Rapid identification of \textit{Legionella} species from a single colony by gas-liquid chromatography with trimethylsulphonium hydroxide for transesterification

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\textbf{Summary.} Transesterification of bacterial fatty acids to methyl esters with trimethylsulphonium hydroxide (TMSH) was compared with a conventional method for the identification of \textit{Legionella} species by capillary gas chromatography. There was an extensive coincidence in the gas chromatographic profiles of bacterial fatty acid methyl esters (FAMEs) obtained by the two methods. However, the TMSH procedure needs less initial material and is much more simple and rapid. The chromatographic pattern of FAMEs obtained from a single colony is sufficient for the identification of the genus \textit{Legionella}, and \textit{L. pneumophila} can be clearly distinguished from other \textit{Legionella} species.

\textbf{Introduction}

The analysis of cellular fatty acid methyl esters (FAMEs) by gas-liquid chromatography (GLC) is a potent method for the identification of \textit{Legionella} at the genus level.\textsuperscript{1,2} Frequently the legionellae can even be identified to species level.\textsuperscript{3,4} \textit{Legionella} spp. differ from other gram-negative organisms by their content of large amounts of branched-chain fatty acids and only smaller or trace amounts of hydroxy acids.\textsuperscript{5} A widely used procedure for sample preparation involves saponification of whole bacterial cells and esterification of the fatty acids and triglycerides with methanol. The resultant FAMEs are extracted with a diethyl ether: hexane mixture, followed by a washing step.\textsuperscript{6} This procedure is time-consuming and laborious, because pure cultures of bacteria are needed and an exact time and temperature protocol has to be followed for transesterification, which takes at least 1 h to perform. Therefore, GLC of bacterial FAMEs has not been used routinely in diagnostic laboratories up to now.

Trimethylsulphonium hydroxide (TMSH), a methylating agent used successfully in organic chemistry,\textsuperscript{7} has been applied in transesterification of fatty acid esters to analyse fats and oils in food chemistry.\textsuperscript{8-10} Recently TMSH was introduced for the assay of bacterial fatty acids.\textsuperscript{11,12} In this technique, cellular fatty acids from lipids of bacteria are converted to FAMEs by TMSH within 1–2 min, even at room temperature, and the sample can be applied directly to the GLC column. In comparison to other techniques, less bacterial material is necessary for the transesterification reaction. The TMSH technique has been tested for several gram-positive and gram-negative bacteria, but not for \textit{Legionella} spp.\textsuperscript{11,12} This report describes the application of the TMSH technique for the GLC identification of \textit{Legionella} spp. from single colonies, even when grown as mixed cultures on primary culture plates. As the assay protocol is much easier than conventional sample preparation procedures, the new method is suitable for the routine diagnosis of legionella infection.

\textbf{Materials and methods}

\textbf{Bacterial strains and growth conditions}

The reference strains \textit{L. pneumophila} serogroup (sg) 1 (ATCC 33152), sg 2 (ATCC 33154), sg 3 (ATCC 33155), sg 4 (ATCC 33156), sg 5 (ATCC 33216), sg 6 (ATCC 33215), sg 7 (ATCC 33823), sg 8 (ATCC 35096), \textit{L. micdadei} (ATCC 33218), \textit{L. bozemanii} sg 1 (ATCC 33217) and sg 2 (ATCC 33256), and \textit{L. dumoffii} (ATCC 33279) were cultured on BCYE\textsubscript{a} agar.\textsuperscript{13} \textit{L. pneumophila} sg 2 (ATCC 33154) was chosen as a model strain for sensitivity testing. Wild-type strains of \textit{L. pneumophila} sg 3 and \textit{L. micdadei} were isolated from potable water with BCYE\textsubscript{a} agar, BMPA\textsubscript{a} agar\textsuperscript{14} and MWY agar.\textsuperscript{15}

In addition to GLC identification, legionellae were identified by direct immunofluorescence with polyclonal antibody conjugates (Mardy Diagnostics Inc., obtained from Viramed, Martinsried, Germany), monovalent antibody conjugates (done by Dr Horbach, Bundesgesundheitsamt, Berlin), and by hybridisation with a DNA probe (\textit{Legionella} Gen-Probe; Gen-Probe Inc., San Diego, obtained from Biermann,

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Bad Nauheim, Germany). For GLC analysis the reference and wild-type strains were cultured at 37°C for up to 4 days.

