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Identification of Vancomycin-Resistant Lactic Bacteria Isolated from Humans

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By using cell morphology, arginine dihydrolase, and gas production in de Man, Sharp, Rogosa broth, 122 isolates of vancomycin-resistant lactic bacteria from humans were assigned to five profiles, allowing us to distinguish *Pediococcus*, homofermentative and heterofermentative *Lactobacillus*, and *Leuconostoc* species. The absence of L-(+)-lactic acid, as detected spectrophotometrically, was confirmatory for *Leuconostoc* species. API 50 CHL panels were useful for the identification of *Lactobacillus* species.

Since the description by Buu-Hoi et al. (3) in 1985 of a case of bacteremia caused by a *Leuconostoc* sp., there have been many reports of gram-positive bacteria isolated from patients with critical infections such as endocarditis, septicemia, meningitis, pneumonia, and odontogenic that have high-level resistance to vancomycin (1, 2, 4, 8, 10, 11). *Leuconostoc* spp., *Pediococcus* spp., and some homofermentative and heterofermentative *Lactobacillus* spp. are now the main members of this group, which are referred to as vancomycin-resistant lactic bacteria (VRLB) (12). The use of conventional tests and commercial panels often results in the misidentification of these bacteria as viridans group streptococci (12). A few studies have emphasized the difficulty in distinguishing some *Lactobacillus* spp., particularly *Lactobacillus confusus*, from *Leuconostoc* spp. (5).

In this report, we propose an identification scheme for the VRLB on the basis of the results obtained with a large number of isolates from humans.

A total of 122 wild strains presumably identified as VRLB were examined. They were isolated from stools or other clinical samples. In addition, the following nine reference strains were included in the study: *Pediococcus acidilactici* NCIB 6990, *Pediococcus pentosaceus* NCIB 12012, *Lactobacillus buchneri* NCIB 8007, *Lactobacillus confusus* NCIB 4037 (= *Lactobacillus coprophilus*), *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293^T, *Leuconosstoc lactis*, ATCC 19256^T, *Leuconostoc paramesenteroides* ATCC 33313^T, *Leuconostoc pseudomesenteroides* ATCC 49371^T, and *Leuconostoc citreum* NCFB 1837^T.

Cellular morphology was observed on Gram staining of cells grown for 24 h in de Man, Sharp, Rogosa (MRS) broth. Coccoid cells were defined as round or ovoid shaped, and the length could not exceed twice the width of the cells. Rod-shaped or bacillary forms were elongated, most of the cells being longer than twice their width. Coccobacillary morphology consisted of a mixture of coccoid and rod-shaped bacteria.

Biochemical reactions. The catalase reaction was performed on colonies grown on blood agar. Gas production from glucose was detected in MRS broth with Durham tubes. Arginine hydrolysis was performed in Moeller decarboxylase medium. Pyrrolidonylarylamidase (PYR) activity was determined by using diagnostic tablets (Rosco Diagnostica, Taastrup, Denmark). Carbohydrate fermentation was tested by using the API 50 CHL System (bioMérieux, Marcy-l'Etoile, France), as recommended by the manufacturer. Acid production from carbohydrates was also tested in MRS broth base without glucose but containing bromcresol purple as the indicator and the substrates at 1% (wt/vol). API 20 Strep panels (bioMérieux) were also used.

L-(+)-Lactate production was detected in a 24-h MRS broth by using a specific lactic acid dehydrogenase (Boerhinger). L-(+)-Lactic acid is oxidized in the presence of L-(+)-lactate dehydrogenase and NAD^+ is oxidized to pyruvate and NADH. The addition of glutamate-pyruvate transaminase in the presence of L-glutamate results in the production of L-alanine and 2-oxoglutarate. The presence of L-(+)-lactic acid was assessed by two methods. (i) The production of 2-oxoglutarate was detected by gas-liquid chromatography (GLC). (ii) The amount of NADH formed in the first reaction described above is stoichiometric with the amount of L-(+)-lactic acid and was detected spectrophotometrically in a 1/50 dilution of a 24-h MRS broth. The increase in the amount of NADH was determined by measuring the A_{340}. The results were expressed as the difference (ΔA) between the absorbance of the sample 120 s after the addition of L-(+)-lactate dehydrogenase and the absorbance before its addition. The mean ΔA of 11 uninoculated MRS broth samples, defined as the background ΔA, was 0.107, and the standard deviation (SD) was 0.23 ΔA. The cutoff was defined as the mean background ΔA + 4 SD, corresponding to a ΔA value of 0.200.

Identification of strains. By using Gram staining, gas production in MRS broth, and the presence of arginine dihydrolase as the first step in identification, the isolates and reference strains, all of which were catalase negative and vancomycin resistant, could be allocated to the following five distinct groups. (i) The first group consisted of coccoid, gas-negative (homofermentative), arginine-positive strains. Two strains that fit this category were discarded from the VRLB group and identified as *Enterococcus* spp. by a positive PYR reaction (5). Thirty-two strains had a characteristic morphology of *Pediococcus* spp., with clumps and tetrads. They were further identified biochemically by the API 50 CHL system as *Pediococcus acidilactici* and *Pediococcus pentosaceus*. Both species could be differentiated by acid production from maltose, the former being negative and the latter being positive.

