Comparison between Rapid ID 32 Strep System, Matrix Assisted Laser Desorption Ionisation–Time of Flight Mass Spectrometry and 16S rRNA gene sequence analysis for the species identification of Enterococcus spp. isolated from water

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ABSTRACT

Matrix-Assisted Laser Desorption Ionisation–Time of Flight Mass Spectrometry (MALDI–TOF MS) has increasingly been used for rapid and reliable identification of clinically relevant micro-organisms. To establish the applicability of this technique in (drinking) water quality analysis, the MALDI–TOF MS identification results for Enterococcus spp. isolated from various water environments were compared with those obtained using the commercial Rapid 32 ID Strep system. One hundred and one bacterial isolates were isolated from various types of water and determined as enterococci on the basis of their growth on Slanetz-Bartley agar in typical colonies. The isolates were identified by MALDI–TOF MS and the commercial biochemical test Rapid 32 ID Strep. Isolates yielding in discrepant identifications were genotyped using 16S rRNA gene sequence analysis. For 86 isolates (85%), the results of Rapid ID 32 Strep were identical to those obtained with MALDI–TOF MS. Six isolates were impossible to be classified by means of the Rapid 32 ID Strep test. And for eight out of a total of nine discrepant results (89%), the 16S analyses confirmed the MALDI–TOF MS identification. MALDI–TOF MS produced highly reproducible results. These results indicated that the use of two different culture media had no effect on the identification. In addition, no significant differences ($p = 0.32; n = 20$) were evident between the scores obtained from a 20-fold measurement of the same isolate. The results of this study showed that MALDI–TOF MS identification (Bruker) is a reliable method for identifying $E. \text{faecium}$, $E. \text{faecalis}$, $E. \text{durans}$, $E. \text{hirae}$ and $E. \text{casseliflavus}$ isolated from water samples. $E. \text{mundtii}$ and $E. \text{moraviensis}$ were not included in the Rapid 32 ID Strep database and could therefore not be identified using that test set. However, MALDI–TOF MS and 16S identified all six isolates as members of these species.

Key words | bacterial identification, drinking water, enterococci, indicator organism, MALDI–TOF MS, source tracking

INTRODUCTION

Enterococci are widely used in drinking water microbiology as indicators for faecal contamination and additionally as indicators for the removal of bacteria by water treatment processes. Their natural habitats are the human and animal intestinal tracts, but they are also found in environmental habitats (Gelsomino et al. 2002). Outside of the host organism, enterococci are able to survive for longer periods under a wide range of temperatures, pH conditions and salinity levels, and they are resistant to the bactericidal effects of detergents (Shepard & Gilmore 2002). Some species, including $E. \text{faecium}$ and $E. \text{faecalis}$, appear to be true faecal indicators. For others, such as $E. \text{gallinarum}$...
and *E. casseliflavus*, it is suggested that grasslands are important habitats (*LeClerc et al. 1996*). Identification of enterococci species level may provide information on the nature of faecal contamination events and the source of faecal contamination.

When the commensal relationship with the host is disrupted, enterococci, particularly *E. faecalis*, may become opportunistic pathogens and cause invasive diseases (*Jett et al. 1994*).

In the laboratory, enterococci are detected by culture methods that use their ability to grow in the presence of azide. Confirmation of presumptive enterococci is performed by testing their ability to hydrolyse aesculin in the presence of bile within 2 h. However, this method gives no information on species composition.

Thus far, identification of cultured microbial isolates in water laboratories relies on methods based on biochemical tests such as Api (bioMérieux). Species identification by means of commercial systems is performed when the results obtained through the use of routine procedures do not provide sufficient information on, for instance, the potential source of contamination or the faecal nature of the contamination. It is often necessary to obtain additional information on the species in question, to collect information on the probability of a true faecal or potential environmental source, or for source tracking studies. The biochemical systems are limited in the sense that they are laborious and less reliable when it comes to environmental isolates. In the last decade, the molecular technique real-time PCR (Polymerase Chain Reaction) has become an important, routinely used detection technique for some of the relevant species for water research (*e.g.* *Escherichia coli*, *Legionella*). Although PCR is accurate, its use for direct detection of (indicator) bacteria is still limited. Reliable quantification at low levels in environmental samples is still difficult, hampering the introduction of PCR in water laboratories. Another drawback of direct PCR determination is the fact that PCR results do not discriminate between viable and nonviable bacteria. PCR is therefore more often used for colony confirmation, whereas species identification and source tracking still requires sequence analysis.

