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Detection of Biomarkers of Pathogenic *Naegleria fowleri* Through Mass Spectrometry and Proteomics

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Abstract

Emerging methods based on mass spectrometry (MS) can be used in the rapid identification of microorganisms. Thus far, these practical and rapidly evolving methods have mainly been applied to characterize prokaryotes. We applied matrix-assisted laser-desorption-ionization-time-of-flight mass spectrometry MALDI-TOF MS in the analysis of whole cells of 18 *N. fowleri* isolates belonging to three genotypes. Fourteen originated from the cerebrospinal fluid or brain tissue of primary amoebic meningoencephalitis patients and four originated from water samples of hot springs, rivers, lakes or municipal water supplies. Whole *Naegleria* trophozoites grown in axenic cultures were washed and mixed with MALDI matrix. Mass spectra were acquired with a 4700 TOF-TOF instrument. MALDI-TOF MS yielded consistent patterns for all isolates examined. Using a combination of novel data processing methods for visual peak comparison, statistical analysis and proteomics database searching we were able to detect several biomarkers that can differentiate all species and isolates studied, along with common biomarkers for all *N. fowleri* isolates. *Naegleria fowleri* could be easily separated from other species within the genus *Naegleria*. A number of peaks detected were tentatively identified. MALDI-TOF MS fingerprinting is a rapid, reproducible, high-throughput alternative method for identifying *Naegleria* isolates. This method has potential for studying eukaryotic agents.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Keywords

Free-living amoebae; matrix-assisted laser-desorption-ionization-time-of-flight mass spectrometry; *Naegleria*; protein fingerprints

NAEGLERIA fowleri is a free-living, amphiozoic, eukaryotic protist that occurs world-wide and can potentially infect humans and other animals (Visvesvara 2013; Visvesvara et al. 2007). Among at least 30 species described in the genus *Naegleria*, *N. fowleri* is the only species that can infect children and young adults causing an acute, fulminant, fatal brain disease known as primary amoebic meningoencephalitis. This protist can be acquired through the exposure to thermally polluted streams, ponds, lakes, or inadequately chlorinated swimming pools. There are well-established morphological, serologic and molecular methods to characterize different species within the genus *Naegleria* as well as intraspecies genetic diversity (Visvesvara et al. 2007; Zhou et al. 2003). However, there are only a few reports on the use of matrix-assisted laser-desorption-ionization-time-of-flight mass spectrometry (MALDI-TOF MS) to characterize this organism (Visvesvara et al. 2007).

Matrix-assisted laser-desorption-ionization-time-of-flight mass spectrometry is a practical and rapidly evolving application of MS for rapid identification of microorganisms and strain differentiation (Fenselau and Demirev 2001; Lay 2001; van Baar 2000). Spectra obtained by MALDI-TOF MS provide characteristic patterns of proteins (fingerprints composed of unique biomarkers) from whole organisms that can be used to identify bacteria, viruses, protozoa and fungi (Amiri-Eliasi and Fenselau 2001; Croxatto et al. 2012; Glassmeyer et al. 2007; Moura et al. 2003; Villegas et al. 2006; Wunschel et al. 2005). Improved algorithms have been developed to interpret MALDI-TOF MS data obtained from whole organisms. MALDI-TOF MS has recently caught the attention of clinical microbiologists as a fast and effective method in identifying microorganisms, and it is now considered a revolution in microbial routine identification (De Bruyne et al. 2011; Seng et al. 2010). There are dedicated instruments with improved databases and the method has been adapted to use in routine clinical microbiology laboratories (Clark et al. 2013; Patel 2013a).

