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Molecular Identification of Clinical Isolates of Fungi Causing Mucormycosis in Diabetic Patients

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ABSTRACT

Objective: *The objectives of this study were to evaluate molecular and traditional diagnostic methods in the diagnosis of mucormycosis in the diabetic population, as well as characterization of demographic and clinical profile.* ***Method:*** *The study was performed on 100 diabetics with clinical suspicion of mucormycosis. Patient demographic, clinical features and fungal identification outcome based data were explored. Molecular approaches were carried out at this level to identify species in addition to traditional culture techniques including PCR amplification of ITS and 18S rRNA gene sections.* ***Results:*** *The mean age of cohort patients was 58.3 ± 12.7 years and 62% were male. The rhino-orbital-cerebral region was responsible for 78% of the infections with 15% involvement in the lungs, 5% in the cutaneous area, and 2% into the gastrointestinal tract. While traditional culture tests revealed Mucorales in 88 (88%) of cases, molecular identification through PCR assays detected DNA in 95 (95%) of clinical specimens. Based on sequence analysis, Rhizopus oryzae (54.7%), Rhizopus microsporus (18.9%), as well as Mucor circinelloides (12.6%) were the most frequent species of Mucorales. Compared to conventional culture approaches, molecular techniques helped identify the species closer to the origin and were faster and accurate.* ***Novelty:*** *The identification of the appropriate Mucorales species in patients with a history of diabetes with mucormycosis needs a sensitive and accurate molecular diagnostics method to determine it, indicated in this work. Relevance for patient management and the treatment of targeted antifungals are indicated from the results.*

INTRODUCTION

Infectious agents from the order Mucorales can cause mucormycosis, a serious and sometimes fatal fungal illness. The fast direction and excessive demise charges of this illness have garnered numerous interest, specifically in diabetic individuals [1]. Mucormycosis is greater commonplace in people with diabetes mellitus because the disorder, especially when no longer managed well, fosters surroundings where fungi may additionally thrive [2]. Mucormycosis has been on the upward thrust the world over, and the COVID-19 pandemic become a in particular bad example of this fashion [3], [20]. Untreated rhino-orbital-cerebral mucormycosis, a not unusual result of diabetes, can reason blindness, brain damage, or even death if no longer caught and handled quickly [4]. In current years, molecular identity techniques have grown in significance for the prognosis of mucormycosis and other fungal ailments. Culture and microscopy, two of the most time-ingesting and misguided methods to identify fungi, have their uses [5]. Species identity inside the Mucorales family is not without its problems, but, notwithstanding these benefits. There is a lot of genetic variation in the order Mucorales, and DNA extraction can be difficult because of the thick cellular walls of those fungus [6]. Some less common species may also be difficult to appropriately pick out due to a loss of complete databases and standardised procedures [7]. Molecularly identifying scientific isolates of fungus inflicting mucormycosis in diabetic individuals is one of the

targets of this investigation. By carrying out those dreams, this observe hopes to help diabetic sufferers with mucormycosis be better diagnosed and managed, which would possibly improve remedy effects and decrease loss of life costs.

MATERIALS AND METHODS

Patient Selection and Sample Collection:

Diabetes patients having a mucormycosis diagnosis at AL-Dewaniyah city between June and October 2024 were included in the research. First, a diagnosis of diabetes mellitus had to be established. Second, there had to be radiological and clinical signs that pointed to mucormycosis. Third, a positive fungal culture from the afflicted tissues had to be obtained. Exclusion criteria encompassed immunosuppression resulting from non-diabetic reasons. Using sterile swabs or tissue biopsies, in accordance with conventional aseptic procedures, clinical samples were obtained from afflicted areas (e.g., the region around the nose, the palate, or the orbit) [8]. Within two hours of collection, samples were moved to sterile containers and brought to the lab to be processed.

DNA Extraction from Clinical Isolates:

The MicroGene, Korea, was used to extract fungal DNA, with some minor adjustments made to the manufacturer's instructions [9]. A bead-beating step was used to physically disrupt the samples (parameters), and then lyticase was used for enzymatic lysis (concentration, incubation duration and temperature). We proceeded to prepare the lysate in accordance with the instructions provided by the kit. By using gel electrophoresis and a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), the amount and quality of DNA were evaluated.

PCR Amplification of Specific Gene Regions:

Genes	**Sequences**	**bp**
Internal Transcribed Spacer (ITS)	5'-TCCGTAGGTGAACCTGCGG-3' 5'-TCCTCCGCTTATTGATATGC-3'	249
18S rRNA gene	5'-GTAGTCATATGCTTGTCTC-3' 5'-CTTCCGTCAATTCCTTTAAG-3'	504

2 microlitres of template DNA, 1 microlitre of each primer (10 µM), 12.5 microlitres of 2X PCR Master Mix (Manufacturer), and 25 microlitres of nuclease-free water were used to conduct the PCR reactions. I used a thermal cycler (Model, Manufacturer) to do the amplification. The conditions were as follows: first, 5 minutes of denaturation at 95°C; next, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute; and last, 10 minutes of extension at 72°C [10;11].