Matrix Assisted Laser Desorption Ionisation–Time of Flight Mass Spectrometry (MALDI–TOF MS) allows the identification of microbial species within a matter of minutes. This is done by analysing mass spectra of peptides and small proteins. Such a pattern was shown to be characteristic for microbial species (*Holland et al. 1996; Krishnamurthy et al. 1996; Fenselau & Demirev 2001; Mellman et al. 2008*). The general principle of MS is to produce, separate, and detect gas-phase ions. The sample is embedded in the crystalline structure of small organic compounds (matrix) and deposited on a conductive sample support. The crystals are irradiated with a laser beam. The laser energy causes structural decomposition of the irradiated crystal and generates a particle cloud from which ions are extracted by an electric field. This results in the disintegration of the crystal. Following acceleration through the electric field, the ions drift through a field-free path before reaching the detector. Ion masses (mass-to-charge ratios [*m/z*]) are calculated by measuring their Time Of Flight (TOF), which is longer for larger molecules than for smaller ones. In recent years, MALDI–TOF MS has been increasingly used to identify clinically relevant bacteria. The advantages of MALDI–TOF MS are short preparation time, rapid measurement, automatic spectrum analysis and no use of consumables. Furthermore, it is possible to add new mass spectra to the database. However, this technique also has two disadvantages: the necessity to culture the micro-organisms before identification can be completed, and the relatively expensive equipment to do so.

In this study, the application of MALDI–TOF MS for water research using *Enterococcus* spp. as a model was examined. Earlier, *Benagli et al. (2011)* proved MALDI–TOF MS to be a fast, reliable and cost-effective technique for the identification of clinically relevant *E. faecalis* and *E. faecium*. We applied the same criteria to the environmental isolates *E. faecium*, *E. faecalis*, *E. hirae*, *E. durans* and *E. casseliflavus*.

**METHODS**

**Bacterial isolates**

All isolates (*n = 101*) were obtained from water samples, collected over a period of 1 year. The origins of the isolates were: surface water, water at different stages of drinking water treatment and drinking water samples from drinking water distribution systems, mainly after repair works. All isolates were cultured using membrane filtration on solid selective
Slanetz and Bartley agar (SBA) for 44 h at 37 °C. To confirm typical colonies, the membrane, with all the colonies, was transferred onto bile-aesculin-azid agar (BEAA) for 2 h at 44 °C (according to NEN-EN-ISO 7899–2:2000). Only confirmed enterococci colonies, which were able to hydrolyse aesculin and induced a brown to black colour in the confirmation test were used in this study. Fresh pure cultures on SBA were made. Furthermore, we took a colony from the SBA plate to inoculate Columbia Sheep-Blood (CSB) agar. Isolates on CSB were cultured for 24 h at 37 °C. Fresh colonies (within 1 h after incubation) on SBA and CSB agar were used for MALDI–TOF MS. The influence of using less fresh colony material was not studied. For the biochemical Rapid ID 32 Strep test (bioMérieux) only colonies on CSB agar were used, as prescribed by the manufacturer.

**Biochemical identification**

For biochemical identification, the Rapid ID 32 Strep tests (bioMérieux) were performed according to the manufacturer's instructions. The identification results were obtained by entering the test results into the Analytical Profile Index WEB (https://apiweb.biomerieux.com/) using the identification software. A reliability percentage of ≥95% was used for species identification.

