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Biochemical and Susceptibility Tests Useful for Identification of Nonfermenting Gram-Negative Rods

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Six hundred nineteen strains of nonfermenting gram-negative rods were tested for alkaline phosphatase, benzyl-arginine arylamidase, pyrrolidonyl arylamidase, ethylene glycol acidification, and susceptibility to desferrioxamine and colistin. The results were highly discriminant. Therefore, the proposed tests may be helpful for the identification of this group of organisms.

Identification of gram-negative nonfermenting rods by conventional methods is often difficult and time-consuming, and commercial systems do not always provide reliable identification, especially of some genera or species (7, 17, 19). Moreover, recent taxonomic studies resulted in the description of an increasing number of new genera and species involved in nosocomial infections and requiring additional tests for identification. This was true, for instance, for the new genus *Ralstonia* (2, 3, 14), the former "Flavobacterium" group (12, 13), and others. In this study, we investigated a large number of strains for six biochemical and susceptibility characteristics that are rarely or not at all included in conventional schemes. Alkaline phosphatase (PHO), benzyl-arginine arylamidase (BAA), and pyrrolidonyl arylamidase (PYR), three enzymatic tests, are sometimes included in commercial systems, but they are not systematically reported in existing identification schemes (4, 6, 11). Susceptibility to desferrioxamine has been proposed for the identification of coagulase-negative staphylococci (9) but not for gram-negative bacilli. Acid production from ethylene glycol was described in corynebacteria (20) but, as far as we know, has not been evaluated in nonfermenters. We have also included susceptibility to colistin, although susceptibility to polymyxin B is often mentioned in conventional schemes (4, 6, 11), but low colistin content disks may yield slightly different results.

Five hundred fifty-three clinical isolates of nonfermenting gram-negative rods and 66 reference strains, including type strains, were tested. They included 41 species or Centers for Disease Control and Prevention (CDC) groups (Table 1). Only fast growers on tryptic soy agar were investigated. *Moraxella* spp. and other fastidious nonfermenters were excluded, as were *Acinetobacter* spp. because the latter can be identified on the basis of a few simple routine tests. All tests and cultures were performed at 30°C. For enzymatic reactions, diagnostic tablets from ROSCO (Taastrup, Denmark) were used in accordance with the manufacturer's instructions with slight modifications. Tablets were put in 0.5 ml of a 3 to 4 McFarland bacterial suspension and incubated for 4 h. Reading of BAA and PYR was done after addition of 1 drop of cinnamaldehyde reagent. PHO turned yellow when positive; no color or a pale yellowish color was considered negative. Determination of acid production from ethylene glycol was performed as previously described (20). Susceptibility to desferrioxamine was determined on Mueller-Hinton agar using 6-mm-diameter paper disks loaded with 250 μg of desferrioxamine (9). Testing for susceptibility to colistin was also carried out on Mueller-Hinton agar using commercial paper disks containing 10 μg of colistin (Becton Dickinson, Cockeysville, Md.). Strains displaying any inhibition of growth were recorded as susceptible. Conventional identification of the strains was performed in accordance with existing schemes (4, 6, 11), including flagellar staining for the vast majority of motile strains and cellular fatty acid analysis where appropriate. Strains showing an unusual profile or one or more atypical characteristics by conventional tests were submitted to 16S rRNA gene sequencing in order to rule out or confirm questionable identifications (8).

Results of the six proposed tests are reported in Table 1. Most of the tests yielded a large number of 100% or 0% positivity rates, resulting in a high discriminant value. However, 13 taxa contained fewer than 10 strains, which does not allow a statistically significant assessment of the results. Nevertheless, if only the 28 species or taxa represented by more than 10 strains are taken into account, tests like BAA achieved a 100% or 0% score for 93% (26 out of 28) of them. The values for the other tests were higher than 80%, except for ethylene glycol, which was only 54%. When considering more than 90% and less than 10% positivity rates as positive and negative results, respectively, a unique profile for the six tests was exhibited by five species: *Burkholderia cepacia*, *Burkholderia pseudomallei*, *Ralstonia pickettii*, *Comamonas terrigena* genomovar 1, and *Weeksella virosa*. Several other profiles were shared by only two taxa. Although these profiles do not constitute an identification system by themselves, they may provide a prominent contribution to a correct identification.

