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Identification of *Enterococcus* Species Isolated from Human Infections by a Conventional Test Scheme

R. R. FACKLAM[1]* AND M. D. COLLINS[2]

Respiratory Diseases Branch, Division of Bacterial Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333,[1] and Department of Microbiology, Institute of Food Research, Agricultural Food Research Council, Reading Laboratory, Shinfield, Reading RG2 9AT, United Kingdom[2]

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Streptococci (206 cultures) previously identified as enterococci were retrieved from storage and reidentified by using tests designed to identify species of the genus *Enterococcus*. Of these 188, 91% were correctly identified as *Enterococcus* species. Of the remaining strains, nine (4%) were unidentified and six (3%) and 3 (1.5%) were identified as *Leuconostoc* sp. and *Lactococcus* sp., respectively. Two new *Enterococcus* species were discovered: *E. raffinosus* and *E. solitarius*. DNA-DNA hybridizations were performed on selected strains to assure correct identification. Cultures representing 10 of the 12 *Enterococcus* species were among the 188 strains identified. An identification system based on the grouping of key reactions of 20 phenotypic characteristics of *Enterococcus* species is described.

In 1972 (7), we published a summary of 26 physiologic tests used to differentiate group D streptococci that had been submitted to our laboratory for species identification. We reported that most of the specimens were *Streptococcus faecalis*, but *S. faecium*, *S. durans*, and *S. bovis* were also present in our collection. No *S. avium*, *S. faecium* subsp. *casseliflavus*, or *S. equinus* isolates were identified. In 1975, Gross et al. (11), using three tests not included in our initial studies (pyruvate utilization, deamination of arginine, and acidification of sorbose broth), documented the occurrence of *S. avium* in humans. We incorporated those 3 tests, together with 14 of our tests, to construct a physiologic identification scheme for group D *Streptococcus* species (8). Other investigators have used similar procedures to identify *S. faecalis*, *S. faecium*, *S. durans*, *S. avium*, and *S. bovis* from humans (12, 16).

The current *Bergey's Manual of Systematic Bacteriology* lists seven genera of facultatively anaerobic, gram-positive cocci (17). Five of these genera, including *Streptococcus*, do not contain cytochrome enzymes and are thus catalase negative. In 1984 (18), the genus *Streptococcus* was split into three genera, *Enterococcus*, *Lactococcus*, and *Streptococcus*. The justification and background for this action were reviewed by Schleifer and Kilpper-Bälz (19). DNA-DNA hybridization studies have confirmed that the following are valid *Enterococcus* species: *E. avium*, *E. casseliflavus*, *E. durans*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae*, *E. malodoratus*, and *E. mundtii* (1, 2, 9, 14, 18). During the course of this study, two new *Enterococcus* species were discovered: *E. raffinosus* and *E. solitarius* (M. D. Collins, R. R. Facklam, J. A. E. Farrow, and R. Williamson, submitted for publication). The DNA-DNA hybridization and penicillin-binding protein patterns of these two strains, as well as those of a third new nonhuman species, *E. pseudoavium*, will be reported elsewhere (Collins et al., submitted). The initial investigations that described the phenotypic characteristics of the new *Enterococcus* species used a combination of miniaturized-dehydrated tests and conventional tests (1, 2, 9, 18). The results of miniaturized-dehydrated tests usually, but not always, correlate with the results of conventional tests (10).

The purpose of this study was to evaluate a procedure based on conventional biochemical tests that will identify the species of the genus *Enterococcus*.

