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ORIGINAL ARTICLE

Comparison of 16S rRNA sequencing with conventional and commercial phenotypic techniques for identification of enterococci from the marine environment

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Abstract

Aims: To compare accuracy of genus and species level identification of presumptive enterococci isolates from the marine environment using conventional biochemical testing, four commercial identification systems and 16S rRNA sequence analysis.

Methods and Results: Ninety-seven environmental bacterial isolates identified as presumptive enterococci on mEI media were tested using conventional and *Enterococcus* genus screen biochemical tests, four commercial testing systems and 16S rRNA sequencing. Conventional and *Enterococcus* genus screen biochemical testing, 16S rRNA sequencing and two commercial test systems achieved an accuracy of ≥94% for *Enterococcus* genus confirmation. Conventional biochemical testing and 16S rRNA sequencing achieved an accuracy of ≥90% for species level identification.

Conclusions: For confirmation of *Enterococcus* genus from mEI media, conventional or genus screen biochemical testing, 16S rRNA sequencing and the four commercial systems were correct 79–100% of the time. For speciation to an accuracy of 90% or better, either conventional biochemical testing or 16S rRNA sequencing is required.

Significance and Impact of the Study: Accurate identification of presumptive environmental *Enterococcus* isolates to genus and species level is an integral part of laboratory quality assurance and further characterization of *Enterococcus* species from pollution incidents. This investigation determines the ability of six different methods to correctly identify environmental isolates.

Introduction

Enterococci are gram-positive cocci that exist as commensal organisms in the intestinal tracts of humans, other mammals and birds. Enterococci are considered opportunistic pathogens of humans and animals (ASM 2002, 2003). They can also be found in soil, plants, insects, invertebrates and water (Sinton *et al.* 1993; Leclerc *et al.* 1996; Manero and Blanch 1999; Devriese *et al.* 2002). Enterococci are now starting to be utilized extensively as indicator bacteria for water quality. In 1986, the United States Environmental Protection Agency (USEPA) set minimum protective standards for enterococci levels in recreational water based on epidemiological studies that showed a strong correlation between enterococci concentrations in recreational waters with gastro-intestinal illness in humans bathing in sewage impacted areas (Cabelli *et al.* 1982; Cabelli 1983; Dufour 1984; Kay *et al.* 1994; WHO 1998). In some locations, testing for enterococci, in addition to total and fecal coliforms, has increased the number of water quality violations and regulatory actions that restrict access to recreational bathing beaches (Kinzelman *et al.* 2003; Noble *et al.* 2003). As a result, studies have focused on determining the contributing sources of

enterococci and to elucidate the ecology of these organisms in the environment (Anderson *et al.* 1997; Byappanahalli *et al.* 2003; Ferguson *et al.* 2005). Accurate identification of *Enterococcus* strains isolated from environmental samples is important for such studies as well as for evaluating accuracy of test methods currently being used or developed to detect and enumerate enterococci in water.

