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Rapid Identification and Speciation of *Haemophilus* Bacteria by Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry

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Several species of the genus *Haemophilus* are well known etiological agents of pneumonia, meningitis, conjunctivitis, epiglottitis and chancroid. However, identification and speciation of *Haemophilus* is both time consuming and labor intensive. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS) has been used by several investigators to profile proteins from intact and disrupted bacteria; consequently, MALDI/TOF-MS has emerged as a powerful tool in diagnostic bacteriology. This paper reports the use of MALDI/TOF-MS as a technique for the rapid identification and speciation of *Haemophilus*. This technique was used to not only identify the pathogen, *H. ducreyi*, but also to determine strain differences from different isolates. Mass spectral 'fingerprints' were obtained which permitted the rapid speciation of not only pathogenic forms of *Haemophilus*, but also those bacteria which are normally regarded as non-pathogenic and members of the normal flora. MALDI/TOF mass spectra can be acquired in 10 min, allowing the identification of *Haemophilus* spp. within 24 h rather than the 48 h or more needed for traditional bacteriological methods. In addition, these are the first mass spectral fingerprints available in the literature for many of these organisms.

KEYWORDS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; *Haemophilus*; bacteria; speciation

INTRODUCTION

Members of the genus *Haemophilus* are pleomorphic, Gram-negative, non-spore-forming coccobacilli which are the etiological agents responsible for a variety of human diseases. Of the 13 species of *Haemophilus*, the primary human pathogens are *H. influenzae*, *H. parainfluenzae*, *H. aegyptius*, *H. aphrophilus* and *H. ducreyi*. *H. influenzae* and the less pathogenic *H. parainfluenzae* are known to cause meningitis, epiglottitis and cellulitis;[1] *H. aegyptius* is the etiological agent of contagious conjunctivitis (pink-eye);[2] *H. aphrophilus* may be involved in infective endocarditis and pneumonia[3] and *H. ducreyi* is the etiological agent of the sexually transmitted genital ulcer disease chancroid, which is now regarded as a significant co-factor in the transmission of HIV.[4,5]

At present, speciation of *Haemophilus* depends upon the requirement for either X factor (hemin) or V factor (nicotinamide adenine dinucleotide) and carbon dioxide to effect optimal growth. Both *H. influenzae* and *H. aegyptius* require X and V factors, *H. parainfluenzae* requires only V factor, while *H. ducreyi* requires only X factor and *H. aphrophilus* requires neither X nor V factor for growth.[6–8] Because these organisms are isolated from body sites which are colonized by a number of other genera, primary isolation followed by determination of the requirement for X and V factors can take up to 48 h to complete. In the case of *H. ducreyi* this time period may be as long as 1 week owing to the fastidious growth requirements of this organism. This time lag may compromise a physician's correct determination of the etiological agent and appropriate treatment. Several of these *Haemophilus* infections require immediate antimicrobial intervention, particularly, *H. ducreyi*, to prevent subsequent dissemination within the community. Consequently, there is a great need to develop procedures that can identify and reliably speciate *Haemophilus* in as short a time as possible.

Although the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS) for bacterial identification has been reported,[9–14] its use in identifying *Haemophilus* spp. has not been demonstrated. Mass spectral analysis has been

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performed in the past on bacterial carbohydrates using gas chromatography/mass spectrometry[15] and on bacterial phospholipids using fast atom bombardment mass spectrometry.[16,17] Unfortunately, these methods can require extensive sample preparation prior to MS analysis. Moreover, they are not preferred techniques for the analysis of large proteins of high molecular mass. In this work, we use MALDI/TOF-MS as a technique for the rapid identification and speciation of *Haemophilus* by obtaining a 'fingerprint' mass spectral pattern of bacterial proteins. These fingerprint patterns allow us to speciate isolates of *Haemophilus* without having to test for the growth requirement of X and V factors. In addition, the mass spectral fingerprint pattern also permits us to identify *Haemophilus* spp. present in a mixture of bacteria, thereby eliminating the need for subculturing.