MATERIALS AND METHODS

Strains. The type strains used to devise the identification scheme were obtained from the American Type Culture Collection (ATCC) (*E. avium* ATCC 14025, *E. casseliflavus* ATCC 25788, *E. durans* ATCC 19432, *E. faecalis* ATCC 19433, and *E. faecium* ATCC 14434) or the National Collection of Dairy Organisms (NCDO) (*E. gallinarum* NCDO 2313, *E. hirae* NCDO 1258, *E. malodoratus* NCDO 846, *E. mundtii* NCDO 2375, and *E. pseudoavium* NCDO 2138). Strains CDC 885-78 and CDC 1739-79 are the type strains of *E. solitarius* and *E. raffinosus*, respectively (Collins et al., submitted). We retrieved 206 cultures of streptococci previously identified as enterococci from storage. Only strains that had one or more atypical reactions based on previous results or were identified as *S. avium*, *S. durans*, or unidentified enterococci were retrieved.

Tests. Gram stains were prepared from thioglycolate broth, resistance to 30-μg vancomycin disks was determined on tryptic soy agar (5), pyrrolidonylarylamidase activity was determined by the broth substrate test (10), and gas production from glucose was determined in Mann, Rogosa, and Sharpe broth (4). Tolerance to bile esculin and growth in 6.5% NaCl broth were determined as previously described (8).

Tolerance to 0.04% tellurite, reduction of 0.1% tetrazolium, hydrolysis of sodium hippurate, and growth at 10 and 45°C were tested as previously described (7).

Carbohydrate fermentation tests were performed in heart infusion broth base with 1% mannitol, sorbitol, sorbose, inulin, arabinose, melibiose, sucrose, raffinose, trehalose, lactose, glycerol, salicin, or maltose (7, 8).

Deamination of arginine was tested in Moellers decarboxylase broth, motility was determined in modified Difco motility medium, and pigmentation was observed after overnight growth on tryptic soy agar. If the culture produced pigment, yellow was observed on a cotton swab that was

* Corresponding author.

TABLE 1. Percentages of positive reactions of study cultures in tests for identification of enterococci

Test	% Positive reactions of strains of the genus:		
	Enterococcus (*n* = 188)	*Lactococcus* (*n* = 3)	*Leuconostoc* (*n* = 6)
Gram-positive cocci	100	100	100
Group D antigen	77	0	17
Bile-esculin reaction	100	100	100
Growth in 6.5% NaCl broth	100	100	100
Vancomycin susceptibility	99	100	0
Gas from glucose	<1	0	100
Pyrrolidonylarylamidase	100	33	0

used to pick up growth from the tryptic soy agar plate. Extracellular polysaccharide production, hydrolysis of esculin, and growth in litmus milk were determined as previously described (7, 8).

Utilization of pyruvate was tested in 1% pyruvate broth (11), and the Coblentz method was used for the Voges-Proskauer tests (10). All of the medium formulations and interpretations of tests used are described in the *Manual of Clinical Microbiology*, 4th ed. (8).

Serogrouping was done by using the Lancefield extraction procedure and Centers for Disease Control group D and Burroughs Wellcome group N antisera (8).

Inoculation and incubation. Single-colony isolates were inoculated into 5 ml of Todd-Hewitt broth which was then incubated overnight at 35°C.

All media were inoculated with a Pasteur pipette with 1 drop of the Todd-Hewitt broth culture. All of the tests were incubated at 35°C, except for growth tests incubated at 10 and 45°C and motility tests incubated at 30°C. The tests were read at 24 h, 48 h, and 7 days.

DNA base composition and DNA-DNA hybridization. Preparations, compositions, and hybridizations were performed by using methods previously described (1).

