Identification of Disseminated Cryptococcosis Using MALDI-TOF MS and Clinical Evaluation

Norihito Tarumoto¹, Jun Sakai¹, Masahiro Kodana², Tohru Kawamura³, Hideaki Ohno¹ and Shigefumi Maesaki²

¹Department of Infectious Disease and Infection Control, Saitama Medical University
²Clinical Laboratory Medicine, Saitama Medical University Hospital
³Department of Infectious Disease and Infection Control, Saitama Medical Center, Saitama Medical University

ABSTRACT

Disseminated cryptococcosis is rare but can often become severe with a poor outcome. Given recent reports that matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyser is useful for Cryptococcus species identification, it was applied retrospectively to past cases of disseminated cryptococcosis at our hospital over the past 10 years, and their clinical courses were reviewed. For each case, the retained Cryptococcus spp. were used for identification using both MALDI-TOF MS and genetic sequencing, as well as for drug susceptibility testing. A total of eight cases were found. Cryptococcus spp. were found in cerebrospinal fluid in 3 cases and blood in 5 cases; anti-HIV antibody was either negative or untested. MALDI-TOF MS identified Cryptococcus neoformans as the pathogen in all 8 cases, but genetic testing identified one of these as Cryptococcus curvatus. The outcome was death within 30 days in 5 of the total 8 cases and in 2 of the 3 cases in which C. neoformans was detected in the cerebrospinal fluid, despite regimens and dosages that followed IDSA Guidelines in all 3 cases. Drug susceptibility testing showed no drug resistance that would have affected the therapy. In conclusion, the outcomes were very poor in these drug-susceptible cases, despite treatment in full accordance with standard guidelines. This study confirmed the need to develop newer therapies as well as the high capability of MALDI-TOF MS for the identification of C. neoformans. Genetic testing, however, may be necessary if non-neoformans Cryptococcus is suspected.

Key words: disseminated cryptococcosis, Cryptococcus curvatus, MALDI-TOF MS, non-neoformans Cryptococcus

Introduction

Disseminated cryptococcosis is an infectious disease that tends to become severe and have a poor prognosis. Some clinical guidelines have been published¹-³, and standardization of its therapy is in progress. Even in developed countries, however, the fatality rate is high at 20%, and early diagnosis and treatment are important⁴,⁵. Among the 70 known species in the Cryptococcus genus⁶, the clinical isolates in cases of cryptococcosis in Japan have for the most part been Cryptococcus neoformans⁷,⁸, with Cryptococcus gattii reported in rare cases⁹-¹¹. Identification has therefore generally been based on morphology in Japan. With reports of infections by species other than C. neoformans rising steadily¹², together with reports of species resistant toazole antifungals¹²,¹³, the importance of species identification using a combination of other methods in addition to morphology has increased.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) can reportedly provide a fast and highly accurate
means of identifying Cryptococcus species\textsuperscript{14, 15}, and its application in clinical laboratories has begun in recent years. For relatively rare species, however, identification by this means has reportedly been hindered by their absence from in-house MALDI-TOF MS libraries\textsuperscript{16}. In light of this, we conducted an investigation to confirm whether there were past cases of disseminated cryptococcosis caused by species other than \textit{C. neoformans} at our hospital through re-identification using MALDI-TOF MS and to assess the clinical courses of these cases.

**Materials and Methods**

From the database of the laboratory in Saitama Medical University Hospital, a general hospital with 972 beds, cases of disseminated cryptococcosis that had occurred during the 10-year period of June 2005 to May 2015 and in which \textit{Cryptococcus} spp. had been detected from blood or cerebrospinal fluid, were extracted. The retained strains were cultured in Sabouraud dextrose agar at 35°C for 2 days and then analyzed. Clinical information was collected retrospectively from the electronic medical records.