For the past 10 yr, we have been using MALDI-TOF MS to characterize different genera of culture-derived bacteria including *Bacillus*, *Coxiellla* and *Streptococcus* (Moura et al. 2003, 2008; Pierce et al. 2007; Satten et al. 2004; Shaw et al. 2004; Williamson et al. 2008; Woolfitt et al. 2011). Consistent and unique spectral patterns were obtained for each organism examined. Using MALDI-TOF MS analysis coupled with statistical analysis we have been able to identify, characterize and differentiate isolates, species, and genera. Examples include discrimination of necrotizing fasciitis-causing invasive group A *Streptococcus* strains from noninvasive strains and identification of specific biomarkers associated with conjunctivitis *Streptococcus pneumoniae* outbreak isolates (Moura et al. 2003; Pierce et al. 2007; Shaw et al. 2004; Williamson et al. 2008; Woolfitt et al. 2011). Among the select agents characterized *Coxiella burnetti* prototype strains isolated from different geographical and/or historical origins were differentiated as well as numerous *Bacillus anthracis* strains (Pierce et al. 2007; Shaw et al. 2004; Woolfitt et al. 2011). Most

organisms studied in our laboratory were bacterial species and only a few microsporidia among eukaryotic organisms have been analyzed and reported (Moura et al. 2003).

We report here the combined development and application of MALDI-TOF MS and statistical analysis as a potential complementary method for *N. fowleri* characterization and strain differentiation. We have applied MALDI-TOF MS with Random Forest analysis, hierarchical cluster analysis, and proteomic database searching to a number of *N. fowleri* isolates. Using a combination of novel data processing methods for visual peak comparison, statistical analysis, and proteomics database searching we were able to demonstrate the power of this combined approach on a number of well characterized *N. fowleri* human and environment isolates. We believe that the combined approach will strengthen the ability of MALDI-TOF MS to differentiate species and isolates of *N. fowleri*.

MATERIALS AND METHODS

Chemicals

All free-living amoebae (FLA) isolates were a gift from Dr. G.S. Visvesvara (DPD, CDC). All chemicals used during this study were purchased from Sigma-Aldrich (St. Louis, MO) except where indicated. Buffers and culture media were obtained from the Scientific Resources Program at the Centers for Disease Control & Prevention (CDC).

Free-living amoebae isolates

We included in this study a total of 24 *Naegleria* isolates, comprising seven species, including 18 *N. fowleri* genotypes I, II, and III from different sources listed in Table 1. These isolates were previously identified by using conventional phenotypic tests and further characterized using the internal transcriber spacer and mitochondrial small subunit rRNA gene (Zhou et al. 2003). For comparison, we included an additional six species described within the genus *Naegleria*. All *Naegleria* spp. used in this study were grown in double-modified Nelson's medium. Growth temperature for *N. jadini* and *N. gruberi* was 25 °C; the other *Naegleria* species were grown at 37 °C (Zhou et al. 2003). All isolates were washed with amoeba saline as described before (Zhou et al. 2003), pelleted and stored at −80 °C. To account for growth variability, we grew, harvested and analyzed separately each organism on different occasions over a 2-wk (batch 2) and 4-wk (batch 3) interval.

Sample preparation

We prepared protein extracts for MS analysis as described previously (Moura et al. 2003, 2008; Shaw et al. 2004; Williamson et al. 2012) with a few modifications. Briefly, extracts were obtained from 100 μl frozen amoeba pellets containing 10^8 trophozoites after several freeze-thaw cycles and extracted with a 10% formic acid-50% acetonitrile solution. Five microliters of each extract were aliquoted and then premixed with equal volumes of the respective matrix solutions just before spotting on the MALDI target. We followed the protocol described previously (Moura et al. 2008) employing MALDI matrices (saturated solutions of α-cyano-4-hydroxycinnamic acid, or 3,5-dimethoxy-4-hydroxycinnamic acid [sinapinic acid]), 192-well stainless steel sample target plates (Applied Biosystems [AB], Framingham, MA) and mass standards for calibration (Sequazyme Peptide Mass Standards

Kit, AB) prior to MALDI-TOF MS analysis. Details of conditions and parameters for MALDI-TOF MS analysis using a MALDI-TOF/TOF mass spectrometer (AB 4700 Proteomics Analyzer) are those described before (Moura et al. 2008).

