ORIGINAL ARTICLE

Molecular Identification of Fungi Causing Tissue Mycoses From Formalin Fixed Paraffin Embedded (FFPE) Archive Specimens

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ABSTRACT

Introduction: Isolation of fungi from tissue specimens using conventional methods is time consuming. However, in some cases, the histopathological examination (HPE) of tissue alone is unable to provide a definite identity of the fungus. Alternatively, a non-culture method, such as polymerase chain reaction (PCR) detecting the internal transcribed spacer (ITS) rRNA genes of the fungi, is a promising diagnostic tool for rapid and accurate diagnosis of tissue mycoses. **Methods:** This work investigated the utility of panfungal PCR in identifying agents of tissue mycoses in 87 FFPE archive specimens. Deoxyribonucleic acid (DNA) extraction was performed on FFPE specimens by using QIAamp DNA FFPE Tissue Kit. The ITS2 region was amplified using ITS3/ITS4 primers. The PCR products were sequenced using the same primers and compared to the NCBI nucleotide database for species identification. **Results:** Fungal DNA was successfully amplified in 52 (59.8%) specimens, from which only 23 (44.0%) fungi were consistent with clinical/HPE findings. The identified fungi were *Aspergillus spp., Candida spp., Penicillium spp., Cryptococcus neoformans, Talaromyces marneffei*, and *Rhizopus oryzae*. A few rare fungi were also identified, such as *Diaporthe longicolla* and fungus-like oomycete such as *Pythium insidiosum* that are commonly associated with plant pathogens. **Conclusion:** Although PCR was able to offer accurate genus/species identification, utilising this method on paraffinised tissue specimens must be evaluated by considering many factors that will reduce its sensitivity and specificity. Therefore, it is important to correlate the PCR results with clinical and HPE findings to obtain a correct diagnosis and adequate treatment for tissue mycoses.
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Keywords: Formalin fixed paraffin embedded tissue, Tissue mycoses, Polymerase chain reaction

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INTRODUCTION

Despite fungal infections showing good response to many antifungal agents, the major challenge in the management of tissue mycoses is making an accurate diagnosis in a timely manner (1). The clinical manifestation of invasive mycoses usually mimics other diseases, especially in the early phase of infection (2). In many diagnostic microbiology laboratories, culture with biochemical and morphological examination is the gold standard method for the diagnosis of fungal infection (1). This conventional method requires viable pathogens in the clinical sample (3). As many fungi are slow growers, a long incubation period is required for their growth. The time-consuming factor in conducting laboratory diagnosis by conventional method further delays the appropriate therapy, resulting in high morbidity and mortality (4).

Fungal infection is usually subtle, and this causes the low index of suspicion among the clinicians. Most tissue biopsy specimens sent to the laboratory are indicated for histopathologic examination without microbiology investigation (5). When the histopathologic features are likely to be fungus, the specimen is no longer suitable for fungal culture due to chemical and physical treatment during sample processing (5-6). Histological examination of tissue mycoses may show granulomatous changes and tissue necrosis with the presence of hyphae or fungal bodies. Tissue staining by Periodic acid–Schiff (PAS) and/or Grocott's methenamine silver (GMS) can detect fungal cell wall component but it is unable to identify the genus/species in some cases (7). Serological test can be used as a predictive marker in diagnosing fungal infection. However, the sensitivity and specificity

vary, and they are greatly dependent on the tested population and type of the fungal species (8-10).

Alternatively, a non-culture method, such as nucleic acid testing using PCR, provides rapid detection of pathogen from fresh clinical specimens, as well as pretreated specimens, including FFPE tissue. This method amplifies the internal transcribed spacer (ITS) regions, which carry different sequences in each fungus (11). The hypervariability of the ITS region has made panfungal PCR with sequence analysis one of the useful methods to diagnose tissue mycoses (12-13). Several international studies have documented the usage and successful performance of panfungal PCR from FFPE samples (6, 12-15). The aim of this study was to determine the applicability of PCR as an alternative diagnostic tool for the diagnosis of tissue mycoses.