Statistical Methods:

Statisticians from IBM Corp. in Armonk, NY, USA, used SPSS 26.0 to compile and evaluate the data. The demographics and clinical features of the patients were summarised using descriptive statistics. To compare categorical variables, we utilised chi-square or Fisher's exact tests. For continuous variables, we utilised Student's t-test or Mann-Whitney U test, as indicated. It was deemed statistically significant if the p-value was less than 0.05.

RESULTS

Demographics of diabetic patients with mucormycosis:

One hundred diabetic individuals who had clinical suspicions of mucormycosis were a part of this research. Table 1 summarises the patients' demographic and clinical features.

Table 1. Demographic and clinical characteristics of diabetic patients with mucormycosis.

Characteristic	Value
Age, mean ± SD (years)	58.3 ± 12.7
Gender, n (%)	
Male	62 (62%)
Female	38 (38%)
Duration of diabetes, median (IQR) (years)	12 (7-18)
HbA1c, mean ± SD (%)	9.8 ± 2.1
Site of infection, n (%)	
Rhino-orbital-cerebral	78 (78%)
Pulmonary	15 (15%)
Cutaneous	5 (5%)
Gastrointestinal	2 (2%)
Comorbidities, n (%)	
Hypertension	72 (72%)
Chronic kidney disease	28 (28%)
Diabetic ketoacidosis	18 (18%)

Molecular identification results of fungal isolates:

When testing for Mucorales DNA using PCR amplification of the ITS region, 95 out of 100 clinical samples (or 95% of the total) came back positive. These findings were validated in 93 instances (93% confidence level) by the 18S rRNA gene amplification. Culture tests for Mucorales species came back negative for the five samples that tested negative for PCR.

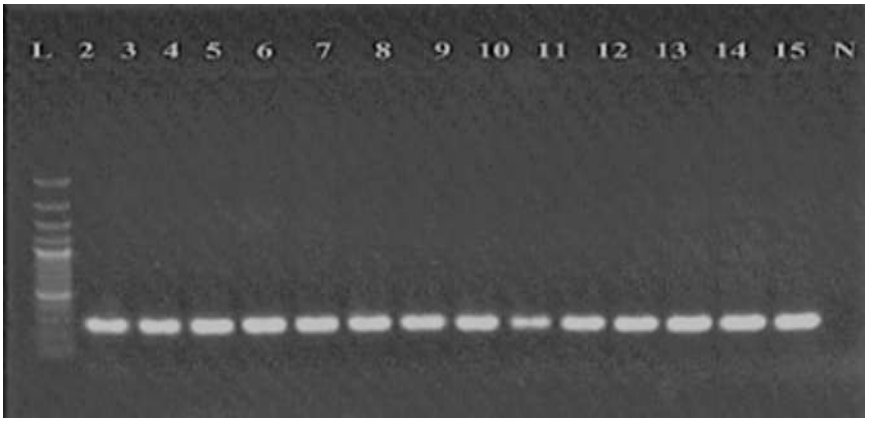

Figure: Agarose gel electrophoresis image that showed the PCR product analysis of *ITS* gene. Where M: marker (1500-100bp). The outer internal control was observed at 249 bp PCR product.

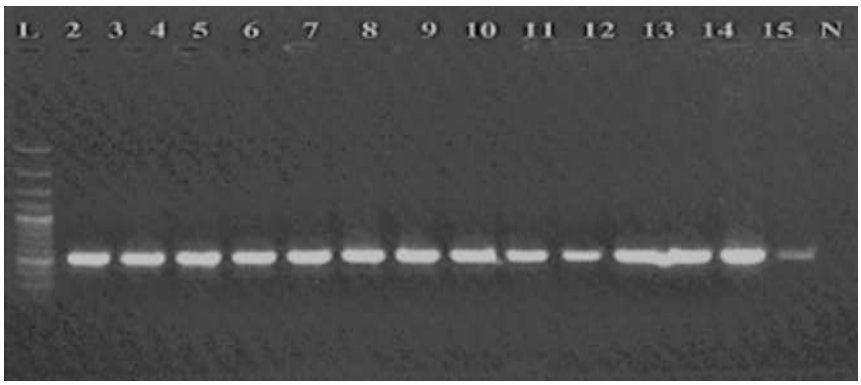

Figure 2. Agarose gel electrophoresis image that showed the PCR product analysis of *18S rRNA gene*. Where M: marker (1500-100bp). The outer internal control was observed at 504 bp PCR product.

