Q-259: Speciation of Environmental *Enterococcus* and Related Species: Comparison of Four Commercial Phenotypic Methods with Biochemical Testing

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I. ABSTRACT

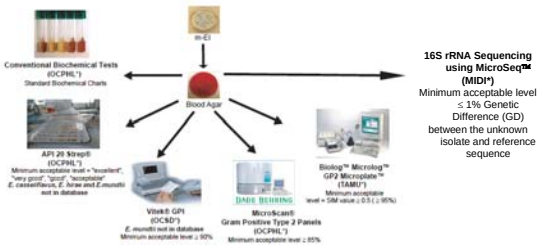
Species identification of enterococci by conventional biochemical methods is challenging and not routinely performed by environmental laboratories. Although automated and rapid identification systems are becoming more widely used, there has not been extensive testing on environmental isolates. Four commercial gram positive cocci identification methods were compared for identifying presumptive environmental enterococci isolates from mEI media: API 20 Strep (bioMérieux), Vitek GPI card (bioMérieux), identification portion of the MicroScan Walk/Away Rapid Positive Combo 12 panel (Dade Behring), and Biolog GP2 MicroPlate (Biolog, Inc.). The methods were compared for identification of *Enterococcus* and closely related, non-*Enterococcus* species, *S. bovis* and *A. viridans* using conventional biochemical testing as the gold standard. The ability to accurately identify typical and atypical strains (N=99) from 9 different species isolated from environmental sources: 66 marine water, 20 sediment, 8 seagull stool and 5 storm drain water samples was determined. Quality control (ATCC) strains were also tested. Without additional biochemical testing, 78.8%, 83.8%, 86.9%, and 87.9% of all isolates were correctly identified to the genus level, and 47.5%, 41.4%, 49.5%, 57.6% strains were correct to the species level, by API, Vitek, MS and Biolog, respectively. For both API and Vitek, additional conventional biochemical testing is suggested. For the species tested in this study, correct identification to species level by Vitek improved from 41.4% to 62.6% with the additional biochemical tests. Accuracy of identification also varied by species; for example, all four methods correctly identified most *E. faecalis* and *E. faecium* isolates, whereas *E. durans* identification was more challenging. Automated or rapid identification systems without additional supplementary biochemical testing should be used with caution for species identification of environmental enterococci strains, as well as *A. viridans* and *S. bovis*.

II. INTRODUCTION

When enterococci levels in water adjacent to the beaches exceeds regulatory limits, local health officials post signs to restrict beach access. Enterococci detected in water using United States Environmental Protection Agency (USEPA) approved methods are identified as "presumptive enterococci". Genus and species identification of environmental enterococci is important for assessing detection methods, method quality control and for investigating potential sources of these organisms to water. Identification is usually determined using phenotypic methods such as commercial rapid test kits, automated systems or conventional biochemical testing. Initially, we assessed the ability of 4 commercial phenotypic systems: API 20 Strep, MicroScan, Vitek and Biolog to accurately identify environmental enterococci using conventional biochemical testing as the standard. The identifications were later confirmed by 16S rRNA sequencing. Correct or final identifications were determined using conventional biochemical test results in conjunction with 16S rRNA sequencing. The accuracy rates using the revised standard is presented in the tables shown here.

III. METHODS

FIGURE 1. Enterococci Identification Methods and Testing Laboratories



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IV. RESULTS

TABLE 1. Summary of conventional and commercial phenotypic methods and 16S rRNA sequencing for identification of presumptive enterococci isolates

Identification system	No. (%) of isolates identified correctly to the following taxonomic level:		
	Genus*	Species†	No Identification‡
Conventional Biochemical Tests	94 (100%)	89 (95%)	0 (0%)
API 20 Strep	74 (79%)	38 (40%)	20 (21%)
API 20 Strep w/supplemental tests	86 (91%)	45 (48%)	8 (9%)
Vitek GPI	85 (90%)	48 (51%)	9 (10%)
Vitek GPI w/supplemental tests	86 (91%)	69 (73%)	8 (9%)
MicroScan Rapid Positive Combo 12	87 (93%)	51 (54%)	7 (7%)
MicroScan Rapid Positive Combo 12 w/supplemental tests	88 (94%)	77 (82%)	6 (6%)
Biolog GP2 MicroPlate	87 (93%)	67 (71%)	7 (7%)
Biolog GP2 MicroPlate w/supplemental tests	87 (93%)	73 (78%)	7 (7%)
16S rRNA Sequencing - MicroSeq	94 (100%)	64 (68%)	0 (0%)

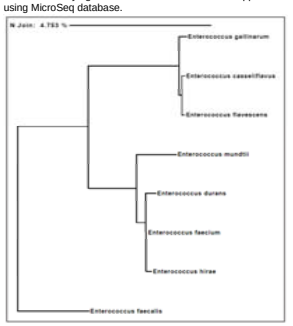
* Accurate result to Genus level above the minimum acceptable level for that test
† Accurate result to Species level above the minimum acceptable level for that test
‡ Incorrect result, identification below acceptable level or no identification

IV. RESULTS (cont'd)

TABLE 2. Accuracy by species of conventional and commercial phenotypic methods and 16S rRNA sequencing for identification of presumptive enterococci isolates to **genus** level

