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ARTICLE

Evaluation of species-specific PCR, Bruker MS, VITEK MS and the VITEK 2 system for the identification of clinical *Enterococcus* isolates

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Abstract The purpose of this investigation was to compare the performance of species-specific polymerase chain reaction (PCR), matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) and phenotypic identification systems for the identification of *Enterococcus* species. A total of 132 clinical isolates were investigated by the following: (1) a multiplex real-time PCR assay targeting *ddl Enterococcus faecium*, *ddl Enterococcus faecalis*, *vanC1* and *vanC2/C3* genes, and a high-resolution melting (HRM) analysis of the *groESL* gene for the differentiation of *Enterococcus casseliflavus* and *Enterococcus gallinarum*; (2) Bruker MS; (3) VITEK MS; and (4) the VITEK 2 system. 16S *rRNA* gene sequencing was used as a reference method in the study. The 132 isolates were identified as 32 *E. faecalis*, 63 *E. faecium*, 16 *E. casseliflavus* and 21 *E. gallinarum*. The multiplex PCR, Bruker MS and VITEK MS were able to identify all the isolates correctly at the species level. The VITEK 2 system could identify 131/132 (99.2 %) and 121/132 (91.7 %) of the isolates at the genus and species levels, respectively. The HRM-*groESL* assay identified all (21/21) *E. gallinarum* isolates and 81.3 % (13/16) of the *E. casseliflavus* isolates. The PCR methods described in the present study are effective in identifying the enterococcal species. MALDI-TOF MS is a rapid, reliable and cost-effective identification technique for enterococci. The VITEK 2 system is less efficient at detecting non-*faecalis* and non-*faecium Enterococcus* species.

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Introduction

Enterococci, Gram-positive facultative pathogens and part of the normal gastrointestinal tract flora have emerged as the major pathogens causing nosocomial and community-acquired infections. *Enterococcus faecalis* is the most frequent enterococcal species isolated from human clinical specimens, followed by *Enterococcus faecium*. However, human infections due to non-*faecalis* and non-*faecium Enterococcus* spp. are increasingly common [1, 2]. Resistance to several commonly used antimicrobial agents is a remarkable characteristic of most enterococcal species. Intrinsic resistance is related to inherent or natural chromosomally encoded characteristics present in all or most of the enterococci. In addition to the intrinsic resistance traits, enterococci have acquired different genetic determinants that confer resistance to several classes of antimicrobial agents. Certain specific mechanisms of resistance to some antimicrobial agents are typically associated with a particular enterococcal species or groups of species. Ampicillin and vancomycin resistance are two of the most problematic resistance profiles in enterococci, which are most commonly associated with *E. faecium*. VanA and VanB are considered to be the most clinically relevant vancomycin-resistant phenotypes and are usually associated with *E. faecium* and *E. faecalis* isolates, while VanC resistance is an intrinsic characteristic of *Enterococcus gallinarum* and *Enterococcus casseliflavus* strains. Accurate identification at the species level among enterococcal isolates is, therefore, important for early appropriate antimicrobial therapy and effective surveillance [3–6].

Conventional culture and biochemical tests, as well as commercially standardised systems such as API and VITEK 2 (bioMérieux, France), have, so far, been used in most clinical microbiology laboratories for the identification of *Enterococcus* spp. Species identification of unusual

enterococci by routine standard methods is not always reliable. Moreover, the occurrence of atypical phenotypic characteristics in some microorganisms may also lead to misidentification [7, 8].

In the last few years, to decrease the possibility of misidentification and obtain more rapid identification, molecular methods such as polymerase chain reaction (PCR)-based diagnostics and 16S ribosomal RNA sequencing have been considered as alternative approaches to the phenotypic methods. The 16S *rRNA* gene sequencing is widely accepted as a tool for identifying bacterial isolates. Besides 16S *rRNA* genes, a variety of species-specific genes, for example, *ddl* genes or *groESL* genes, have been used for the identification of *Enterococcus* species [9–11].

The developments in matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) are rapidly changing the routine diagnostics scene in clinical microbiology laboratories. MALDI-TOF MS has been introduced as a fast, reliable and cost-effective technique for routine application in clinical laboratories [12, 13].

The performance of MALDI-TOF MS and automated phenotypic systems in identifying enterococcal isolates has previously been reported [12–14]. However, information on the performance of the modern methods is scarce in identifying clinical isolates of both the usual *Enterococcus* spp. and non-*faecalis* and non-*faecium Enterococcus* species.

The aim of this study was to compare the performance of species-specific PCR methods, Bruker MS, VITEK MS and the VITEK 2 system for the identification of clinical *Enterococcus* species.

Materials and methods

Bacterial strains

A total of 132 enterococcal isolates were included in the study, consisting of 89 blood isolates and 43 vancomycin-resistant enterococci (VRE) isolates of different clones. *E. faecalis* ATCC29212, *E. faecium* CCUG36804, *E. casseliflavus* CCUG 18657 and *E. gallinarum* CCUG18658 were included as reference strains. All isolates were stored at −70 °C and were subcultured on blood agar plates.