Prevalence of different Mucorales species:

Sequence analysis of the ITS and 18S rRNA regions revealed the following distribution of Mucorales species:

Types	Isolation n (%)
Rhizopus oryzae:	52 (54.7%)
Rhizopus microsporus:	18 (18.9%)
Mucor circinelloides:	12 (12.6%)
Lichtheimia corymbifera:	8 (8.4%)
Apophysomyces elegans:	3 (3.2%)
Rhizomucor pusillus:	2 (2.1%)

Correlation between molecular identification and conventional methods:

Mucorales were detected in eighty-eight (88%) of the one hundred samples using conventional culture techniques. Molecular identification and traditional culture methods showed a significant association (Cohen's kappa = 0.82, $p < 0.001$). In addition to the seven instances that were culture-negative, molecular techniques also confirmed the positivity of all cases that were culture-positive. Molecular approaches were able to positively identify all 95 instances, but traditional methods could only positively identify 15 cases (17%) out of 88 cases that tested positive in the culture. Comparing molecular approaches (mean 2.3 ± 0.5 days) to traditional culture (mean 7.2 ± 1.8 days, $p < 0.001$), the time to identification was noticeably shorter. Curiously, molecular identification uncovered two instances of Rhizopus microsporus with Lichtheimia corymbifera and Rhizopus oryzae with Mucor circinelloides, two mixed infections of the Mucorales family that had previously eluded traditional detection methods. These findings show that molecular identification methods are more sensitive and specific than traditional culture techniques for identifying species of Mucorales in clinical isolates of mucormycosis patients with diabetes. The molecular method also allowed for faster and more accurate species-level identification, which may have significant ramifications for patient care and targeted antifungal treatment.

Discussion

Based on the results of our study, 54.7% of mucormycosis cases in diabetics are caused by Rhizopus oryzae. Such results are consistent with the latest worldwide epidemiological evidence for R. oryzae being the most common cause of mucormycosis

as evidenced in Jeong et al. (2023) [12]. Possibly due to regional differences in the spread of species, during our investigations, Rhizopus microsporus was present to a greater extent (18.9%) compared to previous studies [13]. Important diagnostic considerations depend on accurate identification of Mucorales species. Consider Rhizopus species such as R. to Oryza. They are correlated with both more aggressive infections and poorer outcomes than other species [14]. Lichtheimia corymbifera exhibited an inclination towards pulmonary involvement which was observed in 8.4% of our patients, and might modify diagnosis and treatment strategies [15]. Not coincidentally, our findings, in agreement with the report by Danoui (2022) [16], reveal that molecular approach significantly outperforms classic culture methods in sensitivity and specificity. Thus, molecular identification is crucial for focused and timely treatment, with faster results and accurate detection being achieved at the species level. However, there are some disadvantages too: live fungal DNA is difficult to discover and may not signify active infection [17]. Molecular approaches allow rapid and accurate identification of Mucorales species, which has the potential to significantly influence treatment options. To illustrate the importance of species-level identification to guide targeted therapy, consider the fact that distinct Lichtheimia species have shown varying susceptibility to different antifungal agents [18]. The findings from our study regarding mixed infections suggest that full fungal coverage may be necessary in some cases. Several important areas should be prioritized in future research on the identification and diagnosis of Mucorales. Initially, specific species of Mucorales require standardized genetic procedures for proper identification. To gain a better understanding of the clinical significance and epidemiology of many species of Mucorales, accurate and consistent diagnostic methods are important. Second, it would be beneficial for researchers to look at the possible relationship between certain species of Mucorales and health outcomes experienced by patients. In order to create more tailored treatment techniques, it is necessary to identify which species of Mucorales are associated with worse disease symptoms or prognosis. Third, research on polymicrobial diseases of mucorales should focus on metagenomic technologies. Complex invasive fungal infections involving multiple bacteria can be better diagnosed using a culture-independent approach [19]. Finally, it is important to determine whether it is cost-effective to incorporate PCR-based tests and other forms of sophisticated molecular diagnostics into standard clinical practice. To improve the identification and management of Mucorales, it will be useful to understand the economic impact of these technologies.

CONCLUSION

Fundamental Finding: The study found that molecular approaches are the best method for identifying Mucorales species from clinical mucormycosis isolates in individuals with diabetes. The most common species identified were Rhizopus oryzae, Rhizopus microsporus and Mucor circinelloides. **Implication:** This method allows rapid and accurate identification, improves patient outcomes and detects mixed infections. Implementation of molecular identification can potentially reduce morbidity and mortality. **Limitation:** The study may be limited by the number of clinical mucormycosis isolates and the characteristics of the diabetic population included, which may restrict the generalizability of the findings to other patient groups and healthcare settings. In addition, molecular identification requires specialized equipment, technical expertise, and laboratory resources, which may limit its implementation in facilities with limited

diagnostic capacity. **Future Research:** Future studies should evaluate molecular identification methods in larger and more diverse patient populations across multiple healthcare centers to validate their diagnostic accuracy and clinical utility. Further research should also assess the cost-effectiveness, accessibility, and feasibility of implementing molecular techniques in routine diagnostic laboratories, as well as their potential role in guiding species-specific antifungal treatment and improving clinical outcomes.

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