MATERIALS AND METHODS

Sample collection

In this study, FFPE specimens that were previously analysed by HPE were used. Eighty seven (87) archived FFPE tissue specimens that gave positive reactions with GMS and PAS stains and with histopathological report as "confirmed fungal infection" or "suggestive of fungal infection" were selected. The specimens were obtained from the Pathology laboratory in Hospital Serdang, Malaysia, where the specimens were kept between January 2013 and December 2018. Using sterile microtome blade, the tissue blocks were cut into sizes ranging between 5 and 10 µM and transferred into a 10-ml centrifuge tube before DNA extraction.

DNA extraction, amplification, and amplicon sequencing

DNA extraction was performed on FFPE tissue by using QIAamp DNA FFPE Tissue Kit (Qiagen, U.S.A) following the manufacturer's protocol. Each extracted DNA samples was later subjected to PCR in a 50-µL volume consisting of 25 µL of mastermix (GoTaq® Green PCR master mix, Promega Corporation), 1.5 µl of universal fungal primers targeting ITS2 region; ITS3F, 5'-GCATCGATGAAGAACGCAGC-3'; ITS 4R, 5'TCCTCCGCTTATTGATATGC-3' (16), 12 µL of nuclease free water and 10 µL of DNA. The thermal cycling conditions were 95°C for 10 min, followed by 50 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. Nuclease free water was used as a negative control and *Candida spp.* was used as positive control. The PCR products were separated by 1.5% of agarose gel electrophoresis, stained with Floro+Green DNA gel stain and visualised under ultraviolet (UV) light transillumination (Fig. 1). The PCR products were purified and sequenced using the same PCR primer that amplified the ITS2 region of the fungi. The sequences were compared to the NCBI nucleotide database with the BLAST searching program for species identification (http://www.ncbi.nlm.nih.gov/blast). The blast result of more than 95% similarity was taken as a significant identification of the fungus.

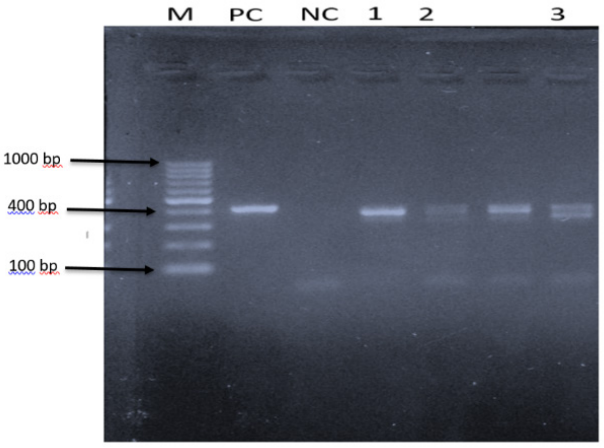

Figure 1: Agarose gel electrophoresis PCR of the ITS 2 region. Lane M :100 bp DNA ladder; lane PC: positive control (*Candida albicans*); lane NC: negative control (nuclease free water); lane 1: single band; lane 2: faint band; lane 3: overlapping double band.

Contamination control

To prevent any potential contamination, the working exterior surfaces and all equipment such as table top, cabinet, pipettes and rack were cleaned with 70% alcohol concentration. Extraction process, PCR mastermix preparation, PCR amplification set up and agarose gel electrophoresis were carried out in a separate room to prevent contamination from carryover. The mastermix was performed in the laminar flow cabinet wiped with 70% alcohol and irradiated with UV for 20 minutes. Filtered micropipette tips were used for the whole process from DNA extraction until PCR.

RESULTS

Out of 87 FFPE tissue samples, 52 gave clear bands, while another 35 produced faint bands. All the 87 PCR products were outsourced for purification and sequencing services. Fifty two (59.8%) clear-band PCR products were successfully sequenced and identified while another 35 (40.2%) faint-band PCR products failed to be identified. Among the 52 clear-band PCR products, 40 showed amplifications of single band, and 12 showed double overlap amplifications. The overlap bands may indicate presence of two fungi in the specimen. However, this work had only managed to sequence one saprophytic fungus from the bands. *Aspergillus tardicrescens* was the most common fungi detected which contributed to 26 (50.0%) of the findings followed by *Candida tropicalis* and *Candida species*, each contributed to 4 (7.7%) samples, *Candida glabrata*, *Candida albicans*, and *Penicillium spp.* detected in 3(5.8%) samples each, *Aspergillus niger* detected from 2 (3.8%) sample. Meanwhile, *Aspergillus fumigatus*, *Candida orthopsilosis*, *Cryptococcus neoformans*,

Diaporthe longicolla, Pythium insidiosum, Rhizopus oryzae and *Talaromyces marneffei* contributed to 1 (1.9%) sample, each (Table I).