Of particular interest are the mass spectral differences of isolates of *H. ducreyi*. Differentiation of *H. ducreyi* isolates is of great importance in epidemiological studies of outbreaks of chancroid and in the identification of infected sexual partners. Previously, strains of *H. ducreyi* have been characterized by plasmid content,[18] ribosomal DNA fingerprinting,[19] indirect immunofluorescence,[20] enzyme profiles[21] and lectin typing.[22] Unfortunately, these methods of analysis are time consuming. MALDI/TOF-MS offers the potential to reduce greatly the time necessary to differentiate strains based upon differences in mass spectral profiles.

RESULTS AND DISCUSSION

The current investigation focuses on four different *Haemophilus* spp.; *H. influenzae*, *H. parainfluenzae*, *H. aphrophilus* and *H. ducreyi*. Two other organisms, *Neisseria gonorrhoreae* and *Actinobacillus actinomycetemcomitans*, were also investigated. *N. gonorrhorreae* was included because it is also a common pathogen found in patients with sexually transmitted diseases. *A. actinomycetemcomitans* was included because it can be confused with *H. aphrophilus*, as both organisms exhibit very similar biochemical and bacteriological profiles. Several isolates of *H. ducreyi* from different patients were also analyzed to determine strain variation which could further establish MALDI/TOF-MS as an epidemiological tool. Bacterial cultures were re-tested after 1 week of refrigeration and no major differences in mass spectral patterns were found.

Figure 1 illustrates the mass spectral profiles of the water–acetonitrile-soluble fractions of *H. influenzae*, *H. aphrophilus*, *H. parainfluenzae* and *H. ducreyi* from bac-

