

Potential Pathogens among Fungi Identified as Nonsporulating Molds from Blood Cultures

Mohd Fuat Abd Razak*, Parameswari Sabaratnam, Rahizan Issa

Infectious Diseases Research Centre, Institute for Medical Research, Kuala Lumpur, Malaysia
Email: *fuat@imr.gov.my

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Abstract

Twenty-eight molds were isolated from clinical blood cultures and were unidentifiable by cellular morphology using conventional microscopy. Using the utility of amplification and direct sequencing of internal transcribed spacer region of ribosomal RNA gene, 93% of these fungi were identified. Seventy-one percents of the molds were found to be associated with plants or soil with no or few published cases of human disease. These include species of basidiomycetes and ascomycetes such as *Botryosphaeria dothidea*, *Phomopsis flavodonflavus*, *Inonotus pachyploeus*, *Earlilella scabrosa*, *Calocybe indica*, *Athelia pellicularis*, *Tinctoporellus epimiltinus*, *Trametes lactinea*, *Coprinellus aureogranulatus* and *Xylaria feejeensis*. Some of the nonsporulating molds were identified as pathogen or potential pathogens in immunocompetent or immunocompromised hosts. These include *Schizophyllum commune* and hyphomycetes such as *Cladosporium cladosporoides*, *Aspergillus niger* and *Fusarium equiseti*. Basidiomycetes and hyphomycetes identified in the current study are ubiquitous in the environment and are almost similar to the species of molds reported from cutaneous and respiratory samples suggesting that the fungi may represent contaminants rather than true fangaemia. Results of this study emphasize the need of an effort to minimise blood culture contamination and support the recommendation to incorporate clinical, radiologic findings and positive blood culture for molds in the diagnosis and management of invasive mycosis.

Keywords

Blood Cultures, Fungi, Nonsporulating Molds, Basidiomycetes

*Corresponding author.

1. Introduction

The incidence of fungal infections by filamentous fungi (molds) is increasing especially among immunocompromised patients. While *Aspergillus*, *Zygomycetes* and *Fusarium* are the most commonly recognized molds, there are large and heterogenous group of molds that are capable causing infection in both immunocompromised and immunocompetent patients. Thus, accurate diagnosis of the causative agents is essential before initiation of antifungal therapy.

Culturing clinical specimens for fungus in the diagnosis of fungal infection is still a gold standard in clinical mycology laboratory. Molds isolated from a positive direct microscopy of clinical specimens are always significant isolates. However, fungal elements in clinical specimens are sparse. Thus, isolation of molds from negative direct microscopy specimen does not rule out fungal infection. It is not uncommon that many clinical mycology laboratories do not perform direct microscopy observation on the clinical specimens.

Molds that grow from clinical specimens are identified by characterizing the reproductive spore under microscope. A mold cannot be identified by microscopic morphology if it does not sporulate in culture media within 2 - 3 weeks. Laboratories report these fungi to clinicians as mycelium sterile or nonsporulating mould (NSM). These reports are rather inconclusive but most clinicians would not treat patient if the NSM are isolated from superficial, cutaneous or respiratory specimens.

In recent years, DNA-based methods have been developed and played a role as adjunctive tool for the identification of pathogenic fungi in certain reference laboratories. Amplification by polymerase-chain-reaction (PCR) and sequencing of the internal transcribed spacer (ITS) regions I and II in the ribosomal RNA operon of fungi have been found to have sequence variability and have been used to identify fungi to species level [1] including NSM [2] [3].

Nonsporulating molds have been isolated and reported from superficial, cutaneous and respiratory tract specimens [4]. To our knowledge no study has attempted to classify NSM fungi associated with systemic clinical samples such as blood. Unlike superficial and cutaneous fungal infections, systemic infections can be life-threatening especially in the immunocompromised patients. Blood samples are the most common clinical samples sent to pathological laboratories to determine systemic fungal infection. The aim of this study was to determine the identity of NSM isolated from clinical blood samples by sequencing of ITS regions in order to detect clinically significant pathogens among these previously unidentifiable fungi.