Existing methods for enumeration of enterococci include EPA Method 1600 using mEI agar (APHA 1998; USEPA 2000), EPA method 1106·1 using mE-EIA (USEPA 2005) and Enterolert™ Quanti-Tray® (IDEXX, Laboratories Inc., Westbrook, Maine). Enterolert uses a defined substrate test that provides a most probable number value of organisms. mEI and Enterolert detect enterococci based on the ability to utilize β-glucosidase in selective media. The mE-EIA method selects for enterococci and bases detection on the organism's ability to hydrolyze esculin. A positive result as defined by any of these methods is considered presumptive for *Enterococcus*. However, some species of *Streptococcus* and *Aerococcus* show up as false positives, compromizing the specificity and accuracy of the tests (Budnick *et al.* 1996; Messer and Dufour 1998; Feerst *et al.* 2002; Kinzelman *et al.* 2003; Ferguson *et al.* 2005). Verification of a percentage of presumptive *Enterococcus* isolates to genus is required for quality control of the membrane filtration method (APHA 1998; USEPA 2000) and recommended for Enterolert (Kinzelman *et al.* 2003). Enterococci can be identified using phenotypic methods such as conventional biochemical tests or commercial test systems or by genotypic methods such as 16S rRNA sequencing (Patel *et al.* 1998; Angeletti *et al.* 2001; ASM 2002, 2003). Conventional biochemical testing is accurate and is still considered the gold standard for speciation of enterococci. However, several *Enterococcus* species vary by only one phenotypic trait, and some of these are variable, leading to possible errors when large numbers of isolates are tested. In addition, this method is laborious and can require between 2 and 10 days to obtain results. Commercial identification systems are less laborious and more rapid with results available in <1–2 day(s) and many are in common use in clinical laboratories. Studies have shown their performance to be variable, with limited accuracy for determining *Enterococci* species except for *Enterococci faecalis*, the most commonly isolated clinical strain (Cartwright *et al.* 1995; Iwen *et al.* 1999; Garcia-Garrote *et al.* 2000; D'Azevedo *et al.* 2001; Ligozzi *et al.* 2002; Bosshard *et al.* 2003, 2004). To our knowledge, no studies have been carried out to determine the accuracy of these systems for testing environmental isolates. 16S rRNA sequencing has been utilized to identify clinical *Enterococcus* isolates (Angeletti *et al.* 2001; Bosshard *et al.* 2004). While this technique is potentially extremely accurate, several *Enterococcus* species have 16S rRNA sequences that are ≥99·9% homologous (Patel *et al.* 1998). This too may lead to identification errors for very closely related species. In addition, we have no knowledge of previous studies comparing the accuracy of sequencing to speciate environmental enterococci strains.

In this study, the ability of six methods to correctly identify environmental enterococci isolates obtained from mEI media was tested. Isolates were selected for each presumptive *Enterococcus* species isolated from the marine environment in our laboratory including species that cause false positive reactions on mEI agar. Accuracy of *Enterococcus* genus confirmation and accuracy of species identification was compared using phenotypic methods including conventional and genus screen biochemical testing, four commercially available identification systems and a genotypic method, 16S rRNA sequencing analysed utilizing MicroSeq and GenBank databases.

Materials and methods

Selection of isolates and testing

Ninety-nine environmental bacterial isolates originally identified as presumptive enterococci by typical reactions on mEI media (colony with blue halo) (USEPA 2000), were obtained from 66 marine water, 20 marine and intertidal sediments, eight seagull stool, and five storm drain water samples. These strains were selected to represent biochemically typical and atypical strains of *Enterococci casseliflavus*, *Enterococci durans*, *E. faecalis*, *Enterococci faecium*, *Enterococci gallinarum*, *Enterococci hirae*, *Enterococci mundtii*, *Aerococcus viridans* and *Streptococcus bovis* biotypes I and II as determined by an initial identification using API 20 Strep Vitek, Biolog and 16S rRNA sequence analysis. All tests were inoculated from the same stock culture. Biochemical testing, MicroScan and API 20 Strep were performed at Orange County Public Health Laboratory, Newport Beach, CA, USA (OCPHL). Vitek, Biolog and 16S rRNA sequencing were performed at Orange County Sanitation District Laboratory, CA, USA; Texas A&M University-Corpus Christi, TX, USA and MIDI Laboratory (Newark, DE), respectively. Results from all laboratories were analyzed at OCPHL.

Identification by conventional and genus screen biochemical testing

Biochemical tests were selected and species identification was performed using published standard biochemical identification charts (Facklam and Collins 1989; Facklam

and Elliott 1995; Facklam 2002; ASM 2003). Conventional biochemical testing included carbohydrate fermentation in brain heart infusion (BHI) broth base with 1% mannitol, sorbitol, arabinose, raffinose, sucrose and lactose, deamination of arginine in Moeller's decarboxylase broth (BBL, Franklin Lakes, NJ, USA), tolerance to bile esculin and growth in 6·5% NaCl all at 35°C, motility test medium w/TTC (triphenyl tetrazolium chloride) incubated at 30°C, growth at 45°C in BHI broth, detection of pyrrolidonyl arylamidase and leucine arylamidase using disk tests (Remel Inc., Lenexa, KS, USA) and 3% catalase. Gram stains were done from BHI broth and pigment production was assessed using Trypticase^{TM} soy agar with 5% sheep blood at 35°C. *Enterococcus* genus screen biochemical testing was a subset of these tests including gram stain, growth in 6·5% NaCl, growth at 45°C in BHI broth, 3% catalase and bile esculin tolerance.

