for the rapid identification of frequently encountered nonfermentative or oxidase-positive gram-negative rods. The system consists of a series of prepackaged biochemical tests commonly used for the identification of these organisms. The tests are divided into two groups, the first of which allows the expedient identification of *Pseudomonas aeruginosa*. The second is used to identify less commonly occurring species or to sufficiently characterize them so that additional tests can be appropriately selected. The physical configuration of the system is similar to that of the Corning Uni-Yeast-Tek System (1).

In the present study 1,289 nonfermentative gram-negative rods were used to evaluate the performance of the individual biochemical tests included in the N/F System for the appearance of timely, appropriate positive and negative reactions and to determine whether the observed results included sufficient key reactions for identification of the organisms within this group. No attempt was made to evaluate the identification schemes or computer code book which are provided with the N/F System.

MATERIALS AND METHODS

Organisms. The organisms included 21 species and 2 CDC unnamed groups of nonfermentative gram-negative rods; 1,266 were clinical isolates from the Clinical Microbiology Laboratories, the Ohio State

University Hospitals, and three other Ohio institutions, and 23 were stock cultures. Organisms from the University Hospitals were consecutive clinical isolates; those from referring laboratories included only isolates other than *P. aeruginosa*. The 23 stock cultures included a single representative of each named and unnamed species in the study group. All strains tested had been previously identified by using established conventional techniques and criteria (2-10; *Identification of Glucose Non-Fermenting Gram-Negative Rods*, American Society for Microbiology, 1975).

N/F System prepackaged media. The N/F System consists of two screening tubes, one constricted (GNF) and one nonconstricted (42P), and a multimedia circular plate (Uni-N/F-Tek). The constricted GNF tube detects glucose fermentation and nitrogen gas production from nitrites in the butt (below the constriction) and fluorescein pigment production on the slant (above the constriction). The nonconstricted 42P tube tests for 42°C tolerance and pyocyanin pigment production. The circular Uni-N/F-Tek plate consists of 11 independently sealed peripheral wells and a central unsealed well, all containing agar-based media for the determination of 12 biochemical reactions. The 11 peripheral wells include five carbohydrates (glucose, xylose, mannitol, lactose, and maltose) to determine oxidative utilization, media for the determination of acetamide assimilation, esculin hydrolysis, production of urease, DNase and β -galactosidase and a carbohydrate control. The center well contains media to test for indole and H₂S production.

Procedure. Organisms were tested in groups of 10 to 20 without knowledge of their identities. An oxidase test was performed on all isolates, using 1.0% tetramethyl-*p*-phenylenediamine dihydrochloride (Marion

Scientific Corporation, Kansas City, Mo.). Oxidase-positive organisms were inoculated into the two screening tubes (GNF and 42P). If they were not identified as *P. aeruginosa* or *P. fluorescens-putida* within 18 to 24 h, the Uni-N/F-Tek plate was inoculated. (There was one exception to the usual procedure. All strains of *P. fluorescens-putida*, including those which were identified by the screening tube reactions, were inoculated into the plates to generate a larger amount of data on the reactions produced by these organisms.) Oxidase-negative isolates were inoculated directly into the Uni-N/F-Tek plates without utilization of the two screening tubes.

Tubes and plates were warmed to room temperature before use. For each test strain, a well-isolated colony from 5% sheep blood agar or MacConkey agar was used to inoculate the screening tubes. The GNF tube was stabbed with a wire through the constriction to the bottom of the tube, and the slant was streaked as the wire was withdrawn. Without flaming the wire, the slant of the 42P tube was streaked. The caps on both tubes were carefully checked to assure that they were loose for proper aeration. To inoculate the plate, a loopful of organisms from the sheep blood agar or MacConkey agar was suspended in 2 ml of sterile distilled water. By using a sterile Pasteur pipette one drop of the turbid suspension was placed on the surface of each peripheral well through the channels at the margin of the plate. The center well was inoculated by placing a drop on the surface of the medium, then stabbing to the bottom of the well with the pipette.