2. Materials and Methods

2.1. Fungal Isolates

Mycology laboratory in Institute for Medical Research, Kuala Lumpur, Malaysia, has been a reference laboratory for identification of fungus isolated from clinical specimens. In 2013, the laboratory received 955 fungal isolates for identification from various hospitals in Malaysia. In the laboratory, the mould submitted as an isolated culture, was first grown on Sabouraud agars (Oxoid), Cornmeal agar (Merck) and Malt extract agar (Oxoid). Microscopic cellular structures were then observed on standard tease slide preparation for up to 21 days. The culture was also grown on Potato-dextrose agar (Oxoid) using the standard slide culture method and sporulation was observed for up to 21 days. Nonsporulating molds were confirmed after the filamentous fungus failed to form any sexual or asexual spores on the media. Out of 955 fungal isolates received, 112 were NSM (32.5%) and 28 of the NSM were from fungal blood culture bottles of automatic blood culture system such as BACTEC. All 28 NSM isolates were grown on Potato dextrose agar for gene amplification and sequencing.

2.2. DNA Extraction

All chemicals and reagents used in the DNA extraction were purchased from Qiagen (Germany). After growth for 7 days on Potato dextrose agar slants, approximately 1 cm² of NSM fungal mycelia were scraped into 500 µl PBS and centrifuged at 18,000 g for 1 min. The supernatant was removed and tissue lysis buffer and proteinase K were added followed by incubation at 55°C for 18 h. Lyticase was added and the reaction mixture was incubated at 37°C for 1 h. The genomic DNA was then extracted and isolated using QIA quick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's instructions.

2.3. Polymerase-Chain Reaction and DNA Sequencing

Polymerase-Chain-Reaction (PCR) was performed using primers ITS1 and ITS4 purchased from Intergrated

DNA Technologies Inc. (IDT, USA). All chemicals and reagents for PCR and preparation of DNA for sequencing were purchased from Qiagen (Germany). A 25 µl-PCR mixture was prepared as follows: 2.5 µl 10× PCR buffer with final MgCl₂ concentration of 1.5 mM, 0.5 µl of 0.2 mM deoxynucleoside triphosphates, 0.25 µl each of the forward and reverse primers, 5 µl template DNA, 0.125 µl Qiagen Hotstar Taq polymerase (5 U·µl⁻¹) and molecular biology grade H₂O was added to a total volume of 25 µl. Amplifications were carried out in thermocycler with the following thermal cycling parameters: initial denaturation at 95°C for 5 min, followed by heat denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. Ten microlitre PCR products were sent to 1st BASE Laboratories, Kuala Lumpur, for sequencing. The sequences were then identified using the BLAST program (<http://www.ncbi.nlm.nih.gov>). Sequence-based identifications were defined by percent identity: species, ≥99%; genus, 94% - 98%; and inconclusive, ≤93%. Molds were classified as fungi often associated with clinical disease (pathogens), fungi with more than three cases reported (potential pathogens), or plant/soil-associated fungi with no published cases of human disease [4].

3. Results

Table 1 shows the identification of the 28 NSM isolates. Twenty-six isolates (93%) were identified to at least genus (19 isolates were identified to species) while 2 being inconclusive. Twenty different genera of molds were

Table 1. Identification of nonsporulating molds from blood cultures by gene amplification and sequencing of ITS region.

Culture code	Closest BLAST match	Identity (%)	GenBank Accession No.	No. of species with ≥99% homology	Microorganism classification
123	<i>Nigrospora</i> spp	100	HQ248210	2	Plant/soil
175	<i>Fusarium equiseti</i>	99	HO718414	4	Potential pathogen
177	<i>Schizophyllum commune</i>	100	AF350925	1	Pathogen
216	<i>Xylariaceae</i> spp.	100	JN418798	2	Plant/soil
263	<i>Cladopsporium cladosporoides</i>	99	EU645693	2	Pathogen
270	<i>Botryosphaeria dothidea</i>	99	AY626347	4	Plant/soil
277	<i>Phomopsis longicolla</i>	100	FJ478126	0	Plant/soil
293-2	<i>Pseudozyma churashimaensis</i>	97	AB548943	0	Plant/soil
313	<i>Aspergillus niger</i>	99	AM745113	1	Pathogen
357-2	<i>Inonotus pachyphloeus</i>	98	AY558635	0	Plant/soil
460-2	<i>Earliella scabrosa</i>	98	JN165006	0	Plant/soil
562	<i>Aspergillus oryzae</i>	100	AKXL01000280	3	Potential pathogen
603	<i>Calocybe indica</i>	88	GQ259881	0	Plant/soil
662	<i>Flavodon flavus</i>	100	FJ478126	2	Plant/soil
710	<i>Tinctoporellus epimiltinus</i>	98	GU731575	0	Plant/soil
739	<i>Trametes lactinea</i>	98	HM756193	0	Plant/soil
340/12	<i>Aspergillus sydowii</i>	98	HO889716.1	0	Potential pathogen
340	<i>Pseudozyma</i> spp.	99	AM176732	3	Potential pathogen
809	<i>Cerrena</i> spp.	99	FJ010208	2	Plant/soil
810	<i>Coprinellus aureogranulatus</i>	99	GO249274	1	Plant/soil
811	<i>Entomocorticium</i> spp.	94	DO118417	0	Plant/soil
813	<i>Fomitopsis ostreiformis</i>	100	FJ372684	3	Plant/soil
876	<i>Penicillium</i> spp.	97	HM773242	0	Plant/soil
903	<i>Schizophyllum commune</i>	99	FJ478109	3	Pathogen
977	<i>Pseudozyma parantarctica</i>	99	AB089356	1	Potential pathogen
983	<i>Coriolopsis polyzona</i>	99	FJ627248	1	Plant/soil
1023	<i>Eutypella scoparia</i>	84	EU436688	0	Plant/soil
1056	<i>Xylaria fejeensis</i>	98	GU322454	0	Plant/soil