MALDI-TOF MS was performed according to the manufacturer’s recommendations. Briefly, the colony grown in the culture medium was suspended in a tube containing 300 µl of distilled water, followed by addition of 900 µl of 99.5% alcohol and centrifugation at 13,000 rpm for 2 min. The supernatant was then removed, and 20 µl of 70% formic acid was added. After vortexing, 20µl of acetonitrile was added, followed by centrifugation at 13,000 rpm for 2 min, placement of 1 µl of the resulting supernatant on a polished steel MSP 48-target plate (Bruker Daltonics, Inc., Billerica, MA), drying, addition of 1µl of matrix, drying, and finally measurement and analysis on a Bruker MALDI Biotyper 4.0 standard library (Bruker Daltonics, Inc.) mass spectrometer. Any score value (SV) of less than 1.7 was deemed insufficient for identification.

In the genetic identification test, sequence analysis was performed for the ITS 5.8S rRNA region using ITS1 (TCCTAGAGGTGAACCTGCGG) and ITS4 (TCCTCGCTTATGGATATGC) primers, and for the D1/D2 region using NL1 (GCATAT CAATAAGCGGAGGAAAAG) and NL4 (GGTCCG TGTTTGAAGACGG) as primers, and the sequences obtained were compared with the GenBank database.

Drug susceptibility was measured in accordance with M27-A3 (method for broth dilution)\textsuperscript{17}, the standard protocol of the Clinical and Laboratory Standards Institute (CLSI), by trace liquid dilution using Yeast-like Fungus DP ‘Eiken (Eiken Chemical, Tokyo, Japan), for amphotericin B (AMPH-B), fluconazole (FLCZ), itraconazole (ITCZ), voriconazole (VRCZ), and flucytosine (5-FC). This study was approved by the local institutional review boards and ethics committees (No. 15-048).

**Results**

In the 8 cases (5 male, 3 female) for which \textit{Cryptococcus} spp. had been detected in the blood (5 cases) or cerebrospinal fluid (3 cases), the mean age was 68.5 years (Table 1). In Cases 4-6 and Case 8, the infection focus was indeterminate, and meningitis could not be ruled out. Case 7 was a soft tissue infection. Blood serum cryptococcal antigen levels were not measured in Case 8, but they were found to be elevated by a factor of 256 or more in all other cases. (1-3)-β-D-glucans (β-Glucan test Wako\textsuperscript{®}, Wako Pure Chemical Industries, Ltd, Osaka, Japan) were measured in 7 cases, and slight elevation was found in 2 cases. Some type of immune deficiency was present in all cases except Case 8. Case 5 had a negative HIV screening test, but none of the other cases were tested for HIV infection.

In the present study, MALDI-TOF MS identified the fungus as \textit{C. neoformans} in 7 of 8 cases, with SVs of 1.9 or more for all strains and matches to the sequence analysis results. In the other case (No. 8), the fungus was not identified with the initial MALDI-TOF MS test, but on retesting it was identified as \textit{C. neoformans} (SV2.016). Sequence analysis for Case 8, however, showed 100% identification in the ITS and D1/D2 regions (597/597 bp and 573/573 bp, respectively) for \textit{Cryptococcus curvatus} (NCBI, accession numbers NR_. 130657 and HQ 323253 for the ITS and D1/D2 regions, respectively).

