Identification of the \textit{Streptococcus anginosus} group' by matrix-assisted laser desorption ionization – time-of-flight mass spectrometry

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) provides rapid, accurate and cost-effective identification of a range of bacteria and is rapidly changing the face of routine diagnostic microbiology. However, certain groups of bacteria, for example streptococci (in particular viridans or non-haemolytic streptococci), are less reliably identified by this method. We studied the performance of MALDI-TOF MS for identification of the \textit{Streptococcus anginosus group} (SAG) to species level. In total, 116 stored bacteraemia isolates identified by conventional methods as belonging to the SAG were analysed by MALDI-TOF MS. Partial 16S rRNA gene sequencing, supplemented with sialidase activity testing, was performed on all isolates to provide ‘gold standard’ identification against which to compare MALDI-TOF MS performance. Overall, 100 % of isolates were correctly identified to the genus level and 93.1 % to the species level by MALDI-TOF MS. However, only 77.6 % were correctly identified to the genus level and 59.5 % to the species level by a MALDI-TOF MS direct transfer method alone. Use of a rapid \textit{in situ} extraction method significantly improved identification rates when compared with the direct transfer method ($P<0.001$). We recommend routine use of this method to reduce the number of time-consuming full extractions required for identification of this group of bacteria by MALDI-TOF MS in the routine diagnostic laboratory. Only 22 % (1/9) of \textit{Streptococcus intermedius} isolates were reliably identified by MALDI-TOF MS to the species level, even after full extraction. MALDI-TOF MS reliably identifies \textit{S. anginosus} and \textit{Streptococcus constellatus} to the species level but does not reliably identify \textit{S. intermedius}.

\textbf{INTRODUCTION}

\textit{Streptococcus anginosus}, \textit{Streptococcus constellatus} and \textit{Streptococcus intermedius} make up the \textit{‘Streptococcus anginosus group’} (SAG). These organisms are associated with purulent infections of the mouth and internal organs, including the central nervous system (Doern & Burnham, 2010; Whiley \textit{et al.}, 1992). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is now being used for routine bacterial identification in many European microbiology laboratories. A number of authors have demonstrated that the use of MALDI-TOF MS in the diagnostic laboratory is an accurate, cost-effective and rapid method for identification of a range of bacteria and fungi (Bizzini \textit{et al.}, 2010; Neville \textit{et al.}, 2011; Seng \textit{et al.}, 2009; van Veen \textit{et al.}, 2010). However, \textit{x}-haemolytic and non-haemolytic streptococci (including the SAG) are less reliably identified by MALDI-TOF MS (Benagli \textit{et al.}, 2011; Friedrichs \textit{et al.}, 2007; Risch \textit{et al.}, 2010; van Veen \textit{et al.}, 2010) and routinely require extraction steps to obtain a reliable identification (van Veen \textit{et al.}, 2010). These extraction steps are time-consuming and labour intensive and reduce the efficiency of MALDI-TOF MS for use in high-throughput diagnostic laboratories. Furthermore, the SAG has been largely under-represented in published studies of MALDI-TOF MS performance for bacterial identification. What data there are have shown species level identification rates varying from 44 to 100 %, and often routine biochemical identification alone has been the comparator for MALDI-TOF MS, only resorting to 16S rRNA gene sequencing if initial results are found to be discordant (Friedrichs \textit{et al.}, 2007; Neville \textit{et al.}, 2011; van Veen \textit{et al.}, 2010). Furthermore, the three species within the

\textbf{Abbreviations:} MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SAG, \textit{Streptococcus anginosus} group.

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SAG have been shown to share 60 % peak MS pattern similarity (Welker & Moore, 2011) and this may hamper MS-based identification of individual species, allowing accurate identification only to the species complex using MALDI-TOF MS.

Given the paucity of data on MALDI-TOF MS performance for identification of the SAG, we analysed bacteraemia isolates identified previously as *Streptococcus milleri*, *S. anginosus*, *S. constellatus* or *S. intermedius* from patients at Guy’s and St Thomas’ Hospitals over a 15-year period using MALDI-TOF MS (Bruker Daltonics Microflex LT). 16S rRNA gene sequencing and determination of sialidase activity were performed on all isolates to provide ‘gold standard’ identification against which to compare the MALDI-TOF MS identification. *S. intermedius* is universally positive for sialidase activity, whilst *S. anginosus* and *S. constellatus* do not produce sialidase (Beighton & Whiley, 1990).