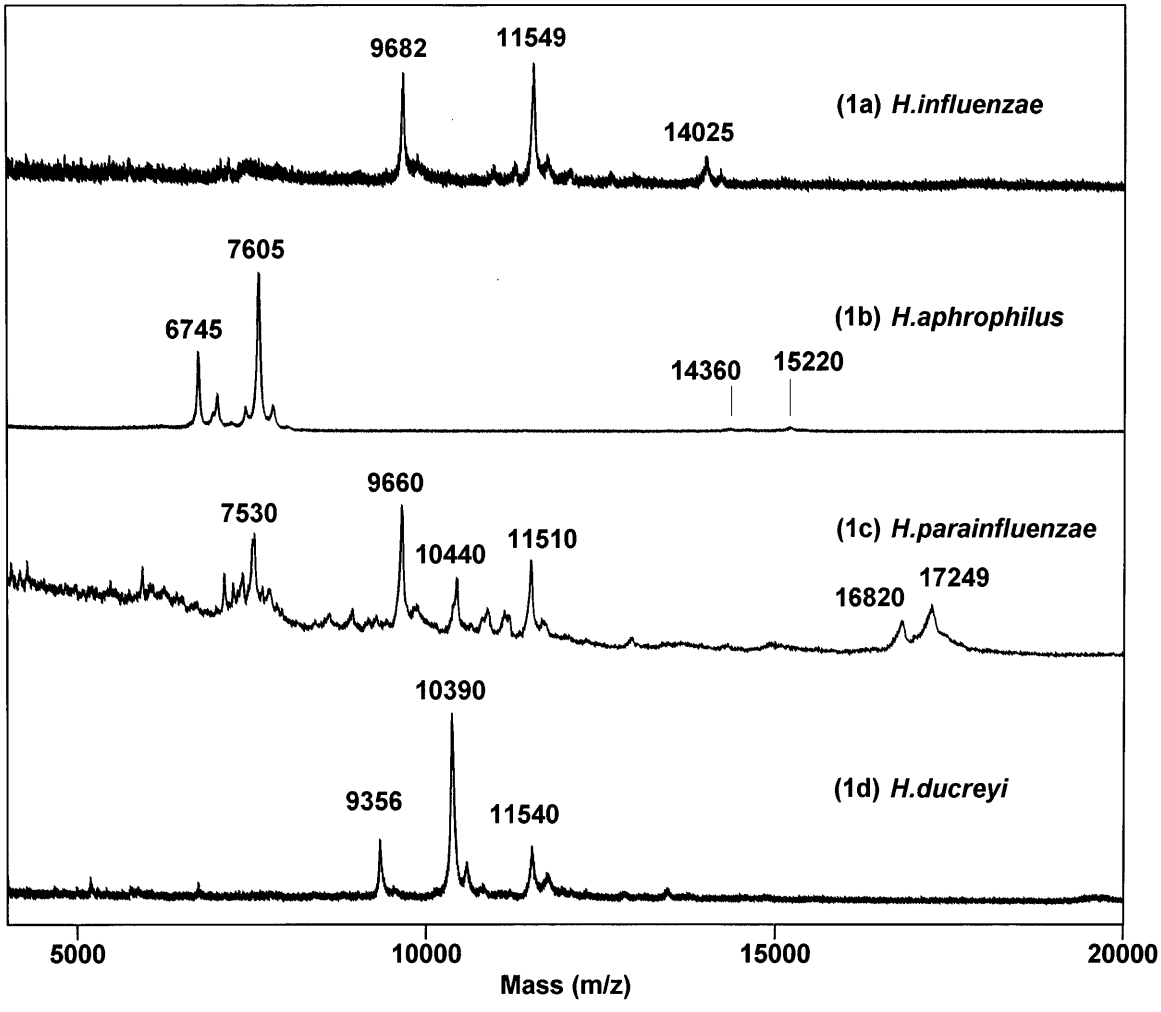

Figure 1. MALDI/TOF mass spectrum of whole cells from several *Haemophilus* species: (a) *H. influenzae*, (b) *H. aphrophilus*; (c) *H. parainfluenzae*, (d) *H. ducreyi*.

teria grown on GC II agar. The cells were suspended in acetonitrile–water and centrifuged prior to MALDI/TOF-MS analysis of the sample supernatant (Fig. 1). Amino acid analysis of the supernatant determined it to be composed almost exclusively of protein. The majority of the mass spectral peaks are within the range of m/z 5000–20 000. In this region, peaks would typically represent singly protonated protein molecules, while most lipoologosaccharides and peptidoglycans would appear at $m/z < 3500$.[23] No mass spectral peaks were observed above m/z 20 000.

One can clearly see differences in the mass spectral patterns of different species of *Haemophilus*. In the case of *H. influenzae*, three major peaks (m/z 9682, 11 549 and 14 025 $\pm$ 0.1%) were always observed [Fig. 1(a)]. One of these three peaks is also close in m/z values to a peak at m/z 11 540 seen in samples of *H. ducreyi* [Fig. 1(d)]. It is not known whether these two peaks at m/z 11 549 and 11 540 arise from identical or related proteins. Two other peaks at m/z 9356 and 10 390 are found to be diagnostic of *H. ducreyi* because they are not found in other species. *H. parainfluenzae* can easily be distinguished from *H. influenzae* by its intense peaks located at m/z 16 820 and 17 249 [Fig. 1(c)]. This is important in that MALDI/TOF-MS peaks for the two species in the region between m/z 9000 and 12 000 have similar m/z values. In the case of *H. aphrophilus*, very intense peaks were found at m/z 6745 and 7605 with only small traces of material observed at m/z 14 360 and 15 220 [the last is probably a non-covalently bound dimer of the molecule at m/z 7605, Fig. 1(b)]. It is the only species that was found to have no apparent peaks from m/z 9000 to 14 000.

Although diagnosis of patients infected with *N. gonorrhoreae* is normally done by visual inspection of the infected area, this bacterium is often present in patients that also have chancroid. Therefore, when trying to isolate *H. ducreyi* from patient exudates grown on artificial media, *N. gonorrhoreae* can often be found as a contaminant. Figure 2(b) shows the mass spectrum obtained from *N. gonorrhoreae* cells. A fairly intense peak can be found at m/z 7075 with lower intensity peaks at m/z 7280 and 8080. Smaller peaks between m/z 10 000 and 15 000 can also be seen, but these were usually only present at slightly above the background level and therefore are not reliable as diagnostic peaks. Figure 2(a) shows the mass spectrum obtained from *A. actinomycetemcomitans*, which is similar in biochemical tests to *H. aphrophilus*. Sharp peaks at m/z 7603 and 11 414 were found with smaller peaks at m/z 10 256 and 11 925.

