Evaluation of Rapid Phenotypic tests, BD Phoenix, VITEK 2, APIweb and Raman spectroscopy for the Identification of Enterococci

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Abstract

A panel of five rapid phenotypic tests (RPT), the Phoenix, the VITEK 2, the API Strep 20 in combination with the new APIweb and a relatively new phenotypic test, Raman spectroscopy, were evaluated for the identification to species level of 95 non-repeat enterococcal isolates, comprising 7 different species. The RPT panel, a newly designed combination of 5 old tests (reduction of litmus milk, acidification of arabinose, hydrolysis of L-arginine, pigment production, and motility) is a cheap and simple method that provides identification within 4 hours. As reference method, a genotypic test based on the sequence of the *rpoA* gene was used. The accuracy to correctly identify enterococci to species level varied from 82% for APIweb, 87% for both automated microbiology systems to 92% for the RPT, which performed significantly better than APIweb (p=0.05). The best method for the identification of *E. faecium* and for discrimination between high-level resistant (*vanA*/*B*) *Enterococcus faecium*/*Enterococcus faecalis* and intrinsic resistant (*vanC*) *Enterococcus casseliflavus*/*Enterococcus gallinarum* was the RPT with a sensitivity and specificity of 94%/100% and 99%/100%, respectively. For the Phoenix these percentages were 85%/96% and 97%/100% respectively, and for the VITEK 2 85%/98% and 99%/92% respectively. The APIweb revealed despite high sensitivities (96% and 99%, respectively), the lowest specificities among the different methods (85% and 42%, respectively). Although not yet commercially available, Raman spectroscopy revealed a promising rapid phenotypic test with high sensitivities (98% and 99%, respectively) and somewhat lower specificities of 94% and 83%, which will probably increase with expansion of the reference database. To conclude, RPT revealed a highly reliable, very fast and cheap method. As a consequence VRE infection control measures (implementation or ending) can be executed one day earlier, resulting in improvement of efficiency and lowering of costs. Furthermore adequate differentiation of (*vanC*) *E. casseliflavus*/*E. gallinarum* from other species is clinically important since vancomycin treatment failure has been associated with the presence of a *vanC* gene.

Introduction

The identification of hospitalized patients infected or colonized with vancomycin-resistant enterococci (VRE) has become an important component of infection-control programs aimed at minimizing cross-transmission of these organisms (1). Nowadays, six phenotypes of glycopeptide resistance have been reported (9), from which the transferable VanA and VanB phenotypes are predominantly found among the clinically relevant *Enterococcus faecalis* and

Enterococcus faecium isolates. VanA phenotype strains are characterized by high-level resistance to both vancomycin and teicoplanin, whereas VanB phenotype strains are resistant to variable levels of vancomycin only. However, the occasionally disease causing *Enterococcus casseliflavus* and *Enterococcus gallinarum* express intrinsically low-level resistance to vancomycin due to a chromosomally located *vanC* gene (9). The differentiation between *E. faecalis*/*E. faecium*, that can serve as host for *vanA*/*B* genes and *vanC*-VRE is relevant since in contrast to *E. faecalis*/*E. faecium*, *vanC*-VRE have not been implicated in outbreaks and appear to be of minimal concern from an infection-control point of view (31). Also from the clinical perspective it is relevant to differentiate *E. casseliflavus* and *E. gallinarum* from other species. Vancomycin treatment failure has been associated with *vanC* gene encoded low-level vancomycin resistance in humans as well as in animal models of experimental endocarditis (14,18,21). Furthermore, several reports have been published reporting failure or breakthrough bacteraemia during vancomycin therapy with *in vitro* susceptible strains (28,29). Consequently, vancomycin therapy for infections caused by *vanC* VRE is generally regarded inadequate (8). Susceptibility testing alone is insufficient to detect VanC-VRE because *vanC* encoded low-level resistance may not be detected using the CLSI breakpoints.

Another reason for adequate species identification is the worldwide increase of *E. faecium* as causative agent of clinical enterococcal infections and hospital outbreaks (35). Population biology and genetic evolutionary studies using multilocus sequence typing (MLST) have identified a distinct *E. faecium* genetic subpopulation, designated complex-17, comprised of highly transmittable isolates well adapted to the hospital environment and characterized by high-level resistance against ampicillin and ciprofloxacin (20,35). Since complex-17 isolates may require specific infection control measures an adequate identification of *E. faecium* is warranted.

In the routine clinical microbiology laboratory of the University Medical Center Utrecht (UMCU) identification of enterococci to species level was only applied to invasive isolates and VRE screening isolates by the API 20 Strep using Apilabplus V3.3.3 for interpretation. Review of the results of the API 20 Strep in combination with the susceptibility patterns revealed an unexpected high number of ampicillin resistant *E. casseliflavus*. Further analysis showed that these isolates were misidentified and appeared to be *E. faecium*. This finding prompted the current study for the evaluation of API 20 Strep in combination with APIweb V1.2.1 for interpretation, a panel of six phenotypic tests, two automated microbiology systems, VITEK 2 and Phoenix, respectively, and a relatively new phenotypic method Raman spectroscopy (19,24-26). A genotypic test based on the sequence of the *rpoA* gene was used as reference method (27).