RESULTS AND DISCUSSION

The tests more commonly used to identify gram-positive bacteria as members of the genus *Enterococcus* are listed in Table 1. Demonstration of group D antigen in extracts of cultures is not specific for enterococci. Not only do cultures of *S. bovis* and *S. equinus* have the group D antigen, but some strains of leuconostocs and most strains of pediococci also have the D antigen (6). Positive reactions in both bile-esculin reaction and salt tolerance tests (6.5% NaCl broth) were the reason that some strains of lactococci and leuconostocs were initially identified as enterococcal streptococci. The lactococci were identified by demonstrating a group N streptococcal antigen rather than group D antigen in Lancefield extracts. The leuconostocs were identified by vancomycin resistance, gas production from glucose, and negative pyrrolidonylarylamidase reactions (6). The vancomycin screening test is a very useful adjunct to other tests for identifying enterococci. Only one *Enterococcus* strain (an *E. faecium* strain) gave no zone of inhibition around a vancomycin disk in the screening tests. Strains of *E. gallinarum* for which vancomycin MICs were 16 μg/ml (13) gave reduced zone diameters (about 12 mm) in response to vancomycin in the screening test but were interpreted as susceptible for identification purposes. It is not known how often vancomycin-resistant enterococci occur in infected humans; however, *E. faecium* strains with high-level resistance to vancomycin (MIC, 1,000 μg/ml) have been isolated in epidemic settings (15, 20). Vancomycin resistance has not been documented among naturally occurring viridans group streptococci. If viridans group streptococci should develop vancomycin resistance, the value of the vancomycin screening test as an aid for differentiating gram-positive cocci would diminish. Gas production from glucose is a very reliable test to help identify gram-positive cocci, since only leuconostocs and about half of the lactobacillus species (which may be confused with gram-positive cocci) produce gas from glucose. One strain of *E. faecalis* produced sufficient gas to generate a positive test in this study. The pyrrolidonylarylamidase test is necessary for accurate identification of the enterococci. All species of enterococci are pyrrolidonylarylamidase positive. Reference 6 should be consulted for a more detailed description of methods to identify different gram-positive cocci. Reference laboratories should include testing for group N antigen, as well as the seven tests included in Table 1 for identification of cultures belonging to the genus.

TABLE 2. Key tests for identification of *Enterococcus* groups

Species	No. of strains	Group	Reaction (% positive)[a]			
			Mannitol	Sorbitol	Sorbose	Arginine
E. avium *E. raffinosus* *E. malodoratus* *E. pseudoavium*	47	I	+ (100)	+ (97)	+ (97)	− (0)
E. faecalis *E. solitarius* *E. gallinarum* *E. faecium* *E. casseliflavus* *E. mundtii*	113	II	+ (99)	V (63)	− (0)	+ (94)
E. durans *E. hirae* *E. faecalis*[b]	28	III	− (7)	− (0)	− (0)	+ (100)

[a] +, Positive reaction; −, negative reaction; V, variable (some strains positive, some strains negative).
[b] Asaccharolytic variant.

The easiest way to identify *Enterococcus* species is to use key phenotypic characteristics to form groups at the species level. Table 2 illustrates how this is done. On the basis of the results of four physiologic tests, the 12 species and 1 variant species were placed in three groups identified as I, II, and III (Table 2). Because exceptions occur with each of the four tests, it is advisable to interpret the key test results as a whole. Note that at least two very different test results are shown for each group. In most cases, three different test results place the enterococci into the correct group. Once placed into group I, II, or III, the unknown organism can be identified to the species level by selected test results described in the following tables.

Nine cultures remained unidentified; of these, five did not fit into any of the three groupings listed in Table 2. Two each fit into groups II and III but did not have characteristics that would place them into any known species listed in Table 3 or 4. The results derived from testing these nine unidentified bacteria are not included in the following tables.

Table 3 lists the *Enterococcus* species and the differential characteristics of each species included in group I. Two of the four species have been recently described on the basis of

TABLE 3. Identification of group I *Enterococcus* species[a]

Species	No. of strains	Reaction (% positive)[b]	
		Arabinose	Raffinose
E. avium	32	+ (99)	− (0)
E. raffinosus	15	+ (100)	+ (100)
E. malodoratus[c]	0	− (0)	+ (100)
E. pseudoavium[c]	0	− (0)	− (0)

[a] Key reactions, Mannitol, sorbitol, and sorbose (+) and arginine (−).
[b] +, Positive reaction; −, negative reaction.
[c] The data for *E. malodoratus* and *E. pseudoavium* are for the type strains (one each) only.

