Identification of the ‘Streptococcus anginosus group’ by matrix-assisted laser desorption ionization – time-of-flight mass spectrometry

Katherine Woods,1 David Beighton2,3 and John L. Klein1

Correspondence
John L. Klein
John.klein@gstt.nhs.uk

1Directorate of Infection, Guys & St Thomas Hospitals NHS Foundation Trust, 5th Floor North Wing, St Thomas’ Hospital, Westminster Bridge Road, London SE1 7EH, UK
2Department of Microbiology, KCL Dental Institute, Floor 17, Guys Tower, London SE1 9RT, UK
3Department of Oral Biology, Leeds Dental Institute, University of Leeds, Clarendon Way, Leeds LS2 9LU, UK

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 ‘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 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 Streptococcus intermedius isolates were reliably identified by MALDI-TOF MS to the species level, even after full extraction. MALDI-TOF MS reliably identifies S. anginosus and Streptococcus constellatus to the species level but does not reliably identify S. intermedius.

INTRODUCTION

Streptococcus anginosus, Streptococcus constellatus and Streptococcus intermedius make up the ‘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 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 et al., 2010; Neville et al., 2011; Seng et al., 2009; van Veen et al., 2010). However, α-haemolytic and non-haemolytic streptococci (including the SAG) are less reliably identified by MALDI-TOF MS (Benagli et al., 2011; Friedrichs et al., 2007; Risch et al., 2010; van Veen et al., 2010) and routinely require extraction steps to obtain a reliable identification (van Veen 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 et al., 2007; Neville et al., 2011; van Veen et al., 2010). Furthermore, the three species within the
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 trfluoroacetic acid, acetonitrile and z-cyano-4-hydroxycinnamic acid) was then applied to each smear and again allowed to dry at room temperature. The target plate spot and allowed to air dry at room temperature for 2 min. Supernatant was removed and the pellet was centrifuged at 13000 r.p.m. for 2 min before being added to air dry prior to MALDI-TOF MS analysis 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 [11.5 µl Reddymix; ABgene, 0.25 µl 9F forward primer (5′-GAGTTTGATCCTGGCT-CAG-3′) and 0.25 µl 907R reverse primer (5′-CGTCAATTCCCTTG-AGTFF-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 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 Microflex LT 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 resuspended in 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 37200 g. 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.

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 93.1% (108/116) of isolates to the species level. Forty-seven isolates (40.5%) required extraction methods, where necessary with Yates’ correction for continuity.
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.

Four of the incorrectly identified intermedius extraction method; seven of these isolates were S. intermedius. Four isolates did not obtain a score value greater than 2.000 using the direct transfer method and underwent further analysis by both in situ formic acid extraction and full extraction.

Reliable, and correct, final identification (i.e. a score value >2.000 on direct transfer method or after extraction, and identification concordant with 16S rRNA gene sequencing) of the different species was as follows: S. anginosus, 98.3%; S. constellatus, 100%; S. intermedius, 22.2% (Table 1). Full extraction did not significantly increase species identification rates compared with the in situ formic acid extraction method except for S. constellatus ($\chi^2 = 9.481; P = 0.002$). The mean score values of the reliable final identification for each species were: S. anginosus, 2.154; S. constellatus, 2.195; S. intermedius, 2.022. Type strains S. anginosus ATCC 33397 (score value = 2.237) and S. constellatus ATCC 27823 (score value = 2.000) were reliably identified using the direct transfer method; however, S. intermedius ATCC 27335 was only reliably identified after full extraction (score value = 2.017). Eight isolates were incorrectly identified by MALDI-TOF MS despite use of the full extraction method; seven of these isolates were S. intermedius (see Table 2). Four of the incorrectly identified isolates had a score value of >2.000 and therefore the identification would have been accepted by the routine diagnostic laboratory and an incorrect identification of the isolate reported. The other four isolates were all identified within the SAG but had scores <2.000. The partial 16S rRNA gene sequencing data obtained in this study did not differentiate S. constellatus to the subspecies level and therefore no comment has been made on the accuracy of the S. constellatus subspecies identifications by MALDI-TOF MS.

**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. anginosus.

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

<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>
</tbody>
</table>

*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.

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</tr>
</thead>
<tbody>
<tr>
<td>S. anginosus</td>
<td>No peaks (&lt;0)</td>
<td>No peaks (&lt;0)</td>
<td>S. constellatus (1.973)</td>
</tr>
<tr>
<td>S. intermedius</td>
<td>S. constellatus (1.674)</td>
<td>S. constellatus (1.962)</td>
<td>S. constellatus (2.114)</td>
</tr>
<tr>
<td>S. intermedius</td>
<td>S. anginosus (1.868)</td>
<td>S. anginosus (1.961)</td>
<td>S. anginosus (2.001)</td>
</tr>
<tr>
<td>S. intermedius</td>
<td>S. intermedius (1.918)</td>
<td>No peaks (&lt;0)</td>
<td>S. intermedius (1.989)</td>
</tr>
<tr>
<td>S. intermedius</td>
<td>S. intermedius (1.937)</td>
<td>No peaks (&lt;0)</td>
<td>S. intermedius (1.989)</td>
</tr>
<tr>
<td>S. anginosus</td>
<td>S. anginosus (1.901)</td>
<td>No peaks (&lt;0)</td>
<td>S. constellatus (2.02)</td>
</tr>
<tr>
<td>S. intermedius</td>
<td>No peaks (&lt;0)</td>
<td>S. anginosus (1.924)</td>
<td>S. intermedius (1.821)</td>
</tr>
<tr>
<td>S. intermedius</td>
<td>S. intermedius (1.814)</td>
<td>S. intermedius (1.877)</td>
<td>S. constellatus (2.045)</td>
</tr>
</tbody>
</table>
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 bacteria (Carbonnelle 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.

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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; 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’.

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REFERENCES