The 42P tube was incubated in a 42°C heating block for 24 h, reactions were recorded, and then the tube was placed in a 37°C room air incubator for an additional 24 h. The GNF tubes and Uni-N/F-Tek plates were incubated at 37°C; results of reactions were recorded at 24 and 48 h.

Test reactions: (i) 42P tube. Obvious growth on the 42P tube within the first 24 h of incubation was indicative of 42°C tolerance. A green-to-dark aqua color on the slant which diffused into the agar indicated the presence of pyocyanin pigment.

(ii) GNF tube. A yellow or greenish-yellow agar slant indicated the presence of fluorescein pigment. An ultraviolet light was used to check for fluorescence when pigment was not readily visualized. A yellow color in the butt of the tube (below the constriction) indicated glucose fermentation. Cracks or bubbles in the agar medium in the butt indicated nitrogen gas production.

(iii) Uni-N/F-Tek plate. All carbohydrate reactions were interpreted against the control (basal medium without carbohydrate). When the control remained neutral (green), changes to varying intensities of yellow indicated a positive reaction. When the control was alkaline (blue), green coloration indicated a weak positive reaction. For the other biochemical tests, the respective color changes which represented positive reactions were: acetamide assimilation, bright blue; esculin hydrolysis, black; urease production, pink; deoxyribonuclease (DNase) production, blue; o-nitrophenyl- β -D-galactopyranoside (ONPG), yellow; and H₂S production, black. The indole test was performed by saturating a cotton swab with Corning Indole reagent (Corning Medical Products, Roslyn,

N.Y.) and rotating the swab in the growth on the surface of the center well. A bright pink color developing in the swab was indicative of a positive reaction.

Identification. Criteria for identification were based on those previously published (2-10; *Identification of Glucose Non-Fermenting Gram-Negative Rods*, American Society for Microbiology, 1975). Oxidative-fermentative carbohydrate criteria were primarily those of Hugh and Gilardi (3), Oberhofer et al. (6), and Tatum et al. (9).

RESULTS AND DISCUSSION

Oxidase tests and screening tubes: (i) test reactions. Table 1 lists all organisms tested and their oxidase test results. Also listed are the 24-h tube reactions for oxidase-positive strains only. All glucose reactions were appropriately neutral or alkaline. At 48 h, some of the strains in the carbohydrate oxidative group produced a rim of acid at the agar-air interface which was not confused with a fermentative reaction.

Nitrogen gas, as expected, was produced by all strains of *P. stutzeri*, 60% of strains of *Alcaligenes denitrificans*, and 79% of strains of *A. odorans*. By 48 h, they were 100, 100, and 79% positive, respectively. The only discrepancies with expected results occurred with the strains of *P. pickettii* and CDC IVc-2, which were uniformly nitrogen gas negative at both 24 and 48 h.

All strains of *P. aeruginosa* grew at 42°C, and 91% produced either fluorescein or pyocyanin pigments, or both, within 24 h; 97% produced pigment by 48 h. None of the *P. fluorescens-putida* group grew at 42°C, and only 19 and 35% at 24 and 48 h, respectively, produced fluorescein pigment. Previous studies (6) have shown that fluorescein pigment production by this group is slower and less intense at 37 than at 25°C and presumably accounted for the low incidence of pigment production in this study.

(ii) Identification. By using the screening tubes, 90% of the strains of *P. aeruginosa* were identified by 24 h, and 97% by 48 h. Only 19 and 35% of the strains of *P. fluorescens-putida* were similarly identified.

Uni-N/F-Tek plate: carbohydrate oxidative group (i) test reactions. The results of the 24-h plate reactions for the carbohydrate oxidative nonfermenters, including the 30 strains of *P. aeruginosa* which were not identified by the screening tubes, are shown in Table 2.