Table I: Species, frequency and result interpretation of fungi from FFPE tissue specimens (n=52)

Fungal species (n=52)	Number of fungi identified	Percentage (%)	Result interpretation
Aspergillus tardicrescens	26	50	Saprophytic fungus
Candida tropicalis	4	7.7	Significant fungus
Candida species	4	7.7	Significant fungus
Candida glabrata	3	5.8	Significant fungus
Candida albicans	3	5.8	Significant fungus
Penicillium species	3	5.8	Saprophytic fungus
Aspergillus niger	2	3.8	Significant fungus
Aspergillus fumigatus	1	1.9	Significant fungus
Candida orthopsilopsis	1	1.9	Significant fungus
Cryptococcus neoformans	1	1.9	Significant fungus
Diaphorthe longicolla	1	1.9	Significant fungus
Pythium insidiosum	1	1.9	Significant fungus
Rhizopus oryzae	1	1.9	Significant fungus
Talaromyces marneffei	1	1.9	Significant fungus

Among the 52 samples, only 23 (44.2%) fungi identified with PCR were consistent with clinical or HPE findings (Table II, Table III), whereby *Candida spp*. were the most common fungi causing tissue mycoses (n = 15/23, 65.5%), followed by *Aspergillus spp*. (n = 3/23, 13.0%), *Talaromyces marneffei*, *Cryptococcus neoformans*, and *Rhizopus oryzae* (n = 1/23, 4.3%, respectively). Tissue mycoses caused by rare fungi, such as *Diaporthe longicolla* and a fungus-like oomycete such as *Pythium insidiosum* (n = 1/23, 4.3%, respectively), were also identified in this study.

DISCUSSION

Tissue mycoses is commonly associated with invasive infections and one of the manifestations of disseminated fungal infection. Early diagnosis of tissue mycoses is critical for effective treatment. Non-specific clinical and radiological findings, as well as difficulties in conventional diagnostic methods may delay in making a correct diagnosis. Nowadays, nucleic acid-based assays have reduced the turn-around time and provided new opportunities for patient care. Various PCR methods are available, such as conventional panfungal PCR with sequencing, semi-nested PCR, and real-time PCR (5,12,14). This study investigated the use of conventional panfungal PCR with sequencing as a diagnostic tool among non-culturable, pretreated specimens. This

Table II: Species, frequency and result interpretation of fungi from FFPE tissue specimens (n=23)

Fungal species (n=23)	Number of fungi identified	Percentage (%)	Result interpretation
Candida tropicalis	4	17.4	Significant fungus
Candida species	4	17.4	Significant fungus
Candida glabrata	3	13.2	Significant fungus
Candida albicans	3	13.2	Significant fungus
Candida orthopsilopsis	1	4.3	Significant fungus
Aspergillus niger	2	8.7	Significant fungus
Aspergillus fumigatus	1	4.3	Significant fungus
Cryptococcus neoformans	1	4.3	Significant fungus
Diaphorthe longicolla	1	4.3	Significant fungus
Pythium insidiosum	1	4.3	Significant fungus
Rhizopus oryzae	1	4.3	Significant fungus
Talaromyces marneffei	1	4.3	Significant fungus

conventional panfungal PCR used primers that targeted the specific hypervariability regions in each fungus, which are known as internal transcribed spacer (ITS) regions (5,14). The sequences of the ITS region in each fungus have made this panfungal PCR with sequence analysis as a promising method to speciate the pathogenic fungi. Several international studies have also demonstrated the usefulness and performance of panfungal PCR from FFPE tissue samples (5-6, 12-15).