Materials and methods

Bacterial isolates and growth conditions

The bacterial isolates used in this study consisted of 34 isolates from our strain collection, including 7 *E. faecium*, 11 *E. faecalis*, 3 *E. casseliflavus* and 5 *E. gallinarum* identified by a species specific PCR targeted on the *ddl* gene as described previously (11,12) and 3 *E. avium*, 2 *E. hirae*, 1 *E. durans* and 2 *Enterococcus* species based on identification using amplified fragment length polymorphism (AFLP) (34). Furthermore, 61 non-repeat clinical isolates were identified as *E. faecium* (25), *E. faecalis* (13), *E. casseliflavus* (16), *E. gallinarum* (1), *E. avium* (5) and *E. durans* (1) using the API 20 Strep (Apilabplus V3.3.3). Twelve of the 16 *E. casseliflavus* isolates as well as the one *E. gallinarum* isolate had been tested in the routine laboratory as ampicillin resistant. All strains were grown overnight on blood agar plates at 37°C.

Reference species identification method

A molecular identification method based on sequence analysis of the α-subunit of bacterial RNA polymerase (*rpoA*) gene (27) was used as reference method with the following modifications. Instead of a time consuming DNA purification step, crude lysates were prepared from bacterial isolates grown overnight on blood agar plates at 37°C. Approximately seven CFUs were suspended in 20 µl lysis buffer (0.25% SDS, 0.05 N NaOH) and incubated at 95°C for 5 min. The cell lysate was spin by short centrifugation and diluted with 180 µl buffer (10 mM Tris-HCl, pH 8.5). After thoroughly mixing, another centrifugation for 5 min at 16,000 x *g* was performed to remove cell debris. Supernatants were frozen at -20°C until further use.

No PCR product was obtained on crude lysates with the previously described primers for the amplification of the *rpoA* gene applied to purified DNA (27). Therefore, PCR conditions were optimized for crude lysates with newly developed primers based on internal fragments of the in Genbank deposited *rpoA* reference sequences, which included 28 different enterococcal species (27). Due to

Table 1. Overview of *rpoA* primers for the multiplex PCR

Primer	Primersequence
rpoA.1F	G G G A A T T C C C T A C G T C G
rpoAefs.1F	C T T T A G G **T** A A **C** T C **T** C T A C G T C G
rpoAegal.1F	G G **A** A A T T C C **T** T A C G T C G
rpoA.1R	T T C G A C C A T G A T T T C A G C
rpoAefs.1R	T T C **C** A C C A T G A T T T C A G C
rpoAegal.1R	T T C **C** A C C A T G A T **C** T C A G C

DNA polymorphisms in the *rpoA* gene a multiplex approach including 3 different primer sets was developed (Table 1). Five µl of the crude lysate was used in the PCR reaction. Reactions were performed in 25 µl volumes with HotStarTaq Master Mix buffers from Qiagen (Qiagen inc.), including 15 pmol per primer. The PCR program comprised of 15 min at 95°C, 30 cycles of 30 sec at 94°C, 30 sec at 52°C, 1 min at 72°C and finally 7 min at 72°C. Five µl of the PCR mix was checked on agarose gel. Without any further purification, 1 µl PCR product was subsequently sequenced, both strands, using ABI BigDye Terminator Cycle sequencing Ready reaction mix version 3.1 (Applied Biosystems, Foster City, USA) and run on an ABI PRISM 3100 Genetic analyzer (Applied Biosystems) according to manufacturers instructions. Raw data were analyzed using BioNumerics software (version 4.5; Applied Maths, Sint-Martens-Latem, Belgium).

To standardize the *rpoA* sequence analysis, a fragment of 430 bp with fixed start and end points (Table 2) was chosen for both the Genbank reference sequences as well as the test isolates. Sequence alignment and cluster analysis by Unweighted Pair Group Method using Arithmetic averages (UPGMA) of the 430 bp fragments of the Genbank *rpoA* reference sequences resulted in a 100% identical species identification as compared to the entire *rpoA* gene (data not shown). The similarity matrix from the UPGMA clustering was used as reference library for the identification of test isolates.