Data processing, peak matching and database searching

We processed mass spectra from three harvestings as described before with a few modifications (Moura et al. 2008; Satten et al. 2004). Briefly, we exported profile spectral data as text-format *m/z*-intensity lists. The text data were further processed and viewed by use of a suite of custom Microsoft Visual Basic.NET (VB.NET) programs, the VBA macros and VB.NET programs "MultiSpec Processor" and "MultiSpec Viewer". We normalized these spectra to the base peak, smoothed and finally standardized and denoised by use of a custom Fortran program (Satten et al. 2004). We used PAST software v1.34 (http://folk.uio.no/ohammer/past/doc1.html) for hierarchical cluster analysis, with the single summed spectra (one spectrum representing each organism) for input. We used Random Forest (RF) v5.1 (http://www.stat.berkeley.edu/users/breiman/RandomForests/cc_home.htm) for classification and identification, in this case, with ~9 summed spectra from three separated harvestings (2 wk apart) of each organism as a training set and ~3 summed spectra as unknowns. The recompilation of the Fortran RF code for each experimental condition was automatically driven by VB.NET programs, and custom viewing applications were developed to aid in the interpretation of the RF results.

In addition, we imported the summed spectra into the BioNumerics software v7.1 (Applied Maths Inc., Sint-Martens-Latem, Belgium) that were preprocessed using default parameters. A similarity matrix was generated using Ranked Pearson Correlation and provided the basis for constructing Neighbor Joining trees. Next, we submitted the extracted peak lists from all studied *Naegleria* to an in-house script for tentative protein identification. We used a total of 31,833 *Naegleria* spp. proteins entries available at GenBank (http://www.ncbi.nlm.nih.gov/genbank/) as of January, 2014. The Pepstats software from the European Molecular Biology Open Software Suite (EMBOOS) (Olson 2002) was used to retrieve protein database information and to calculate statistics of protein properties. We subtracted the molecular mass of the N-terminal methionine (131.00 Da), as described by before (Moura et al. 2008) and subtracted the proton charge (1 Da) as well. We matched the observed peaks (*m/z* minus 1 Da) and the predicted protein MW proteins in the database using an in-house script considering ±3.00 Da and posttranslational modifications such as the oxidation of methionine (+16 Da). Finally, the list of possible proteins for each peak was manually curated in order to exclude partial or fragment proteins and hypothetical proteins.

RESULTS

Visual analysis revealed differences among species and isolates of Naegleria

Extracts from three batches of the FLA species and isolates described in Table 1 were analyzed by MALDI-TOF MS. Spectra obtained revealed complex spectral patterns with 20–40 peaks in the *m/z* range corresponding to 1,000–6,000 Da or 2,000–14,000 Da. The mass range corresponding to 2,000–14,000 Da was preferred for this study because the peaks in this region were more abundant and more consistent. Spectra of amoeba protein

extracts revealed reproducible patterns and differences among species could be detected visually after analyzing several runs. As expected, only minor variation from batch to batch and from well to well was detected in the analysis. Careful analysis of the spectra in Fig. 1 reveals that several peaks desorbed from extracts of *Naegleria* species and isolates are markedly similar to each other. However, there are specific peaks that can be used to easily differentiate among the different species although isolate differentiation would require more effort.

Statistical analyses improved interspecies and strain discrimination in Naegleria