Identification by commercial test systems

The API 20 Strep kit (bioMérieux Vitek, St. Louis, MO, USA) is comprised of 20 biochemical test reactions to identify gram-positive cocci, including enterococci and streptococci, to genus or species level. Identification is obtained using a database based on the biochemical reactions of 45 gram-positive cocci, including five *Enterococcus* spp. The MicroScan Pos Combo Type 12 panel of the MicroScan WalkAway 96 System (Dade Behring Inc., Sacramento, CA, USA) contains 27 dried biochemical tests. The software database includes 42 gram-positive cocci, including seven *Enterococcus* spp. Identification using the Vitek GPI (bioMérieux Vitek) card is determined using 29 tests and a database of 50 gram-positive cocci, including seven *Enterococcus* spp. The Biolog GP2 MicroPlate Panels of the MicroLog (Biolog, Hayward, CA, USA) provides 95 tests and a database with reactions for 339 gram-positive cocci, including 18 *Enterococcus* spp.

Inoculum preparation and incubation was performed as per manufacturer's instructions. Identification was scored with the test panel results provided by the system. Supplemental tests, as recommended by manufacturer's instructions, were performed by OCPHL for API, MicroScan and Vitek. For Biolog, although supplemental tests were not recommended, pigment production and motility testing was performed for each isolate and the Biolog results modified utilizing standard biochemical tables. For each commercial test, the result was considered acceptable if it met the following minimum criteria: (i) API: acceptable identification (corresponding to 80% identification), (ii) MicroScan: 85% identification, (iii) Vitek: 90% and (iv) Biolog: a similarity index of value ≥0·5 (corresponding to a ≥95% confidence level).

16S rRNA sequencing

A 500-bp 16S rRNA gene sequence, corresponding to *Escherichia coli* positions 005–531, was amplified and the PCR products were purified and sequenced using the MicroSeq 500 Gene Kit protocols. Cycle sequencing of the 16S rRNA amplification products was carried out using AmpliTaq FS DNA polymerase and dRhodamine dye terminators. Applied Biosystems MicroSeq^{TM} microbial analysis software and database were utilized to perform the identification. The top ten alignment matches were presented according to percent genetic difference (%GD) when aligned to minimize sequence gaps. The species with the lowest %GD was considered the identification. Identification utilizing GenBank database was carried out by internet-based 16S rRNA gene sequence comparison software utilizing the basic local alignment search tool with default settings. The closest species level match (% identity) was considered the identification.

Quality control reference strains

The following reference type strains were also analysed with all test systems: *E. casseliflavus* (ATCC#700327), *E. durans* (ATCC#6056), *E. faecalis* (ATCC#29212), *E. faecium* (ATCC#35667), *E. gallinarum* (ATCC#700425), *A. viridans* (ATCC#700406), and *S. bovis* (ATCC#49147).

Data analysis

The final identification for each isolate was assigned using a gold standard consisting of conventional biochemical testing in conjunction with 16S rRNA sequencing. The results obtained by both standard methods agreed for 85 of 97 (88%) isolates with 12 discrepancies. Discrepancies were resolved based on a perfect 16S rRNA sequence match with the reference strain or by positive key biochemical reactions. Three were identified as *Desemzia incerta*, a newly reclassified species (Stackebrandt *et al.* 1999) by 16S rRNA sequencing based on a perfect sequence match to reference strain and a sequence difference of ≥8·75% to any other strains. One discrepancy was due to a nonmotile *E. casseliflavus* strain that was initially identified as *E. mundtii*. The remaining eight were strains with sequences that were too close to allow discrimination by16S rRNA sequencing and were differentiated using positive key phenotypic traits including pigment production (5 *E. casseliflavus*) and positive mannitol and arabinose fermentation (three *E. faecium*). Two isolates that had inconclusive identifications using both reference tests and were removed from further analysis. Results for each separate test system were compared to the final identification to calculate percentage of correct identifica-