Table 2. Start and end points for the analysis of the *rpoA* sequences

Startpoint *rpoA* sequence	Endpoint *rpoA* sequence	Species
TCAAAGGTGT	GAAGCAATGAG	*E. faecium, E. dispar, E. mundtii,*
		E. ratti, E. phoeniculicola
	GAAGCATTGAG	*E. avium, E. raffinosus, E. pseudoavium,*
		E. malodoratus, E. hermanniensis
	GAAGCATTAAG	*E. gilvus*
	GATGCTATGAG	*E. columbae*
TAAAAGGTGT	GAAGCATTAAG	*E. canis*
	GATGCAATGAG	*E. sulfureus*
TTAAAGGTGT	GAAGCCCTTAG	*E. haemoperoxidus, E. moraviensis*
	GAAGCAATGAG	*E. durans, E. villorum*
	GATGCTATGAG	*E. columbae*
TTAAGGGTGT	GAAGCAATGAG	*E. casseliflavus*
	GACGCAATGAG	*E. saccharolyticus*
	GATGCAATGAG	*E. italicus, E. saccharominimus*
TTCCAGGTGT	GAAGCACTAAG	*E. faecalis*
TTAAGGGCGT	GAAGCAATGAG	*E. gallinarum*
TTAAAGGGGT	GAAGCAATGAG	*E. hirae*
TTGTAGGTGT	GACGCAATGAG	*E. cecorum*
TCAAAGGCGT	GACGCGTTAAG	*E. solitarius*

API 20 Strep

All isolates were tested using API 20 Strep system (BioMérieux, S.A. Marcy-l'Etoile, France) according to manufacturers instructions. Identification was performed using APIweb V1.2.1. Identification (ID) was regarded excellent when the probability was ≥ 99.9% and the T index ≥ 0.75, very good ≥ 99% and T ≥ 0.50, good ≥ 90% and T ≥ 0.25 and acceptable ≥ 80% and T ≥ 0. The identification was not reliable when probability was below 80%.

Automated microbiology systems

Two different systems were included in the evaluation, the Phoenix (BD Biosciences, USA) and the VITEK 2 (BioMérieux S.A. Marcy-l'Etoile, France).

PMIC/ID-50 Phoenix panels (combined susceptibility and identification card) were inoculated according to manufacturers recommendations. For this study software version V5.03E/V4.11B was used. Biochemical reactions contained on the panel are read at 20-minute intervals by the instrument. As soon as, as a function of time, the biochemical profile of the test strain compared to the profiles from the Phoenix database is above the 90% probability value, identification results of bacteria are given. The Phoenix does not categorize the accuracy of identification above the 90% probability.

The VITEK 2 system was used as specified by the manufacturer. Specific Gram-positive cards (Vitek 2 GP card) were used for identification and results were interpreted using software version VT2-R04.01. Identification was regarded excellent when the probability was between 96% and 99%, very good between 93% and 95%, good between 89% and 92%, acceptable between 85% and 88% and low when the probability was below 85%.

Phenotypic tests

In total six different phenotypic tests were evaluated. (i) Hydrolysis of L-arginine was determined using arginine dihydrolase (ADH) diagnostic tablets (Rosco, Taastrup, Denmark) and (ii) acidification of arabinose (ARA) (Rosco, Taastrup, Denmark) according to manufacturers instructions. (iii) Reduction of lithmus milk (LM) (Becton Dickinson, Le Pont de Claix, France) was performed as described by Hanson *et al.* (16) and interpreted after 4 and 24 hours. (iv) Acidification of methyl-α-D-glucopyranoside (MGP) (ICN, Ohio, USA) was determined as described by Carvalho *et al.* (6). (v) Motility was determined as previously described using *Enterobacter aerogenes* as positive motile control and *Klebsiella pneumoniae* as nonmotile negative control (32,33). (vi) Yellow pigment was observed using a cotton swab (5). The reaction patterns for the 7 different enterococcal species included in this study were interpreted according to literature (Table 3).

Raman spectroscopy

Raman spectroscopy is a non-destructive optical technique that can be used to analyze the (bio-) chemical composition of a wide variety of samples. The usefulness of this technique to study microbiological samples has been shown earlier (26). For the measurements, all isolates were grown on Mueller Hinton agar (Beckton Dickinson, Franklin Lakes, NJ, USA) for ~24 hours at 35°C. A calibrated 1μl loop full of CFU was suspended in 7.5 μl of sterilized distilled water, 4 μl of this suspension was transferred to a quartz slide (Hellma Benelux, Rijswijk, The Netherlands) and allowed to dry.

Raman spectra were collected using a High Performance Raman Module 2500 (River Diagnostics BV, Rotterdam, The Netherlands) coupled to an automated XYZ-stage (River Diagnostics) and operated using RiverICon software, version 1.63. Samples were excited using a 785 nm diode laser (Sacher Lasertechnik, Marburg, Germany), delivering approximately 150 mW on the sample. The spectrometer was calibrated using the manufacturers guidelines, and the spectral region of 400 to 1800 cm^{-1} with a resolution of ~2 cm^{-1} was used for the analysis. Data analysis was performed using MATLAB version 7.1 (The Mathworks, Natick, MA, USA).

For each isolate, 50 spectra were collected from different locations, using 1 second exposure time on each location. An average of the 50 spectra was used in the analysis.