Because spectral visual analysis is not an easy task, we preferred to employ a statistical analysis of the MALDI-TOF MS data to quickly reveal significant differences among the strains analyzed. *Naegleria fowleri* could be discriminated from each other by Random Forest using summed spectra from three harvestings of each organism as a training set and ~3 summed spectra as unknowns. With low estimated classification error (0.75%) the RF algorithm successfully classified all the *Naegleria* species and the majority of the isolates in the training set (Fig. S1). Two isolates (CDC:V019 and CDC:V551) shared several peaks and the RF algorithm was unable to differentiate this subset. Results with three clustering algorithms within PAST, such as paired group, single linkage, and Ward's method, using similarity measure methods such as Euclidean, Dice, and Jaccard, among others, were analogous and quite consistent regardless of the analysis method applied, so that the different *Naegleria* species and isolates studied could be reliably and reproducibly separated from each other (Fig. S2). As expected, *N. fowleri* isolates were clustered together, and even sub-clusters could be detected. Interestingly, two isolates (CDC:V020 and CDC:V518) were clustered with other species within the genus *Naegleria. Naegleria lovaniensis* was clustered with *N. fowleri* isolates CDC:V551 while *N. australiensis* is the closest species to the *N. fowleri* isolates. Even more, isolates were mainly clustered according to the species in Neighbor Joining trees constructed using BioNumerics (Fig. 2). *Naegleria fowleri* isolates could be distinguished from related species, such as *N. dunnebackei*, *N. gruberi*, *N. jadini*, and *N. italica*. In this analysis, *N. australiensis* was closely related to a *N. fowleri* isolate recovered from water (CDC:V518) and *N. lovaniensis* was closely related to a *N. fowleri* isolate recovered from CSF (CDC:V551). Among *N. fowleri* strains, various subgroups were observed.

Tentative identification of peaks using an in-house script disclosed a large number of small proteins

The peak lists compared with proteins of *Naegleria* spp. in the database revealed 51 distinct matches among all *Naegleria*, including peaks corresponding to 13 ribosomal proteins (Table 2). Considering the peaks observed among all 18 *N. fowleri* isolates studied plus the strain HB3, a mean of 37 proteins were identified. The isolate that generate the smaller number of peaks was CDC:V511 with only 27 proteins identified. A total of 42 of the 51 proteins identified were found in *N. fowleri* HB3. Nevertheless, none of the 19 *N. fowleri* studied presented all the 51 proteins identified, although 29 proteins were identified in 14 isolates.

DISCUSSION

Emerging methods based on MS can be used to rapidly identify microorganisms. In particular, MALDI-TOF MS detects small acidic proteins and peptides desorbed from whole cells. Observed mass spectra are believed to consist primarily of protonated peptide and protein signals, although some signals below *m/z* 4,000 may represent other materials. The method is applicable to the identification of microorganisms at different levels (Clark et al. 2013; Sandrin et al. 2013). After spectra collection, multiple types of data analysis can be applied to discriminate genus, species, and even strains of the microorganisms. MALDI-TOF MS is making a fundamental shift in the routine practice of clinical microbiology (Patel 2013a,b). With over 500 publications each year in the past 5 yr, thus far these practical and rapidly evolving methods have been applied mostly to characterize and identify prokaryotes and clinically relevant fungi (Cayrou et al. 2010; Clark et al. 2013).

In this study, we used a high-resolution MALDI-TOF MS instrument and processing methods that we have developed (Moura et al. 2008; Satten et al. 2004; Woolfitt et al. 2011) to successfully detect differences and similarities among species and isolates of the genus *Naegleria*. Our work was expedited by use of improved in-house developed software that combined mass spectral visual analysis, statistical analysis using Random forest and tentative peak identification by database search and proteomics strategies (Moura et al. 2008; Pierce et al. 2007; Satten et al. 2004; Williamson et al. 2008; Woolfitt et al. 2011). In addition, we were able to apply commercial software (BioNumerics) that added another layer of certainty to the analysis. Concerns from early studies done with prokaryotes about MALDI-TOF reproducibility, the nature of the observed peaks and the best data analysis algorithms have been addressed in a number of publications (De Bruyne et al. 2011). However, there are still limitations of the MALDI method that need to be addressed. For example, the variability presented in MALDI chromatograms can be minimized by acquiring a number of spectra and can use the averaged spectrum for identification. In this study, complex eukaryotic organisms from the genus *Naegleria* were analyzed and different MALDI preparations varying solvents, matrices, plating techniques, and mass ranges were tested. Several factors can influence the composition of the MALDI-TOF spectra resulting from whole organism analysis. Although the metabolic state of the organism cannot be ruled out, MALDI identification is based largely on small conserved proteins such as the ribosomal proteins. In addition, due to the complexity of the material analyzed, which is composed of an expressive number of proteins with a large dynamic range, and without any analytical separation, it is not expected that all peaks be detected in each species in each analysis. The way to compensate for the variability presented in MALDI chromatograms, which may be a limitation of the MALDI analysis, is to run the samples several times acquiring a number of spectra and to apply statistical analysis. However, as even after these analyses the total number of expected peaks is generally not achieved for each species, a common criteria for microorganism identification by MALDI is to accept a percentage of representative peaks. For example, the two MALDI instruments in the market dedicated for bacterial identification (Bruker Biotyper and bioMerieuex Vitek-MS) assign species identification with high confidence when over 70% of the peaks of the unknown organism match the species in their database. Overall, in this study, the spectra of replicate samples