The patterns of carbohydrate reactions observed at 24 h were usually typical of those observed with conventional oxidative-fermentative sugars, and certain uniquely characteristic combinations of reactions were readily appreciated: *P. maltophilia* produced a strong maltose reaction (99%) and a weak (80%) or negative

TABLE 1. Nonfermentative gram-negative rods tested, results of oxidase tests, and screening tube reactions at 24 h

Organism	No. tested	Oxidase (% positive)	Tube test reactions (% positive) ^a				
			GNF tube			42P tube	
			Glucose fermented	N ₂	Fluorescein	42°C	Pyocyanin
Carbohydrate oxidative							
<i>P. aeruginosa</i>	921	100	0	72	84	100	72
<i>P. fluorescens-putida</i> group	26	100	0	0	19	0	0
<i>P. maltophilia</i>	78	6	0	0	0	100	0
<i>P. cepacia</i>	17	82	0	0	0	94	0
<i>P. stutzeri</i>	13	100	0	100	0	92	0
<i>P. pickettii</i>	7	100	0	0	0	100	0
<i>P. vesicularis</i>	2	100	0	0	0	100	0
<i>A. calcoaceticus</i> subsp. <i>anitratus</i>	64	0	NT	NT	NT	NT	NT
<i>A. xylosoxidans</i>	50	100	0	4	0	2	0
Glucose weakly or nonoxidative							
<i>P. acidovorans</i>	8	100	0	0	0	50	0
<i>P. testosteroni</i>	3	100	0	0	0	100	0
<i>P. alcaligenes</i>	14	100	0	7	0	93	0
<i>P. pseudoalcaligenes</i>	5	100	0	0	0	100	0
<i>P. putrefaciens</i>	5	100	0	0	0	80	0
<i>P. diminuta</i>	4	100	0	0	0	100	0
<i>A. calcoaceticus</i> subsp. <i>lwoffii</i>	28	0	NT	NT	NT	NT	NT
<i>Moraxella</i> sp.	3	100	0	0	0	33	0
<i>A. faecalis</i>	6	83	0	0	0	100	0
<i>A. denitrificans</i>	5	100	0	60	0	20	0
<i>A. odorans</i>	19	100	0	79	0	100	0
<i>B. bronchiseptica</i>	7	100	0	0	0	100	0
CDC IVc-2	1	100	0	0	0	0	0
CDC IVe	3	100	0	0	0	67	0

^a Values indicate percent positive among oxidase-positive strains. NT, Not tested.

TABLE 2. Results of plate reactions at 24 h; carbohydrate-oxidative group

Organism	No. of strains tested	Uni-N/F-Tek plate reactions (% positive)											
		Glucose	Xylose	Mannitol	Lactose	Maltose	Acetamide	Esculin	Urease	DNase	ONPG	Indole	H ₂ S
<i>P. aeruginosa</i> ^a	30	100	87	83	0	0	90	0	70	10	10	0	0
<i>P. fluorescens-putida</i> group	26	100	100	46	8	12	0	0	35	0	0	0	0
<i>P. maltophilia</i>	78	80	18	3	35	99	0	95	3	99	71	0	0
<i>P. cepacia</i>	17	100	88	94	94	88	24	65	76	0	35	0	0
<i>P. stutzeri</i>	13	100	85	62	8	85	0	0	8	0	0	0	0
<i>P. pickettii</i>	7	100	100	0	0	0	0	0	100	0	0	0	0
<i>P. vesicularis</i>	2	100	50	0	0	100	0	100	0	100	0	0	0
<i>A. calcoaceticus</i> subsp. <i>anitratus</i>	64	100	100	0	94	6	3	0	2	0	0	0	0
<i>A. xylosoxidans</i>	50	92	98	0	0	0	4	0	0	0	0	0	0

^a Non-pigmented strains.