(ii) The second group comprised rod-shaped, gas-negative (homofermentative), arginine-negative strains. Fifty-five strains belonged to *Lactobacillus* spp. according to the API

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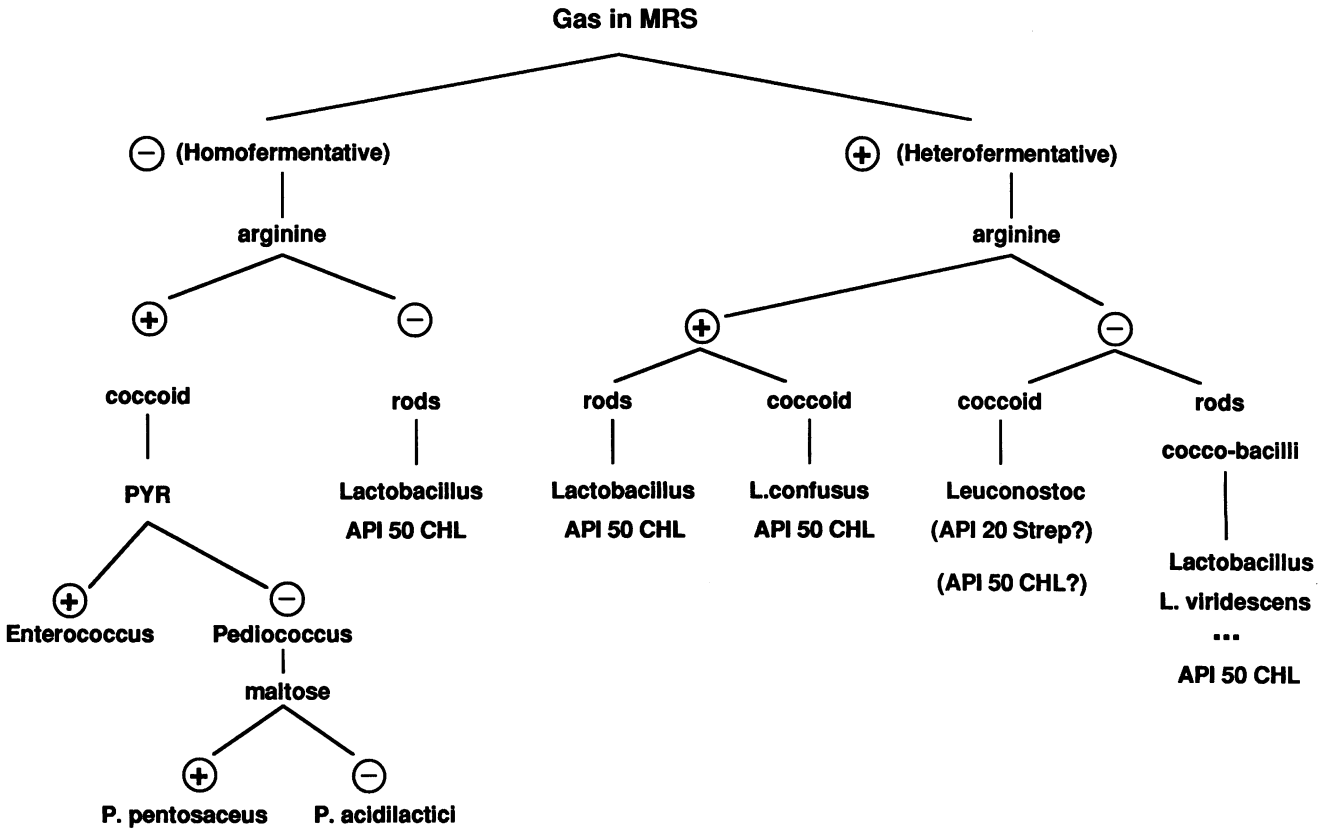

FIG. 1. Practical identification scheme for VRLB of human origin.

50 CHL system, which allowed recognition of only three species: *Lactobacillus casei* subsp. *casei* and *Lactobacillus casei* subsp. *rhamnosus*, *Lactobacillus plantarum*, and *Lactobacillus salivarius*.

(iii) The third group consisted of rod-shaped, gas-positive (heterofermentative), arginine-positive strains. Thirteen strains were *Lactobacillus* spp., and the API 50 CHL system identified eleven strains as *Lactobacillus fermentum* (= *Lactobacillus cellobiosus*) or *Lactobacillus buchneri*. Two strains could be identified only to the genus level. The lactic acid produced by these strains revealed, as expected, the presence of the L-(+) form.

(iv) The fourth group was made up of coccoid, gas-positive (heterofermentative), arginine-positive strains. Only three strains fit this category, and on Gram staining, members of this group resembled *Leuconostoc* spp. The API 50 CHL system clearly identified them as *Lactobacillus confusus* (= *Lactobacillus coprophilus*), which is known to be morphologically similar to *Leuconostoc* spp. The lactic acid analysis, however, detected the production of L-(+)-lactic acid, allowing a definite distinction with *Leuconostoc* spp. which produce only D-(−)-lactic acid.