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tion. Isolates identified as *Enterococcus flavescens* were considered equivalent to *E. casseliflavus* since they are the same species (Teixeira *et al.* 1997; Carvalho *et al.* 1998; Patel *et al.* 1998; Baele *et al.* 2000; Poyart *et al.* 2000; ASM 2002, 2003; Facklam 2002; Tyrrell *et al.* 2002; Dutta and Reynolds 2003). Likewise, identifications of *Streptococcus equinus* were considered equivalent to *S. bovis* (Schlegel *et al.* 2003). Results with more that one species' identification with equal probability (e.g. *E. durans* identified as *E. durans*/*E. hirae*) were considered correct to genus level only. For calculating accuracy of *Enterococcus* genus confirmation, only the correct identification to *Enterococcus vs* any other genus was scored.

Results

The summary of the accuracy of conventional and genus screen biochemical testing, four commercial testing systems, and 16S rRNA sequencing for identifying 97 presumptive enterococci isolates is summarized on Table 1 for both *Enterococcus* genus confirmation and species identification. Detailed speciation accuracy rates by species of final identification are presented in Table 2. Conventional and *Enterococcus* genus screen biochemical testing were very accurate for *Enterococcus* genus confirmation with 100% and 99% accuracy, respectively, and conventional biochemical testing was very accurate for species identification with 96% accuracy. The four isolates not speciated correctly were one nonmotile *E. casseliflavus* identified as an *E. mundtii* and identification of three *D. incerta* isolates as *A. viridians*. All ATCC quality control strains were identified correctly. Commercial test systems did not perform as well as biochemical testing for either *Enterococcus* genus confirmation or species level identification. Their accuracy for *Enterococcus* genus confirmation was 79–94% without supplemental biochemical tests and 91–96% with supplemental tests (Table 1). The accuracy of identification to the species level for all commercial test systems was lower, ranging from 39 to 73% without supplemental tests and 46–80% with supplemental tests.

The API 20 Strep accuracy for *Enterococcus* genus confirmation was 79%. Overall accuracy for speciation was 39% without supplemental testing (Tables 1 and 2). Fifty-seven (59%) of isolates required supplemental tests as specified by the manufacturer's directions. This included a total of 35 pigment production and glycerol acidification tests, 30 45°C growth tests and one to six other tests (growth in 6·5% NaCl and 40% bile, growth at 10°C, milk and arbutin acidification, salicin and levan production). After supplemental testing, *Enterococcus* genus confirmation was improved to 91% and speciation accuracy

Table 1 Overall accuracy of conventional and commercial phenotypic methods and 16 sequencing for identification of 97 presumptive enterococci isolates

Identification system	No. (%) isolates	
	Correct confirmation of enterococcus genus	Correct to Species
Conventional biochemical test	97 (100%)	93 (96%)
Genus screen biochemical tests*	96 (99%)	N/A
API 20 strep	77 (79%)	38 (39%)
API 20 strep w/supplemental tests†	88 (91%)	45 (46%)
Microscan rapid positive combo 12	91 (94%)	50 (52%)
Microscan rapid positive combo 12 w/supplemental tests†	93 (96%)	78 (80%)
Vitek GPI	86 (89%)	48 (49%)
Vitek GPI w/supplemental tests†	89 (92%)	70 (72%)
Biolog GP2 microplate	91 (94%)	71 (73%)
Biolog GP2 microplate w/supplemental tests‡	91 (94%)	73 (75%)
16S rRNA sequencing – microseq database	97 (100%)	89 (92%)
16S rRNA sequencing – microseq database w/supplemental tests§	97 (100%)	97 (100%)
16S rRNA sequencing – GenBank database	97 (100%)	87 (90%)
16S rRNA sequencing – GenBank database w/supplemental tests§	97 (100%)	97 (100%)

*Growth in 6·5% NaCl and at 45°C, bile esculin, catalase reaction, and gram stain.
†As recommended by manufacturer.
‡Motility and pigment production.
§Pigment production (for *Enterococcus casseliflavus* and *Enterococcus gallinarum*); sucrose, arabinose, and mannitol fermentation (for *Enterococcus faecium*, *Enterococcus hirae*, and *Enterococcus durans*).