Organism (No. isolates)	No. (%) isolates with the following identifications					
	Conventional Biochemical Tests	API 20 Strep	Vitek GPI	MicroScan Rapid Positive Combo 12	Biolog GP2 MicroPlate	Sequencing 16S rRNA
E. gallinarum (10)	10 (100%)	7 (70%)	8 (80%)	9 (90%)	10 (100%)	10 (100%)
E. casseliflavus (12)	12 (100%)	11 (92%)	10 (83%)	10 (83%)	12 (100%)	12 (100%)
E. mundtii (7)	7 (100%)	7 (100%)	7 (100%)	7 (100%)	7 (100%)	7 (100%)
E. durans (8)	8 (100%)	6 (75%)	8 (100%)	8 (100%)	8 (100%)	8 (100%)
E. faecium (14)	14 (100%)	7 (50%)	14 (100%)	14 (100%)	13 (93%)	14 (100%)
E. hirae (8)	8 (100%)	6 (75%)	8 (100%)	8 (100%)	8 (100%)	8 (100%)
E. faecalis (10)	10 (100%)	6 (60%)	10 (100%)	10 (100%)	9 (90%)	10 (100%)
A. viridans (5)	5 (100%)	5 (100%)	1 (20%)	3 (60%)	0 (0%)	5 (100%)
S. bovis (20)	20 (100%)	19 (95%)	17 (85%)	18 (90%)	20 (100%)	20 (100%)

FIGURE 1. Phylogenetic tree of the *Enterococcus* spp. in this study using MicroSeq database.



The species relationships for the isolates tested are similar to those published elsewhere. *E. gallinarum* and *E. casseliflavus/flavescens* form a distinct group, as do *E. durans, E. faecium* and *E. hirae*. *E. faecalis* (and to a lower extent *E. mundtii*) do not demonstrate a specific relationship (sequence relatedness) to any other species.

TABLE 3. Accuracy by species of conventional and commercial phenotypic methods and 16S rRNA sequencing for identification of presumptive enterococci isolates to **species** level

Organism (No. isolates)	No. isolates with the following identifications					
	Conventional Biochemical Tests	API 20 Strep	Vitek GPI	MicroScan Rapid Positive Combo 12	Biolog GP2 MicroPlate	Sequencing 16S rRNA
E. gallinarum (10)	10 (100%)	4 (40%)	0 (0%)	6 (60%)	6 (60%)	6 (60%)
E. casseliflavus (12)	11 (92%)	0 (0%)*	0 (0%)	2 (17%)	12 (100%)	7 (58%)
E. mundtii (7)	7 (100%)	0 (0%)*	0 (0%)*	1 (14%)	7 (100%)	7 (100%)
E. durans (8)	8 (100%)	3 (38%)	4 (50%)	0 (0%)	2 (25%)	8 (100%)
E. faecium (14)	10 (71%)	4 (29%)	11 (79%)	11 (79%)	9 (64%)	11 (79%)
E. hirae (8)	8 (100%)	0 (0%)*	8 (100%)	0 (0%)	2 (25%)	6 (75%)
E. faecalis (10)	10 (100%)	4 (40%)	10 (100%)	10 (100%)	9 (90%)	10 (100%)
A. viridans (5)	5 (100%)	4 (80%)	0 (0%)	3 (60%)	0 (0%)	3 (60%)
S. bovis (20)	20 (100%)	19 (95%)	15 (75%)	18 (90%)	20 (100%)	6 (30%)

*Species not included in the test database

V. DISCUSSION

- For accurate identification of environmental enterococci, we used the results from a combination of conventional biochemical testing and 16S rRNA sequencing as the standard due to phenotypic and genotypic method limitations that became apparent in this study.
- Phenotypic methods are not always able to distinguish different *Enterococcus* species due to variable/aberrant phenotypic reactions and/or loss of specific phenotypic traits over time and passage.
- 16S rRNA sequencing was unable to distinguish some strains of closely related species that clustered together as shown in the phylogenetic tree (FIG. 1).
- 16S rRNA sequencing was useful for identifying new species currently not available in the phenotypic databases tested. Three isolates originally identified as *A. viridans* had sequences that were perfectly matched with *Desemzia incerta* and distantly related to enterococci (data not shown).
- There is still no consensus on the minimum degree of genetic difference between an unknown and the reference strain which would define them as the same species, further complicating identification of closely related species. A genetic difference of 0.5 to 1% is often used to define a species; we used ≤ 1% as recommended by the manufacturer and published elsewhere.

VI. CONCLUSIONS

For identification of environmental *Enterococcus* and related species, conventional biochemical testing was the best method for discriminating most species as compared to 16S rRNA sequencing and commercial phenotypic tests. Most isolates could be reliably identified to genus based on gram strain, growth in 6.5% NaCl, hydrolysis of bile esculin and growth at 45°C. Accurate identification to species was obtained using additional biochemical tests based on standard biochemical identification charts (Facklam and Collins 1989 *J Clin Microbiol* ; Manual of Clinical Microbiology 2003 *ASM Press*).

Most of the automated methods tested were acceptable for genus-level identification (>90% probability). Biolog was best for species-level identification (without supplemental biochemical tests).

Supplementary biochemical tests, such as motility, pigment production and sucrose fermentation, is needed for accurate identification of *E. casseliflavus*, *E. durans*, *E. faecium* and *E gallinarum* using commercial phenotypic identification systems.

API Strep 20 (without supplemental biochemical testing) accurately identified most *A. viridans* and *S. bovis* isolates but was unreliable for identification of most *Enterococcus* species tested here.

16S rRNA sequencing was useful for identifying commonly isolated species of enterococci, such as *E. faecalis* and *E. faecium* than for species which differed by just a few base pairs such as *E. gallinarum* and *E. casseliflavus*.

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