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Standardization of An in-House Conventional Pcr Assay for The Identification of *Bipolaris* And *Curvularia* Species From Clinical Isolates

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ABSTRACT
Bipolaris and *Curvularia* spp. are the common dematiaceous molds causing phaeohyphomycosis in humans. Accurate identification is essential for treating the patients with phaeohyphomycosis. Hence, this study standardizes a Polymerase Chain Reaction (PCR) assay for a reliable detection using specific primers, providing early diagnosis in infected patients. A total of 20 known archived isolates of *Bipolaris* and *Curvularia* from clinical isolates were taken up for standardization of the assay. Phenotypic identification was done by microscopy and cultural characteristics. PCR was performed using self-designed in-house primers for *Bipolaris* and *Curvularia* species. Sequence confirmed *Bipolaris* species and ITCC *Curvularia lunata* 6257 were used as the reference strain for PCR standardization. The assay was tested for other fungi of interest to check for the specificity. Of the 20 isolates, 17 isolates sporulated and 3 were non-sporulating. Among the 17 isolates, 12 were identified as *Curvularia* species and 5 were phenotypically identified as *Bipolaris*. The *Bipolaris* species produced a band at 243 bp, and the *Curvularia* species produced a band at 430 bp. Other dematiaceous and non-dematiaceous fungi did not produce any band. The PCR assay using in-house primers accurately identified all the *Bipolaris* and *Curvularia* species within 3 hours. Even the non-sporulating colonies can be identified using this PCR without any delay in diagnosis. It also helps to ascertain the prevalence database for epidemiological purposes.

INTRODUCTION:

Dematiaceous fungi are melanized environmental moulds commonly found in soil, decaying vegetation, and plant material. Owing to the presence of dihydroxynapthalene (DHN) melanin in their cell walls these fungi exhibit characteristic darkly pigmented hyphae and conidia and are capable of causing a wide range of infections collectively known as phaeohyphomycosis, chromoblastomycosis and eumycetoma. [1] Among the clinically significant dematiaceous fungi, *Bipolaris*, *Curvularia*, *Cladophialophora*, *Exophiala*, and *Alternaria* are frequently implicated in human disease [2]. Although distributed worldwide, these fungi are more prevalent in tropical and subtropical regions. In India, particularly in the southern states, *Curvularia* and *Bipolaris* species are increasingly recognized as

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important cause of fungal keratitis, sinusitis, allergic fungal rhinosinusitis, and cutaneous and subcutaneous infections likely due to favourable climatical conditions and increased environmental exposure [2].

Culture remains the gold standard for laboratory diagnosis, despite the availability of various diagnostic techniques for the detection of dematiaceous fungi. However, culture-based identification is often limited by various factors such as long turnaround times, variable sporulation and the possibility of contamination by environmental fungi. Accurate identification highly depends on the presence of characteristic reproductive structures, which may not easily develop in all isolates under routine laboratory conditions. Moreover, the slow growing nature and variable growth patterns of certain dematiaceous fungi in vitro can make accurate identification even more difficult.

For many clinically important mould infections genus-level identification can provide useful information for initial patient management. Delays in laboratory identification may result in inappropriate antifungal therapy, prolonged disease progression, and adverse clinical outcomes, especially in invasive infections and fungal keratitis. As conventional phenotypic methods often require prolonged incubation for definitive identification, there is a need for rapid and reliable molecular techniques to enable early detection and accurate differentiation of clinically important dematiaceous fungi.

In view of the limitations associated with conventional phenotypic identification, the present study was undertaken to standardize a conventional PCR assay for the rapid and accurate identification of *Bipolaris* and *Curvularia* species from clinical isolates. The assay was developed with the aim of providing reliable differentiation between these closely related dematiaceous fungi, particularly in situations where morphological identification is difficult because of overlapping phenotypic features or inadequate sporulation. The development of a standardized molecular approach may help in timely identification and serve as a useful adjunct to conventional methods in the diagnostic mycology laboratory.

MATERIALS AND METHODS:

Laboratory-based assay standardization study was conducted in the Department of Microbiology, Sri Ramachandra Institute of Higher Education and Research, Chennai. A total of 20 archived, sequence-confirmed clinical isolates belonging to the genera *Bipolaris* and *Curvularia,* recovered from various clinical specimens, were included for the standardisation of the in-house conventional PCR assay.