(v) The fifth group consisted of coccoid or coccobacillary, gas-positive (heterofermentative), arginine-negative strains. Seventeen strains of this group were presumptively identified as *Leuconostoc* spp. However, production of L-(+)-lactic acid by two strains, as evidenced by ΔA values of 0.482 and 0.400, respectively, permitted them to be identified as *Lactobacillus* spp. The biochemical study of the first strain by the API 50 CHL system resulted in the identification of *L. viridescens*, another *Lactobacillus* spp. that, although it is rod shaped, may be misleading in the differential identification with *Leuconostoc* spp. The second strain was *Lactobacillus confusus* (= *Lactobacillus coprophilus*), which, although usually arginine positive, had a slightly delayed arginine reaction in this test.

In contrast, the 15 other strains had a mean ΔA value of 0.113 (range, 0.075 to 0.165), indicating a lack of L-(+)-lactic acid production, consistent with the identification of the genus *Leuconostoc*, in which the presence of only D-(−)-lactic acid is a salient feature. These strains were poorly identified by the API 50 CHL system. Five of the 15 wild strains were identified as *Lactococcus* spp. or *Lactobacillus* spp. The 10 remaining strains were identified as *Leuconostoc mesenteroides* or *Leuconostoc lactis*; for 5 of the strains, however, the discriminatory capacity was low. The type strains of the five *Leuconostoc* species tested by the API 50 CHL system were recorded as follows: *Leuconostoc mesenteroides* ATCC 8293^T = API *Leuconostoc mesenteroides*, 73.4% (dubious profile); *Leuconostoc paramesenteroides* ATCC 33313^T = API *Leuconostoc mesenteroides*, 99.9% (dubious); *Leuconostoc pseudomesenteroides* ATCC 49371^T = API *Lactobacillus pentosus* (unacceptable); *Leuconostoc lactis* ATCC 19256^T = API *Aerococcus viridans*, 99.9% (dubious); *Leuconostoc citreum* NCFB 1837^T = API *Leuconostoc citreum*, 98.4% (dubious).

The first step in the identification of VRLB is vancomycin resistance. Outside VRLB and the rarely encountered *Erysipelothrix rusiopathiae*, among the catalase-negative, gram-positive bacteria, only *Enterococcus* species may exhibit resistance to this antibiotic. Gas production in MRS broth and arginine dihydrolase production have been advocated as

salient features in the identification of VRLB (9). Arginine dihydrolase is by no means a distinctive character for the differentiation of genera of lactic bacteria except *Leuconostoc* spp. Since variable results occur in both *Pediococcus* and *Lactobacillus* spp. according to the species. However, the occurrence of only a few species of these genera in humans makes this test suitable for such purposes. Indeed, within the homofermentative category (gas production negative in MRS broth), the two *Pediococcus* species so far found in humans, *Pediococcus pentosaceus* and *Pediococcus acidilactici*, are arginine positive (12) and the *Lactobacillus* spp. are arginine negative. Similarly, in the heterofermentative group, arginine provides an easy diagnostic test for the recognition of *Leuconostoc* spp., which are always negative. However, some heterofermentative lactobacilli, mainly *Lactobacillus viridescens*, may be arginine negative, but either they are rod shaped or the bacillary cells usually outnumber the coccoid forms.

Nevertheless, the most reliable feature for use in differentiating *Leuconostoc* spp. from *Lactobacillus* spp. is the detection of L-(+)-lactic acid production by the latter, whereas *Leuconostoc* spp. only produce D-(−)-lactic acid (9). All of our strains identified as *Leuconostoc* spp. failed to produce L-(+)-lactic acid, whereas the arginine-positive heterofermentative strains and the arginine-negative *Lactobacillus viridescens* strain were positive in this test. Among the *Lactobacillus* strains tested, the mean ΔA value was 0.396 (range, 0.275 to 0.642; SD, 0.100), which was significantly higher than the value for the *Leuconostoc* group ($P = 0.0001$; two-tailed t test). In this respect, the GLC method is generally recommended (6, 9). The method that we proposed, which is based on the photometric detection of NADH, has, as far as we know, not yet been evaluated for this purpose. In our experience, this technique provides a rapid method for the detection of L-(+)-lactic acid in MRS broth, and the results are easier to interpret than those obtained by GLC (data not shown).

The poor results of genus and species identification of *Leuconostoc* by commercial panels is supported by the dubious or erroneous identifications obtained with reference strains. However, some *Leuconostoc* strains were correctly identified at the genus level by the API 20 Strep panel. Results of identification of our *Leuconostoc* strains to the species level by conventional tests were often inconsistent with those obtained by using the existing schemes, which also present discrepancies, according to the authors and the methods used, or are based on tests with too few isolates (5, 7, 9, 13).

A practical identification scheme for VRLB for application in the clinical laboratory is presented in Fig. 1. Although it could not be recommended routinely, spectrophotometric detection of L-(+)-lactic acid may provide a reliable confirmatory test for *Leuconostoc* spp. identification within the heterofermentative group of VRLB when arginine or cell morphology results are equivocal.

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