**Preparation of trimethylsulphonium hydroxide**

TMSH is not available commercially, but can easily be prepared from trimethylsulphonium iodide (E. Merck, Darmstadt, Germany). A TMSH solution (approximately 0.2 mol/L) was synthesised according to the method of Schulte and Weber by the following procedure: 20 g of strong basic ion exchanger (Amberlyst A-26, E. Merck) was suspended in distilled water and introduced into a glass column (inner diameter approximately 0.2 mol/L) was synthesised according with methanol (100 ml). Trimethylsulphonium iodide (4.1 g) was dissolved in 60 ml of methanol at 50°C in a water bath and the solution was applied to the ion exchange column in portions of 5–10 ml. Meanwhile, the solution was always returned to the water bath to avoid recrystallisation of trimethylsulphonium iodide in the container or column. Finally, the ion exchange resin was washed with 40 ml of methanol. The eluate (approximately 100 ml) was stored in a capped vessel at 4°C. At this temperature the TMSH solution proved to be stable for more than 1 year.

**TMSH technique for FAME preparation**

The whole procedure was performed in a single gas chromatographic (GC) vial (32 mm × 11 mm, Hewlett Packard). Between one and five colonies (c. 3 × 10⁸–1.5 × 10⁹ cfu) grown for 3 or 4 days were picked from the culture plates with a 1-μl disposable loop (Nunc). The bacteria were suspended in 10 μl of distilled water and 30 μl of methanolic TMSH solution (0.2 mol/L) was added. During mixing (Vortex, 5 s), the bacterial cells were lysed by the strongly alkaline TMSH and the suspension was converted into a homogeneous, transparent phase. This viscous fluid was completely dried in a nitrogen stream and 200 μl of a mixture of tert-butylmethyl ether and methanol 10:1 was added as the parent phase. This viscous fluid was completely dried in a nitrogen stream and 200 μl of a mixture of tert-butylmethyl ether and methanol 10:1, v:v) was added to the remainder. After shaking thoroughly to extract the FAMEs, the sample was applied directly to the GLC column.

**Conventional technique for FAME preparation**

The procedure described by Miller and Berger was chosen for comparison, because it is frequently used for FAME preparation. Additionally, this procedure is used in commercially available Microbial Identification Systems (Microbial Identification System, Microbial ID Inc., Newark, DE, USA). All parameters of the method including reagent concentrations, reaction temperatures and times must be kept strictly constant (e.g., 80 ± 1°C for methylation). The protocol of sample preparation strictly followed the procedure described by Miller and Berger, except the slight modification that a mixture of tert-butylmethyl ether and n-hexane (1:1, v:v) was used instead of diethyl ether.

**Gas chromatographic equipment and parameters**

Gas-liquid chromatography of bacterial FAMEs was performed with an HP 5890 gas chromatograph (Hewlett Packard, Avondale, USA) equipped with a flame ionisation detector (FID), split injection and automatic sample injection. The FAMEs were separated on a 25-m fused-silica capillary column, SE 54 (crosslinked 5% phenylmethyl silicone, Ultra 2 No. 19091 B 102, Hewlett Packard), i.d. 0.2 mm, film thickness 0.33 μm. The carrier gas was 0.7 bar hydrogen, sample volume 2 μl, split 1:30. The injector temperature was maintained at 250°C, and the FID temperature was maintained at 300°C. Column temperature was 170–270°C with a heating rate of 5°C/min. GC-analysis time was 20 min.

**Standards**

The bacterial FAMEs were identified by calculating the equivalent chain length with a FAME standard mixture (Supelco, No. 4-7080, Bellafonte, USA). FAMEs not present in the standard mixture were obtained by preparing bacterial reference strains.