Identification of isolates is based on comparison of the obtained spectra with a reference database of spectra. Since this method is under development and not commercially available, we used internal validation to estimate the method's identification capabilities. Briefly, the similarity between each sample pair is calculated using the squared Pearson correlation coefficient (R^2) and multiplied by 100 to be expressed as percentages. For each individual sample, the predicted species (i.e. identification) was similar to the sample with the highest R^2 value. Cluster analysis on 83 spectra was performed using MATLAB version 7.1 (The Mathworks, Natick, MA, USA) by the Ward's algorithm.

For practical reasons only the most prevalent species were analyzed using Raman spectroscopy; *E. avium*, *E. durans*, and *E. hirae* were omitted from the analysis.

Table 3. Results of phenotypic tests

Phenotypic test		ADH		ARA		LM		Pigment		Motility		MGP	
Incubation time		4 hours		4 hours		4 hours		4 hours		4 hours		24 hours	
Species	No. of isolates	Expected[a]	% Correctly identified	Expected[b]	% Correctly identified	Expected[c]	% Correctly identified	Expected[d]	% Correctly identified	Expected[e]	% Correctly identified	Expected[f]	% Correctly identified
E. faecium	48	+	100	+	94	-	98	-	100	-	100	-	100
E. faecalis	23	+	100	-	96	+	96	-	100	-	100	-	100
E. gallinarum	8	+	100	+	100	+	88	-	88	+	100	+	100
E. casseliflavus	4	+	50	+	75	+	75	+	100	+	100	+	100
E. avium	9	-	100	+	78	-	100	-	100	-	100	+	67
E. durans	1	+	100	-	100	-	100	-	100	-	100	-	100
E. hirae	2	+	100	-	100	-	100	-	100	-	100	-	100

[a] some *E. faecalis*, *E. casseliflavus* and *E. gallinarum* isolates are ADH negative (6)

[b] some *E. casseliflavus* and *E. gallinarum* isolates are ARA negative (6,16)

[c] some *E. casseliflavus* isolates are LM negative (16)

[d] some *E. casseliflavus* don't produce pigment (6)

[e] some *E. casseliflavus* and *E. gallinarum* isolates are nonmotile (6)

[f] some *E. faecium* and *E. faecalis* isolates are MGP positive, while some *E. gallinarum* isolates are MGP negative (6,7,16)

Abbreviations: ADH, arginine dihydrolase; ARA, arabinose; LM, lithmus milk; MGP, methyl-α-D-glucopyranoside

Table 4. Identification results

		No. of strains that were:				
Method	Species	Tested	Correctly identified	Identified with low discrimination[a]	Misidentified	Accuracy (%)
APIweb vs 1.2.1	*E. faecium*	48	46		*E. gallinarum* (1); *E.hirae*/*E. durans* (1)	96
	E. faecalis	23	23			100
	E. casseliflavus	4			*E. faecium* (2); *E. gallinarum* (1); *Aerococcus viridans* (1)	0
	E. gallinarum	8		3	*E. faecium* (5)	0
	E. avium	9	8	1		89
	E. durans	1	1			100
	E. hirae	2		2		0
Total						82

Phoenix	*E. faecium*	48	41		*E. faecalis* (2); *E. casseliflavus /E. gallinarum* (1); *E.hirae* (2); *E. durans* (2)	85
	E. faecalis	23	21		*E. faecium* (1); *E. casseliflavus /E. gallinarum* (1)	91
	E. casseliflavus [b]	4	4			100
	E. gallinarum [b]	8	8			100
	E. avium	9	7		*E. raffinosus* (1); *Streptococcus uberis* (1)	78
	E. durans	1	1			100
	E. hirae	2	1		*E. faecium* (1)	50
Total						87
VITEK 2	*E. faecium*	48	41	2	*E. faecalis* (2); *E. durans* (3)	85
	E. faecalis	23	23			100
	E. casseliflavus	4	3	1		75
	E. gallinarum	8	7		*E. faecium* (1)	88
	E. avium	9	7		*Pediococcus pentosaceus* (1); *Streptococcus alactolyticus* (1)	78
	E. durans	1	1			100
	E. hirae	2	1		*E. faecalis* (1)	50
Total						87
Rapid Phenotypic tests	*E. faecium*	48	45		*E. faecalis* (1); *E.hirae /E. durans* (2)	94
	E. faecalis	23	21		*E. gallinarum* (1); *E.hirae/E. durans* (1)	91
	E. casseliflavus	4	4			100
	E. gallinarum	8	7		*E. casseliflavus* (1)	88
	E. avium	9	7		*E.* species (2)	78
	E. durans	1	1			100
	E. hirae	2	2			100
Total						92[c]
Raman spectroscopy	*E. faecium*	48	47		*E. gallinarum* (1)	98
	E. faecalis	23	22		*E. faecium* (1)	96
	E. casseliflavus	4	3		*E. gallinarum* (1)	75
	E. gallinarum	8	6		*E. faecium* (1); *E. faecalis* (1)	75