(20%) glucose reaction, and *Achromobacter xylosoxidans* produced a strong xylose reaction (98%) and a weak glucose reaction (92%). Although most carbohydrate reactions were strong at 24 h, *P. cepacia* and *P. stutzeri* displayed the largest number of weak reactions. At 48 h, however, all weak reactions were frankly positive. Discrepancies in expected 24-h reactions were

seen with two strains of *P. maltophilia* which were mannitol positive and one strain of *P. stutzeri* which was lactose positive. These reactions represented neutral carbohydrate reactions with an alkaline carbohydrate control; at 48 h, all three reactions were alkaline and read as negative. Discrepancies at 48 h were seen with 8 strains of *P. maltophilia* which were weakly

glucose-positive at 24 h but reverted to negative at 48 h.

Acetamide alkalization, as expected, was positive with nonpigmented strains of *P. aeruginosa* (90% at 24 h and 97% at 48 h), infrequently positive with *Acinetobacter calcoaceticus* subsp. *anitratus* (3% at 24 and 48 h), and negative with *P. fluorescens-putida*, *P. maltophilia*, *P. stutzeri*, *P. pickettii* and *P. vesicularis*. The percentages of positive reactions for *P. cepacia* (24% at 24 h and 41% at 48 h) and *A. xylosoxidans* (4% at 24 h and 8% at 48 h) were lower than expected. We suspected that this was due to a skewed population of strains studied.

Esculin and urea hydrolysis reactions gave expected results. Reliability for the former was important in the identification of *P. maltophilia*, *P. cepacia*, and *P. vesicularis*. Reliability of the latter was important in the identification of *P. pickettii*.

DNase production was, as expected, positive with *P. maltophilia* and negative with most of the other species in this group.

ONPG reactions were, as expected, positive primarily with strains of *P. maltophilia* and *P. cepacia*. Positive reactions increased from 71 to 92% and 35 to 76%, respectively, at 48 h.

Indole and hydrogen sulfide production were appropriately negative with all strains tested.

(ii) Identification. By using the 24-h results of biochemical reactions, 95% of the oxidative nonfermenters studied were identified without the use of supplemental tests. Although more reactions were positive after 48 h, particularly with *P. maltophilia* and *P. cepacia*, only two additional strains in this group, both *P. cepacia*,

were identified by using the 48-h results. There were no misidentifications based on discrepancies of observed individual test reactions with expected results.

Uni-N/F-Tek plate: carbohydrate weakly or nonoxidative group: (i) test reactions. The results of the 24-h plate reactions for the carbohydrate weakly and nonoxidative nonfermenters are shown in Table 3. Positive reactions produced by organisms in this group were infrequent, as expected.

Carbohydrate reactions, when present, were weak in comparison to those produced by the oxidative nonfermenters but were easily read against the strongly alkaline control and typical for given species: *P. acidovorans* was mannitol positive (50% at 24 h and 63% at 48 h), *P. pseudoalcaligenes* was glucose positive (60% at 24 and 48 h) and maltose positive (20% at 24 and 48 h).

Acetamide alkalization was usually positive with *P. acidovorans* (88% at 24 and 48 h) and *A. odorans* (84% at 24 and 48 h), occasionally positive with *A. calcoaceticus* subsp. *anitratus* (4% at 24 and 48 h), and usually negative with the other species, as expected. The only discrepancies with expected results were with the strains of *P. alcaligenes*, which were acetamide positive (14% at 24 h and 21% at 48 h).