were similar, with a common subset of specific biomarkers found consistently in all studied conditions. Only minor differences were detected from analyses on different days.

It is important to note that the spectra obtained from different *Naegleria* spp. could be easily set apart from each other by visual inspection of their spectra. Because visual analysis was not an ideal approach we applied the previously reported Random Forest method for spectral standardizing and denoising to differentiate species within the genus *Naegleria* (Satten et al. 2004). In addition, the combined algorithms for data processing applied in this study proved to be useful. Random Forest is a robust method which can be used to differentiate all *Naegleria* species and separate most of the strains tested. More importantly, results from Neighbor Joining trees constructed using a similarity matrix generated with Ranked Pearson Correlation revealed similar results to outcomes using Random Forest. In concordance with PAST analysis, *N. australiensis* was closely related to a *N. fowleri* isolate recovered from water. Interestingly, *N. australiensis* can cause infection in mice after intranasal or intracerebral inoculation. Likewise, *N. lovaniensis* was closely related to a *N. fowleri* isolate (CDC:V551) recovered from CSF; the close relationship between these two species was previously reported (Zhou et al. 2003). Among *N. fowleri* strains, the various subgroups observed may reflect either genotype distribution or epidemiological characteristics of the strains, including year of isolation and geographical origin. Moreover, while some genotype I and III isolates were gathered in the same *N. fowleri* subgroup, strains of genotype II were more distantly related, in agreement with previous results of mtSSU-rRNA gene and ITS analyses (Zhou et al. 2003). Still, discrimination among isolates belonging to the same genotype was also possible. Within three *N. fowleri* branches, isolates from environmental sources were clustered together with those from human sources. When considered collectively, these data confirmed the potential of MALDI-TOF MS for discrimination among species, and strains.

Challenges in tentative peak identification in this study exist because *Naegleria gruberi*, a nonpathogenic species is the only representative within the genus that had its genome completely sequenced (Fritz-Laylin et al. 2010). Only the mitochondrial genome and a 60-kb segment of nuclear genome from *N. fowleri* have been sequenced and assembled (Herman et al. 2013). However, we were able to retrieve 31,833 *Naegleria* spp. protein sequences available in GenBank that were tentatively matched to our results using a combination of an in-house script with the EMBOOS software. The complex procedure was fruitful since we have been able to tentatively identify over 50 small proteins or peptides occurring in the different *Nagleria* species studied. As expected, a number of them corresponded to ribosomal proteins which are extremely ancient molecules, and consequently one-third of ribosomal protein families are conserved among Bacteria, Archae, and Eucharia (Lecompte et al. 2002). These proteins are used to identify distinct microorganisms using MALDI-TOF spectra and these approaches are largely in current used (Hamprecht et al. 2014; Moura et al. 2008; Seng et al. 2010).

Matrix-assisted laser-desorption-ionization-time-of-flight mass spectrometry-based methods have been generally used for genus and species differentiation, and in a few cases for strain characterization (Sandrin et al. 2013). We report here one of the first uses of MALDI-TOF MS for analyzing *Naegleria* species and *N. fowleri* strains. Species within the genus and

most of the strains were discriminated by MALDI-TOF MS. Although MS-based methods are sensitive and specific the present MALDI-TOF MS application was designed for microbial identification and not for microorganism detection, as there is a requirement of ~5,000 organisms per well for the observation of a clean and meaningful spectra.