[a] Only APIweb and VITEK 2 provide identification (ID) message

[b] BD Phoenix doesn't discriminate between *E. casseliflavus* and *E. gallinarum*

[c] Significant with APIweb (p =0.05)

Results

rpoA sequence identification

Sequencing of the 430 bp *rpoA* gene fragment of 95 enterococcal isolates revealed a clear discrimination between 7 different species (Figure 1). Using the similarity matrix of the reference sequences, 5 sequences differing in only 1 or 2 bp were identified as originating from *E. faecium*, with one predominant sequence accounting for 40/48 (83%) of the *E. faecium* isolates. Three other sequences with either 1 or 2 bp differences were identified as *E. casseliflavus* specific, while 4/9 isolates that could be identified as *E. avium* based on *rpoA* sequences differed only in 1 bp. *E. hirae* and *E. durans*, *E. faecalis* and *E. gallinarum* were represented by only one *rpoA* sequence and could be clearly distinguished from the other enterococcal species.

No discrepancies between identification based on the *ddl* gene and *rpoA* sequencing were observed. From the isolates identified by AFLP, one *E. durans* isolate revealed by *rpoA* sequencing an *E. faecium* and two *E.* species revealed to be *E. durans* and *E. gallinarum* isolates, respectively.

From the 61 clinical isolates identified by API 20 Strep in the routine clinical laboratory, *rpoA* sequencing confirmed the identification for all *E. faecium* (25) and *E. avium* (5) isolates. Only 1/23 *E. faecalis* was misidentified as *E. faecium*. However, 15/16 (94%) of *E. casseliflavus* isolates were misidentified and revealed twelve *E. faecium* (ampicillin resistant), one *E. avium* and two *E. gallinarum* isolates, respectively, also the single *E. gallinarum* was misidentified as *E. faecium*.

For the evaluation of the different methods the *rpoA*-based species identification of isolates was used.

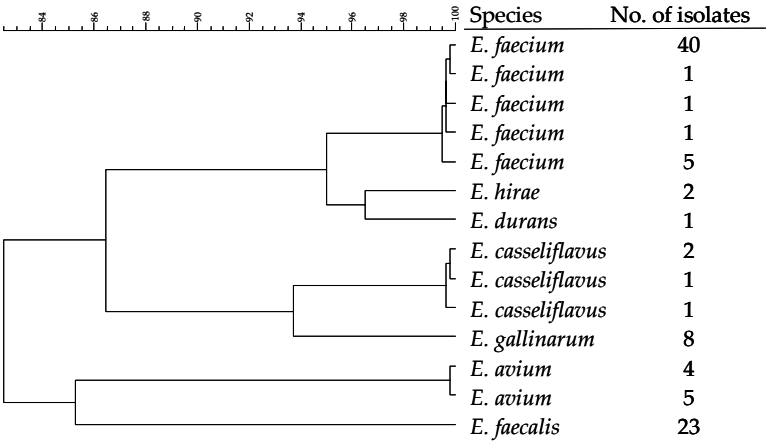

Figure 1. UPGMA clustering of 14 representative rpoA sequences. For each rpoA sequence numbers of isolates are depicted.

API 20 Strep using APIweb

The APIweb performed very well in the identification with an accuracy of *E. faecium* of 96% and *E. faecalis* of 100% (Table 4) The 12 by the Apilabplus V3.3.3 misidentified ampicillin resistant *E. casseliflavus* isolates, were now correctly identified as *E. faecium*. However, the APIweb did not correctly identify all *E. casseliflavus* (4) and *E. gallinarum* (8) isolates. Seven of the *E. casseliflavus* and *E. gallinarum* were identified as *E. faecium*. Despite the high accuracy for the identification of *E. faecium* en *E. faecalis*, APIweb appeared to be the least accurate method for identification of enterococci in general (82%).

Automated microbiology systems

The Phoenix and VITEK 2 appeared to be comparable in their accuracy to identify enterococci, both 87% (Table 4). Both systems misidentified seven *E. faecium* isolates, of which two strain were misidentified by both methods. Incidentally, the two automated systems identified enterococci as other Gram-positive bacteria, mainly streptococci. Furthermore, the Phoenix misidentified 2/23 *E. faecalis* as either *E. faecium* or *E. casseliflavus*/*E. gallinarum* and one *E. hirae* as *E. faecium*. The VITEK 2 misidentified one *E. gallinarum* as *E. faecium* and one *E. hirae* as *E. faecalis*. Finally, both systems were unable to identify 2/9 *E. avium* isolates.

