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Evaluation of Three Disk Tests for Identification of Enterococci, Leuconostocs, and Pediococci

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Simple rapid tests for presumptive identification of catalase-negative non-beta-hemolytic cocci (i.e., enterococci, leuconostocs, and pediococci) have not previously been available. Seven hundred thirty-four strains of aerobic and facultatively anaerobic, catalase-negative, non-beta-hemolytic gram-positive cocci were tested for susceptibility to vancomycin (Van^s) by a screening procedure and production of leucine aminopeptidase (LAPase) and pyrrolidonylarylamidase (PYRase) in disk tests. Three unique patterns of activity in response to the three disks (30 μg of vancomycin, PYRase, and LAPase) can be used to presumptively identify the vancomycin-resistant (Van^r) enterococci (Van^r and PYRase and LAPase positive), leuconostocs (Van^r and PYRase and LAPase negative), and pediococci (Van^r, PYRase negative, and LAPase positive). The results indicate that, together with Gram stain characteristics and the catalase test, the vancomycin, LAPase, and PYRase disk tests can be used to presumptively identify Van^r strains of enterococci as well as *Leuconostoc* and *Pediococcus* strains from human infections.

Vancomycin-resistant (Van^r), facultatively anaerobic and aerobic gram-positive cocci, including *Enterococcus*, *Leuconostoc*, and *Pediococcus* species, are being isolated with increasing frequency from patients (1, 9, 10, 13, 14). The tests used to identify these three Van^r genera are complex and sometimes require several days to complete (6, 11, 15). Simple and accurate diagnostic tests are needed to shorten the time required to identify Van^r bacteria, because patient management methods may differ according to the infecting organism.

Disk tests such as those using bacitracin and optochin have been used for years to identify group A streptococci and pneumococci, respectively. They are simple to perform and require only isolated colonies on the primary culture plate and an additional blood agar plate for the test. In most cases, the test can be interpreted after overnight incubation. A disk test for determining the presence of pyrrolidonylarylamidase (PYRase) has been previously described for the identification of group A streptococci as well as enterococci (6, 8, 17). We have previously described a vancomycin susceptibility (Van^s) screening test to aid in the identification of viridans group streptococci (4). A disk test, the IDENTICULT-AE test (PML Microbiologicals, Tualatin, Oreg.), which combines esculin hydrolysis and PYRase production into a single disk to identify the enterococci and group A streptococci, has also been described (3).

This study describes the results of testing of clinical isolates submitted to our laboratory for identification from 1989 to 1993 with three disk tests, consisting of (i) Van^s screening, (ii) production of leucine aminopeptidase (LAPase), and (iii) production of PYRase.

MATERIALS AND METHODS

Bacterial cultures were received from city and state health departments in the United States from 1989 to 1993. The strains were isolated from a variety of human sources, including blood and cerebrospinal fluid cultures, wound infections, abscesses, and peritoneal and synovial fluid cultures. Bacterial cultures were identified by previously described procedures (5, 7).

Vancomycin disks and trypticase soy–5% sheep blood agar plates were obtained from BBL Microbiology Systems, Inc., Cockeysville, Md.

PYRase disks, LAPase disks, and the detection reagent (*p*-dimethylaminocinnamaldehyde) were obtained from Carr Scarborough Microbiological, Inc., Stone Mountain, Ga.

Bacterial strains were cultured on trypticase soy–5% sheep blood agar plates. The inoculum was spread very thickly over one-half of the plate and spread thinly over the remainder of the plate. The vancomycin disk was placed on the section of the plate with the heaviest growth. The plates were incubated overnight in a CO_2 incubator at 35 to 37°C. Growth on the thinly spread area of the plate was examined for culture purity after incubation. After incubation, the LAPase and PYRase disks were placed on an area of the plate with little or no growth. One or two large loopfuls of bacteria from the overnight growth were then transferred to the surface of each disk. If the growth was scant, several loopfuls of bacteria were transferred to each disk. The reaction was allowed to proceed at room temperature for 10 min, at which time 1 drop of the detection reagent was added to each disk.