Colistin (polymyxin E) and polymyxin B have similar activities, and the interpretive diameters of resistance and susceptibility for therapeutic purposes are approximately the same when 10-μg colistin disks and 300-U polymyxin B disks are used (1). In this study, however, the results obtained for some

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TABLE 1. Percentages of positive results obtained with the six proposed tests[a]

Organism (no. of strains)	% of results positive					
	PHO	BAA	PYR	EGL	DEF	COL
Pseudomonas aeruginosa (35)	3	97	77	94	0	100
Pseudomonas fluorescens (21)	0	100	62	38	0	100
Pseudomonas putida (20)	0	100	0	100	0	100
Pseudomonas stutzeri (27)	0	100	0	100	0	100
Pseudomonas mendocina (3)	66	100	0	100	0	100
Pseudomonas alcaligenes/pseudoalcaligenes (17)	0	100	0	94	59	100
CDC group 1[c] (3)	0	100	0	100	0	100
Pseudomonas (*Chryseomonas*) *luteola* (10)	30	100	100	90	0	100
Pseudomonas (*Flavimonas*) *oryzihabitans* (17)	7	100	100	100	0	100
Burkholderia cepacia complex (23)	87	0	0	4	13	0
Burkholderia pseudomallei (3)	100	0	0	100	100	0
Ralstonia pickettii (22)	0	0	100	0	100	0
Ralstonia mannitolytica (4)	0	0	100	0	0	0
Ralstonia paucula (9)	22	0	100	0	0	100
Ralstonia gilardii (5)	100[b]	0	0	60	0	100
Brevundimonas diminuta (25)	100	100	12	52	92	0
Brevundimonas vesicularis (10)	100	100	0	70	100	0
Delftia (*Comamonas*) *acidovorans* (28)	0	0	96	39	0	0
Comamonas testosteroni (15)	0	0	100	20	0	100
Comamonas terrigena genomovar 1 (7)	0	0	100	0	100	100
Comamonas terrigena genomovar 2/3 (27)	0	0	0	0	100	100
Stenotrophomonas maltophilia (31)	100	100	0	0	0	38
Ochrobactrum anthropi/intermedium (31)	0	100	100	100	0	93
Agrobacterium radiobacter (19)	0	100	100	100	21	53
Shewanella spp. (6)	100	100	100	100	0	100
Sphingomonas paucimobilis (16)	100	100	25	69	0	19
Achromobacter (*Alcaligenes*) *xylosoxidans* subsp. *xylosoxidans* (29)	0	0	100	100	0	69
Achromobacter (*Alcaligenes*) *xylosoxidans* subsp. *denitrificans* (14)	0	0	100	100	0	100
Achromobacter (*Alcaligenes*) *piechaudii* (14)	0	0	100	86	0	86
Alcaligenes faecalis (21)	5	28	0	81	100	100
Bordetella bronchiseptica (14)	0	0	0	0	0	100
Bordetella hinzii (5)	0	0	100	100	0	100
Chryseobacterium meningosepticum (16)	100	100	100	100	0	0
Chryseobacterium indologenes (24)	100	100	100	54	0	0
Empedobacter brevis (2)	100	100	100	0	0	0
Sphingobacterium multivorum (10)	100	100	100	0	0	0
Myroides spp. (12)	100	100	100	0	17	0
CDC group EO-2[d] (6)	0	0	0	100	0	100
Oligella urethralis (2)	0	0	0	100	100	100
Weeksella virosa (13)	100	100	100	0	100	100
Bergeyella zoohelcum (3)	100	100	0	0	100	0

[a] EGL, ethylene glycol acidification; DEF, desferrioxamine susceptibility; COL, colistin susceptibility.
[b] Reaction weak or delayed.
[c] Reference 6.
[d] Reference 10.