Phenotypic identification:

All isolates were sub-cultured on Sabouraud Dextrose Agar (SDA), Potato Dextrose Agar (PDA), and Oatmeal agar (OA) supplemented with antibiotics and without cycloheximide. The cultures were incubated at 25°C for 3–7 days and further extended until adequate sporulation was achieved. Colony characteristics showed colony texture, pigmentation, growth rate were assessed. Microscopic examination was performed using slide culture and banana peel culture methods to preserve the natural arrangement of fungal structures. Lactophenol Cotton Blue (LPCB) mounts were prepared and examined to assess conidial morphology, septation, conidiophore features and the pattern of conidial arrangement [3]

Isolates producing curved, multicellular conidia with a prominent enlarged central cell along with geniculate conidiophores were identified as *Curvularia* species, whereas isolates showing straight to slightly curved conidia without a swollen central cell were identified as *Bipolaris* species.[2]

Molecular identification:

DNA extraction:

Genomic DNA was extracted from fungal cultures using the phenol-chloroform-isoamyl alcohol extraction method with minor modifications. DNA quality and concentration were assessed before amplification. [4]

Pan-Fungal PCR Amplification:

All isolates were initially screened using pan-fungal primers targeting the internal transcribed spacer (ITS) region. The pan fungal primers used were ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′ & ITS4: 5′-TCCTCCGCTTATTGATATGC-3′. PCR amplification was carried out with an initial denaturation 95°C for 7 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 10 minutes. Amplified products were analysed by agarose gel electrophoresis and visualised under a UV transilluminator.

Sequencing and Species Confirmation:

The amplified ITS products from all isolates were subjected to bidirectional sequencing for species-level identification. The obtained sequences were analysed and compared with reference sequences available in the NCBI GenBank database using BLAST. Sequence identify was further verified using the MycoBank, ISHAM Barcoding Database, and CBS (Westerdijk Fungal Biodiversity Institute)

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databases. Isolates demonstrating ≥98% sequence similarity with reference strains were considered confirmed and included for further analysis.

Primer Design and In-Silico Validation:
Reference sequences for the ITS region of *Curvularia* (GenBank accession no. MN053005) and the translation elongation factor-1α (TEF-1α) gene of *Bipolaris* (GenBank accession no. OQ857394) were retrieved from the NCBI GenBank database. Multiple sequence alignment was performed in MEGA version 11 to identify conserved and genus-specific regions suitable for primer design. Genus-specific primers targeting *Curvularia* and *Bipolaris* were manually designed based on sequence variations identified during alignment. Primer specificity was evaluated using the NCBI Primer-BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi), followed by in silico PCR analysis (http://insilico.ehu.es/PCR/) to assess potential cross-reactivity with closely related fungal species and to predict expected amplicon sizes. The primers are listed in Table 1 below.

Table 1 – Primers for identification of *Bipolaris* and *Curvularia*

BPF	5` - TTGGTGTCAAGCAGCTCATC – 3`
BPR	5` - TCTTACCGGTGGACTTGGAC – 3`
CUF	5` - CACTTGTTGTTTCCTGGGCGGGTTCGCTCG - 3`
CUR	5`- CCGAGGTCAAACGTGAGAAG – 3`

Conventional PCR standardization:
All isolates were initially screened using pan-fungal ITS primers to confirm the presence of fungal DNA. Subsequently, genus-specific PCR assays targeting *Bipolaris* and *Curvularia* were standardized using the designed primer sets. Optimization of the assay was performed by gradient PCR to determine the optimal annealing temperature and amplification conditions.

The PCR protocol consisted od an initial denaturation at 95°C for 7 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension step at 72°C for 10 minutes. Amplified products were separated by agarose gel electrophoresis and visualized under ultraviolet illumination using a 100–1200 bp DNA ladder as a molecular size marker.

Sequence-confirmed *Bipolaris* isolates and *Curvularia lunata* ITCC 6257 were included as positive controls during assay standardization and validation.

Statistical Analysis:
Data were analysed using descriptive statistics. Categorical variables were summarized as frequencies and percentage. The performance of the genus-specific PCR assay was evaluated by comparing the PCR results with sequencing-based identification. Concordance between the two methods was assessed descriptively. As the primary objective of the study was assay development and standardization using archived isolates, inferential statistical analyses were not performed.

RESULTS:

A total of 20 archived clinical isolates of *Bipolaris* and *Curvularia* species were evaluated. On PDA and OA, *Curvularia* isolates exhibit olive-grey, woolly to cottony colonies, whereas *Bipolaris* isolates produce grey to brownish colonies with a similar texture.

Microscopic examination of LPCB mounts prepared from slide culture and banana peel culture showed characteristics morphological features. *Curvularia* species demonstrated curved, multicellular conidia with an enlarged central cell and geniculate conidiophores, whereas *Bipolaris* species revealed straight to slightly curved conidia lacking a distinctly swollen central cell.

Among the 20 isolates, 17 (85%) produced adequate sporulation, whereas 3 (15%) remained non-sporulating in spite of prolonged incubation. Based on phenotypic characteristics, 12 of the 17 sporulating isolates (70.6%) were identified as *Curvularia* species and 5 (29.4%) as *Bipolaris* species.