Table 2 Accuracy by species for identification of presumptive enterococci isolates

Organism (no. isolates)	No. (%) isolates with correct identifications												
	Conventional biochemical tests	API	API and suppl.†	Microscan	Microscan and suppl.†	Vitek	Vitek and suppl.†	Biolog	Biolog and suppl.†	Sequencing microseq	Microseq and suppl.†	Sequencing GenBank	GenBank and suppl.†
Enterococcus casseliflavus (16)	15 (94%)	0 (0%)*	0 (0%)*	2 (13%)	14 (88%)	0 (0%)	15 (94%)	16 (100%)	16 (100%)	11 (69%)	16 (100%)	16 (100%)	16 (100%)
Enterococcus durans (8)	8 (100%)	3 (38%)	8 (100%)	0 (0%)	8 (100%)	4 (50%)	4 (50%)	2 (25%)	2 (25%)	8 (100%)	8 (100%)	0 (0%)	8 (100%)
Enterococcus faecalis (10)	10 (100%)	4 (40%)	5 (50%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	9 (90%)	9 (90%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
Enterococcus faecium (14)	14 (100%)	4 (29%)	4 (29%)	11 (79%)	11 (79%)	11 (79%)	13 (93%)	9 (64%)	11 (79%)	11 (79%)	14 (100%)	12 (86%)	14 (100%)
Enterococcus gallinarum (6)	6 (100%)	4 (67%)	4 (67%)	5 (83%)	6 (100%)	0 (0%)	5 (83%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)
Enterococcus hirae (8)	8 (100%)	0 (0%)*	0 (0%)*	0 (0%)	7 (88%)	8 (100%)	8 (100%)	2 (25%)	2 (25%)	8 (100%)	8 (100%)	8 (100%)	8 (100%)
Enterococcus mundtii (7)	7 (100%)	0 (0%)*	0 (0%)*	1 (14%)	1 (14%)	0 (0%)*	0 (0%)*	7 (100%)	7 (100%)	7 (100%)	7 (100%)	7 (100%)	7 (100%)
Aerococcus viridans (5)	5 (100%)	4 (80%)	4 (80%)	3 (60%)	3 (60%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	5 (100%)	5 (100%)	5 (100%)	5 (100%)
Streptococcus bovis (20)	20 (100%)	19 (95%)	20 (100%)	18 (90%)	18 (90%)	15 (75%)	15 (75%)	20 (100%)	20 (100%)	20 (100%)	20 (100%)	20 (100%)	20 (100%)
Desemzia incerta (3)	0 (0%)*	0 (0%)*	0 (0%)*	0 (0%)*	0 (0%)*	0 (0%)*	0 (0%)*	0 (0%)*	0 (0%)*	3 (100%)	3 (100%)	3 (100%)	3 (100%)

*Species not included in the test database.
†With supplemental tests.