**METHODS**

A database containing all clinically significant bloodstream infection episodes at St Thomas’ Hospital and Guy’s Hospital was searched to identify all SAG isolates saved between January 1997 and December 2011. The frozen (–80 °C) isolates were retrieved and subcultured onto fastidious anaerobe agar with 5% horse blood (FHB) and incubated anaerobically for 48 h at 35–37 °C. Single colonies were picked off, resubcultured onto FHB and incubated anaerobically at 35–37 °C for 24 h to obtain fresh pure cultures. Type strains (*S. anginosus* ATCC 35397, *S. constellatus* ATCC 27823 and *S. intermedius* ATCC 27335) were included as positive controls.

**MALDI-TOF MS.** All MALDI-TOF MS identifications were carried out on fresh 24 h cultures by a single investigator (K.W.). The direct transfer method (touching a single colony with a cocktail stick and making a thin film of the organism on a single spot of a 96-spot ‘target’ plate) was used first. A ‘double spot’ (use of a single cocktail stick to touch a colony and then spot onto two ‘target’ spots in succession) was used in order to optimize the smear technique. The bacteria were allowed to air dry on the target plate at room temperature. One microlitre of matrix solution (composed of trifluoroacetic acid, acetonitrile and x-cyano-4-hydroxycinnamic acid) was then applied to each smear and again allowed to dry at room temperature. The target plate was then inserted into the Bruker Daltonics Microflex LT and the mass spectra generated were analysed using the Biotyper 3.0 database. Identifications with a score of 2.0–3.0 were accepted as reliable identification to the species level. A score of 1.700–1.999 indicated only probable genus identification, and a score of <1.700 indicated no identification. Isolates with a score of <2.0 after the direct transfer method underwent extraction and were then reanalysed by MALDI-TOF MS. An on-plate *in situ* formic acid extraction method (Haigh et al., 2011), identical to the direct transfer method with the additional step of applying 1 µl 70 % formic acid to the spot on the target plate and leaving to air dry prior to adding matrix solution, as well as the standard full extraction method recommended by Bruker Daltonics, was used. For the full extraction method, 1 µl bacteria was suspended in 300 µl sterile water and 900 µl absolute ethanol was then added prior to centrifugation at 15000 g for 2 min. Supernatant was removed and the pellet was recentrifuged at 13000 r.p.m. for 2 min. Any remaining ethanol was removed by pipette and then by evaporation (leaving the sample tube open on the bench for 10 min). Next, 10–50 µl 70 % formic acid and acetonitrile (in a 1:1 ratio) were added according to pellet size, and the resuspended sample was then centrifuged for 2 min at 13000 r.p.m. One microlitre of supernatant was then pipetted onto the target plate spot and allowed to air dry at room temperature (approx. 10 min); 1 µl matrix was added and allowed to air dry prior to MALDI-TOF MS analysis as for the direct smear method.

**16S rRNA gene sequencing.** DNA was extracted from the 24 h pure cultures as follows: two to three colonies were suspended in 100 µl sterile Ultra High Quality water, vortexed, heated at 95 °C for 12 min (Microtherm microtube incubator; Camlab) and then cooled on ice for 1–2 min. Samples were centrifuged at 13000 r.p.m. for 1 min, and the supernatants (containing extracted bacterial DNA) were used directly as templates for PCR. The 12.5 µl PCR mixture consisted of: 0.5 µl DNA extract and 12 µl master mix (1.15 µl Reddymix; ABgene, 0.25 µl 9F forward primer (5′-GAGTITGATCTGCGTCACT-3′) and 0.25 µl 907R reverse primer (5′-CGTCAATTCCTTGAGTTF-3′)). PCR conditions were as follows: 10 min denaturation at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 49 °C and 90 s at 72 °C, with a final 5 min at 72 °C. Cleaned PCR products were sequenced in a forward direction using the 9F primer and the Applied Biosystems Big Dye reaction (Do et al., 2010). Sequenced products were cleaned using the Big Dye terminator clean-up method. The DNA sequence was then determined by the Sanger dideoxy method using a 3730xl DNA analyser (Applied Biosystems). A minimum sequence read of 500 nt for each isolate was accepted; however, the majority (114/116) of reads were >650 nt. Sequences were checked visually for levels of background noise, edited and aligned using BioEdit software. Individual sequences were subjected to BLAST searching using the Human Oral Microbiome Database website (http://www.homd.org) to obtain identification. A minimum of >99% sequence similarity was the criterion used for species identification.