Table 5. Rapid phenotypic test panel

ADH	ARA	LM	Pigment	Motility	Identification
+	+	-	-	-	*E. faecium*
+	-	+	-	-	*E. faecalis*
+	+	+	-	+	*E. gallinarum*
+	+	+	+	+	*E. casseliflavus*
+	-	-	-	-	*E. durans* group[a]
-	+	-	-	-	*E. avium* group[b]
-	-	-	-	-	*E.* species

[a] *E. durans* , *E. dispar* and *E. hirae*

[b] *E. avium* , *E. raffinosis*

Abbreviations: ADH, arginine dihydrolase;

ARA, arabinose; LM, lithmus milk

Phenotypic tests

The phenotypic test results per species are shown in Table 3. All six tests were very easy to perform and interpret. The LM reaction was easy to interpret after 4 hours of incubation, but became unreliable after 24 hours of incubation.

Analysis of the 95 isolates combining ADH, ARA, LM, pigment and motility, further referred to as the rapid phenotypic test panel (RPT) (Table 5) resulted in identification within 4 hours with an overall accuracy of 92% (Table 4), which was better than the automated microbiology systems, but only significant better when compared to APIweb (p =0.05). The MGP test, which needs an overnight incubation step did not enhance the accuracy and was therefore not included in the RPT.

In some cases the individual phenotypic tests revealed a number of a-typical reacting isolates (Table 3). Most pronounced incongruent, though previously described results (6,16) included *E. casseliflavus* isolates that were ADH negative (2), ARA negative (1) or LM negative (1), but combination with the motility and pigment test of the RPT scheme resulted in correct identification of these a-typical reacting *E. casseliflavus* isolates. Other unexpected a-typical reacting isolates were two ARA and three MGP negative reacting *E. avium* isolates, three ARA negative and one LM positive reacting *E. faecium* isolates and one pigment producing *E. gallinarum* isolate (Table 3). Due to these atypical reactions 8 isolates were misidentified, including 3/48 *E. faecium* identified as either *E. faecalis* or *E. hirae*/*E. durans*, 2/23 *E. faecalis* as either *E. gallinarum* or *E. hirae*/*E. durans*, 1/8 *E. gallinarum* identified as *E. casseliflavus* and 2/9 *E. avium* identified as *E.* species (Table 4).

Raman spectroscopy

Clustering based on the averaged spectra of the isolates revealed species-specific clusters, except for 2 isolates (Figure 2), though based on the highest R^2 value 5 isolates were misidentified (Table 4). Based on the highest R^2 value 1/4 (25%) *E. casseliflavus* and 2/8 (25%) *E. gallinarum* were misidentified as *E. gallinarum* and *E. faecium*/*E. faecalis*, respectively. The as *E. faecium* misidentified *E. gallinarum* isolate clustered together with another *E. faecium* isolate and as a consequence, based on the highest R^2 value, this *E. faecium* isolate was misidentified as *E. gallinarum*, although this isolate clustered among the other *E. faecium* isolates. Finally, one *E. faecium* isolate was misidentified as *E. faecalis*, while all *E. faecalis* isolates were correctly identified.

The relatively high accuracy for the identification of *E. faecium* and *E. faecalis* was with 98% and 96%, respectively, comparable to APIweb, but higher when compared to both automated microbiology systems and RPT (Table 4). The relatively low accuracies for *E. casseliflavus* and *E. gallinarum*, both 75%, were probably due to low numbers of isolates tested and hence the limited reference spectra available. The overall accuracy was excluded for comparison with the other identification methods, while *E. avium*, *E. hirae* and *E. durans* were not tested with Raman spectroscopy.