was improved to 46%. API identified 95% of *S. bovis* correctly and was the only method able to correctly differentiate *S. bovis* as biotypes I or II. Six of seven ATCC quality control strains were identified correctly. The *E. casseliflavus* strain was inconclusive. MicroScan accuracy for *Enterococcus* genus confirmation was 94%. Overall accuracy for speciation was 52% without supplemental testing (Tables 1 and 2). Fifty (52%) isolates required additional tests recommended by the manufacturer including a total of 35 pigments production and motility tests and 15 sucrose fermentation tests. The results from these tests improved the speciation accuracy by 29–80%, utilizing manufacturer's identification tables. *Enterococci faecalis* and *S. bovis* were identified with ≥90% accuracy. *Enterococci casseliflavus*, *E. durans*, *E. hirae* and *E. mundtii* were identified with ≤25% accuracy. With supplemental testing, four of seven ATCC quality control strains were identified correctly. The *E. faecium* and *E. gallinarum* results were inconclusive and the *A. viridans* result was incorrect. The Vitek accuracy for *Enterococcus* genus confirmation was 89%. Overall accuracy for speciation was 49% without supplemental testing (Tables 1 and 2). Thirty-seven (38%) of isolates required pigment production and motility tests recommended by the manufacturer. These additional tests improved the *Enterococcus* genus confirmation accuracy to 92% and the speciation accuracy to 72%, utilizing the manufacturer's identification tables. *Enterococci faecalis* and *E. hirae* were identified with an accuracy of 100%. *Enterococci casseliflavus*, *E. gallinarum* and *E. mundtii* and *A. viridans* were identified with an accuracy of 0%. *Enterococci mundtii* could not be identified because the species is not included in the database, and *A. viridans* was not considered identified to the species level because the database contained '*Aerococcus* species' only. With supplemental tests, five of seven ATCC quality control strains were identified correctly. *Aerococcus viridans* could not have been identified and *S. bovis* was reported with 80% confidence, below the 90% confidence level selected for this method. Biolog had an accuracy of 94% for *Enterococcus* genus confirmation. The overall speciation accuracy was 94%. Biolog did not recommend or have provisions for including supplemental test results. The addition of pigment production and motility test results for all isolates and correction of the Biolog results utilizing standard biochemical tables resulted in no increase in *Enterococcus* genus confirmation accuracy and a 2% increase in speciation accuracy. *Enterococcus casseliflavus*, *E. faecalis*, *E. gallinarum*, *E. mundtii* and *S. bovis* were identified with ≥90% accuracy. *Enterococcus durans*, *E. hirae* and *A. viridans* were identified with an accuracy of ≤25%. Six of seven ATCC quality control strains were identified correctly. 'No growth' was reported for the *A. viridans* control strain.

16S rRNA sequencing had an accuracy of 100% for *Enterococcus* genus confirmation utilizing both GenBank and MicroSeq databases (Table 1). Ninety-two percent and 90% of isolates were correctly speciated using the MicroSeq and GenBank databases, respectively (Tables 1 and 2). The MicroSeq analysis discrepancies were due to five *E. casseliflavus* isolates that could not be differentiated from *E. gallinarum* and three *E. faecium* isolates that could not be differentiated from *E. durans*. GenBank analysis discrepancies were due to eight *E. durans* isolates that could not be discriminated from *E. faecium* and two *E. faecium* isolates that could not be discriminated from *E. durans*. The performance of selected phenotypic tests improved the speciation accuracy to 100% using either database. This required testing for pigment production for any isolate identified as *E. gallinarum* or *E. casseliflavus* and sucrose, mannitol and arabinose fermentation tests for any isolate identified as *E. faecium*, *E. durans* or *E. hirae* (Tables 1 and 2) and utilizing standard biochemical tables to obtain the identification.

The per cent genetic difference (%GD) between the isolate tested and the closest MicroSeq match ranged from 0 to 3% for *Enterococcus* species and 0–2·8% for the non *Enterococcus* species. *E. faecalis* and *E. mundtii* were well differentiated from other pecies with a difference in %GD between the first match and the second match of 6·6% and 1·7%, respectively. All other *Enterococcus* species were differentiated from the second most related species by 0–0·37% GD. Identical %GD from two different species resulted in inability to accurately discriminate to species level for seven isolates (five *E. casseliflavus* and two *E. faecium*). Non *Enterococcus* species had a difference in %GD between the closest and second closest match of 1·9–8·8% GD. For GenBank analysis the %GD for the closest match ranged from 0 to 0·6% except for two *A. viridians* with 3·2%GD. *Enterococci durans* could not be differentiated from *E. faecium* due to identical %GD with the two different species. Two *E. faecium* isolates could not be differentiated from *E. durans* for the same reason.

Discussion

Accurate identification of presumptive enterococci isolates to genus level is required as a routine quality assurance or quality control test to assure analytical procedures are working and that the method is detecting *Enterococcus*. Accurate identification to the species level is now often being used for assistance in determination of the source of an environmental pollution problem, ecological studies of *Enterococcus* in the environment or as a first step in further genetic or phenotypic analysis of environmental isolates. The goal of this study was to determine the accuracy of tests used for enterococci identification in environmental monitoring laboratories. Bacterial strains that had been isolated by our laboratory from the environment and identified as presumptive enterococci in the standard membrane filter mEI screen method were used in the study. The results indicate that acceptable accuracy (≥90%) for *Enterococcus* genus confirmation can be accomplished using several methods, including a simple genus screen set of five phenotypic tests. However, accurate identification to the species level was accomplished by only conventional biochemical testing or 16S rRNA sequencing.