In previous reports,[9–13] it was not determined whether proteins observed by MS using whole cells were from proteins bound to the surface of the cell or

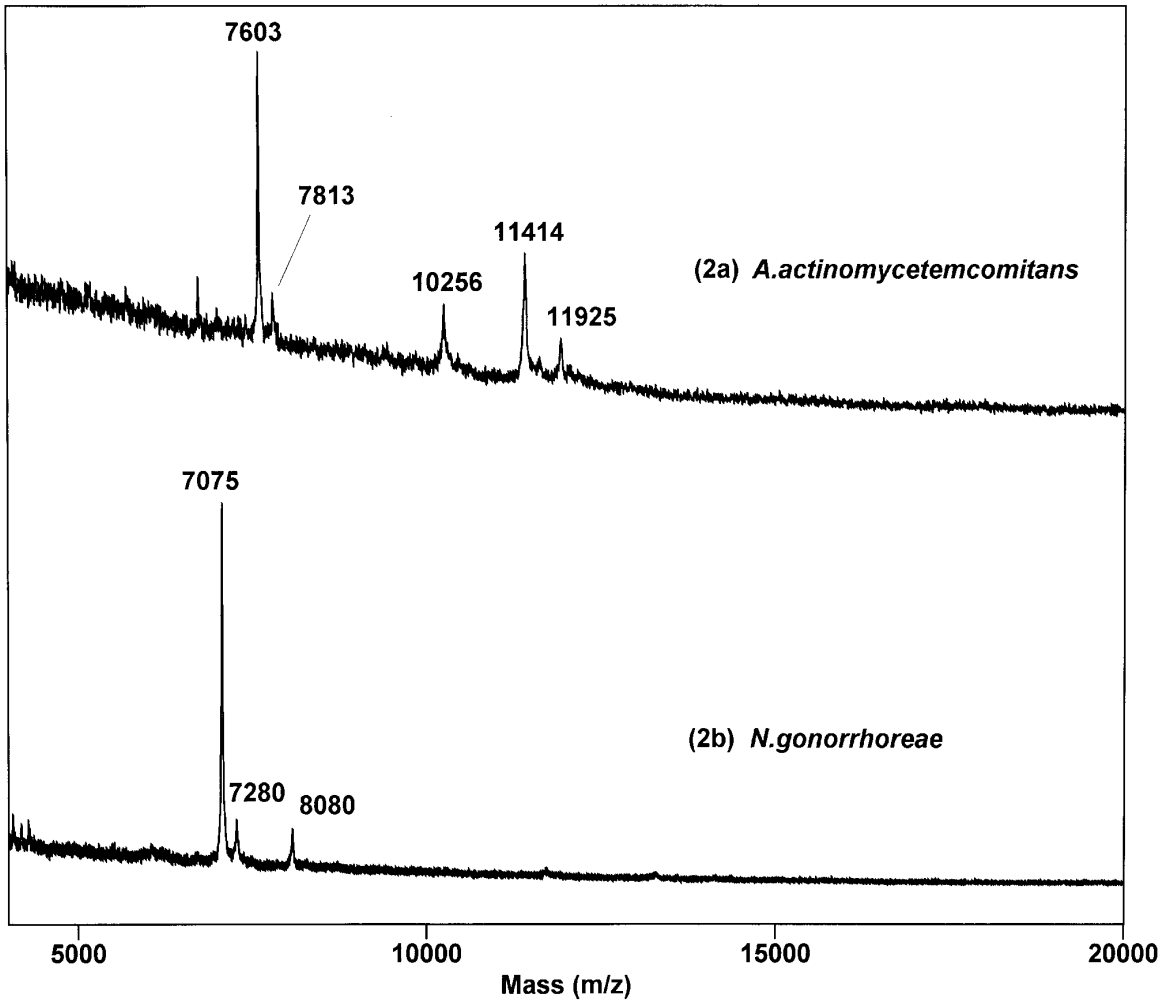

Figure 2. Mass spectrum of (a) the bacterium *A. actinomycetemcomitans*, which is similar in biochemical tests to *H. aphrophilus*; and (b) *N. gonorrhoreae*, which is a common sexually transmitted disease that can also appear in patients infected with *H. ducreyi*.

whether the proteins were merely excreted by the cells. In the current study, samples were first analyzed using whole cells in which colonies removed from the growth media were mixed directly with the sample matrix to obtain a mass spectral profile. The supernatant from the same cells was then used for a second analysis. This time the cells were washed with acetonitrile–water (2:1) followed by centrifugation to remove the cells. The supernatant was then added to sample matrix in preparation for MALDI/TOF-MS analysis. In both cases, the mass spectral patterns were identical, i.e. no major differences were observed on comparing whole cells and supernatant. This evidence suggests that the observed proteins originating from near the cell surface were not strongly bound.