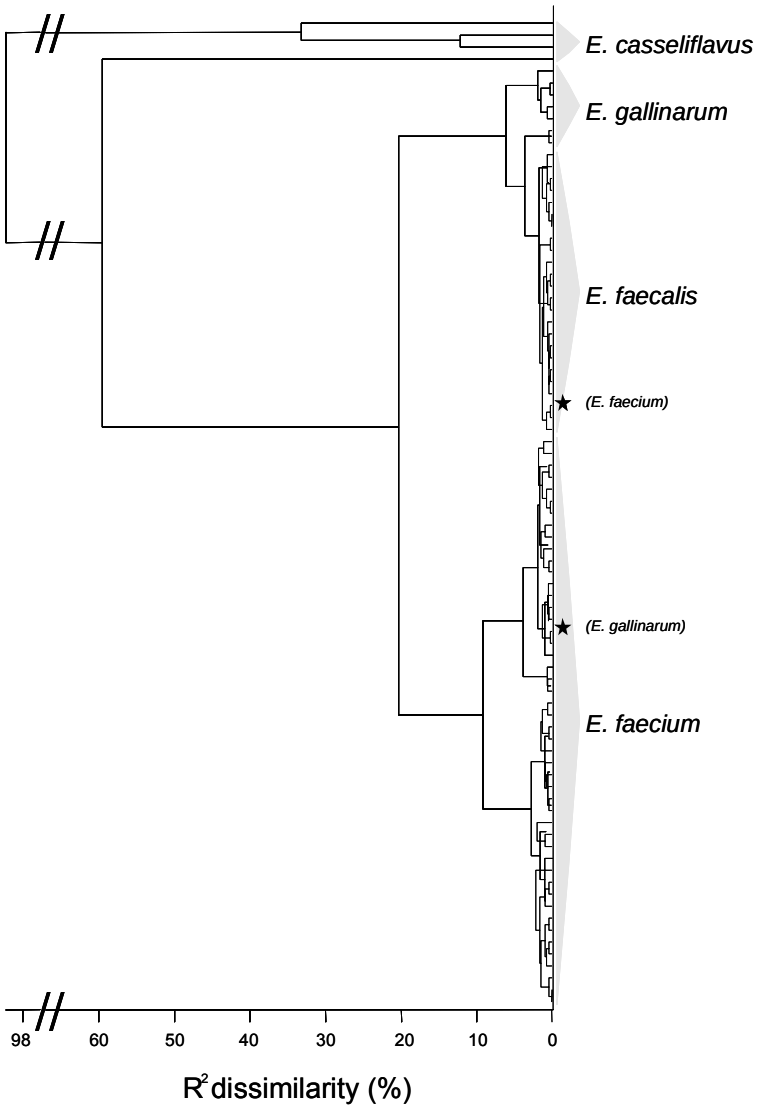

Figure 2. Cluster analysis of 83 Raman spectra using Wards algorithm. Marked isolates were misidentified

Comparative analysis

Comparison of the characteristics of the different identification methods revealed a relatively comparable hands-on time, including preparation of suspensions and interpretation of identification result (Table 6). For the automated microbiology systems and RPT the hands-on time was 4 to 5 minutes, while Raman spectroscopy and API Strep 20 needed 10 to 15 minutes. In contrast, the incubation time varied from 1 minute for Raman spectroscopy to overnight incubation for API Strep 20 and the Phoenix. Technically, for Raman spectroscopy

Table 6. Comparison identification methods

				Identification of *E. faecium*		*E. faecium* /*E. faecalis* vs *E. casseliflavus*/*E. gallinarum*	
Method	Hands-on time	Incubation time	Costs ($)[a]	Sensitivity	Specificity	Sensitivity	Specificity
APIweb	15 Minutes	4-24 Hours	8.10	96	85	99	42
Phoenix	5 Minutes	~24 Hours	6.72	85	96	97	100
VITEK 2	4 Minutes	~ 5 Hours	9.18	85	98	99	92
Rapid phenotypic tests	5 Minutes	4 Hours	1.30	94	100	99	100
Raman spectroscopy	10 Minutes	1 minute	[b]	98	94	99	83

[a] Only materials, purchase of VITEK 2, Phoenix and Raman spectrometer excluded

[b] Unknown, method under development

there is no incubation time, just the measurement time. Only materials costs were included for the comparison and revealed that, compared to RPT both identification cards and API Strep 20 were relatively expensive. Raman spectroscopy was not included, since this method is not yet commercially available.

Identification of *E. faecium*

Sensitivities and specificities of the different methods to identify *E. faecium* revealed that both the RPT (94% and 100%, respectively) and Raman spectroscopy (98% and 94%, respectively) performed very well (Table 6). Both automated microbiology systems revealed the lowest sensitivity with 85% due to high numbers of false-negative *E. faecium* identifications. Although the sensitivity of APIweb was high (96%), the specificity was the lowest (85%) among the methods due to high numbers of false-positive *E. faecium* identifications.

Differentiation between *E. faecalis*/*E. faecium* and *vanC*-VRE

The RPT was superior to the other methods in distinguishing *E. faecalis*/*E. faecium* from *vanC* positive *E. casseliflavus*/*E. gallinarum*, with a sensitivity of 99% and a specificity of 100% (Table 6). The Phoenix also exhibited 100% specificity but a lower sensitivity (97%). Although, Raman spectroscopy revealed a comparable sensitivity (99%), the specificity was relatively low (83%) due to false-positive *E. gallinarum* and *E. casseliflavus* isolates. Despite the high sensitivity of APIweb (99%), the specificity was very low, 42%.

Discussion

Here, we report on the performance of API 20 Strep using APIweb, VITEK 2, Phoenix, Raman spectroscopy and a new developed rapid phenotypic test panel (RPT) to identify enterococci to species level.

For identification the RPT scored better than the automated microbiology systems, but only significant better when compared to APIweb. Furthermore, of

the routine laboratory tests the RPT was the most rapid and reliable method to distinguish *E. faecium*/*E. faecalis* from *E. casseliflavus*/*E. gallinarum* as well as the most simple and cheapest method.