Multiplex real-time PCR

A multiplex real-time PCR assay directed to the *ddl E. faecalis*, *ddl E. faecium*, *vanC1* and *vanC2*/*C3* genes was established on the ABI 7500 Fast System (Applied Biosystems, USA). The primer sequences used were as follows: *ddl E. faecalis* 5′-GTG GCT TAA GTC GCT GTG AT-3′ and 5′-AGG CAT GGT GTT CAA TTC AT-3′; *ddl E. faecium* 5′-TTT ACA AGC TGC TGG TGT GC-3′ and 5′-AAC CCA TAT TCG CAG GTT TG-3′; *vanC1* 5′-TGC TTG TGA TGC GAT TTC TC-3′ and 5′-ATC GCT CCT TGA TTG GTG AC-3′; *vanC2*/*C3* 5′-GGG AAG ATG GCA GTA TCC AA-3′ and 5′-GCA GCA GCC ATT TGT TCA TA-3′ [15]. PCR amplification reactions were performed in a volume of 20 μL containing 10 μL 2×MeltDoctor™ HRM Master Mix (Applied Biosystems, USA), 0.75 μM of primers for *vanC1*, 0.5 μM of each of the other primers and 5 μL of DNA templates. Cycling parameters were as follows: an initial denaturation at 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 60 s; and one cycle of 95 °C for 10 s, 60 °C for 60 s, 95 °C for 15 s and 60 °C for 15 s.

HRM analysis of the *groESL* gene

High-resolution melting (HRM) analysis of the *groESL* gene was designed for the differentiation of *E. casseliflavus* and *E. gallinarum*. Primers were based on the nucleotide sequences of *groESL* genes (nucleotide accession code: AF417584, AF417587) from *E. casseliflavus* ATCC25788 and *E. gallinarum* ATCC49573 [11]. The sequences of the primers are as follows: forward primer 5′-GAA TAT TTG ATC GTT GCT GCA AAA-3′ and reverse primer 5′-CGA TCG CTT GTG TTA GCA ATG-3′. The PCR was performed in a volume of 20 μl containing 10 μL 2×MeltDoctor™ HRM Master Mix (Applied Biosystems, USA), 0.5 μM of each primer and 5 μL of DNA templates. Cycling parameters were as follows: an initial denaturation at 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 55 °C for 60 s; and one cycle of 95 °C for 10 s, 60 °C for 60 s, 95 °C for 15 s and 60 °C for 15 s.

MALDI-TOF Bruker MS

Measurements were performed with a Microflex LT mass spectrometer (Bruker Daltonik, Germany) using FlexControl software (version 3.3). The spectra were imported into the integrated MALDI Biotyper software (version 3.0) and were analysed by standard pattern matching with default settings.

The strains were tested without pretreatment. A colony from the blood agar plate was directly spotted on the MALDI plate, and then overlaid with 1 μL of matrix solution and air-dried. The loaded plate was then applied to the instrument according to the manufacturer's instructions. The spectrum of each isolate was compared with those in the database and identification was provided with a score of reliability. A score ≥1.7 and <2.0 is considered to be identification at the genus level, while scores ≥2.0 indicated identification at the species level.

MALDI-TOF VITEK MS

VITEK MS (bioMérieux, France) is another automated microbial identification system based on MALDI-TOF

technology. Myla™, a web-based middleware application integrated in the VITEK systems, provides a platform for both slide composition consultation and results consultation for VITEK MS.

The strains were tested by depositing one bacterial colony on the target slide, followed by the addition of matrix solution (VITEK MS-CHCA) and air-drying. The loaded slide was then inserted into the VITEK MS system. Microbial identification is achieved by obtaining spectra using MALDI-TOF technology and analysing the spectra with the VITEK MS database. The peaks from these spectra are compared to the characteristic pattern for a species, genus or family of microorganism, thus, resulting in organism identification. The organisms were reported with a percentage-scaled confidence value as well as a confidence level.

The VITEK 2 system

Isolates were inoculated into the GP cards, which were then run on the VITEK 2 Compact system (bioMérieux, France).

16S *rRNA* gene sequencing

16S *rRNA* gene sequencing was used as a reference method when discordant results were obtained with the investigated methods. The 16S *rRNA* genes were amplified and sequenced by using universal 16S rRNA-specific primers. Forward primer 5′-AGA GTT TGA TCM TGG CTC AG-3′ and reverse primer 5′-CCG TCA ATT CMT TTR AGT TT-3′ produced a fragment of about 900 bp spanning from *Escherichia coli* position 8 to 926 [16, 17]. The sequences obtained were blasted against databases in GenBank, EMBL, DDBJ and PDB via http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Results

The 132 isolates included in the study were identified as 32 *E. faecalis*, 63 *E. faecium*, 16 *E. casseliflavus* and 21 *E. gallinarum* (Table 1). The final identification of the isolates with discordant results by the investigated methods was reached by the reference method, 16S *rRNA* sequencing.

PCR assays

The multiplex PCR assay used in the study was able to identify all the isolates belonging to *E. faecalis*, *E. faecium*, *E. casseliflavus* and *E. gallinarum* correctly. The HRM-*groESL* assay identified all (21/21) *E. gallinarum* isolates and 81.3 % (13/16) of the *E. casseliflavus* isolates.