**Results**

The chromatogram shown in fig. 1A was from a single colony of *L. pneumophila* sg 2 (ATCC 33154) harvested from a 4-day culture and treated with TMSH. The amount of FAMEs extracted from this single colony (c. 3 × 10⁹ cfu) was sufficient to yield a GC profile, which is nearly identical with the GC profile obtained from three colonies of the same strain after growth for 3 days (fig. 1B). There are only minor differences between the profiles in figs. 1A and B—nonadecanoic acid (C₁₉:₀), 14-methyl pentadecenoic acid (C₁₆:₁) and pentadecanoic acid (C₁₅:₁), present in slightly higher amounts in fig. 1A, and mainly 12-methyl tetradecanoic acid (C₁₃:₀) present in higher amount in fig. 1B—due to the different growth times of the cultures.

The extensive conformity of the TSMH procedure with the conventional method of GC analysis is demonstrated in figs. 1B and C. These profiles were obtained with *Legionella* colonies from the same culture plate incubated for 3 days. Three colonies (c. 1 × 10⁹ cfu) of this culture were harvested and treated with TMSH. The remainder of the same culture, at least 10–20 times the amount taken for the TMSH procedure, was used for FAME preparation by the conventional method. The FAME profiles yielded by both procedures are almost congruent. The same is
RAPID IDENTIFICATION OF LEGIONELLA BY GLC

RNA

A

B

C

Fig. 1A. TMSH-FAME profile of L. pneumophila sg 2 (ATCC 33154), obtained with one colony after growth for 4 days. Designations of fatty acids—the number before the colon refers to the number of carbon atoms, the number after the colon indicates the number of double-bonds, the exponent indicates the position of the double-bond; Δ = cyclopropane, a = ante-iso, i = iso fatty acid. B. TMSH-FAME profile of L. pneumophila sg 2 (ATCC 33154) obtained from three colonies after growth for 3 days. C. FAME profile of L. pneumophila sg 2 (ATCC 33154) obtained by conventional sample treatment from the same culture plate as in B.

true for the FAME profiles obtained with all the reference strains of L. pneumophila sg 1-8, L. micdadei, L. bozemanii sg 1 and 2 and L. dumoffii, tested with both sample preparation methods.

As random examples, the profiles of L. pneumophila sg 3 (ATCC 33155, fig. 2B) and L. micdadei (ATCC 33218, fig. 3B) obtained with the TMSH technique were compared with the corresponding profiles obtained by conventional sample treatment (figs. 2C and 3C). The samples of the reference strains were prepared as described for fig. 1B and C. It is evident that both procedures produce profiles which coincide extensively, except that eicosanoic acid (C20:0) shown in fig. 3B was detected only in a trace amount in fig. 3C (conventional method).

The potency of the new transesterification method was tested with colonies of two primary isolates from tap water, which were identified presumptively to genus level by their ability to grow on selective MWY agar. After 3 days, five colonies (c. 1.5 x 10⁹ cfu, fig. 2A) and three colonies (c. 9 x 10⁸ cfu, fig. 3A) were harvested from the primary culture plate and the FAMEs were liberated by means of TMSH. Both wild strains could be identified by their FAME patterns (figs. 2A and 3A) as Legionella strains, because their profiles were in good agreement with the patterns of the corresponding reference strains (figs. 2B and 3B).

In addition, the GLC identification was confirmed by hybridisation with a Legionella-specific DNA probe and by serotyping with monovalent antibody conjugates. The two wild strains were serotyped as L. pneumophila sg 3 (fig. 2A) and L. micdadei (fig. 3A). There was a minor qualitative difference between the wild-type strain of L. pneumophila sg 3 (fig. 2A) which contained 14-methyl hexadecenoic acid (C₁₇:₁) and the reference strain of L. pneumophila sg 3 (ATCC 33155, fig. 2B and C), in which C₁₇:₁ fatty acid was not detected.

Comparison of figs. 1 and 2 with fig. 3 demonstrates that L. pneumophila can clearly be distinguished from L. micdadei by GLC analysis. The same is true for other non-L. pneumophila reference strains tested, i.e., L. dumoffii and L. bozemanii sg 1 and 2, which both show a predominant peak of C₁₅:₀ fatty acid. Differentiation of L. pneumophila serogroups by GLC was not possible.