DNA-DNA hybridization studies (Collins et al., submitted). The identities of 11 strains (5 *E. avium* strains and 6 *E. raffinosus* strains) were confirmed by DNA-DNA hybridization studies (Collins et al., submitted). No *E. malodoratus* or *E. pseudoavium* strains were identified among the 197 enterococcal strains in this study. This group is characterized by acidification of mannitol, sorbitol, and sorbose broths, as well as failure to deaminate arginine. The species were differentiated from each other by acidification of arabinose and raffinose broths (Table 3).

Table 4 lists the enterococcal species and the differential characteristics of each species included in group II. One new species is included in this group (*E. solitarius*). The identities of 15 strains, including 4 *E. faecalis* strains, 1 *E. solitarius* strain, 4 *E. gallinarum* strains, 5 *E. faecium* strains, and 1 *E. casseliflavus* strain, were confirmed by DNA homology studies (Collins et al., submitted). *E. solitarius* is differentiated from *E. faecalis* by the failure of *E. solitarius* to form acid in lactose broth. However, other differences, which are discussed below, exist between the two species. The other four species listed in Table 4, *E. gallinarum*, *E. faecium*, *E. casseliflavus*, and *E. mundtii*, are differentiated by motility and pigmentation.

Table 5 lists the *Enterococcus* species and the differential characteristics of each species identified in group III. One variant strain is included in this group, the asaccharolytic variant of *E. faecalis*, which failed to form acid in mannitol, sorbitol, lactose, sucrose, or glycerol broth; typical *E. faecalis* strains formed acid in all five broths. The identities of five strains (one *E. durans* strain, three *E. hirae* strains, and one *E. faecalis* asaccharolytic variant) were confirmed by DNA homology studies. *E. durans* was differentiated from *E. hirae* by failure of the former to form acid in either sucrose or raffinose broth. The *E. faecalis* asaccharolytic variant usually utilizes pyruvate (like *E. faecalis*), whereas *E. durans* and *E. hirae* do not.

TABLE 4. Identification of group II *Enterococcus* species[a]

Species	No. of strains	Reaction (% positive)[b]				
		Arabinose	Sorbitol	Lactose	Motility	Pigment
E. faecalis	55	− (0)	+ (96)	+ (100)	− (0)	− (0)
E. solitarius	4	− (0)	+ (100)	− (0)	− (0)	− (0)
E. gallinarum	13	+ (100)	− (0)	+ (100)	+ (100)	− (0)
E. faecium	31	+ (100)	V (29)	+ (97)	− (0)	− (0)
E. casseliflavus	8	+ (100)	V (50)	+ (100)	+ (100)	+ (100)
E. mundtii	2	+ (100)	V (50)	+ (100)	− (0)	+ (100)

[a] Key reactions, Mannitol and arginine (+) and sorbose (−).
[b] +, Positive reaction; −, negative reaction; V, variable (some strains positive, some strains negative).

TABLE 5. Identification of group III *Enterococcus* species[a]

Species	No. of strains	Reaction (% positive)[b]		
		Sucrose	Raffinose	Pyruvate
E. durans	6	− (0)	− (0)	− (0)
E. hirae	8	+ (88)	+ (75)	− (0)
E. faecalis[c]	14	− (0)	− (0)	+ (64)

[a] Key reactions, Mannitol, sorbitol, and sorbose (−) and arginine (+).
[b] +, Positive reaction; −, negative reaction.
[c] Asaccharolytic variant.

The percentages of positive reactions of the 12 species and the one variant strain in 15 additional tests are listed in Table 6. Although most of the tests were not useful for differentiating most of the *Enterococcus* species, some helped in identifying and differentiating some species. *E. casseliflavus* is the only *Enterococcus* species to form acid in inulin (six of eight strains). Also, *E. faecalis* tolerated 0.04% tellurite (93%), but *E. solitarius*, which was very similar in other phenotypic characteristics, did not (0%). The reduced overall percentage of enterococcal strains that gave positive group D antigen test results (77% [Table 1]) is due to our failure to demonstrate group D antigen from strains of *E. avium* and *E. raffinosus* (32 and 40% positive, respectively [Table 6]).

