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Methods for Distinguishing Gram-Positive from Gram-Negative Bacteria

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Lysis by KOH and hydrolysis of L-alanine-4-nitroanilide were compared with the Gram reaction of aerobic, microaerophilic, and anaerobic bacteria. Both tests correlated well with the Gram reaction with nonfermentative bacilli and *Bacillus* species, whereas they did not correlate with nonsporulating anaerobes. Only campylobacteria were KOH positive and L-alanine-4-nitroanilide and gram negative.

Members of the genera *Bacillus*, *Erysipelothrix*, *Lactobacillus*, and *Listeria*, among others, are classified in part on the basis of their positive reaction in Gram stain. Occasionally, however, isolates are encountered in the clinical laboratory that appear to be gram variable or gram negative (3, 5). Some of these are later shown, by obtaining gram-positive stains with young cultures or by demonstrating the presence of spores, to be compatible with *Bacillus* species. With others, an aberrant staining reaction may lead to misidentification or inability to generate a compatible biochemical profile. Indeed, errors in determining the Gram reaction are among the most frequent causes of mistaken identification (3). Furthermore, recognition of a clinical isolate as a *Bacillus* species rather than an "unidentified gram-negative rod" is important since the former are now known to be opportunistic pathogens (4, 9, 10).

Particularly because of problems in recognizing *Bacillus* species, tests have been sought which will correlate with the Gram reaction. The data of Gregersen (6) suggest that dissolution of the cell wall and cytoplasmic membrane by 3% KOH should be a reliable index for Gram negativity and that failure to dissolve should be an index for Gram positivity. However, Blachman et al. (1) and Halebian et al. (7) have reported that the KOH test does not precisely correlate with the Gram reaction. Yet another test, that for the presence of cell wall aminopeptidase, has been reported by Cerny (2). Working with aerobic and facultative organisms, he found complete correlation between the presence of aminopeptidase and Gram negativity.

We report here an examination of the Cerny test and its parameters (2) and a comparison of this test with the KOH test of Gregersen (6). Except for *Campylobacter* species, all strains used for this study were obtained from the UCLA and Wadsworth Veterans Administration Hospital stock culture collections and from other regional hospitals. *Campylobacter* species were obtained from fresh clinical isolates provided by the UCLA clinical laboratories and from the stock culture collection of the U.S. Food and Drug Administration, Los Angeles district. Anaerobic species were incubated for 48 to 72 h on anaerobic blood agar (Clinical Standards, Los Angeles, Calif.) in an anaerobic GasPak system (BBL Microbiology Systems, Cockeysville, Md.) at 35°C. Campylobacteria were grown on blood agar plates (Clinical Standards) under microaerophilic conditions in an anaerobic jar at 35°C. All other cultures to be tested were taken from 24- to 48-h blood or chocolate agar plates incubated at 35°C. The procedure for the KOH test was that of Halebian et al. (7). All Gram stains were prepared according to the method of Paik (8) with modified Huckers crystal violet (Fisher Scientific Co., Fair Lawn, N.J.), safranine O (Manufacturing Chemists, Norwood, Ohio), and 95% ethyl alcohol. For the L-alanine-4-nitroanilide (LANA) test, we used (i) the reagent of Cerny (2), namely, 4% LANA (EM Laboratories Inc., Elmsford, N.Y.) in 50 mM Tris-maleate buffer (pH 7.0); (ii) LANA at concentrations from 0.25 to 4%; (iii) Tris-maleate buffer at several concentrations and from pH 5 to 9; and (iv) LANA in phosphate rather than Tris-maleate buffer. To perform the LANA test, we used the "spot" procedure of Cerny (an opaque suspension of bacteria in 2 drops of 4% reagent on a glass slide or in the lid of a petri plate) and tube tests (0.5 to 1.0 ml in each tube [13 by 100 mm]. The spot and tube tests were examined for up to 60 min before being discarded. All tests, unless otherwise specified, were performed at room temperature.