The sensitivity of the TMSH method in comparison with the conventional method is greater if an identical amount of bacterial culture (plate counts of 8 x 10⁸–1 x 10⁹ cfu) is used as initial material. The reproducibility of the TMSH procedure is very good; 15–20 serial subcultures of the Legionella reference strains yielded qualitatively identical FAME patterns over a period of >1 year.

For the quantitative evaluation of reproducibility, GC profiles of the L. micdadei reference strain were chosen. In contrast to L. pneumophila, FAME patterns of this strain are influenced less by different growth times of the cultures. Seven cultures of L. micdadei were grown for 3–4 days within 2 weeks. The FAMEs
Fig. 2A. FAME profile with TMSH of a wild *L. pneumophila* isolate of serogroup 3. The FAME profiles of the corresponding reference strain *L. pneumophila* sg 3 (ATCC 33155) are shown in B (TMSH technique) and in C (conventional technique). The FAMEs were prepared from cultures after growth for 3 days.

Fig. 3A. FAME profile of a wild non-pneumophila *Legionella* isolate identified serologically as *L. micdadei*. The FAME patterns of the corresponding reference strain (ATCC 33218) are shown in B (TMSH technique) and C (conventional technique). The FAMEs were prepared from bacteria after growth for 3 days.

of 5–8 colonies each were prepared and analysed. In each profile, the areas of the seven major fatty acids were taken as 100% and the relative percentages of these fatty acids were calculated. The mean values and standard deviations of the relative area percentages of these peaks are presented in the table. It is evident that the variation from day to day did not influence the result. In *L. micdadei* a15:0 fatty acid is the predominant fatty acid. The quotient of the relative area percentages of a15:0 and i16:0 in *L.*
Table. Reproducibility of FAME analysis by TMSH (n = 7) of L. micdadei ATCC 33218 independently cultured seven times within 2 weeks

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Mean (SD) relative percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>a15:0</td>
<td>38.4 (2.3)</td>
</tr>
<tr>
<td>15:0</td>
<td>3.4 (0.9)</td>
</tr>
<tr>
<td>i16:0</td>
<td>13.2 (1.2)</td>
</tr>
<tr>
<td>16:1</td>
<td>9.8 (2.4)</td>
</tr>
<tr>
<td>16:0</td>
<td>8.3 (3.0)</td>
</tr>
<tr>
<td>a17:1</td>
<td>5.4 (1.4)</td>
</tr>
<tr>
<td>a17:0</td>
<td>21.5 (1.8)</td>
</tr>
</tbody>
</table>

micdadei, which is a criterion for differentiation from L. pneumophila, is always > 1, whereas in L. pneumophila it is < 1.

Reproducibility of the TMSH method was even better when FAMEs were prepared from cultures grown under exactly equal conditions. Three sample preparation procedures of FAMEs from L. micdadei grown in parallel showed negligible inter-analysis variations. The standard deviation of relative area percentage of the seven major peaks in these analyses did not exceed 0.8%.

When stored at 4°C, the FAME extracts were stable for more than a year without a change in the FAME patterns. The GLC profiles of the TMSH procedure were not influenced by the agar medium (BCYEa, BMPA or MWY) used for cultivation of the strains.

The TMSH reagent is stable for more than a year if stored at 4°C. A comparison of bacterial FAME patterns produced with freshly prepared as well as with TMSH solution stored at 4°C for 1 year showed that the reagent was stable. No change either in the yield of FAME or in FAME profiles was detected.

Discussion

Legionella isolates are usually identified presumptively at the genus level by their ability to grow on selective MWY and BMPA agar, and by absence of growth on media without iron and cysteine. Additional tests such as nucleic acid hybridisation, direct immunofluorescence or GLC are necessary for definitive identification.

GLC analysis of FAMEs has become a useful method for the identification of Legionella isolates even to the species level and there are more than 10 different FAME preparation methods with several modifications. Recently it was shown that only three of 11 procedures for bacterial FAME preparation are reliable for the identification of bacteria.

One of these is sample preparation by the method of Miller and Berger which was chosen here for comparison with the TMSH technique. All the usual sample preparation methods, including the method chosen here, are complicated by a temperature and time protocol which must be strictly obeyed. Additionally, these procedures require large volumes of solvent and several extraction procedures. Therefore, a relatively large amount of bacterial culture is necessary. Usually these conventional techniques require enrichment of bacteria by subcultivation from a primary isolate to obtain sufficient cell mass for FAME preparation.