Esculin hydrolysis was negative for all organisms tested, and urea hydrolysis was usually negative except for strains of *Bordetella bronchiseptica*, CDC IVc-2, and CDC IVe, which were consistently urease positive. The characteristic "instant" urea reaction previously reported with CDC IVe organisms (9) was not

TABLE 3. Results of plate reactions at 24 h; glucose weakly or nonoxidative group

Organism	No. of strains tested	Uni-N/F-Tek plate reactions (% positive)											
		Glucose	Xylose	Mannitol	Lactose	Maltose	Acetamide	Esculin	Urease	DNase	ONPG	Indole	H ₂ S
<i>P. acidovorans</i>	8	0	0	50	0	0	88	0	0	0	0	0	0
<i>P. testosteroni</i>	3	0	0	0	0	0	0	0	0	33	0	0	0
<i>P. alcaligenes</i>	14	0	0	0	0	0	14	0	0	0	0	0	0
<i>P. pseudoalcaligenes</i>	5	60	0	0	0	20	0	0	20	0	0	0	0
<i>P. putrefaciens</i>	5	0	0	0	0	0	0	9	0	80	0	0	100
<i>P. diminuta</i>	4	0	0	0	0	0	0	0	0	75	0	0	0
<i>A. calcoaceticus</i> subsp. <i>lwoffii</i>	28	0	0	0	0	0	4	0	0	0	0	0	0
<i>Moraxella</i> sp.	3	0	0	0	0	0	0	0	0	0	0	0	0
<i>A. faecalis</i>	6	0	0	0	0	0	0	0	0	0	0	0	0
<i>A. denitrificans</i>	5	0	0	0	0	0	0	0	0	0	0	0	0
<i>A. odorans</i>	19	0	0	0	0	0	84	0	0	11	0	0	0
<i>B. bronchiseptica</i>	7	0	0	0	0	0	0	0	100	0	0	0	0
CDC IVc-2	1	0	0	0	0	0	0	0	100	0	0	0	0
CDC IVe	3	0	0	0	0	0	0	0	100	0	0	0	0

observed, although the reaction was positive at 0.5 to 1 h.

DNase production was, as expected, usually positive with *P. putrefaciens* (80% at 24 h and 100% at 48 h) and *P. diminuta* (75% at 24 h and 100% at 48 h) and usually negative with other species.

ONPG, indole, and H₂S reactions gave expected negative results with all of the organisms in this group with the exception of *P. putrefaciens* which, as expected, was consistently H₂S positive.

(ii) **Identification.** By using the 24-h results of biochemical reactions, only 26% of the weak and nonoxidative nonfermenters studied were identified. Although more reactions were positive after 48 h, no additional strains were identified with those results. The low percentage of identification was expected because of the general nonreactivity of these organisms with the tests included in the N/F System. Positive reactions, when present, were consistent with expected conventional results and were useful for identification: 63% of the strains of *P. acidovorans* were mannitol and acetamide positive, 75% of the strains of *P. diminuta* were DNase positive, 100% of the strains of *P. putrefaciens* were H₂S positive, and 84% of the strains of *A. odorsans* were acetamide positive and nitrogen gas producers (GNF tube). Negative reactions were similarly consistent with expected conventional results and also useful in the identification process. Once it became apparent that organisms were in this group, supplemental tests could have been selected which would have resulted in definitive identification. Gram stain morphology and hanging drop motility could have identified the nonmotile, coccobacillary *Acinetobacter lwoffii* and *Moraxella* species to the genus level. Flagella staining could have identified the remaining members of the group with the exception of the peritrichously flagellated, urea-positive strains of *B. bronchiseptica*, CDC IVc-2 and CDC IVe. The first two could have been distinguished from each other on the basis of nitrate reaction and growth on Salmonella-Shigella agar and the strains of CDC IVe could have been identified by the "instant" urea reaction on Christensen's urea.

In conclusion, the N/F System was found to be a conveniently prepackaged series of selected biochemical tests which, when used to identify

nonfermenters, gave a high frequency of expected reactions within 24 h. The screening tubes effectively identified *P. aeruginosa*, the most common clinical isolate in this group, and the biochemical plate effectively identified most oxidative strains. Weakly and nonoxidative strains were usually not identified but were readily characterized as such, so that appropriate supplemental tests could be performed. Sufficient numbers of key biochemicals were provided so that discrepancies in expected reactions did not lead to misidentification.

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