From 87 PCR sample results, 35 (40.2%) produced faint bands which failed to be sequenced in this work. As this research used the archival FFPE specimen, there was a possibility that the quality of the DNA was compromised (crosslinking and fragmented) by the routine HPE processing procedure, especially, the duration of the tissue contact time with formalin prior to be embedded in the paraffin (14,17). Lau et al. and Moncada et al. reported that fresh tissue specimens gave higher detection rates of 96.8% and 97.3%, respectively, than FFPE specimens at 87.5% and 70.0%, respectively (13-14). Insufficient amount of DNA may also affect the amplification process, as reported in a study by Lau et al., whereby in eight negative-PCR specimens, histological examination showed scanty fungal elements seen in the tissue sections (14). Result in this study showed that out of 35 faint bands-PCR specimens, ten had scanty fungal elements upon histological examination.

With only 44.2% (23 samples) positive-PCR results that were consistent with clinical or histological diagnoses, the rate was much lower in comparison with studies done by Lau et al. (94.4%) (14). It can be postulated that the 55.8% (29 samples) discordant results between HPE and PCR were due to high contamination rate by

Table III: Type of tissue, diagnosis, HPE findings and PCR result interpretation of fungi from FFPE tissue specimens (n=52)

No.	Type of tissue	Clinical Diagnosis	HPE findings	PCR band	Sequencing result	Result interpretation
1.	Cervix	Cervicitis	Numerous hyphae. Consistent with fungal infection.	Single band	*Candida albicans*	Significant fungus
2.	Esophagus	Upper gastrointestinal bleeding	Budding spores seen. Consistent with fungal infection	Single band	*Candida tropicalis*	Significant fungus
3.	Esophagus	Not stated in the request form	Suggestive of penicilliosis or histoplasmosis.	Single band	*Talaromyces marneffei*	Significant fungus
4.	Inner ear	Chronic suppurative otitis media	Consistent with fungal infection.	Single band	*Candida orthopsilopsis*	Significant fungus
5.	Lung	Esophageal carcinoma	Fungal infection consistent with candidiasis.	Single band	*Candida glabrata*	Significant fungus
6.	Lung	Pulmonary tuberculosis	Encapsulated budding yeast seen. Suggestive of cryptococcosis.	Single band	*Cryptococcus neoformans.*	Significant fungus
7.	Lung	Not stated in the request form	Features are suggestive of pulmonary aspergillosis.	Single band	*Aspergillus niger*	Significant fungus
8.	Nasal cavity	Acute fulminant fungal sinusitis	Fungal hyphae seen. Consistent with fungal sinusitis.	Single band	*Rhizopus oryzae*	Significant fungus
9.	Nasal cavity	Acute fulminant fungal rhinosinusitis	Fungal hyphae seen. Consistent with fungal infection.	Single band	*Pythium insidiosum*	Significant fungus
10.	Nasal cavity	Right fungal sinusitis	The fungal hyphae is septated and have acute angle branching. Consistent with fungal infection	Single band	*Aspergillus fumigatus*	Significant fungus
11.	Nasal cavity	Invasive fungal sinusitis	Abundant hyphae. Consistent with fungal infection.	Single band	*Aspergillius niger*	Significant fungus
12.	Product of conception	Complete miscarriage	Fungal yeast and hyphae seen. Consistent with fungal infection.	Single band	*Candida species*	Significant fungus
13.	Skin	Subcutaneous fungal infection	Numerous fungal bodies in yeast form. Consistent with fungal infection.	Single band	*Candida species*	Significant fungus