In contrast, the TMSH technique described here is far more convenient. In the case of Legionella spp., a single colony, even from a primary isolation plate, may yield enough FAMEs for identification. Thus, time consuming subcultivation of slow-growing Legionella spp. can be avoided. The loss of material is minimised, because phase separations are not necessary and the reaction is done in the GC vial. If a fatty acid present in only small amounts is to be detected, e.g., cyclo-propane heptadecanoic acid (C_{17:0} fatty acid), which is in the range 0-1% in L. micdadei, the sensitivity can be improved if the FAMEs are concentrated by evaporating the FAME-solvent with nitrogen. Sample preparation by TMSH is far more rapid than in the conventional method. A FAME extract can be completed within 1-2 min. The reaction is performed at room temperature and does not require a water bath or a block heater as in the conventional method. During transesterification with TMSH, volatile by-products of the reaction and of excess reagent, mainly dimethyl sulphide (boiling point 38°C) and methanol, are formed. These had no harmful effect on columns or detector when the injector temperature was maintained at or above 250°C.

As in the conventional method, the TMSH technique yielded all the FAMEs that are important for the identification of Legionella spp., i.e., straight, branched, saturated, unsaturated, and cyclopropyl FAMEs. FAME patterns of L. pneumophila strains were differentiated clearly from those of non-L. pneumophila species like L. micdadei (see figs. 1 and 3), L. bozemanii sg 1 and 2, and L. dumoffii, which were tested by the TMSH technique and which show a predominant C_{15:0} fatty-acid peak. The labile cyclopropyl fatty acids are preserved by the new technique because of the low reaction temperature. Hydroxy fatty acids are unimportant for the identification of Legionella spp. but are generally liberated by TMSH in species containing hydroxy fatty acids, e.g., in Bacteroides thetaiotaomicron. Both kinds of fatty acids are often difficult to detect. In addition, hydroxy fatty acids can cause problems on account of their chemical lability (catalytic and thermal); 3-OH FAMEs may be converted into the corresponding fatty aldehydes with two carbon atoms less in chain length if glass wool packed inlets at low split flows are used for sample injection.

The chromatograms obtained in the analysis of FAMEs from Legionella spp. after TMSH treatment, are consistent, reproducible, and correspond well with GC data from conventional sample preparation methods. Nevertheless, confusion may arise from the variable amounts of C_{16} fatty acids detected.
by GC analysis. For example, the relative amounts of 14-methyl pentadecanoic acid (C16:1\(\alpha\)) and 9-hexadecenoic acid (C16:1\(\beta\)) in fig. 2A are quite different from those in fig. 2B. These differences in relative peak areas of fatty acids are due to slightly shorter incubation of the culture used for the analysis shown in fig. 2A and not to the fact that a wild-type strain was analysed. A change in the relative peak areas occurs during the first 4 days of culture. Generally, as the culture ages, the relative amount of C16:1\(\alpha\) acid decreases while that of C16:1\(\beta\) acid increases. This emphasizes the importance of considering the physiological age of the culture if FAME patterns from different samples are to be compared. This is currently under investigation. These age-dependent differences in fatty-acid content have not been reported previously for \textit{L. pneumophila}. Similar results for \textit{L. feeleii} have been reported previously.\textsuperscript{28}

The TMSH procedure may have the disadvantage that it requires a reagent not available commercially. For \textit{Legionella} spp. and some other gram-negative bacteria, e.g. \textit{B. thailandensis}, \textit{Escherichia coli}, \textit{Pseudomonas aeruginosa}, the commercially available methylating agents TMAH (trimethyl-anilinium hydroxide), Macherey-Nagel GmbH and Co. KG, Duren, Germany) and Meth-Prep II (m-trifluoromethylphenyl trimethylammonium hydroxide; Alltech GmbH, Unterhaching, Germany), were tested with the same protocol as TMSH. These reagents yielded FAME profiles which were qualitatively and quantitatively identical to those of TMSH.

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\textbf{References}