**MALDI–TOF MS analysis**

A colony of a freshly cultured isolate on SBA was thinly smeared onto a steel target plate (Bruker Daltonik) using a wooden toothpick. Each colony was overlaid with 1 μl of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, and 2.5% trifluoroacetic acid) and dried at room temperature. Measurements were performed using a Microflex mass spectrometer (Bruker Daltonik, Bremen, Germany) using FlexControl software (version 2.0). Spectra were recorded in the positive linear mode (laser frequency, 60 Hz; ion source 1 voltage, 20.0 kV; ion source 2 voltage, 16.7 kV; lens voltage, 7.0 kV; mass range, 2,000–20,000 Da). For each spectrum 240 shots in 40-shot steps from different positions of the target spot (automatic mode) were collected and analysed. The BioTyper database contains spectra of approximately 34 Enterococcus species and is regularly updated by Bruker Daltonik.

The software compared the obtained spectrum with those present in the database. During the matching process the obtained spectra were scored on the basis of three matching criteria: (1) the position of peaks in the measured spectrum and how well they matched the position of peaks in the spectrum from the database, (2) overall match between the measured spectrum and the spectrum in the database, and (3) the height of the peaks in both spectra. For each criterion a maximum score of 10 could be obtained. An identical match would give a score of 1,000 (10 × 10 × 10).

The final results of the mass spectra matching process were expressed in scores ranging from 0 to max 3 (10/log1000). For each isolate, the highest score of a match against a spectrum in the database was used for identification. For reliable species identification, only scores of ≥2.0 were used, which has been established as a criterion in the validation of MALDI–TOF MS (Moliner et al. 2010; Risch et al. 2010). In each target plate run we used E. coli Bacterial Test Standard (BTS) provided by the manufacturer to control the quality of the measurement. According to the manufacturer’s instructions only the measurements with BTS reliability of ≥2.2 were used in this study.

**16S analyses**

In case of discrepant results between Rapid ID 32 Strep and MALDI–TOF MS identifications, additional identification was performed by 16S rRNA whole gene sequencing analyses after PCR using 8F: AGAGTTTGATCATGGCTCA and 1592R: ACGGCGGTGTGGTACA primers. A sequence was attributed to a certain species when similarity (>99%) was obtained using BLAST (Basic Local Alignment Search Tool) software.

**Reproducibility test**

Three isolates per species (n = 15) (E. faecium, E. faecalis, E. durans, E. hirae and E. casseliflavus), originating from different water samples were used for the MALDI–TOF MS reproducibility test and Rapid 32 ID Strep identification. From each isolate, a pure culture was made on SBA and identified by MALDI–TOF MS. Subsequently, we took a colony from SBA and inoculated the CSB agar to obtain the colonies which were identified using Rapid 32 ID
Strep. We repeated the same procedure after a week with the same isolates, using fresh pure cultures.

**Influence of culture media on MALDI–TOF results**

Since the manufacturer of Rapid 32 ID Strep prescribes the use of CSB agar which is not routinely used in the water laboratory, we examined the influence of this culture medium on MALDI–TOF MS identification. From almost all isolates included in this study \((n = 93)\) pure cultures were made on SBA. These pure cultures were subsequently identified by MALDI–TOF and further used to inoculate CSB agar (bio-Mérieux) for 24 h at 37°C. The colonies were then analysed by MALDI–TOF.

During the study we verified whether or not the amount of colony material influenced the reliability. We repeated the identification of a randomly chosen *E. faecium* 20-fold. Ten times the colony was smeared on the target plate in a thin layer and another 10 times it was smeared in a thick layer. As only one isolate was used for the 20-fold test, these results are not representative for all isolates used in this study. However, the results may provide information on the sensitivity of the analysis and the way the analysis was conducted. The robustness of the analysis is important if the method is going to be used routinely.

**RESULTS AND DISCUSSION**

We obtained 101 isolates of positive enterococci on BEAA agar from water samples and identified them using Rapid ID 32 Strep system and MALDI–TOF MS.