14.	Skin	Plaque psoriasis	Fungal bodies seen. Consistent with superficial fungal infection.	Single band	*Candida glabrata*	Significant fungus
15.	Skin	Post inflammatory hypopigmentation	Fungal bodies seen. Consistent with superficial skin infection.	Single band	*Candida glabrata*	Significant fungus
16.	Skin	Giant Cell Tumour of tendon sheath	Fungal hyphae seen. Consistent with fungal infection.	Single band	*Diaphorthe longicolla*	Significant fungus
17.	Skin	Acute on chronic spongiotic dermatitis to rule out fungal infection	To rule out fungal infection.	Single band	*Candida species*	Significant fungus
18.	Stomach	Perforated gastric ulcer	Numerous fungal elements are seen. Consistent with fungal infection.	Single band	*Candida species*	Significant fungus
19.	Stomach	Perforated gastric tumour	Present of fungal bodies. Consistent with fungal infection.	Single band	*Candida tropicalis*	Significant fungus
20.	Stomach	Perforated gastric ulcer	Budding yeast. Consistent with fungal infection.	Single band	*Candida tropicalis*	Significant fungus
21.	Stomach	Perforated gastric ulcer	Many fungal hyphae seen. Consistent with fungal infection.	Single band	*Candida albicans*	Significant fungus
22.	Trachea	Tracheal mass	Numerous fungal bodies and hyphae. Consistent with fungal infection.	Single band	*Candida tropicalis*	Significant fungus
23.	Vagina	Vaginal wart	Multiple fungal bodies seen most likely candidiasis.	Single band	*Candida albicans*	Significant fungus
24.	Breast	Granulomatous mastitis	Granulomatous mastitis, suggestive fungal infection.	Single band	*Aspergillus tardicrescens*	Saprophytic fungus
25.	Bronchus	Thymoma type A	Fungal bodies seen. Fungal infection most likely candidiasis.	Single band	*Aspergillus tardicrescens*	Saprophytic fungus
26.	Endometrium	Menorrhagia	Budding yeast seen. Suggestive of candidiasis.	Single band	*Aspergillus tardicrescens*	Saprophytic fungus
27.	Esophagus	Not stated in the request form	Budding yeasts consistent with candidiasis.	Double band	*Aspergillus tardicrescens*	Saprophytic fungus
28.	Inner ear	Cholestetoma	Pigmented fungal bodies and hyphae. Consistent with fungal infection.	Single band	*Penicillium species*	Saprophytic fungus
29.	Lung	Right pleural effusion	Numerous fungal hyphae. Consistent with fungal infection.	Single band	*Aspergillus tardicrescens*	Saprophytic fungus
30.	Lung	Pneumonia	Fungal ball seen. Suggestive of mucormycosis.	Double band	*Aspergillus tardicrescens*	Saprophytic fungus
31.	Nasal cavity	Polypoidal mass for investigation	Fungal spores seen. Suggestive of fungal infection	Double band	*Penicillium species*	Saprophytic fungus
32.	Nasal cavity	Non-invasive left maxillary fungal sinusitis	Fungal hyphae seen. Suggestive of fungal infection.	Double band	*Aspergillus tardicrescens*	Saprophytic fungus
33.	Penile and scrotum	Penile ulcer	Fungal spore seen. Suggestive of fungal infection.	Single band	*Aspergillus tardicrescens*	Saprophytic fungus
34.	Rectum	Haemorrhoid	Fungal spore seen. Suggestive of fungal infection.	Single band	*Aspergillus tardicrescens*	Saprophytic fungus
35.	Skin	Superficial fungal infection	Fungal hyphae seen. Suggestive of fungal infection.	Single band	*Aspergillus tardicrescens*	Saprophytic fungus
36.	Skin	Not stated in the request form	Fungal spores. Suggestive of fungal infection.	Double band	*Penicillium species*	Saprophytic fungus