Another problem that has not been addressed in previous reports is whether there are mass spectral differences between lysed and unlysed cells. Because the proteins from whole cells are mostly those of excreted proteins, it would be of interest to determine whether there are any mass spectral differences between these whole cells and those that have been lysed, as many cellular proteins and plasmids remain within the cell. The lysed cells were prepared by first washing whole cells with water and then lysing them with 0.1% sodium dodecyl sulfate (SDS). SDS was later removed by desalting and the resulting solution was analyzed by MALDI/TOF-MS. Figure 3 compares the MALDI/TOF mass spectra of *H. ducreyi* cells that have been lysed [Fig. 3(b)] and those that are unlysed [whole, Fig. 3(a)]. The mass spectrum of unlysed cells reveals proteins that have been excreted by the cell. Notably, the peaks in the range *m/z* 9000–12 000 observed for whole cells [Fig. 3(a)] are no longer present in lysed cells [Fig. 3(b)]. Several lower mass peaks located at *m/z* 4139, 5350 and 6500 are observed for lysed samples. The signal intensities for these peaks are lower than those for whole cells since far less material was available. The mass spectra obtained from lysed cells can provide additional identification information to supplement data obtained from whole cells.

To determine whether there were any differences in the MALDI/TOF mass spectral profiles arising from samples of *H. ducreyi* obtained from different sources, numerous samples originating from different patients were analyzed. It was found that three peaks were consistently present in the MALDI/TOF-MS analysis of unlysed *H. ducreyi* cells (*m/z* 9356, 10 390 and 11 540). Previous reports of differences in *H. ducreyi* strains were based on plasmid analyses. Many of the samples exhibiting strain differences by plasmid analysis were examined by MALDI/TOF-MS. The mass spectral patterns obtained, however, showed few fluctuations with plasmid variability. There were however, some mass