This is the first study, where the routine phenotypic tests as API or automated microbiology systems were compared to a sequence-based reference method with a proven high discriminatory power to differentiate enterococcal species (28). This reference method was a modification of a previously described sequence-based method targeted on the *rpoA* gene using degenerated primers on purified DNA (27). For this study the protocol was modified for crude DNA lysates to reduce time-consuming DNA purification steps using a multiplex PCR approach including species-specific primers. DNA sequencing of 95 isolates and cluster analysis of an internal fragment with fixed start- and endpoints of *rpoA* clearly discriminated the different species in concordance with the previous results (27). Often 16S rRNA sequencing is used for the identification of bacterial species, but this method is less suitable for enterococci due to low discrimination among the so called *E. faecium* group, comprising *E. faecium*, *E. durans*, *E. hirae* and *E. mundtii* (36). In contrast, the *rpoA* sequencing clearly discriminated the different species from the 16S rRNA *E. faecium* group. Alternatively, a multiplex PCR based on the *ddl* and *van* genes (11) has been used to differentiate between enterococcal species. This PCR, however, is limited to the identification of *E. faecium*, *E. faecalis*, *E. casseliflavus* and *E. gallinarum* only. The identification based on *ddl* gene of the 26 isolates included in this study was confirmed by *rpoA* sequencing. In conclusion, the modified *rpoA* sequencing method is the most accurate molecular method to identify enterococci to species level.

Using the API 20 Strep system in combination with the Apilab V3.3.3 a high number of *E. faecium* isolates were misidentified as *E. casseliflavus* (12/16, 75%) confirming the results of a previous study (37). In the current study, the new APIweb version misidentified only 4% of *E. faecium* as *E. casseliflavus*/*E. gallinarum*. However, now 58% of the *E. casseliflavus*/*E. gallinarum* were incorrectly identified as *E. faecium*. So, introduction of the new interpretation scheme has resulted in an improvement of the identification of *E. faecium* but a deterioration of the identification of *E. casseliflavus*/*E. gallinarum* strains, yielding the API 20 Strep/APIweb system less suitable for species identification in the clinical laboratory.

In this study, the accuracy of 87% for both the Phoenix and VITEK 2 to identify enterococci was either comparable (10,15,23) or lower when compared to other studies (2-4,13,30) and confirmed the reported relatively high degree of false negative *E. faecium* identifications by both Phoenix and VITEK 2 (2,3,10,13,15,23). The relative low accuracy found in this study in comparison with previous studies may be explained by the different reference methods used. All of the

aforementioned studies, except one (4), used API as reference identification method often complemented with one or more phenotypic tests despite previous reports on the poor results by the API systems on enterococcal identification. (17,22,37).

The RPT yielded the highest accuracy in identification (92%). Furthermore, sensitivities and specificities of both the identification of *E. faecium*, 94% and 100% respectively, and the distinction between *E. faecium*/*E. faecalis* and *E. casseliflavus*/*E. gallinarum*, 99% and 100% respectively were superior when compared to the Phoenix and VITEK 2. Only API revealed comparable sensitivities, but due to the high degree of false-positive *E. faecium* identifications this would result in unnecessary infection control measures. Therefore, we propose to use the rapid phenotypic test panel for identification of enterococci as an alternative for the more expensive automated microbiology systems and the expensive as well as time-consuming API. A limitation of this scheme is its inability to discriminate between the genetically related *E. hirae* and *E. durans* or between *E. avium* and *E. raffinosis*. However, in the routine setting differentiation between these species is not clinical relevant. Another advantage of the test is the rapid result within 4 hours. As a consequence VRE infection control measures (implementation or ending) can be executed one day earlier, resulting in improvement of efficiency and lowering of costs. The same is the case for the switch from empirical to directed antibiotic therapy.

Raman spectroscopy is a method under development, but was included in this study to compare its abilities in the identification of enterococci in general, the identification of *E. faecium* and the distinction between *E. faecium*/*E. faecalis* and *vanC* positive isolates to other conventional phenotypic tests. Identification of isolates is based on comparison of spectra of the test isolate with a reference database comprising representative spectra of different species (26). In a previous study, only low numbers of enterococcal isolates were included (19) and therefore the reference database had to be build from the isolates included in current study. The clinically less relevant species with low numbers were therefore excluded in the analysis.

Cluster analyses based on the phenotypic spectra revealed species specific clustering. Compared to the other methods a relatively high accuracy was found to identify *E. faecium* and *E. faecalis*, while a much lower accuracy was found for *E. casseliflavus*/*E. gallinarum*, which was probably due to low numbers of isolates in the reference database. As a result, although the sensitivity for the distinction between *E. faecium*/*E. faecalis* and *vanC* positive isolates was almost 99%, the specificity was relatively low with 83%. In contrast, the sensitivity and specificity to identify *E. faecium* was comparable with RPT. In conclusion, Raman spectroscopy is a promising tool to identify enterococci to species level.

Expansion of the reference database including also the less clinical relevant isolates will further improve identification of enterococci. Finally, further studies are needed to evaluate its capacity to identify outbreak related isolates.

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