In conclusion, we have successfully applied MALDI-TOF MS with statistical analysis to differentiate among different *Naegleria* species and isolates. Visual analysis of multiple spectra was sufficient to differentiate among the different species analyzed. However, the combined MS visual analysis, fingerprinting and proteomics approach yielded more robust biomarker identification by use of a database search, which permitted isolate differentiation among *N. fowleri* isolates. Small ribosomal proteins were consistently found among biomarkers detected in this study. We believe that this method is a rapid, reproducible, high-throughput alternative for the characterization of microorganisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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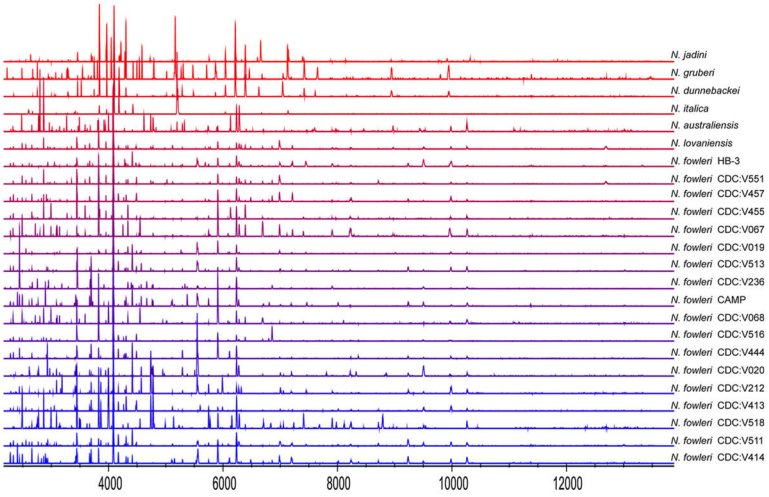

Figure 1.

MALDI-TOF analysis of different *Naegleria* species. MultiSpec Viewer depicting denoised peaks (m/z 2,000–14,000 Da) from extracts of vegetative forms that are consistently seen in all wells and reveal differences among species and isolates.

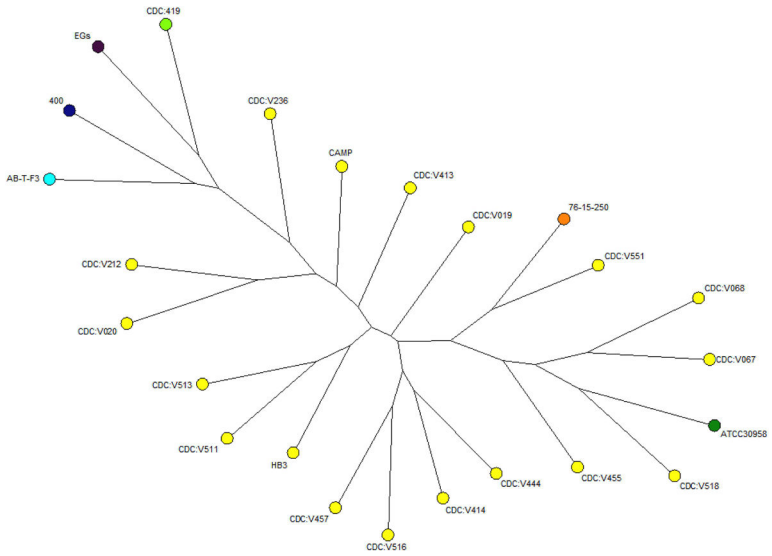

Figure 2.

Neighbor joining tree obtained after BioNumerics analysis. The images are color-coded according to the *Naegleria* species analyzed. *Naegleria* fowleri isolates could be distinguished from the other species in the genus.