conclusively identified. Fifteen of the 28 isolates were basidiomycetes which include *Botryosphaeria dothidea*, *Phomopsis flavodonflavus*, *Inonotus pachyphloeus*, *Earlilella scabrosa*, *Calocybe indica*, *Athelia pellicularis*, *Tinctoporellus epimiltinus*, *Trametes lactinea*, and *Coprinellus aureogranulata*. Seven isolates were hyphomycetes such as *Cladosporium cladosporoides*, *Aspergillus niger*, *Aspergillus sydowii*, *Aspergillus oryzae*, *Fusarium equisetii*, *Nigrospora* species and *Penicillium* species. Six ascomycetous fungi such as *Xylaria feejeensis*, *Phomopsis longicolla* and *Pseudozyma* were also identified.

4. Discussion

Majority of molds grew in the blood cultures (71%) were associated with plants or soil with no published cases of human disease. These include filamentous basidiomycetes such as *Botryosphaeria dothidea*, *Phomopsis flavodonflavus*, *Inonotus pachyphloeus*, *Earlilella scabrosa*, *Calocybe indica*, *Athelia pellicularis*, *Tinctoporellus epimiltinus*, *Trametes lactinea*, and *Coprinellus aureogranulata*, and ascomycetes such as *Xylaria feejeensis* and *Phomopsis longicolla*. However, there are more than 70 reported cases of human infection (mostly through respiratory tract) by the basidiomycetous fungi *Schizophyllum commune* [5]. Invasive infections of the lung, brain and palate in immunocompromised and immunocompetent patients have been described [6].

Basidiomycetes have been known not to produce recognizable spores on mycological media commonly used for culturing medically important fungi such as Sabaouraud dextrose agar, Malt extract agar and Potato dextrose agar. Microscopic examination revealed hyaline, septate hyphae without any particular features. Some isolates showed clamp connections which indicate it belongs to *Basidiomycetes*. The presence of clamp connections and lateral, short, thin truncate hyphal tubercles are diagnostic of *S. commune*. However, clamp connections and truncate hyphal tubercle may be overlooked or may not develop in certain isolates [7]. Isolation of *Pseudozyma parantarctica* in blood samples was first reported in 2003 and there has been no report of human infection by *P. churashimaensis*. More than 10 species of *Pseudozyma* have been described to-date. However, human infections have been more frequently associated with *P. aphidis* than any other species of *Pseudozyma*. *Pseudozyma aphidis* has recently been implicated in potentially fatal disorders ranging from subcutaneous mycoses to disseminated infections [8].