The reaction to vancomycin was recorded as positive if any zone of inhibition was present. Zones of inhibition were not measured. The LAPase and PYRase tests were recorded as positive if a pink or red color developed within 3 min of the addition of the detection reagent. A yellow color or no color change after 3 min indicated a negative reaction.

RESULTS

All of the non-beta-hemolytic streptococci tested (beta-hemolytic strains were not tested) were Van^s in the vancomycin screening test (Table 1). The majority of streptococci produced LAPase, but only the nutritional variant streptococci produced PYRase. Two reaction patterns were identified among the enterococcal strains: Van^r and Van^s. All of the Van^r strains produced both LAPase and PYRase. Some of the enterococcal strains, notably *Enterococcus avium* and *Enterococcus raffinosus*, failed to produce LAPase. If Van^r is detected in these species, a new pattern of reactions to the three disks will result when tested: Van^r, LAPase negative, and PYRase positive. The *Lactococcus* cultures gave nearly the same results as the Van^s enterococci. The *Globicatella* cultures were all Van^s and produced PYRase but did not produce LAPase. The *Leuconostoc* cultures were all Van^r and failed to produce LAPase or PYRase.

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TABLE 1. Percent positive reactions of gram-positive cocci for three diagnostic disk tests

Organism	No. tested	% of organisms		
		Van^s	LAPase positive	PYRase positive
Gram-positive cocci in chains				
Streptococcus spp.				
Viridans group	282	100	98	0
S. bovis	10	100	100	0
NVS[a]	22	100	76	77
Enterococcus spp.				
Van^s	98	100	90	99
Van^r	62	0	100	100
Lactococcus spp.	39	100	95	83
Globicatella spp.	7	100	0	100
Leuconostoc spp.	53	0	0	0
Gram-positive cocci in pairs, tetrads, and clusters				
Aerococcus spp.	28	100	0	100
ALO[b]	7	100	100	0
Helcococcus spp.	7	100	0	100
Alloiococcus spp.	23	100	100	100
Gemella spp.	34	100	100	94
Pediococcus spp.	21	0	100	0
Unidentified	41	90	63	58

[a] NVS, nutritional variant streptococci.
[b] ALO, *Aerococcus*-like organisms.

Among the gram-positive cocci that did not form chains (Table 1), only the pediococci were Van^r. The aerococci and helcococci were all susceptible to vancomycin and produced PYRase but not LAPase. The *Aerococcus*-like organisms were also Van^s but produced LAPase but not PYRase. The alloiococci and gemellae were Van^s and produced both LAPase and PYRase. In addition to being Van^r, the pediococci produced LAPase but not PYRase.

The unidentified gram-positive cocci were those that did not fit the genus description given in reference 5. Cultures in this category had at least one reaction that was atypical for genus identification. For example, one vancomycin-resistant gram-positive coccus formed chains and produced gas like a *Leuconostoc* strain; however, this organism also produced LAPase, which is unlike the leuconostocs. This strain appears to be nearly identical to the type strain of *Leuconostoc paramesenteroides*, which is also LAPase positive (14). Confirmation of the identity of this strain is pending. Other unidentified gram-positive cocci had two or more characteristics that prevented them from being placed into a genus identification.

DISCUSSION

The isolation of additional genera and species of gram-positive cocci has made it necessary for clinical laboratories to expand their diagnostic capacities to identify leuconostocs, pediococci, and helcococci (1, 5). In the majority of cases in which microbiologic tests are applied to aid in the identification of an unknown culture, it is necessary to have some knowledge of the culture's Gram-staining characteristics, cellular arrangement, and hemolytic and catalase activity. This is the case for the presumptive tests described here.

Leuconostoc and *Pediococcus* species are intrinsically resistant to vancomycin, while some species of enterococci have acquired Van^r (16). There is a need for quick, simple, and accurate tests to identify Van^r gram-positive cocci. Disk tests are easy to perform and generally require only overnight growth of the bacteria for interpretation. Perhaps the best examples of these tests are the bacitracin and optochin tests for group A streptococci and pneumococci, respectively. Neither requires a standardized inoculum, and in both tests, identification of each is determined by inhibition of growth around the disk after overnight incubation. Some investigators have described a combination of PYRase and esculin hydrolysis for rapid identification (within 30 min of obtaining isolated colonies) of the enterococci (3). This combination of reactions should accurately identify the enterococci but will not identify the other genera of gram-positive cocci. Other investigators have advocated the use of Gram staining, gas formation from glucose broth, hydrolysis of arginine, PYRase production, and Van^r to identify the Van^r strains of enterococci, leuconostocs, lactobacilli, and pediococci (11). Although these tests are simple, they require 2 days or more for a final reading.

