Evaluation of early detection of mucorales by rapid one step off- plate protein extraction using maldi -tof ms

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Abstract---Aim: To validate a simple one step method of protein extraction of moulds for routine early detection of fungi using automated instrument like MALDI- TOF MS. Methodology: A total of 123 clinical mould isolated were tested in Department of Microbiology, Indira Gandhi Institute of Medical Sciences, Patna, Bihar, India during period of 3 months (August 2021-October 2021) with the routine Laboratory and culture investigations. Out of these specimens, approximately 50 specimens were suspected of Mucorales based on their growth timing, colony morphology, history of patient, character of tissue or sample, and corresponding KOH mount findings having hyphae in tissues or samples were included in study. Samples were inoculated on routine fungal culture media like SDA. The plates were examined at earliest for appearance of any growth and the process of protein extraction was done to be analysed in MALDI- TOF MS and compared with KOH preparation findings of sample. The culture were again incubated further for appearance of mature colony

and later on isolates were identified by LPCB mount examination. Results: Around 70% growth of mucorales could be identified by this method and 30% could not be identified. Though this method is not 100% effective for early mucorales identification but still it is helpful as 70 % mucorales are identified. Conclusion: MALDI-TOF MS has contributed to improving the laboratory diagnosis of infections by filamentous fungi in terms of rapidity and accuracy of identification. As fast and precise recognition of a fungal pathogen can result in a significant benefit for optimal patient treatment, MALDI-TOF MS-based identification should be integrated into antifungal stewardship strategies.

Keywords---Mucorales, Rhizopus, spectrometry, mucormycosis.

Introduction

The *Mucorales*, which is the core group of the traditional *Zygomycota* [1–3], have been recently reclassified into the subphylum *Mucoromycotina* of the *Glomeromycota* phylum of the kingdom Fungi [4]. The *Mucorales* are characterized by aseptate (coenocytic) hyaline hyphae, sexual reproduction with the formation of zygospores, and asexual reproduction with nonmotilesporangiospores. They are ubiquitous in nature, being found in food, vegetation, and soil [1–3]. The majority of the invasive diseases are caused by genera of the *Mucoraceae*family, and the resulted disease is called mucormycosis [1–3, 5–6].

Risk factors for invasive diseases include uncontrolled diabetes mellitus, haematological malignancies, bone marrow and solid organ transplantation, deferoxamine therapy, corticosteroid therapy, or other underlying conditions impairing the immune system [7]. Limited activity of some principal classes of antifungal drugs (i.e., echinocandins and azole derivatives) as well as vascular invasion and neurotropic activity could explain the high mortality seen in mucormycosis [8, 9]. Mucormycosis can be classified in rhinocerebral, pulmonary and disseminated, abdominal-pelvic and gastric, and cutaneous or chronic subcutaneous diseases. Common features of rhinocerebral, pulmonary, and disseminated diseases include blood vessel invasion, hemorrhagic necrosis, thrombosis, and a rapid fatal outcome.

Histology and culture are still the most important diagnostic approaches for mucormycosis because of the lacking of molecular diagnosis methods standardized or commercially available. Moreover the β-1–3 glucan detection is not useful in this kind of infection due to the extremely low content of this molecule in the *Mucorales* [10, 11]. Timely diagnosis of invasive mucormycosis is essential due to the rapid progression of the disease, and because signs and symptoms of the infection could mimic other invasive fungal infections. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has recently revolutionized the diagnostic mycology workflow [11, 12]. With MALDI-TOF MS, cultured yeasts may be correctly identified in minutes without apriori knowledge of organism-type; since it doesn't matter whether a bacterium or yeast is being tested, the decision-making procedure characteristically

surrounding differentiation of bacteria or yeasts growing on solid media prior to selecting further testing is obviated. Filamentous fungi can also be identified, though usually their processing prior to MALDI-TOF MS analysis typically takes longer than yeasts. MALDI-TOF MS is enabling implementation of total laboratory automation in clinical microbiology laboratories, allowing automated specimen processing, plating, incubation, plate reading using digital imaging, and spotting to MALDI-TOF MS plates. Early growth detection by digital imaging, paired with MALDI-TOF MS may result in earlier detection of fungi than conventional techniques [13].