Biochemical testing was the most accurate single identification method for the majority of the environmental isolates. Accurate confirmation of *Enterococcus* genus was achieved by a genus screen set of five phenotypic traits; typical gram stain reaction, positive catalase, growth in 6·5% NaCl, and at 45°C and tolerance to bile esculin. Accurate species identification was obtained using 16 phenotypic and physiological tests selected according to standard identification charts designed by Facklam and Collins (Facklam and Collins 1989). The only discrepancies were due to one of two causes. First was a variation in phenotypic traits (one nonmotile *E. casseliflavus*). The second was an overlap in phenotypic traits for a new species not found on standard biochemical charts. In this case, three *D. incerta* isolates were identified as *A. viridians*. Conventional biochemical testing was very successful in this study, however phenotypic variability of some species has been recognized and may reduce overall accuracy on continued routine use. This includes mannitol-negative *E. faecium* strains (Facklam and Collins 1989; Teixeira *et al.* 1995; Angeletti *et al.* 2001; ASM 2003; Carvalho *et al.* 2004); nonmotile *E. gallinarum* strains misidentified as *E. faecium* (Vincent *et al.* 1991; Carvalho *et al.* 1998; Patel *et al.* 1998; ASM 2002; Facklam 2002); and nonpigmented *E. casseliflavus* and *E. mundtii* strains (Teixeira *et al.* 1997; Carvalho *et al.* 1998; ASM 2002; Facklam 2002).

While accurate, conventional biochemical testing is both time consuming and laborious. This study demonstrated some of the limitations of automated and faster commercial identification systems for identification of environmental *Enterococcus*. For confirmation of *Enterococcus* genus, two systems, Biolog and MicroScan, had acceptable (≥94%) accuracy without supplemental tests while Vitek had an accuracy of 89%. With additional supplemental testing, Vitek achieved an accuracy of 92% and API 20 Strep, 91%. Overall accuracy for speciation ranged from 39 to 73%, which was increased to 46–80% by performing recommended supplemental tests. The lack of accuracy is partially due to small databases. All systems had only limited representation of *Enterococcus* species in their databases (5–18 strains) while two systems (API and

Vitek) did not have one to three *Enterococcus* species tested here in their database. In addition, the performance of commercial identification systems is dependent on the types and number of tests used. Vitek, API and MicroScan identify bacteria using a combination of enzymatic and carbohydrate reactions for up to 29 tests while Biolog is a sole-carbon source test that includes 95 different carbon sources. With the exception of Biolog, a shortcoming of all commercial systems is the dependence on supplemental testing. Supplemental testing was limited to pigment, motility and sucrose fermentation testing for Vitek and MicroScan. API required many biochemical tests not readily available in the typical laboratory such as growth at 10°C, arbutin and levan. For MicroScan and Vitek, supplemental testing increased the speciation accuracy substantially (29 and 23%, respectively). However, performance of supplemental tests somewhat defeats the purpose of utilizing a commercial system, which is to save time and labour. One possible future improvement may be to include the pigment and motility results in the product databases up front, so they can be included in the initial identification. These tests could be easily done as part of the setup for inoculation and could potentially increase initial accuracy. Pigment production and motility are particularly useful in discriminating *E. casseliflavus*, *E. gallinarum* and *E. mundtii*. Environmental laboratories can utilize these test results with standard tables to clarify a result from a commercial system. However, even with supplemental testing, the overall speciation accuracy for commercial systems did not exceed 80%, lower than needed for routine use. For *E. faecalis*, all systems except API achieved a speciation accuracy of ≥90%. For all other species, accuracy varied by test system. Similar to results here, studies in clinical settings have shown variable accuracy depending on the species of *Enterococcus* included in the study. All studies have shown good accuracy identifying *E. faecalis* and good or reasonable accuracy for *E. faecium*, the two most commonly identified clinical strains. The identification accuracy for all other species is low and performing supplemental tests to achieve sufficient accuracy is recommended (Cartwright *et al.* 1995; Iwen *et al.* 1999; D'Azevedo *et al.* 2001; Kirschner *et al.* 2001; Ligozzi *et al.* 2002; Bosshard *et al.* 2004).