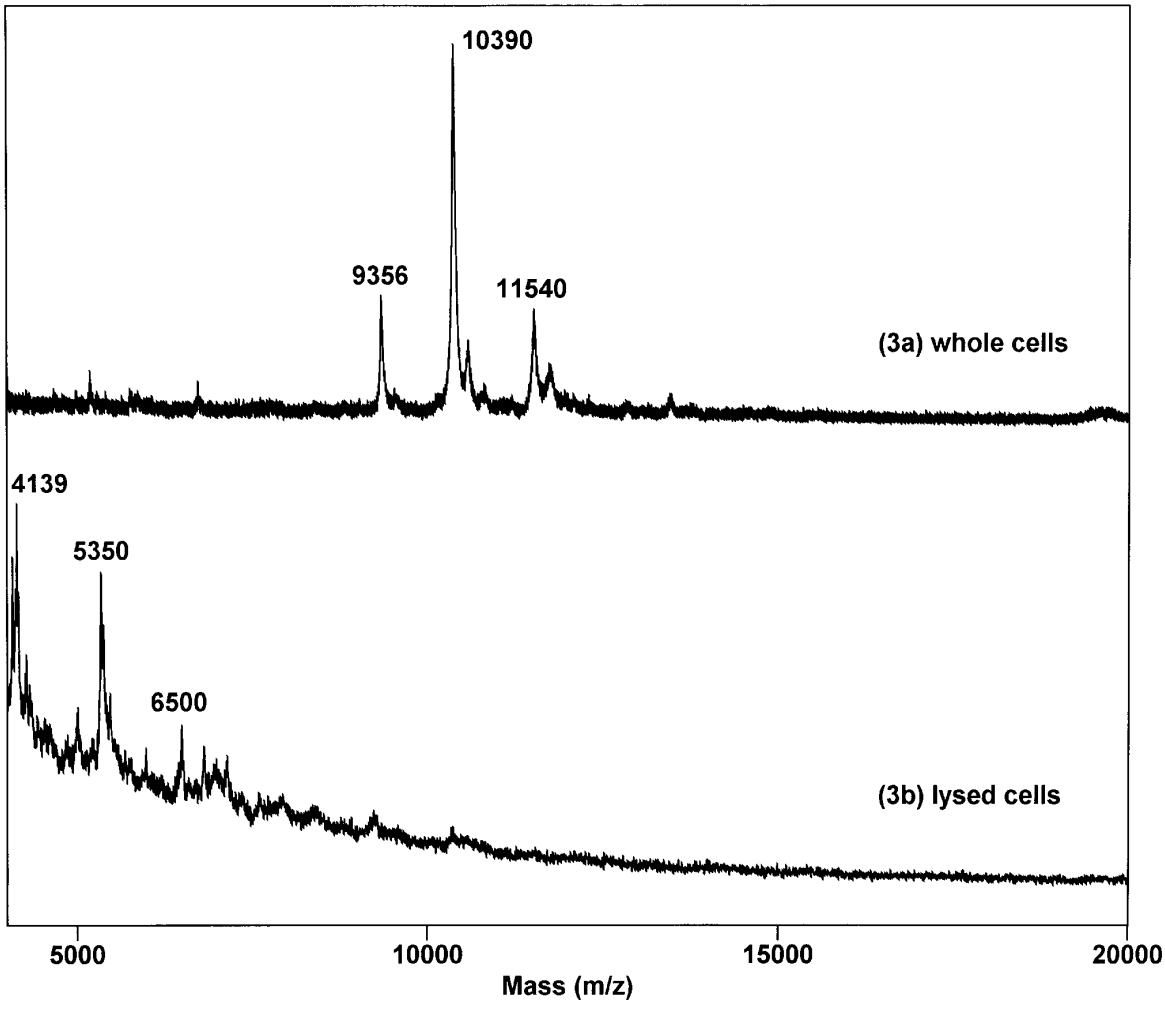

Figure 3. Mass spectrum of whole cells *vs.* lysed cells.

spectral differences found in *H. ducreyi* samples depending on the date on which the chancroid infection occurred. Table 1 shows the presence of mass spectral peaks of *H. ducreyi* based on the date at which patient chancroid diagnosis was made. Figure 4 shows the MALDI/TOF mass spectral patterns of several *H. ducreyi* isolates revealing some differences in mass spectral peaks. It was found that earlier *H. ducreyi* exudates contained a peak located at *m/z* 7326. This peak was not observed in samples obtained at later dates. Instead, samples taken at later dates showed peaks at *m/z* 5526 and 6926 that were absent in the earlier analysis. *H. ducreyi*, strain 35 000, did not yield any of the three lower mass peaks (i.e. *m/z* 7326, 5526 or 6926) that were found in patient exudates. Based solely on the mass spectral profiles, one can conclude that strain differences exist, and these are represented in Fig. 5. MALDI/TOF-MS can therefore be used to determine strain differences and to monitor the epidemiology and dissemination of *Haemophilus* bacteria during an outbreak.

A major noteworthy advantage is that, in all cases, it took less than 10 min to obtain the MALDI/TOF mass spectrum to enable a positive identification to be made of any species of *Haemophilus* grown on culture plates. With the use of MALDI/TOF-MS, the total time

Table 1. *m/z* values for *H. ducreyi* patient isolates

Isolate number	*m/z* 7326	*m/z* 5526	*m/z* 6926	*m/z* 9356	*m/z* 10 390	*m/z* 11 540	Date
HD37	×	—	—	×	×	×	25 Jan 1989
HD67	×	—	—	×	×	×	26 Jan 1989
HD72	×	—	—	×	×	×	26 Jan 1989
HD42	×	—	—	×	×	×	27 Jan 1989
HD52	×	—	—	×	×	×	6 Feb 1989
HD45	—	—	—	×	×	×	31 Mar 1989
HD14	×	—	—	×	×	×	10 Oct 1989
HD15	×	—	—	×	×	×	13 Oct 1989
HD18	×	—	—	×	×	×	31 Oct 1989
HD30	—	×	—	×	×	×	30 Jan 1990
HD169	—	×	—	×	×	×	5 Jul 1990
HD233	—	×	×	×	×	×	18 Oct 1991
HD138	—	×	×	×	×	×	15 Oct 1992

[a] × = peak present; —=peak not found. The presence or absence of peaks at *m/z* 7326, 5526 and 6926 is dependent on the date at which the patient isolates were taken.