There are different protocols for protein extraction, as developed by different researchers. In this study we are going to follow a simple one step rapid extraction method and compare our result with sample preparation method for moulds as recommended by manufacturer of VITEK MS, bioMERIEUX. As Mucorales are rapidly growing, the protein extraction of moulds grown on SDA culture plate will be done as early as possible, and the result will be analysed in MALDI TOF MS. For this early examination of culture plate will be done for appearance of any growth and then will be preceded for protein extraction, and identification. As our hospital is COVID hospital and currently we are diagnosing lots of mucormycosis cases by conventional fungal culture method, application of MALTI -TOF MS in this process will help early and accurate species speciation of Mucorales, and this will certainly help in early intervention and institution of appropriate therapeutic measures for mucormycosis patients in future waves of COVID pandemic. This will validate a simple one step method of protein extraction of moulds, to be processed in MALTI-TOF MS system. As routine extraction of protein from clinical filamentous fungi is time consuming and slightly cumbersome, there is need to follow a simple and accurate protein extraction method for routine early detection of fungi using automated instrument like MALDI- TOF MS.

Materials and Methodology

Methodology

A total of 123 clinical mould isolated were tested in Department of Microbiology, Indira Gandhi Institute of Medical Sciences, Patna, Bihar, India during period of 3 months (August 2021 - October 2021) with the routine laboratory and culture investigations. Out of these specimens, approximately 50 specimens were suspected of Mucorales based on their growth timing, colony morphology, history of patient, character of tissue or sample, and corresponding KOH mount findings having hyphae in tissues or samples were included in study. All mixed growth and contaminated growth over culture media, were excluded from the study.

Procedure

As samples received from COVID hospital is mostly tissues, it was properly processed for routine fungal culture plating. Sputum or other body fluids were similarly inoculated on routine fungal culture media like SDA and PDA. The plates were examined at earliest for appearance of any growth and the process of protein extraction was done to be analysed in MALDI- TOF MS and compared

with KOH preparation findings of sample. The culture were again incubated further for appearance of mature colony and later on isolates were identified by LPCB mount examination.

For rapid one step protein extraction, a readymade extraction solution was made by mixing acetonitrile, 70% formic acid and distilled water in a ratio of acetonitrile 50%, formic acid 35% and distilled water 15%. 5 mm piece of mould growth was removed from culture plate using wet swab and put in to 2 ml tube containing 50 microlitre of silica beads and 100 microlitre of above readymade protein extraction solution. Tube was then vortexed properly for 3 minutes and then centrifuged for 3 minutes at 13000 rpm. One microlitre of supernatant was put over the spot on target plate and was allowed to dry, and then 1 microlitre of CHCA matrix was overlaid over spot and dried. Then the target plate was placed in ionisation chamber of MALDI -TOF MS to be analysed automatically. MALDI TOF identification was later on confirmed by phenotypic method to judge the effectiveness of the study.

Results

During the study period, out of 123clinical molds isolated, 50 specimens suspected of Mucorales spp.wereanalyzed by MALDI-TOF MS and protein extraction method and later confirmed by phenotypic method i.e. by slide culture technique as most of the mucorales grow early within 24 hrs but could not be identified accurately as their phenotypic characters (like rhizoides, collumella) were not well developed. Results for this method was categorized by logarithmic score cut-offs assigned by the database of bioMERIEUXspectra.

Current practice by the NIH Microbiology Service dictates that a score of ≥2.00 is required for reporting MALDI-TOF MS results to the species or genus level, depending on the organism. For this study, isolates were sorted into two different score thresholds: scores ≥2.00 and score < 2.00. Using these criteria, 36 (72%) of the clinical isolates achieved scores at the reporting level (≥2.00). Scores less than 2.00 were produced by 14 (28%) isolates. Spectra generated from all three extraction methods were analyzed using only the bioMERIEUX supplied database to determine whether the rapid extractions allowed for identifications without a user supplemented database. So, all the mucorales isolated could not be identified by MALDI TOF using protein extraction. Around 70% growth of mucorales could be identified by this method and 30% could not be identified.