**Sialidase enzyme test.** A simple fluorescence method was used for demonstrating sialidase activity, as described previously (Beighton & Whiley, 1990). A positive control (*S. intermedius* ATCC 27335) was included in each batch of sialidase tests.

**Statistical analysis.** Statistical analyses used χ² tests for comparison of species identification rates by direct transfer and extraction methods, where necessary with Yates’ correction for continuity.

**RESULTS**

In total, 177 putative SAG bacteraemia episodes were identified between January 1997 and December 2011. In 37 of the episodes isolates were not found, six isolates did not grow on subculture and 18 isolates were identified on 16S rRNA gene sequencing as not belonging to the SAG. The remaining 116 SAG isolates [identified by 16S rRNA sequencing as *S. anginosus* (n=59), *S. constellatus* (n=48) and *S. intermedius* (n=9)] were included for analysis. Only the *S. intermedius* isolates were positive for sialidase activity.

Overall, MALDI-TOF MS correctly identified 100 % of isolates to the genus level and 93.1 % (108/116) of isolates to the species level after full extraction. Using the direct transfer method alone, only 77.6 % (90/116) were correctly identified to the genus level and 59.5 % (69/116) to the species level. Forty-seven isolates (40.5 %) required extraction to MALDI-TOF MS analysis as for the direct smear method.
Differentiate rRNA gene sequencing data obtained in this study did not, within the SAG but had scores is isolate reported. The other four isolates were all identified diagnostic laboratory and an incorrect identification of the identification would have been accepted by the routine isolates had a score value of (see Table 2). Four of the incorrectly identified intermedius S. intermedius extraction method; seven of these isolates were identified by MALDI-TOF MS despite use of the full extraction. This is consistent with identification difficulties of S. intermedius encountered with conventional identification methods, and misidentification of S. intermedius, as S.

<table>
<thead>
<tr>
<th>Organism (16S rRNA)</th>
<th>MALDI-TOF MS direct</th>
<th>MALDI-TOF MS in situ extraction (no. correct/no. extracted)</th>
<th>MALDI-TOF MS full extraction (no. correct/no. extracted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. anginosus (n=59)</td>
<td>38</td>
<td>19/21</td>
<td>20/21</td>
</tr>
<tr>
<td>S. constellatus (n=48)</td>
<td>30</td>
<td>9/18</td>
<td>18/18</td>
</tr>
<tr>
<td>S. intermedius (n=9)</td>
<td>1</td>
<td>0/8</td>
<td>1/8</td>
</tr>
<tr>
<td>Total (n=116)</td>
<td>69</td>
<td>28/47*</td>
<td>39/47*</td>
</tr>
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Forty-seven isolates did not obtain a score value >2.000 using the direct transfer method and underwent further analysis by both in situ formic acid extraction and full extraction.

Table 1. Number of isolates correctly identified to the species level by the different methods

DISCUSSION

Overall species-level identification of SAG bacteria in our study (93.1%) is better than in other studies that have also used Bruker Daltonics Microflex MS; for example, 77.8% found by van Veen et al. (2010) using FlexControl software version 3.0 and 44–67% found by Neville et al. (2011) using Biotyper 2.0 software version 3.1.1.0. Both of these studies had small numbers of isolates from the SAG (9 and 17, respectively), neither had any S. intermedius isolates, and 16S rRNA gene sequencing was only used to confirm identification in the latter study, and even then only for results discordant with conventional testing. Friedrichs et al. (2007) claimed 100% identification to the species level of 23 isolates (19 S. anginosus, two S. constellatus and two S. intermedius) with MALDI-TOF MS; however, comparison was with biochemical identification to SAG level rather than species identification or 16S rRNA gene sequence data. The reported accuracy of the SAG species-level identification in all these studies must therefore be regarded with caution.

Our data show that the performance of MALDI-TOF MS is not equal across all three species within the SAG. Consistently accurate results were obtained for S. anginosus and S. constellatus; however, S. intermedius was poorly identified to the species level by MALDI-TOF MS even after full extraction. This is consistent with identification difficulties of S. intermedius encountered with conventional identification methods, and misidentification of S. intermedius, as S.

Table 2. Discrepant results after full extraction (score value given in parentheses)

http://jmm.sgmjournals.org
anginosus or S. constellatus may account for the lack of S. intermedius isolates in other studies.