species with the 10-μg colistin disks for diagnostic purposes sometimes differed from those reported for polymyxin B. Many *Agrobacterium radiobacter* strains were resistant and displayed full growth at the edge of the disk, whereas they have been reported to be susceptible to polymyxin B (11). *Ochrobactrum anthropi* strains were susceptible overall, but results for some strains were difficult to interpret because there was only partial inhibition around the disk. As expected, the type strain of *O. intermedium* was clearly resistant, as was one clinical isolate confirmed as *O. intermedium* by 16S gene sequencing (18). All *Delftia* (formerly *Comamonas*) *acidovorans* isolates were uniformly resistant, while the related species *Comamonas testosteroni* and *C. terrigena* were susceptible.

Some tests or combinations of tests allowed us to rule out some genera or species, or they were highly suggestive for a group of organisms. For example, BAA activity and susceptibility to colistin are always present in *Pseudomonas* species but never in *Burkholderia* spp. or in *Ralstonia pickettii* or *Ralstonia mannitolytica*. BAA activity with a strong PHO reaction, resistance to colistin, and susceptibility to desferrioxamine in motile

nonfermenters are almost always specific to *Brevundimonas* spp. The three enzymatic reactions PHO, BAA, and PYR are, as a rule, positive in members of the former "Flavobacterium" group, including *Chryseobacterium*, *Empedobacter*, *Myroides*, and *Sphingobacterium* spp. Among the two *Myroides* species (12), *Myroides odoratimimus* is more frequently isolated in humans than is *M. odoratus*. In our series, only one clinical isolate was identified as *M. odoratus* by cellular fatty acid analysis and 16S gene sequencing. It is noteworthy that only this isolate and the type strain of *M. odoratus* were susceptible to desferrioxamine, in contrast to *M. odoratimimus* strains, but this should be confirmed in the future with a larger number of strains. Susceptibility to desferrioxamine allows us to distinguish easily between *Comamonas terrigena* and *Comamonas testosteroni*. Moreover, among the three genomovars of *Comamonas terrigena* outlined by Willems et al. (21), genomovar 1 is positive for PYR activity but genomovars 2 and 3 are negative.

Correct identification of *Burkholderia cepacia* is often considered difficult (5, 7, 17). This species is of prominent importance in cystic fibrosis patients. Recent studies have proved the heterogeneity of the species both genomically and phenotypically, and several new species have been proposed within the former group (5, 15, 16). Phenotypic profiles may be confused with those of similar species. In this respect, all of our strains failed to exhibit the two enzymatic activities BAA and PYR, and although the number of strains was limited, this might be helpful in distinguishing the *Burkholderia cepacia* complex from *Burkholderia gladioli* (results not shown), *Ralstonia pickettii*, and *Ralstonia mannitolytica*, which are all PYR positive.

Kiska et al. evaluated four different commercial systems for the identification of *Burkholderia cepacia* and other nonfermenting gram-negative bacilli recovered from patients with cystic fibrosis: RapID NF Plus (Innovative Diagnostic Systems, Norcross, Ga.), API Rapid NFT (bioMérieux-Vitek, Hazelwood, Mo.), Vitek Auto/Microbic System GNI (bioMérieux-Vitek), and Uni-N/F Screen (Remel, Lenexa, Kans.). They reported results ranging from 43 to 86% for *Burkholderia cepacia* and from 57 to 80% for all nonfermenters (7). Furthermore, in a study of the Crystal Enteric/Non-Fermenter system (Becton Dickinson), only 75% of the nonfermenters were correctly identified and a similar identification score was achieved by the API 20NE system (bioMérieux, Marcy l'Etoile, France) (19). Hence, commercial identification systems do not always provide satisfactory results for the identification of gram-negative nonfermenting rods.

The tests proposed in this study may improve the identification of gram-negative nonfermenting rods either by their inclusion in conventional schemes or as complementary tests when results obtained by other methods are ambiguous or questionable.

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