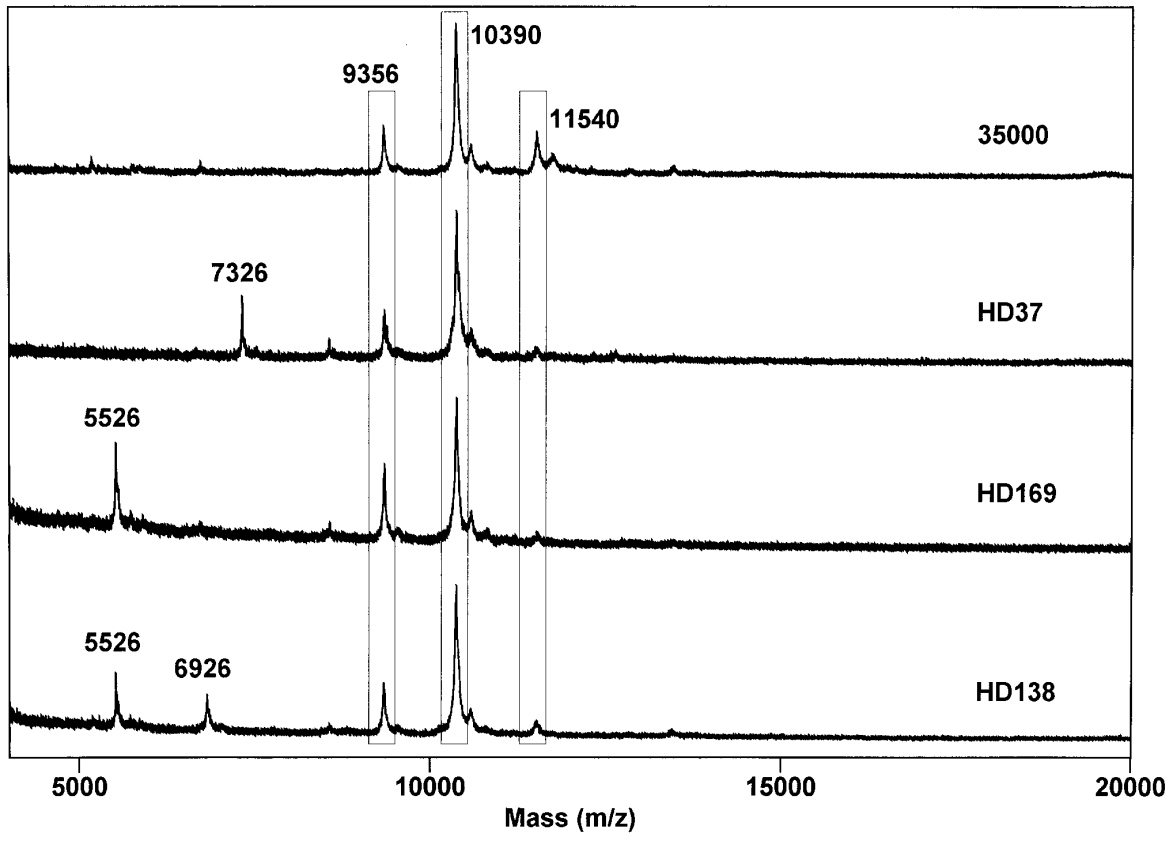

Figure 4. Mass spectrum of *H. ducreyi* from different patients isolated at different time periods. Patient isolates show peaks not found in strain 35 000.