Review of the cases showed that, except for 1 case (No. 6) in which death occurred before diagnosis, antifungal treatment was performed in all cases, consisting of liposomal amphotericin B (L-AMB) plus 5-FC in 3 cases and AMPH-B, L-AMB, FLCZ, or VRCZ in 1 case each. In 2 of the 3 cases of cerebrospinal meningitis (Cases 1-3), the out-
<table>
<thead>
<tr>
<th>No</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Locus detected</th>
<th>BDG (pg/ml)</th>
<th>Antifungal agent</th>
<th>Daily dose</th>
<th>Outcome</th>
<th>Underlying disease</th>
<th>HIV status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>69</td>
<td>F</td>
<td>CSF</td>
<td>&lt; 6</td>
<td>L-AMB + 5-FC</td>
<td>4.9 mg/kg + 100 mg/kg</td>
<td>Death</td>
<td>Systemic lupus erythematosus, Sjögren syndrome</td>
<td>Not tested</td>
</tr>
<tr>
<td>2</td>
<td>74</td>
<td>F</td>
<td>CSF</td>
<td>&lt; 6</td>
<td>L-AMB + 5-FC</td>
<td>4.0 mg/kg + 103 mg/kg</td>
<td>Death</td>
<td>Systemic lupus erythematosus, Diabetes mellitus</td>
<td>Not tested</td>
</tr>
<tr>
<td>3</td>
<td>67</td>
<td>F</td>
<td>CSF</td>
<td>&lt; 6</td>
<td>L-AMB + 5-FC</td>
<td>5.4 mg/kg + 108 mg/kg</td>
<td>Alive</td>
<td>Autoimmune hepatitis, Multiple myeloma</td>
<td>Not tested</td>
</tr>
<tr>
<td>4</td>
<td>78</td>
<td>M</td>
<td>Blood</td>
<td>41</td>
<td>FLCZ</td>
<td>400 mg</td>
<td>Death</td>
<td>Bullous pemphigoid, Diabetes mellitus</td>
<td>Not tested</td>
</tr>
<tr>
<td>5</td>
<td>67</td>
<td>M</td>
<td>Blood</td>
<td>23.5</td>
<td>L-AMB</td>
<td>2.6 mg/kg</td>
<td>Death</td>
<td>Autoimmune hepatitis, Liver cirrhosis</td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>71</td>
<td>M</td>
<td>Blood</td>
<td>NA</td>
<td>(none)</td>
<td></td>
<td>Death</td>
<td>Anca-related nephritis</td>
<td>Not tested</td>
</tr>
<tr>
<td>7</td>
<td>91</td>
<td>M</td>
<td>Blood</td>
<td>&lt; 6</td>
<td>VRCZ</td>
<td>400 mg</td>
<td>Alive</td>
<td>Diabetes mellitus</td>
<td>Not tested</td>
</tr>
<tr>
<td>8</td>
<td>29</td>
<td>M</td>
<td>Blood</td>
<td>&lt; 6</td>
<td>AMPH-B</td>
<td>0.3 mg/kg</td>
<td>Alive</td>
<td>(None)</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

Abbreviations: CSF, cerebrospinal fluid; BDG, (1-3)-β-D-glucan; HIV, human immunodeficiency virus; L-AMB, liposomal amphotericin B; 5-FC, flucytosine; FLCZ, fluconazole; VRCZ, voriconazole; AMPH-B, amphotericin B; NA, not available
come was death despite drug therapy in accordance with the IDSA Guidelines\(^1\). In Cases 5 and 6, polyene antifungal plus 5-FC therapy was planned, but both passed away before it was started. The overall 30-day death rate was 62.5% (5 of 8 cases). On drug-susceptibility testing, all strains were found to be susceptible to all antifungal drugs tested, except for 5-FC resistance in only one strain (No. 6), which was deemed not of a degree that would affect the therapy (Table 2).

### Discussion

The several guidelines currently available for treatment for disseminated cryptococcosis\(^{1-3}\) recommend initial treatment of cryptococcal meningitis with a combination of polyene antifungal and 5-FC. Among the 8 cases reviewed in this study, the 3 cases in which *C. neoformans* was detected in cerebrospinal fluid were treated with the recommended doses in accordance with the guidelines, but 2 of the 3 nevertheless ended in death within 30 days, thus conforming to the known tendency of cryptococcal meningitis to deteriorate rapidly.

The guidelines recommend that the same regimen as for meningitis be followed in cases of non-meningitis and non-pulmonary inflammation and in cases where meningitis cannot be ruled out\(^1\). In Case 4, meningitis was not ruled out, and a better outcome might have been achieved if the combination therapy of polyene antifungal plus 5-FC had been chosen. Cases 5 and 6 died before treatment with an appropriate regimen. It may be that death could have been avoided had early diagnosis and appropriate treatment been performed.