All isolates yielded amplification with the pan-fungal ITS primers and were subsequently subjected to genus-specific PCR using the in-house designed primers. The *Curvularia*-specific assay generated an amplicon of 430 bp, while the *Bipolaris*-specific assay produced an amplicon of 243 bp. No amplification was observed with other dematiaceous fungi (*Phialophora*, *Alternaria*, *Exserohilum*, and *Fonsecaea*) or non-dematiaceous fungi (*Fusarium*, *Aspergillus*, Mucorales, and *Acremonium*), indicating high analytical specificity of the assay.

Genotypic identification classified 14 (70%) isolates as *Curvularia* species and 6 (30%) as *Bipolaris* species. Species-level identification by sequencing revealed 7 isolates as *Curvularia lunata*, 4 as *Curvularia geniculata*, 3 as *Curvularia spicifera*, 1 as *Bipolaris maydis*, and 5 as *Bipolaris* species. The results obtained by genus-specific PCR showed complete concordance with sequencing-based identification.

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Curvularia lunata	7
Curvularia geniculata	4
Curvularia spicifera	3
Bipolaris maydis	1
Bipolaris species	5

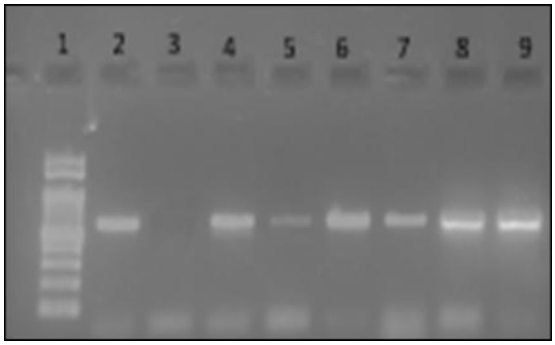

Figure 1 – Agarose gel electrophoresis showing conventional PCR using genus specific *Bipolaris* and *Curvularia* primers (Lane 1- Ladder, Lane 2- PC- *Curvularia* spp., Lane 3- NC, Lane 4-6- *Curvularia* spp., Lane 7-9- *Bipolaris* spp.)

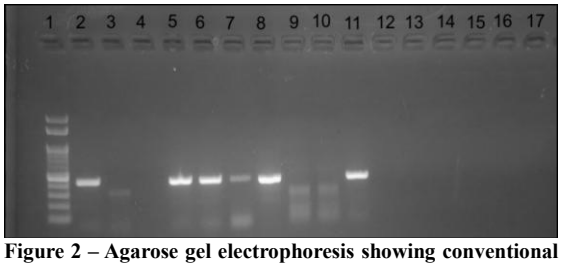

Figure 2 – Agarose gel electrophoresis showing conventional PCR using genus specific *Bipolaris* and *Curvularia* primers (Lane 1- Ladder, Lane 2- PC- *Curvularia* spp., 3- PC- *Bipolaris* spp., Lane 4- NC *Candida* ATCC, Lane 5 – 8, 11 - *Curvularia* spp., Lane 9 & 10 - *Bipolaris* spp., Lane12- *Mucor* spp., Lane 13- *Aspergillus* spp., Lane 14- *Fonsecaea* spp., Lane 15- *Fusarium* spp., Lane 16- *Exserohilum* spp., Lane 17- *Alternaria* spp.)

Due to the considerable morphological overlap between Curvularia and Bipolaris species, accurate differentiation based solely on phenotypic characteristics may be challenging. The development of genus-specific primers provides a rapid and reliable approach for distinguishing these closely related fungi. In the present study, the results were obtained by conventional PCR were in complete agreement with sequencing-based identification, demonstrating full concordance between phenotypic and genotypic methods for the isolates evaluated.

DISCUSSION:

The accurate identification of dematiaceous fungi remains a challenge for clinical microbiology owing to overlapping of morphological features and variation in phenotypic expression. Identification is primarily based on sporulation and on recognition of microscopic features such as conidial curvature, septation and hilum morphology. [2] However these features may not be expressed consistently under routine laboratory culture conditions. In the present study, 17 out of 20isolates were identified at the genus level based on morphology and the remaining 3isolates were non-sporulating after prolonged incubation. Such observation has been reported earlier in which non-sporulating dematiaceous fungi posed a major diagnostic challenge and were often misidentified as sterile mycelia resulting in delay or incomplete identification. [5]

The increase in recognition of *Curvularia* and *Bipolaris* infections especially in tropical and subtropical regions mainly highlights the importance of accurate diagnostic technique. *Curvularia* species are one of the common causes of dematiaceous fungal keratitis in India. *Bipolaris* species have been implicated in keratitis, sinusitis and allergic fungal rhinosinusitis and invasive infections particularly in immunocompromised individuals. Thus, precise identification is important not only for patient management but also for the understanding of the epidemiology and emerging trends of these infections. [6]