For 86 isolates (85%), the results of Rapid ID 32 Strep were identical to those obtained with MALDI–TOF MS. 16S rRNA analysis was performed on nine isolates with discrepancies in identification between the two methods used to compare the corresponding identification by means of MALDI–TOF and Rapid ID 32 Strep (see Table 1). Five strains that showed identical identification with MALDI–TOF and Rapid ID32 were subjected to 16S rRNA analysis to confirm the identification.

Discrepant results were observed in isolates identified as *E. gallinarum* by Rapid ID 32 Strep, where all six isolates were identified and confirmed as *E. faecium* using MALDI–TOF MS and 16S analysis (shown in Table 1). One out of a total of 14 isolates identified by Rapid ID 32 Strep as *E. faecium* was identified by MALDI–TOF MS as *E. faecalis* and one out of a total of 22 isolates identified by Rapid ID 32 Strep as *E. hirae* was identified as *E. durans*. In both cases, the 16S rRNA gene analysis confirmed the MALDI–TOF MS identification.

For *E. faecalis* and *E. durans* the agreement between Rapid 32 ID and MALDI–TOF MS results was 100%. A noteworthy

<table>
<thead>
<tr>
<th>No. of isolates</th>
<th>Rapid ID 32 Strep</th>
<th>MALDI–TOF MS</th>
<th>16S rRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td><em>E. faecium</em></td>
<td><em>E. faecium</em></td>
<td><em>E. faecium</em>^a^</td>
</tr>
<tr>
<td>26</td>
<td><em>E. faecalis</em></td>
<td><em>E. faecalis</em></td>
<td><em>E. faecalis</em>^a^</td>
</tr>
<tr>
<td>21</td>
<td><em>E. hirae</em></td>
<td><em>E. hirae</em></td>
<td><em>E. hirae</em>^a^</td>
</tr>
<tr>
<td>Agreement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td><em>E. durans</em></td>
<td><em>E. durans</em></td>
<td><em>E. durans</em>^a^</td>
</tr>
<tr>
<td>15</td>
<td><em>E. casseliflavus</em></td>
<td><em>E. casseliflavus</em></td>
<td><em>E. casseliflavus</em>^a^</td>
</tr>
<tr>
<td>3</td>
<td>No reliable identification^b^</td>
<td><em>E. mundtii</em></td>
<td><em>E. mundtii</em></td>
</tr>
<tr>
<td>3</td>
<td>No reliable identification^b^</td>
<td><em>E. moraviensis</em></td>
<td><em>E. moraviensis</em></td>
</tr>
<tr>
<td>1</td>
<td><em>E. faecium</em></td>
<td><em>E. faecium</em></td>
<td><em>E. faecalis</em></td>
</tr>
<tr>
<td>Disagreement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td><em>E. gallinarum</em></td>
<td><em>E. faecium</em></td>
<td><em>E. faecium</em></td>
</tr>
<tr>
<td>1</td>
<td><em>E. hirae</em></td>
<td><em>E. durans</em></td>
<td><em>E. durans</em></td>
</tr>
<tr>
<td>1</td>
<td><em>E. casseliflavus</em></td>
<td><em>E. cecorum/E. casseliflavus</em>^c^</td>
<td><em>E. casseliflavus</em></td>
</tr>
<tr>
<td>Total</td>
<td>((n = 101))</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^aNot all, but one randomly chosen isolate was identified by 16S rRNA.

^bSpecies not included in database of Rapid 32 ID test.

^cBy repeating the measurement twice, different identifications were obtained: *E. cecorum* and *E. casseliflavus*. Both times the reliability score was \( \geq 2.0 \).
result was observed for *E. casseliflavus*. Using MALDI–TOF MS one of the *E. casseliflavus* isolates was identified as *E. ceceorum*. When the analysis was repeated, it was identified as *E. casseliflavus*. The 16S rRNA gene analysis identified *E. casseliflavus*.

Once the correct (defined as identical identification by at least two methods) identity was determined, the list of species included in this study was made.

The number of isolates per species is listed in Table 2.