We have reported that the 30-μg vancomycin disk test could be used to help identify gram-positive cocci (4, 5). All viridans group streptococci and aerococci gave zones of inhibition around the vancomycin disk. At this time, among the catalase-negative, gram-positive cocci and coccobacillary bacteria, only *Leuconostoc* spp., *Pediococcus* spp., some *Enterococcus* strains, and some *Lactobacillus* strains are resistant to vancomycin (16). This indicates that, although the vancomycin screening test does not selectively identify any given genus or species, it may be used in combination with other tests to help identify gram-positive cocci.

The PYRase test has been a useful test to aid in the identification of enterococci and other gram-positive cocci (4, 8, 17). Of more than 500 strains of enterococci (160 in this study) tested in the past few years, we have identified only 1 strain that failed to give a positive PYRase reaction. Many species of other genera of gram-positive cocci have PYRase activity, including the majority of strains belonging to the *Lactococcus*, *Globicatella*, *Aerococcus*, *Alloiococcus*, and *Gemella* genera (Table 1). However, one of the more useful aspects of the PYRase tests is the failure of the viridans group streptococci and *Streptococcus bovis* strains, leuconostocs, and pediococci to show any PYRase activity. This "all or none" aspect of the PYRase test makes it attractive for differentiating the gram-positive cocci.

The LAPase test has been a component in the Rapid Strep identification system (Biomerieux, Hazelwood, Mo.) and has only recently become available as a separate test. This is the first report of an evaluation of the LAPase test as an independent test. As with PYRase, many gram-positive cocci have LAPase activity. The most useful aspect of the LAPase test is the failure of all strains of aerococci, globicatellae, helcococci, and leuconostocs to have LAPase activity. Only one species of the *Leuconostoc* genus, *L. paramesenteroides*, is reported to have LAPase activity (14). Not only is this strain atypical in that it is not genetically closely related to the other *Leuconostoc* species (2, 12), but it is found only rarely among strains identified from human sources. Among 100 strains of leuconostocs isolated from human sources, we have confirmed only 1 strain that resembles *L. paramesenteroides.*

TABLE 2. Genus identification by three disk tests

Organism	Response to test[a]		
	Vancomycin	LAPase	PYRase
Enterococcus spp. (Van^s) *Lactococcus* spp. *Streptococcus pyogenes* NVS[b] *Gemella* spp. *Alloiococcus* spp.	S	+	+
Streptococcus spp. (not GAS[c] or NVS) ALO[d]	S	+	−
Aerococcus spp. *Globicatella* spp. *Helcococcus* spp.	S	−	+
Enterococcus spp. (Van^r)	R	+	+
Pediococcus spp.	R	+	−
Leuconostoc spp.	R	−	−

[a] S, susceptible; R, resistant; +, positive; −, negative
[b] NVS, nutritionally deficient streptococci.
[c] GAS, group A streptococci.
[d] ALO, *Aerococcus*-like organisms.

Three unique patterns of activity in response to the three disks (30 μg of vancomycin, PYRase, and LAPase) can be used to presumptively identify the Van^r enterococci (Van^r and PYRase and LAPase positive), leuconostocs (Van^r and PYRase and LAPase negative), and pediococci (Van^r, PYRase negative, and LAPase positive) (Table 2).

We also suggest that the viridans group streptococci and *Streptococcus bovis* strains together can be presumptively identified by the combination of reactions Van^s, LAPase positive, and PYRase negative. Among these strains, there have been no exceptions in any of these three tests (Tables 1 and 2). However, remember that these identifications are assisted by knowledge that the strains are catalase negative, gram-positive cocci.

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