Use of an azole antifungal agent is generally considered for cryptococcosis other than meningitis\(^1\). In our cases, FLCZ was used in Case No. 4, but no resistance to azole antifungal agents was observed. Drug susceptibility might not correlate with treatment failure in vivo, and clinical breakpoints have not been clearly defined for *Cryptococcus* spp.\(^{18-20}\). However, low MIC value was correlated with successful treatment for cryptococcosis in the other studies\(^{21-23}\). Additionally, low-dose preemptive therapy over multiple weeks led to the selection of resistant organisms\(^{24}\). It is therefore presumably desirable to perform drug susceptibility testing in cases of disseminated cryptococcosis and unfavorable progress inazole monotherapy.

Generally, accurate and rapid identification of pathogens is needed in order to shorten the time from fungal disease onset to start of appropriate therapy. Recently, in addition to the usual MALDI-TOF MS method used for cultured colonies, the MALDI Sepsityper method was reported for directly using specimens such as blood\(^{25}\). Perhaps, when there is enough inoculum, *Cryptococcus* spp. could be directly detected from cerebrospinal specimen. Moreover, the MALDI-TOF MS method was recently proposed to rapidly assess the drug susceptibility of isolated fungi\(^{26,27}\). In this way, MALDI-TOF MS methods may contribute to early management of fungal diseases. Indeed, use of MALDI-TOF MS with antimicrobial stewardship team intervention decreased the time to organism identification and improved patient outcomes\(^{28}\). Further use of the MALDI-TOF MS method is recommended to im-

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**Table 2. Antifungal susceptibility for 8 strains of Cryptococcus spp.**

<table>
<thead>
<tr>
<th>Antifungal agents</th>
<th>Number of isolates inhibited at MIC or MEC (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>AMPH</td>
<td>1</td>
</tr>
<tr>
<td>FLCZ</td>
<td>3</td>
</tr>
<tr>
<td>ITCZ</td>
<td>1</td>
</tr>
<tr>
<td>VRCZ</td>
<td>1</td>
</tr>
<tr>
<td>MCFG</td>
<td>8(&gt;16)*</td>
</tr>
</tbody>
</table>

Abbreviations: MIC, minimum inhibitory concentration; MEC, minimum effective concentration; AMPH-B, amphotericin B; 5-FC, flucytosine; FLCZ, fluconazole; ITCZ, itraconazole; VRCZ, voriconazole; MCFG, micafungin. * is MIC for *C. curvatus*.
prove prognosis of cryptococcosis.

One of the points that should be taken into consideration regarding identification by MALDI-TOF MS is that difficulties have been noted in regard to its use for identification of relatively rare species of clinical isolates. In the present study, one strain identified as C. neoformans by MALDI-TOF MS was shown to be C. curvatus by genetic testing. As one of the biochemical features for the discrimination between C. curvatus and C. neoformans, C. curvatus is able to assimilate some substances, such as lactate, lactose, and glycerol, that C. neoformans cannot assimilate. To the best of our knowledge, this report is the first one to report misidentification of C. curvatus by MALDI-TOF MS.

The incidence of C. curvatus infection is unknown, but it has reportedly been detected in cases of vaginitis and myeloradiculitis, and strains exhibiting azole resistance have also reportedly been observed. Given the existence of species other than C. neoformans that exhibit drug resistance and an increasing incidence of infection by such species, it has become increasingly important to rapidly and accurately identify species. Cryptococcosis exhibits various degrees of severity depending on the species, and the species cannot be inferred from the clinical presentation. Non-neoformans Cryptococcus may tend to become negative to serum cryptococcal antigens, and this may be an important point in diagnosis. Cryptococcal antigens were not measured in the Case 8 reviewed here, but it appears that, in cases where antigen testing is negative, genetic testing will be necessary in addition to a MALDI-TOF MS.

In summary, this report showed that, although therapy was performed in accordance with recognized guidelines for cases with drug susceptibility, the outcomes were poor, and this confirms the importance of developing newer therapeutic agents. The results of the present study also show that the capability of MALDI-TOF MS for identification of C. neoformans is high, but they also indicate that, if Cryptococcus spp. is suspected morphologically, then, for cases such as those in which cryptococcal antigen testing is negative, genetic testing and consideration of its results are needed.

Conflict of Interest

Self-declared COI content: none

References