Another important finding of our study is that, in order to obtain accurate species identification for SAG bacteria with MALDI-TOF MS, a high proportion of isolates required extraction. Full extraction is laborious and time-consuming, especially when required on a large number of isolates. We demonstrate that a simple, rapid, in situ formic acid extraction method significantly improved identification rates of SAG bacteria and reduced the number of isolates requiring full extraction. Given the low identification rates using the direct transfer method for SAG bacteria, as well as other α-haemolytic streptococci (Neville et al., 2011; van Veen et al., 2010), we argue that the direct transfer method should be abandoned and the in situ formic acid extraction method used as the first-line method for MALDI-TOF MS identification of viridans group streptococci. Those isolates identified only to the genus level as members of the SAG could be tested for sialidase activity to determine whether they are S. intermedius.

SAG organisms are associated with purulent infections of the mouth and internal organs, including the central nervous system (Doern & Burnham, 2010; Whiley et al., 1992). Some studies have shown that the three species have a propensity for abscess formation at differing anatomical sites (Clarridge et al., 2001; Whiley et al., 1992). At present, identification of S. intermedius by MALDI-TOF MS is not good enough to allow reliable conclusions regarding clinical associations of the different species to be made from routine clinical laboratory data. Further studies are needed before evidence-based decisions about the clinical relevance of pursuing a species, rather than group (SAG), level identification within the routine diagnostic laboratory is necessary.

An essential pre-requisite for accurate identification of bacteria by MALDI-TOF MS is a comprehensive range of species isolates within the database in order to cover the natural diversity of any species (Keys et al., 2004; Welker & Moore, 2011), and any one species should be represented by multiple strains, ideally 10 or more (Seng et al., 2009). Only five SAG strains (two S. anginosus, one S. constellatus ssp. constellatus, one S. constellatus ssp. pharyngis, one S. intermedius) are included within the Bruker Daltonics Biotyper 3.0 database. This is a far more limited dataset than that for many of the other commonly isolated organisms, including many of the streptococci, in the Biotyper 3.0 database. Whether supplementing the database with additional strains would overcome the problems with identification of S. intermedius or whether this difficulty is inherent in the high degree of similarity between mass spectra within the SAG requires further investigation.

Our study has some limitations. The use of FHB agar and anaerobic incubation in this study was designed to optimize growth conditions for the SAG and maximize identification potential by MALDI-TOF MS from a 24 h culture. However, these conditions may not be representative of SAG isolates identified in the routine diagnostic laboratory and may have positively skewed our results in favour of MALDI-TOF MS. However, whilst agar type and incubation conditions may vary, the mass spectra of a strain the peak patterns obtained are generally stable (Carbonnelle et al., 2011; Welker & Moore, 2011) and MALDI-TOF MS identification has been shown to be robust over a range of medium and culture conditions (Claydon et al., 1996; van Veen et al., 2010).

The use of the ‘double spot’ technique, which is recommended when training in MALDI-TOF MS, is not employed in routine practice in our laboratory. Significantly more isolates would have required extraction for reliable identification (67 vs 47, P=0.0126) if a single, rather than double, spot technique direct transfer method had been used in our study. At a species level, a statistically significant effect on the number of isolates requiring extraction, when compared single with double spots, was only seen with S. anginosus (34 vs 21, P=0.0164). Therefore, whilst overall identification rates (i.e. reliable identification either with the direct transfer method or after any necessary extractions) were not affected, the performance of the direct transfer method may be significantly less within the routine laboratory setting than was found in our study. This lends further weight to our argument for employing the in situ formic acid extraction method as the initial test for this group of organisms.

In conclusion, MALDI-TOF MS is accurate and performs well in the routine diagnostic laboratory for identification of S. anginosus and S. constellatus to the species level, achieving species-level identification rates comparable to those found in studies analysing MALDI-TOF MS performance across a range of bacteria (Carbonnelle et al., 2011; Neville et al., 2011; Seng et al., 2009; van Veen et al., 2010). However, a high proportion of isolates require extraction to obtain reliable identification, and we recommend routine use of the in situ formic acid extraction method to reduce the number of time-consuming full extractions required. S. intermedius is poorly identified by MALDI-TOF MS. Finally, although identification of the S. anginosus group to the species level is important to establish the pathological relationships between individual species and the isolation site, whether such speciation is clinically important in bloodstream isolates has not been established conclusively. It may therefore be appropriate currently for routine diagnostic laboratories to report such isolates as ‘S. anginosus group’.

**ACKNOWLEDGEMENTS**

We would like to thank Lisa Bryan and Matt Readwin for their guidance and patience facilitating the use of the MALDI-TOF MS system, Steven Gilbert for his assistance with the partial 16S rRNA gene sequencing and Amita Patel for helping retrieve the frozen isolates from our laboratory.

**REFERENCES**