Although reports on human infections by hyphomycetes *Cladosporium cladosporoides* are rare, *C. cladosporoides* were reported as etiologic agents of systemic phaeohyphomycosis in immunocompetent [9]. *Aspergilli* normally sporulate on Sabaouraud agar or Malt extract agar but were observed as hyaline mycelium in the current study. Reports on nonsporulating *Aspergilli* were not uncommon [2]. Three species of *Aspergillus* were identified from the blood cultures but only *A. niger* could be considered as pathogen especially in otitis media and cutaneous infections. *Aspergillus niger* is considered to be a fungus of low virulence and was not frequently implicated in invasive disease in immunocompetent patients. The advent of organ transplants and immunosuppressive therapy has shown that *Aspergillus niger* has the ability to initiate invasive disease in this group of patients [10]. *A. sydowii* and *A. oryzae* may cause invasive infection in those with more profound immunosuppression [11]. When not sporulating on medical mycology laboratory growth media, hyphomycetes will be observed as septate hyaline or dematiaceous mycelium without any particular features.

Despite method such as PCR may lead to the identification of the NSM, the significance of isolation of the NSM need to be confirmed. Rate of isolation of filamentous molds in commercial blood culture systems that represented true fungemia has been very low and involved fungi mostly in the genera of *Fusarium* and *Aspergillus* [12]. Molds other than *Fusarium* and *Aspergillus* spp. represented 1% of all positive fungal blood cultures in a study of 185,000 blood cultures [13]. The blood culture growth medium was found not supportive for growth of many molds including some common hyphomycetes such as *Penicillium* species, *Exophiala dermatidis*, *Phialophora verrucosa*, *Acremonium* species and *Alternaria alternata* [14]. It may also not supportive for growth of basidiomycetes as little or no reports on basidiomycetes were isolated from blood cultures. Numerous reports on systemic infections by basidiomycetes and other filamentous molds showed that blood cultures were mostly negative and infections were detected by radiographic procedures [15] [16]. Most laboratories withdraw some culture broth using hypodermic syringe from blood culture detected for growth by BACTEC machine and culture this broth onto growth agar media. However, NSM are not likely to be grown on subculture from culture broth withdrawn with hypodermic syringe because clumps of mycelium would not rapidly travel down into the neck of the bottle when inverted. As such, no hyphae would be drawn out during the subculture of the blood onto solid media [14] [17]. In a report, growth of filamentous fungi in blood culture was confirmed by visualization

of hyphae in the culture broth after repeated subcultures of culture broth on agar plates yielded no growth [18].

Although *S. commune*, *C. cladosporoides* and *A. niger* are pathogenic and clinically significant pathogenic fungi, these fungi and all other basidiomycetes, ascomycetes and hyphomycetes identified in the current study are ubiquitous in the environment and are almost similar to the species of NSM reported from cutaneous and respiratory samples elsewhere [3] [4] suggesting that the fungi may represent contaminants rather than true fungemia. Contaminant fungi may be introduced into blood culture bottle from blood samples (contaminants on the skin or sampling devices), inoculation of blood sample into blood culture bottle through rubber septum and during subculture of culture broth from blood culture bottle onto Mycological growth media such as Brain-heart-infusion agar (laboratory contaminants) after positive growth signal was detected by the BACTEC machine. Even though aspergillemia is one of the most common invasive filamentous molds infections and have been detected by blood culture, a study on 300,000 blood samples in wards of cancer patients showed that majority of Aspergilli isolated from these patients (solid tumours and hematologic malignancies) represented contamination [19]. In a study of high-risk patients including cancer patients, 70% of the positive blood cultures for molds were false-positive [13].

In the past decade, filamentous basidiomycetes have been reported as invasive pathogens in immunosuppressed patients, such as patients with hematological malignancies and solid-organ transplant recipients. Furthermore, the resistance to amphotericin B reported for some NSM emphasizes the need for minimizing the rate of false-positive blood culture. Eliminating sources of blood culture contamination requires an effort of both laboratory and ward personnel. Aseptic techniques should be strictly adhered in sampling and inoculating blood samples into blood culture bottles. There is a need for a good quality control of mycological media and inoculating devices including biosafety cabinet. Laboratory personnel should from time to time determine the load of fungal spores and the most common contaminants in the laboratory. They should be cautious if the same species of environmental fungi including NSM was isolated from several blood cultures. The European Organization for Research and Treatment of Cancer incorporate clinical, radiologic, microscopic and culture results in the diagnosis and management of systemic infections associated with filamentous fungi [20]. These criteria are recommended in the cases of positive blood cultures associated with NSM.

5. Conclusion

Results of the current study show that all NSM isolated from the blood cultures were environmental fungi with only 3 species having been reported to be pathogenic. Species of the NSM were similar to most NSM identified from cutaneous and respiratory specimens and isolation of these isolates may not reflect true fungemia.

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