MALDI-TOF MS

MALDI-TOF MS was performed in parallel on two systems, Bruker MS (Bruker Daltonik, Germany) and VITEK MS (bioMérieux, France). Bruker MS and VITEK MS gave identical results for the 132 isolates (Table 1).

VITEK 2

VITEK 2 could identify 131/132 (99.2 %) and 121/132 (91.7 %) of the isolates at the genus and species levels, respectively. One *E. faecalis* isolate was reported as low discrimination from *Lactococcus garvieae*. One *E. faecium* isolate was misidentified as *E. gallinarum*. Nine isolates were not differentiable between *E. casseliflavus* and *E. gallinarum*.

Time to identification

For PCR assays and VITEK 2, it took 2.5 h and 3–8 h, respectively. Regarding MALDI-TOF, the results were available in 5–10 min.

Discussion

Enterococci have become the second or third leading cause of nosocomial urinary tract infections, wound infections and bacteraemia in the United States [3]. Moreover, the emergence and spread of glycopeptide resistance in enterococci has become of significant clinical concern and VRE are now an increasingly important infection control issue in hospitals worldwide [4–6, 18]. Accurate and rapid identification of enterococcal isolates at the species level is, therefore,

Table 1 Clinical *Enterococcus* isolates correctly identified at the species (genus) level by Bruker MS, VITEK MS, VITEK 2 and the multiplex polymerase chain reaction (PCR) assay

Bacteria	No. of isolates	Bruker MS	VITEK MS	VITEK 2	Multiplex PCR
E. faecalis	32	32 (32)	32 (32)	31 (31)	32
E. faecium	63	63 (63)	63 (63)	62 (63)	63
E. casseliflavus	16	16 (16)	16 (16)	11 (16)	16
E. gallinarum	21	21 (21)	21 (21)	17 (21)	21
Total	132	132 (132)	132 (132)	121 (131)	132

essential in making early clinical decisions and in the infection control of VRE [5, 19–23].

In this study, the most relevant diagnostic methods available in the field of clinical microbiology were compared for the identification of enterococci.

The multiplex PCR, Bruker MS and VITEK MS identified all isolates belonging to *E. faecalis*, *E. faecium*, *E. casseliflavus* and *E. gallinarum* correctly. In contrast, VITEK 2 could identify 91.7 % of these isolates at the species level, which is in accordance with the findings by Jin et al., who reported an identifying rate of 92.3 % for *Enterococcus* spp. [14].

The HRM assay developed in the present study is better than VITEK 2 in identifying *E. casseliflavus* and *E. gallinarum*. VITEK 2 gave correct results for 76 % (28/37) of *E. casseliflavus* and *E. gallinarum* isolates at the species level, while, 34 of the 37 isolates (92 %) belonging to *E. casseliflavus* or *E. gallinarum* were correctly identified by the HRM assay.

In the study, we also observed an enterococcal isolate of unusual species, *Enterococcus gilvus* (data not shown). Correct identification at the species level for the *E. gilvus* isolate was achieved by only Bruker MS among the four methods compared in the study. Since the species *E. gilvus* is not included in the target panel of the PCR assays, the strain was not detectable by PCR, as expected. VITEK 2 and VITEK MS could identify the strain at the genus level, but misidentified it to *E. avium* or *E. raffinosus*, which are two species closely related to *E. gilvus*. The lack of identification of *E. gilvus* by VITEK MS points to the fact that spectra for this species are not carried in the database. *E. gilvus* has been previously reported from human sources and could be clinically relevant [24].

Regarding the turn-around-time (TAT) of the various methods, MALDI-TOF is fast and PCR results are available within hours.

On the aspect of cost, the material cost of the in-house-developed PCR assay is inexpensive as compared to the commercial kit, according to our experience [18, 19]. The cost of MALDI-TOF MS identification (including consumables, salaries and depreciation of the apparatus over 5 years), according to Seng et al. [25], is one-quarter of that of phenotypic identification (2.44 € with MALDI-TOF MS vs. 4.60–13.85 € with an automated identification system). A similar cost reduction was estimated by Cherkaoui et al. [26]. Bizzini and Greub [12] have also observed other aspects of MALDI-TOF-based identification which can lead to further cost reduction, such as multiple growth media required in phenotypic methods.

Although the drift cost for the PCR assay is less economical than those for MALDI-TOF and VITEK 2, the PCR assay does not require expensive equipment as in the latter methods. Therefore, in those laboratories where advanced instruments such as MALDI-TOF are not yet available and laboratories with small amounts of samples, the PCR assay described in the present study might still be a cost-effective method in the rapid identification of enterococci.

In conclusion, the PCR methods described in the present study are effective in identifying the enterococcal species. MALDI-TOF MS is a rapid, reliable and cost-effective identification technique for *Enterococci* spp. The VITEK 2 system is less efficient in detecting non-*faecalis* and non-*faecium Enterococcus* species.

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Conflict of interest The authors declare that they have no conflict of interest.

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