It is not possible to compare our findings with those of others, because the cultures of bacteria used in this study were selected on the basis of unusual phenotypic characteristics and our criteria for species identification were different. We have established the value of this identification scheme by documenting serial isolation of *E. gallinarum* (13). We identified more than 200 additional typical enterococcus cultures, mostly *E. faecalis* and *E. faecium*, in our laboratory after completion of this study. In their comprehensive review of streptococcal infections, Parker and Ball (16) listed 15 strains, 7 of which were from endocarditis patients, as "other enterococci." More than likely, some of those other enterococci were species that could be identified by this system. Colman and Ball (3) reported *E. durans* and *E. avium*, as well as *E. faecalis* and *E. faecium*, in their collection of strains originally isolated from humans. They also speculated that they had *E. gallinarum* isolates; however, on the basis of the description given for their "atypical enterococci," at least three of the isolates were probably *E. raffinosus*.

Of the strains identified in this study, only *E. mundtii* has not been isolated from human blood cultures. Endocarditis was given as a clinical diagnosis of patients from whom *E. avium*, *E. casseliflavus*, *E. durans*, *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. raffinosus* were isolated. Seven of the 14 *E. faecalis* asaccharolytic variants were isolated from patients with endocarditis. The non-blood culture enterococcal strains were isolated from sources such as urine, pleural fluid, wounds, and abscesses.

Although published studies have reported the distribution of *Enterococcus* species identified from human infections, it is believed that most of the infections are caused by *E. faecalis* (3, 7, 12, 16). *E. faecium* is the second most commonly identified species, and all of the other species probably make up only 10% or less of all enterococcal isolates from infected humans. *E. faecium* cultures are more resistant than *E. faecalis* to several commonly used antimicrobial agents, making the identification of *Enterococcus* species useful for aiding in managing patients with enterococcal infections (12, 13, 15, 20).

TABLE 6. Percentages of positive reactions in additional tests used to differentiate *Enterococcus* species[a]

Strain	% Positive for:									
	Acid formation				Hippurate hydrolysis	Growth tolerance			VP	Group D antigen
	Glc	Inu	Mel	Tre		10°C	Tell	Tetr		
E. avium	44	0	50	100	0	66	0	78	62	32
E. raffinosus	93	0	100	100	33	27	0	33	66	40
E. malodoratus	0	0	100	100	0	100	0	0	0	100
E. pseudoavium	0	0	0	100	0	100	0	0	0	0
E. faecalis	93	0	4	99	24	99	93	99	89	91
E. solitarius	100	0	25	100	100	75	0	50	75	100
E. gallinarum	0	0	100	100	62	100	8	46	62	100
E. faecium	0	0	81	100	23	97	3	19	89	68
E. casseliflavus	63	75	100	100	0	75	0	63	25	100
E. mundtii	0	0	100	100	0	100	50	100	100	100
E. durans	0	0	75	100	33	100	0	17	100	75
E. hirae	0	0	88	100	13	100	0	38	100	63
E. faecalis[b]	0	0	0	75	100	100	0	100	0	100

[a] Glc, Glycerol; Inu, inulin; Mel, melibiose; Tre, trehalose; Tell, 0.04% tellurite; Tetr, 0.25% tetrazolium; VP, Voges-Proskauer. None of the cultures hydrolyzed starch or produced extracellular polysaccharides in 5% sucrose broth or 5% sucrose agar. All of the strains formed acid in salicin and maltose broths, grew at 45°C, and formed acid in litmus milk; the clot reaction in litmus milk was variable.

[b] Asaccharolytic variant.

The identification system described in this study will enable microbiologists to accurately identify most *Enterococcus* species with a minimal number of conventional physiologic tests.

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