TABLE 1. Comparison of Gram stain with KOH and LANA tests[a]

Species	No. of strains	Gram stain	LANA test	KOH test[b]
Aerobic				
Achromobacter xylosoxidans	1	−	+	+
Achromobacter var. Vd-2	1	−	+	+
Acinetobacter anitratum	7	−	+	(1)
Acinetobacter lwoffii	1	−	+	+
Alcaligenes denitrificans	7	−	+	+
Alcaligenes faecalis	5	−	+	+
Alcaligenes odorans	5	−	+	+
Bordetella bronchiseptica	6	−	+	+
Escherichia coli	1	−	+	+
Flavobacterium meningosepticum	3	−	+	+
Flavobacterium multivorum (var. IIk-2)	6	−	+	+
Flavobacterium odoratum	2	−	+	+
Flavobacterium var. IIb	5	−	+	+
Flavobacterium var. IIf	1	−	+	+
Moraxella nonliquefaciens	2	−	+	(1)
Moraxella osloensis	3	−	+	(1)
Moraxella phenylpyruvica	2	−	+	(1)
Moraxella urethralis	5	−	+	+
Pseudomonas acidovorans	4	−	+	+
Pseudomonas aureofaciens	2	−	+	+
Pseudomonas alcaligenes	4	−	+	+
Pseudomonas cepacia	2	−	+	+
Pseudomonas diminuta	8	−	+	+
Pseudomonas fluorescens	5	−	+	+
Pseudomonas stutzeri	1	−	+	+
Bacillus cereus	2	+	−	(1)
Bacillus globigii	1	+	−	−
Bacillus licheniformis	1	+	−	−
Bacillus subtilis	1	+	−	−
Microaerophilic and anaerobic				
Bacteroides fragilis	1	−	−	−
Bacteroides melaninogenicus subsp. *intermedius*	1	−	+	−
Bacteroides ruminicola subsp. *ruminicola*	1	−	+	−
Bacteroides vulgatus	1	−	−	−
Campylobacter fetus subsp. *fetus*	5	−	−	+
Campylobacter fetus subsp. *jejuni*	12	−	−	+
Campylobacter fetus subsp. *coli*	5	−	−	+
Campylobacter fetus subsp. *venerealis*	1	−	−	+
Campylobacter sputorum subsp. *sputorum*	1	−	−	+
Veillonella parvula	1	−	−	−
Clostridium difficile	1	+	−	−
Clostridium perfringens	1	+	−	−
Clostridium sporogenes	1	+	−	−
Clostridium tetani	1	+	−	−
Lactobacillus catenaforme	1	+	−	−

[a] For the KOH test, 3% KOH was used; a positive reaction was the dissolution of cells with release and stringing of DNA. For the LANA test, 0.5 ml of 1% LANA–50 mM Tris-maleate buffer (pH 7.0) per tube was used. Each inoculum was ca. 3 mm^3 of cell paste; incubation was for 30 min at room temperature. +, Positive reaction; −, negative reaction.

[b] Number within parentheses is the number of negative strains.

We found that phosphate buffer was unacceptable for the LANA reagent; its shelf life was less than 1 week at 4°C, in contrast to LANA in Tris-maleate, which remained nearly colorless after storage for 8 weeks at 4°C. A 1% solution gave results in the tube tests comparable to those with the 4% reagent used by Cerny. With concentrations below 0.5%, the positive (yellow) tests were less intense. The molarity of the Tris-maleate buffer was not critical; comparable re-

sults in tube tests were obtained with concentrations from 12.5 to 200 mM. However, the intensity of the yellow color in positive tests was significantly less at pH 6 than that at pH 7 to 9. In tube tests, inocula of 10^9 to 10^{10} bacteria (ca. 3 mm^3 of cell paste) gave positive tests (with positive strains) within 30 min, and most strains gave positive tests within 5 min. Moraxellae were an exception; some strains failed to give positive spot tests within 30 min and required 15 to 20 min for positive tube tests. This time could be significantly decreased by deviating from the normal procedure and incubating the tubes at 35°C.

Our results from Gram stains, KOH tests, and LANA tube tests are shown in Table 1. Nonfermentative gram-negative bacteria were emphasized in this study since their appearance with Kligler's iron agar medium can be mimicked by *Bacillus* species. All of the 88 strains of nonfermentative gram-negative bacteria gave positive LANA tests, but 4 strains gave negative KOH tests. Four strains of *Bacillus* species gave negative KOH and LANA tests, but one strain of *Bacillus cereus* gave a positive KOH test. Gregerson (6) found that one strain of *Bacillus macerans* also gave a positive KOH test.

Of all gram-negative bacteria examined in this study, only the microaerophilic campylobacteria were KOH positive but LANA negative. Our results with nonsporulating anaerobic bacteria, like the results of Halebian et al. (7), did not show good correlation among Gram reaction, lysis by KOH, and hydrolysis of LANA. Only two of the four *Bacteroides* species gave positive LANA tests, and none gave a positive KOH test. Susceptibility to vancomycin and colistin disks, although not as rapid as KOH and LANA tests, can aid in the determination of Gram positivity and Gram negativity with anaerobic bacteria.

We conclude that both the KOH and LANA tests may be useful adjuncts for characterizing clinical isolates and therefore merit further evaluation.

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