Table 1

Characteristics of the *Naegleria* spp. *isolates* included in the present

Number	Isolate no.	State/or country/year	Sex/age/source	Species/genotype	References
1	CDC:V414	FL, 1998	M/16/CSF	*N. fowleri,* I	Zhou et al. (2003)
2	CDC:V511	GA, 2002	M/11/CSF	*N. fowleri,* I	Zhou et al. (2003)
3	CDC:V518	AZ, 2002	Water filter 2	*N. fowleri,* I	Zhou et al. (2003)
4	CDC:V413	TX, 1998	M/17/CSF	*N. fowleri,* I	Zhou et al. (2003)
5	CDC:V212	AL, 1990	M/?/CSF	*N. fowleri,* I	Zhou et al. (2003), Herman et al. (2013)
6	CDC:V020	TX, 1984	M/12/CSF	*N. fowleri,* I	Zhou et al. (2003)
7	CAMP	CA, 1978	F/8/CSF	*N. fowleri,* II	Zhou et al. (2003)
8	CDC:V236	CA, 1991	M/29/CSF	*N. fowleri,* II	Zhou et al. (2003)
9	CDC:V444	CA, 1999	F/Cow/Brain	*N. fowleri,* I	Visvesvara et al. (2005)
10	CDC:V513	GA, 2002	Water	*N. fowleri,* III	Zhou et al. (2003)
11	CDC:V019	TX, 1984	M/25/CSF	*N. fowleri,* III	Zhou et al. (2003)
12	CDC:V516	AZ, 2002	Water filter 4	*N. fowleri,* I	Zhou et al. (2003)
13	CDC:V068	AZ, 1987	Water	*N. fowleri,* I	Zhou et al. (2003)
14	CDC:V067	AZ, 1987	M/30/CSF	*N. fowleri,* III	
15	CDC:V455	NV, 2000	M/??/CSF	*N. fowleri,* III	Zhou et al. (2003)
16	CDC:V457	TX, 2000	M/12/CSF	*N. fowleri*	Visvesvara, G.S. (unpubl. data)
17	CDC:V551	Colombia, 2004	M/??/CSF	*N. fowleri*	Visvesvara, G.S. (unpubl. data)
18	76-15-250	Belgium, 1976	Water	*N. lovaniensis*	Stevens et al. (1980) AUTHOR: Stevens et al. (1980) has not been included in the Reference List, please supply full publication details.
19	ATCC30958	Australia	Water	*N. australiensis*	De Jonckheere, (1981)
20	AB-T-F3	Italy, 1982	Thermal mud	*N. italica*	Scaglia et al. (1983)
21	CDC:419	CA, 1999	Water	*N. dunnebackei*	Visvesvara et al. (2005)
22	EGs	CA, 1964	Soil	*N. gruberi*	Schuster (1964)
23	400	Belgium, 1973	Water	*N. jadini*	Willaert et al. (1980)
24	HB3	Czechoslovakia, 1969	M/??/CSF	*N. fowleri*	Cerva et al. (1969)

Table 2

Tentative identification of detected biomarkers of the studied *Naegleria* spp. isolates