Using the MALDI–TOF MS database, we were able to identify 34 *Enterococcus* species, while we could only identify nine using the Rapid 32 ID Strep system. Some *Enterococcus* species, which are not included in the list of species that can be identified by Rapid ID 32 Strep, such as *E. mundtii* and *E. moraviensis*, were identified during our study using MALDI–TOF MS. Three isolates of these two species were compared to the 16S sequencing results. For all six isolates the 16S confirmed the identifications obtained by MALDI–TOF MS. In this case the Rapid 32 ID Strep identifications were considered unreliable.

The MALDI–TOF MS appeared to be very time efficient; 2 minutes were needed to prepare the isolate for the measurement and another 2 minutes were needed to identify it. The preparation of one isolate using Rapid ID Strep required 5 minutes to prepare the test and after the 4.5-h incubation an additional 10 minutes were needed to read and interpret the result.

Reproducibility test

With regard to both methods (Rapid 32 ID Strep and MALDI–TOF MS respectively), no discrepancy has been observed between identification of all 15 isolates obtained on a single day and the duplicate test repetition performed on another day. Using Rapid 32 ID Strep, on both days one of the *E. hirae* isolates was identified with a low reliability percentage (77.3%), but confirmed by MALDI–TOF MS and 16S rRNA gene sequence analysis as *E. hirae*.

Use of a different culture medium

To examine if the reliability of MALDI–TOF MS identification depended on the use of a different cultivation medium, the results obtained using CSB agar were compared with those obtained using SBA agar. There was no difference found in the identification between the two culture media.

Using the Wilcoxon signed-rank test, no significant difference was found either between the reliability score obtained using SBA and using CSB agar; *p* = 0.304 (*n* = 93). However, when the same test was carried out per species, a significant difference was found for *E. hirae* (*p* = 0.021). The reliability score obtained for *E. hirae* with CSB agar was higher than with SBA, but we have not been able to explain the cause of this variation. As the observed difference did not influence the identification, only the reliability of the identification, this result may be less significant.

Reliability

The identification score of the 10-fold measurement of thinly smeared *E. faecium* varied between 2.399 and 2.540, and when the same isolate was smeared thickly, the score varied between 1.998 and 2.457. Using the Wilcoxon signed-rank test, we found a significant difference (*p* = 0.01, *n* = 10) between the reliability scores obtained from 10 thickly and 10 thinly smeared colonies. In all cases *E. faecium* was identified.

The MALDI–TOF MS and Rapid 32 ID Strep identification methods were compared for the identification of *Enterococcus* species isolated from surface water, process and drinking water samples. 16S rRNA gene sequencing was used to verify the correct identification. The 16S rRNA gene sequencing has been shown to be a reliable and universal technique for species identification in clinical microbiology (Clarridge 2004; Vargha et al. 2006).

The results of this study show that MALDI–TOF MS can be used to efficiently identify enterococci (*E. faecium, E. faecalis, E. durans, E. hirae, E. casseliflavus*) isolated from water samples. For *E. mundtii* (*n* = 3) and

<table>
<thead>
<tr>
<th>Enterococcus spp.</th>
<th>(n – 101)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecium</em></td>
<td>19</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>27</td>
</tr>
<tr>
<td><em>E. hirae</em></td>
<td>21</td>
</tr>
<tr>
<td><em>E. durans</em></td>
<td>12</td>
</tr>
<tr>
<td><em>E. casseliflavus</em></td>
<td>16</td>
</tr>
<tr>
<td><em>E. mundtii</em></td>
<td>3</td>
</tr>
<tr>
<td><em>E. moraviensis</em></td>
<td>3</td>
</tr>
</tbody>
</table>
E. moraviensis \((n=3)\) we did not have enough isolates to show the reliability of MALDI–TOF MS, although the 16S rRNA gene sequencing confirmed the MALDI–TOF MS identification for six isolates in 100% of the cases.

To explain the discrepancy between two MALDI–TOF MS identification results of E. casseliflavus, additional research is needed.