Identification utilizing 16S rRNA sequencing to either genus or species level was very accurate with 100% *Enterococcus* genus confirmation accuracy and 90–92% speciation accuracy utilizing the GenBank and MicroSeq databases, respectively. A major advantage is the ability to identify species not normally seen or expected. In this case, three *D. incerta* isolates thought to be *A. viridans* were identified. The shortcomings of this technique were due to its inability to discriminate species with sequences differing by just a few base pairs, namely, *E. casseliflavus* and *E. gallinarum;* and *E. durans*, *E. faecium* and *E. hirae*. In our study, the MicroSeq database was unable to discriminate five *E. casseliflavus* from *E. gallinarum*. However, utilizing the GenBank database all environmental isolates had the lowest genetic difference (%GD) to two of the 82 sequence entries for *E. casseliflavus* strains in the database. *E. durans vs E. faecium* errors for both databases were also due to isolates being equally related to two different species, or in one case, matching the incorrect species. The MicroSeq database contains over 1400 strains, comprised of well-documented reference type strains, with only one representative per species while the GenBank database contains over 90 000 16S rRNA gene sequences with multiple entries per species. However, the identification of the species may not be correct or complete (Clarridge 2004). For this study, the accuracy of identification for both databases would be improved by an increase in the number of database strains, accurately identified, that capture the genetic variation of these species and matched our isolates better. Alternatively, a laboratory-specific supplementary database of well-characterized strains would also increase accuracy.

Accuracy of genetic based tests may also be improved by (i) the addition of limited supplementary biochemical testing, (ii) sequence analysis of other genes, such as the *atpA* gene (Naser *et al.* 2005), the *rpoB* gene (Drancourt *et al.* 2004), as well as sequencing the entire 1500 bp region of the 16S rRNA (Ozawa *et al.* 2000; Poyart *et al.* 2000; Clarridge 2004; Devulder *et al.* 2005; Kiratisin *et al.* 2005), or (iii) the use of site-specific mutations.

While sequence-based identification worked well in this study, there is currently no consensus on the exact sequence difference that defines a species. Less than half of *Enterococcus* species examined in literature have 16S rRNA sequences differences greater than 3% from the sequences of the nearest neighbours, which means that the sequence must match exactly to the known strain in order to be identified with a high degree of confidence (Williams *et al.* 1991; Patel *et al.* 1998; ASM 2002). It has been suggested that using a single value for the definition of a genus or species on the basis of the 16S rRNA gene sequence may not be appropriate for all genera (Clarridge 2004) and that the cut-off value should be based on laboratory-created databases. This seems to be the case for the environmental enterococci tested here.

This study utilized common presumptive enterococci species found in the marine or near marine environment in our geographic area. One of the limitations is that not all environmental species were represented and that the overall accuracy is dependent on the mix of species included in the study. While we endeavoured to include both typical and atypical strains of each species, no attempt was made to obtain strains from differing geographical

areas. While additional studies are needed to determine the accuracy of identification for other enterococci species, species from other sites, or species that may be isolated using a different method, we believe the overall results of this study are clear and will remain the same.

Since the final species identifications obtained by the six methods evaluated here were compared to a combination of biochemical testing and sequencing as the gold standard, this may have contributed somewhat to the higher accuracy rates obtained using each of these two methods separately. However, since all but 12 isolates were identified similarly using biochemical testing and sequencing, we do not feel that this would have made a significant difference in the accuracy levels obtained.

For presumptive *Enterococcus* isolates from mEI agar, accurate *Enterococcus* genus confirmation can be accomplished utilizing a simple *Enterococcus* genus screen phenotypic test panel, two commercial test kits without supplemental testing (MicroScan or Biolog) or 16S rRNA sequence analysis. For accurate identification to the species level, conventional biochemical testing or 16S rRNA sequence analysis with either MicroSeq or GenBank analysis has sufficient accuracy. If the 16S rRNA sequences for two or more species are >99·7% similar, sequence analysis may have difficulty differentiating species and additional biochemical or molecular testing may be necessary to provide accurate and reproducible results.

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