Accession number	Description	Protein MW (Da)[a]	N. fowleri CDC:V414	N. fowleri CDC:V511	N. fowleri CDC:V518	N. fowleri CDC:V413	N. fowleri CDC:V212	N. fowleri CDC:V020	N. fowleri CAMP	N. fowleri CDC:V236	N. fowleri CDC:V444	N. fowleri CDC:V513	N. fowleri CDC:V019	N. fowleri CDC:V516	N. fowleri CDC:V068	N. fowleri CDC:V067	N. fowleri CDC:V455	N. fowleri CDC:V457	N. fowleri CDC:V551	N. fowleri HB3	*N. lovaniensis*	*N. australiensis*	*N. dunnebacki*	*N. gruberi*	*N. italica*	*N. jadini*	# *N. fowleri* [a]	# All [c]
EFC43258.1	Acyl carrier protein	13054																									15	20
EFC45268.1	Acyl CoA binding protein	9,788.26																									16	18
AF288092_20	ATP synthase F0 subunit 8	13,459.44																									13	16
AFP72311.1	ATP synthase F0 subunit 8 (mitochondrion)	13,364.49																									14	17
AF288092_45	ATP synthase F0 subunit 9	7,401.11																									14	18
AFP72336.1	ATP synthase F0 subunit 9 (mitochondrion)	7,429.12																									17	22
P22067.2	ATP synthase subunit a	13,959.83																									5	8
AAA31968.2	ATPase subunit 6	13,959.83																									5	8
EFC46184.1	Autophagy-related protein ATG8	13,708.71																									14	19
EFC35858.1	CHY zinc finger domain-containing protein	13,742.97																									17	20
EFC49888.1	CKS domain-containing protein	12,479.01																									12	14
EFC42349.1	Cyclin-dependent kinase regulatory subunit-like protein	12,160.75																									15	19
EFC45605.1	Cysteine protease inhibitor	12,818.4																									10	13
EFC35480.1	Cytochrome *b*5	10,987.17																									11	15
EFC50108.1	Cytochrome *c*	12,041.97																									14	19
ADK09904.1	Cytochrome oxidase subunit 3	3,584.11																									11	13
EFC47363.1	Ferredoxin	13,090.99																									14	18
Q9NH76.1	Hemerythrin-like protein	13,272.22																									15	18
EFC48882.1	Histone H3	12,740.99																									7	10
EFC38679.1	Histone H4	11,375.41																									12	16
P83726.1	Isocitrate dehydrogenase [NADP]	2,091.3																									11	14
ACA13169.1	Meiosis-specific MutS-like protein	8,216.72																									15	20
ABD62823.1	Mitochondrial single-subunit RNA polymerase	7,110.55																									17	21
AF230370_1	Myohemerythrin	13,272.22																									15	18
AF288092_5	NADH dehydrogenase subunit 4L	9,832.18																									13	17
AFP72297.1	NADH dehydrogenase subunit 4L (mitochondrion)	9,664.03																									16	19
EFC45267.1	NADH-ubiquinone oxidoreductase	13,243.27																									16	19
AAT92053.1	**Nfa1**	13,272.22																									15	18
EFC44447.1	Nuclear transport factor 2	13,820.84																									12	16
EFC44334.1	Peptidyl-prolyl isomerase	12,729.81																									15	19
EFC50153.1	**Profilin**	13,572.52																									13	15
EFC49299.1	Ras family small GTPase	13,918.47																									7	10
EFC35562.1	Rho family small GTPase	11,485.04																									16	19
AFP72318.1	Ribosomal protein L14 (mitochondrion)	13,923.9																									14	19
EFC45499.1	Ribosomal protein L29-like protein	7,119.39																									12	14
EFC40133.1	Ribosomal protein L30	13,462.67																									13	17
EFC41887.1	Ribosomal protein L34e	13,883.63																									15	17
EFC43240.1	Ribosomal protein L36E	12,513.94																									9	10
EFC48875.1	Ribosomal protein P1	11,782.32																									15	20
AFP72313.1	Ribosomal protein S10 (mitochondrion)	9,550.43																									11	13
AF288092_29	Ribosomal protein S14	12,042.61																									14	18
AFP72320.1	Ribosomal protein S14 (mitochondrion)	11,973.41																									16	21
P51429.1	Ribosomal protein S15	13,187.73																									14	18
AF288092_24	Ribosomal protein S19	10,404.64																									12	17
AFP72315.1	Ribosomal protein S19 (mitochondrion)	10,289.36																									17	22
EFC45174.1	Ribosomal protein S21	8,865.29																									13	17
EFC45948.1	RRM domain-containing protein	10,975.41																									14	19
EFC43571.1	S25 ribosomal protein	7,119.39																									13	14
AAB01771.1	Thioredoxin homolog	11,048.64																									15	19
EFC39089.1	TM2 domain-containing protein	11,669.66																									15	18
EFC35327.1	WD40 repeat domain-containing protein	12,310.02																									10	14
	Total number of proteins tentatively identified in each isolate/species		37	27	39	32	35	40	37	31	39	36	36	30	37	38	33	37	37	41	33	34	35	37	38	34		

[a] Protein MW expected without N-terminal methionine (Da).

[b] Number of *N. fowleri* isolates where the tentatively identified proteins were found.

[c] Number of *Naegleri* spp. where the tentatively identified proteins were found.