Discrepancy between MALDI–TOF MS and Rapid 32 ID Strep was observed in six isolates of E. faecium identified by Rapid 32 ID as E. gallinarum. When the Rapid 32 ID test for these isolates was repeated, different identification results were obtained: one E. gallinarum was identified this time as E. faecium. We also noticed that one of the biochemical tests (raffinose) has an important influence on the biochemical discrimination between these two species. A positive raffinose test indicated E. gallinarum, whereas a negative test indicated E. faecium. For all six isolates, 16S rRNA gene sequencing confirmed E. faecium as the identity.

Variation of scores obtained in 20 consecutive runs for one randomly chosen isolate showed good repeatability. However, the preparation of the sample influences the reliability score and should therefore be taken into consideration when implementing MALDI–TOF MS in routine testing. We observed lower scores when running a thick layer of colony material.

It was shown that the use of two different culture media, Slanetz & Bartley and CSB agar, did not influence the MALDI–TOF MS identification.

Several studies have reported on the use of MALDI–TOF MS for specific micro-organisms and yeast obtained from reference collections and clinical isolates (Seng et al. 2009; van Veen et al. 2010). These studies concluded that MALDI–TOF MS can replace conventional systems for identification of bacteria in a conventional clinical laboratory.

Until now, only a few studies included isolates obtained from environmental samples. A recent study by Moliner et al. (2010) using a large collection of clinical and environmental Legionella strains, demonstrated that MALDI–TOF is a reliable tool for the rapid identification of Legionella isolates at the species level. However, for the most common pathogenic species, L. pneumophila, it could not discriminate among serogroups, using the present database.

Identifying enterococci on species level requires a new approach for enterococci analysis within a water laboratory and interpretation of the results. The advantage of rapid identification is the possibility of a rapid response in the case of a true-positive result. Rapid identification also avoids a response based on a ‘false-positive’ test result. As published previously (Health Protection Agency 2007), some strains of Aerococcus viridans are also bile-aesculin positive and can therefore be confused with Enterococcus spp. in water testing. A. viridans has been observed in many non-faecal environments (Facklam & Elliott 1995), it does not possess Lancefield group D antigen and cannot be used to indicate faecal pollution.

Beside rapid identification there are also other possible applications of MALDI–TOF MS which are more suitable for research purposes than for routine use. For example, trained personnel could extend the database, add mass spectra to the database and exchange data with other users. This is especially relevant for environmental isolates. However, good quality assurance on newly entered spectra is of great importance. The Microflex database currently consists of 34 Enterococcus clinically relevant species. Although this number may seem relatively small, the identification with MALDI–TOF is specific enough to discriminate between these clinical and our environmental strains and hence between true faecal species and potential environmental species.

As suggested earlier (Giebel et al. 2008), MALDI–TOF MS-based fingerprinting of environmental isolates of faecal indicators as shown in this study for environmental isolates of enterococci, has the potential to become a tool for bacterial source tracking (BST). To establish the value of such a tool, a large number of environmental isolates need to be analysed.

**CONCLUSIONS**

It has been shown that MALDI–TOF MS can be used for rapid, efficient and reliable identification of E. faecium, E. faecalis, E. durans, E. hirae and E. casseliflavus isolated from water samples. To establish the identification reliability of two other species, E. mundtii and E. moraviensis, more isolates needed to be tested. We obtained high reproducibility of the identification and obtained the same result when using two different culture media.
To further advance the implementation of this identification technique in (drinking) water laboratories requires the validation of MALDI–TOF MS for other microorganisms isolated from water.

ACKNOWLEDGEMENTS

We would like to thank the Streeklaboratorium voor de Volksgezondheid in Haarlem (Regional Public Health Laboratory) for allowing us to make use of the MALDI-TOF MS apparatus for the purpose of this study. Special thanks go out to Kim Jansen and Paul van Beers for their unstinting assistance in conducting the measurements. Last but not least, we also thank the management team of Het Waterlaboratorium (Water Laboratory) for giving us the opportunity to study this interesting subject.

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First received 15 May 2012; accepted in revised form 12 September 2012. Available online 14 September 2013