Table 1: Identification of species by routine method and MALDI-TOF MS

Species	MALDI-TOF MS	By phenotypic method
Rhizopusarrhizus	17	21
Rhizopus microsporus	8	10
Mucorracemosus	3	5
Rhizomucorpusillus	3	4
Lichtheimiacorymbifera	3	6
Cunninghamellabertholletiae	2	4

Discussion

MALDI-TOF MS identification is slowly gaining popularity as a fast and inexpensive method for the routine identification of clinical mould isolates. Unlike bacteria and yeast, which can easily be prepared for analysis in one extraction step [14, 15], the current cell lysis extraction procedures for filamentous fungi require a multitude of washing, inactivation, and chemical extraction steps [16]. Benefits of this study is to identify mucorales family infection (Mucormycosis), especially in covid era. While conventional method of identification of mould infection is by KOH preparation and confirmation by fungal culture. After fungal culture species identification can be done eitherby phenotypic identification by slide culture technique, use of LPCB mount etc. Early detection of rapidly growing fungus like mucor group of fungus (Zygomycosis or mucormycosis) can be done if their protein is recovered at earliest of their growth on SDA media. Conventional method of identification is time consuming. Rhinocerebral mucormycosis is an emergency. So there is need of developing a method of identification up to species level.

Many of the studies that examined MALDI-TOF MS for the identification of moulds used different extraction protocols, including commercially-developed and laboratory-developed procedures [16-21]. After growth, mould samples are inactivated via immersion in ethanol prior to analysis to prevent potential laboratory contamination. Following inactivation, a chemical solution is applied to extract the fungal proteins. To aid in the extraction process, manual and/or mechanical disruption of the fungal cell wall may be performed. Extraction protocols may involve multiple steps (i.e. wash, inactivation, sequential extraction with solvents) and require anywhere from minutes [19] to over an hour to perform [16, 22] before the sample can be analysed via MALDI-TOF MS. Due to the time burden of published extraction procedure (requiring a minimum of 35 minutes per clinical isolate, with approx. 1 min added for each additional sample), mold identification by MALDI-TOF MS had to be batched and was performed only twice weekly at NIH.

In order to study all the moulds isolated which shows growth on SDA plate within 12 to 24 hrs, a rapid, one-step extraction procedure was used for the identification of clinical mould isolates by MALDI-TOF MS. Analysis of clinical mould isolates found that the rapid extraction method reduced the 35 minutes per isolate processing time to only 5 minutes per isolate, with only a few seconds more for each additional sample [23]. Intact cell extraction methods, in which spores and mycelial material are deposited directly on the target plate, offer reduced extraction times [16, 19, 24, 25].

Over the last 5 years, accumulated experience clearly shows that MALDI-TOF MS holds promise as an accurate mould identification tool, particularly with common filamentous fungal pathogens. Conventional phenotypic methods to identify filamentous fungi are relatively inexpensive but have a turnaround time of several days because of the time taken for fungal growth, which in certain groups of fungi (e.g., dermatophytes) can be very long. As a result, initial antifungal therapy of infection is empirical, and appropriate therapy may be delayed if antifungal resistance is not suspected. The turnaround time has been reduced by the

introduction of MALDI-TOF MS instruments in the clinical laboratory routine, although these instruments continue to rely on fungal cultures. In this context, the MALDI-TOF MS technology is also being exploited to analyze patient specimens directly, completely bypassing the need for fungal growth [11]. In the meantime, rapid and accurate identification of fungi can be achieved directly from positive blood cultures.

Conclusion

MALDI-TOF MS has contributed to improving the laboratory diagnosis of infections by filamentous fungi in terms of rapidity and accuracy of identification. As fast and precise recognition of a fungal pathogen can result in a significant benefit for optimal patient treatment, MALDI-TOF MS-based identification should be integrated into antifungal stewardship strategies. Future studies are required to estimate the real impact of MALDI-TOF MS identification results on the clinical and therapeutic management of mould diseases.

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