Molecular methods have greatly enhanced the accuracy and reliability of fungal identification. Among these PCR based assays targeting the Internal Transcribed Spacer (ITS) region are widely used because of their reproducibility and their ability to discriminate between closely related fungal species. [7] Furthermore multi-locus phylogenetic studies have demonstrated that reliable differentiation within the *Bipolaris-Curvularia* complex frequently requires the analysis of multiple genetic loci, such as Internal Transcribed Spacer (ITS), large subunit (LSU) and various protein coding genes. [6] Although these approaches provide a high level of taxonomic resolution, their routine use may be constrained by increased cost, increased technical expertise, longer turnaround times which can limit their applicability in routine clinical diagnosis.

Several molecular techniques, including Restriction Fragment Length Polymorphism (RFLP), have been utilized for differentiation of fungal species. However, their routine application is often limited by the need for additional post amplification processing steps and the longer turnaround time needed to obtain results [8]. Multiplex PCR assays offer the advantage of detecting multiple fungal pathogens in a single reaction but achieving consistent specificity and reproducibility requires careful optimization. This requirement may pose challenges for their adoption, particularly in laboratories with limited resources. [9]

In the present study, a conventional PCR assay using in-house designed genus-specific primers was standardized for the differentiation of *Bipolaris* and *Curvularia* species. The assay produced amplicons

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of distinct sizes measuring 243 bp for *Bipolaris* and 430 bp for *Curvularia* enabling reliable differentiation between the two genera. No amplification was detected with other dematiaceous fungi, including *Phialophora, Alternaria, Exserohilum and Fonsecaea*, or with non-dematiaceous fungi such as *Fusarium, Aspergillus, Mucorales* and *Acremonium.* These findings indicate a high degree of analytical specificity for the assay.

A notable advantage of the assay was its ability to identify isolates that failed to sporulate under culture conditions. Non-sporulating dematiaceous fungi often present a challenge for identification by conventional phenotypic methods and may be reported as sterile mycelia. In the present study, the PCR-based approach enabled reliable identification irrespective of sporulation status, thereby addressing an important limitation of culture-based identification. [5, 10]. In addition, the assay could be completed within approximately three hours, resulting in a substantially shorter turnaround time compared with conventional culture-based identification and sequencing methods.

Accurate and timely identification of dematiaceous fungi has important therapeutic implications, as antifungal susceptibility profiles may vary among species. Delays in laboratory diagnosis often necessitate empirical treatment, which may contribute to prolonged disease progression, repeated surgical interventions, prolonged hospital stay and poorer clinical outcomes, especially in cases of fungal keratitis. Earlier identification of the causative fungus may assist in the timely selection of appropriate antifungal therapy and thereby support improved clinical management.

Mythili et al. reported that ITS- based PCR followed by sequencing was useful for the accurate species-level identification of dematiaceous fungi [11]. Although sequencing provides definitive identification and remains an important taxonomic tool as its routine use is often limited by the need for specialized infrastructure, technical expertise, and longer turnaround times. In comparison, the conventional PCR assay standardized in the present study provides a simpler, rapid and cost-effective approach for the differentiation *Bipolaris* and *Curvularia* isolates at the genus-level identification.

The present study was subject to certain limitations, including the relatively small number of isolates examined and the inclusion of only selected *Bipolaris* and *Curvularia* species. Further validation using a larger and more diverse collection of clinical and environmental isolates is required to establish the broader applicability and diagnostic performance of the assay.

The results of this study suggest that genus-specific conventional PCR assay can serve as a valuable complement to phenotypic methods for the identification of dematiaceous fungi. The important advantage of this assay is its ability to identify isolates irrespective of their sporulation characteristics addresses an important limitation of conventional culture-based methods. The use of such a molecular approach may enable earlier laboratory identification and support clinical management, particularly in laboratories where sequencing facilities are not readily available. However, evaluation of the assay using a larger number of clinical isolates from different geographic regions would be useful in assessing the wider applicability of this assay in diagnostic laboratories.

CONCLUSION:

In the present study an in-house conventional PCR assay was standardized for the identification of *Bipolaris* and *Curvularia* species from clinical isolates. This assay accurately differentiated the two genera and the results were in complete agreement with sequencing based identification, including non-sporulating isolates that could not characterized phenotypically. Due to its rapid turnaround time, specificity, and ease of implementation, this assay may serve as a useful tool for routine identification of these clinically important dematiaceous fungi in diagnostic mycology laboratories.

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CONFERENCE PRESENTATION: This study has been presented at MYCOCON 2026 at Chandigarh.

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