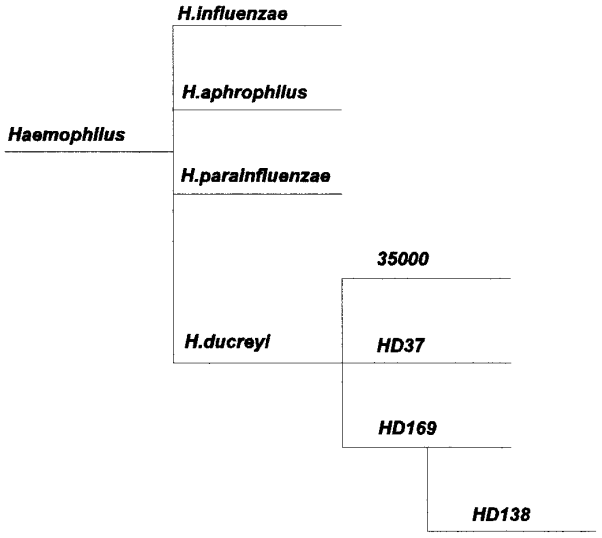

Figure 5. Categorization of strains of *H. ducreyi* (35 000, HD37 and HD169) and sub-strains (HD138) based on mass spectral analysis.

needed for analysis from the start of culturing is $\leqslant$24 h because the lengthy subculturing step following initial growth is no longer required. This represents a significant improvement over the normal amount of time required for identification and speciation of *Haemophilus* by the conventional approach (i.e. $\geqslant$48 h from the time that culturing begins).

CONCLUSION

We have shown that MALDI/TOF-MS can be used as an efficient method to identify and determine the species of *Haemophilus* present in both pure and mixed cultures owing to its ability to detect 'fingerprint' proteins. MALDI/TOF-MS may also be used as a rapid screening technique for *Haemophilus* because analysis may be performed immediately after growth occurs, thus eliminating the necessary additional subculturing needed to perform biochemical studies. This is especially advantageous in the diagnosis of chancroid because current testing is both time consuming and laborious. Mass spectrometry will provide inexpensive testing while also allowing the rapid analysis of many samples in short periods of time. We have also demonstrated the use of MALDI/TOF-MS as a rapid method for categorizing different strains of *H. ducreyi* based on variations in the profiles of proteins originating from near the surface of the cell. This capability can greatly aid cluster studies of chancroid outbreaks. Future studies will investigate the use of MALDI/TOF-MS for the analysis of clinical samples which would eliminate the need for culture of fastidious organisms.

EXPERIMENTAL

Bacterial cultures

A. actinomycetemcomitans, *N. gonorrhoreae* and all species of *Haemophilus* were cultivated on GC II agar (BBL/Beckton Dickinson, Cockeyville, MD, USA) supplemented with 1% fetal bovine hemoglobin, 5% fetal bovine serum, 1% IsoVitalex and 3 μg ml^{-1} vancomycin at 35 °C in a humidified candle jar for 24 h. Patient exudates of *H. ducreyi* were first subcultured to obtain pure cultures.

Sample preparation for MALDI/TOF-MS

After incubation, several colonies were removed using an inoculating loop and placed in a vial containing 30 μl of water–acetonitrile (2:1). The entire mixture was vortex mixed for 1 min and then centrifuged at 14 000 rpm for 1 min. The supernatant was removed and added to 30 μl of a saturated sinapinic acid solution. Sinapinic acid (Aldrich, Milwaukee, WI, USA) was dissolved in water–acetonitrile (2:1) until saturation occurred. Samples were spotted on an autosampler plate and allowed to air dry. In the case of samples that were lysed, several colonies of *Haemophilus* were first washed with acetonitrile–water (2:1) and then lysed with 10 μl of a 200 mM 0.1% SDS solution. The samples were then desalted by spotting on a 0.25 μm Millipore membrane floating on distilled water. After 30 min the solution was removed and 10 μl were added to 10 μl of the saturated sinapinic acid solution as before. Sample spotting and drying were performed as before.

MALDI/TOF-MS analysis

Samples were analyzed on a PerSeptive Biosystems (Framingham, MA, USA) Voyager-DE MALDI/TOF-MS mass spectrometer using 337 nm light from a nitrogen laser. The mass spectrometer was operated in the positive ion mode and 50 laser shots were averaged in each acquisition. An accelerating voltage of 20 kV was used and a two-point external calibration was performed using horse heart myoglobin and sinapinic acid as the calibrants. Two separate cultures of each sample were prepared and each cultured sample was analyzed in triplicate by MALDI/TOF-MS. Reported m/z values can vary by $\pm$0.1%.

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