Table III: Type of tissue, diagnosis, HPE findings and PCR result interpretation of fungi from FFPE tissue specimens (n=52) (continued....)

No.	Type of tissue	Clinical Diagnosis	HPE findings	PCR band	Sequencing result	Result interpretation
37.	Skin	Left back papilloma	Fungal bodies seen. Suggestive of fungal infection.	Double band	*Aspergillus tardicrescens*	Saprophytic fungus
38.	Skin	Tinea corporis	Fungal hyphae seen. Consistent with tinea infection.	Double band	*Aspergillus tardicrescens*	Saprophytic fungus
39.	Skin	Penicilliosis with bone marrow infiltration	Fungal bodies seen. Consistent with penicilliosis	Single band	*Aspergillus tardicrescens*	Saprophytic fungus
40.	Soft tissue	Left gluteal abscess	Suggestive of fungal infection.	Single band	*Aspergillus tardicrescens*	Saprophytic fungus
41.	Soft tissue	Left maxillary mycetoma	Pigmented fungal hyphae seen. Consistent with fungal infection.	Double band	*Aspergillus tardicrescens*	Saprophytic fungus
42.	Soft tissue	Subcutaneous mycoses	Fungal hyphae and spores seen. Consistent with fungal infection.	Double band	*Aspergillus tardicrescens*	Saprophytic fungus
43.	Stomach	Not stated in the request form	Fungal hyphae and spore seen. Consistent with fungal infection.	Double band	*Aspergillus tardicrescens*	Saprophytic fungus
44.	Stomach	Not stated in the request form	Fungal hyphae and budding yeast seen. Consistent with fungal infection.	Double band	*Aspergillus tardicrescens*	Saprophytic fungus
45.	Stomach	Perforated gastric ulcer	Invasive fungal infection. Suggestive of Candida.	Double band	*Aspergillus tardicrescens*	Saprophytic fungus
46.	Stomach	Perforated gastric ulcer	Fungal hyphae and budding seen. Suggestive of candidiasis.	Single band	*Aspergillus tardicrescens*	Saprophytic fungus
47.	Stomach	Upper gastrointestinal bleeding	Fungal spores seen. Consistent with fungal infection.	Single band	*Aspergillus tardicrescens*	Saprophytic fungus
48.	Stomach	Gastritis	Fungal bodies seen. Consistent with fungal infection.	Single band	*Aspergillus tardicrescens*	Saprophytic fungus
49.	Stomach	Perforated gastric ulcer	Perforated ulcer with fungal infection most likely Candida.	Single band	*Aspergillus tardicrescens*	Saprophytic fungus
50.	Stomach	Perforated gastric ulcer	Fungal bodies seen. Consistent with fungal infection.	Single band	*Aspergillus tardicrescens*	Saprophytic fungus
51.	Stomach	Perforated gastric ulcer	Suggestive of fungal infection.	Single band	*Aspergillus tardicrescens*	Saprophytic fungus
52.	Tonsil	Right tonsil hypertrophy	Fungal hyphae seen. Consistent with fungal infection.	Single band	*Aspergillus tardicrescens*	Saprophytic fungus

saprophytic fungi spores acquired from the environment. Contamination of FFPE specimens by environmental fungal spore might have occurred following improper storage of the specimen or during the PCR process (6,12,14). However, contamination during PCR process was less possible in this study, as we took strict preventive measures by using hyper-filtered tips during the DNA extraction process apart from performing the test in laminar airflow cabinet. The fungal contaminants can grow on the surface or within the paraffin. Long-term storage of the paraffin tissue blocks may cause high contamination rate if no specific measures are employed (12). This was supported by the high amplification rate of *Aspergillus tardicrescences* (50.0%) among discordant PCR results (Table I). Frickmann et al. reported that contamination by saprophytic fungi was also found in FFPE specimens (12).

Candida spp. (65.5%) was identified as the most common cause for tissue mycoses in this study, which is mainly from stomach and genitourinary specimens known as the common sites for colonisation of *Candida spp*. Even though *Candida spp*. is a normal commensal in the gastrointestinal tract, many studies reported on association of Candida with perforated gastric ulcer (18-19). Nakamura et al. reported that 44.0% of resected specimens were positive for Candida. High stomach pH, prolonged antacid use, and increased sugar level were the favouring factors for *Candida spp*. growth (18). This study also found that *Aspergillus spp*. (11.5%) was the second most common fungi identified causing tissue mycoses from respiratory sample, in which *Aspergillus spp*. were commonly associated with fungal sinusitis and pulmonary aspergillosis. These findings are similar with a study by Komkrit et al., whereby they also reported that the majority of the *Aspergillus spp*. infection had involved the respiratory system (15). *Aspergillus spp*. are ubiquitous saprophytic fungi whose conidia are easily spread in the airborne, later inhaled by human, and subsequently deposited in the bronchiolar and alveolar space (20).

There was also an uncommon fungus, *Diaporthe longicolla*, detected from a skin specimen. This fungus is widely known as a plant pathogen and is extremely rare to cause human infection. However, there are a few reports regarding *Diaporthe spp*. causing cutaneous, subcutaneous, and invasive mycoses (21-24). This work also identified *Pythium insidiosum* and *Rhizophus oryzae* in two cases of acute fulminant fungal sinusitis. As reported in the literature, *Pythium insidiosum* can cause massive tissue destruction due to arterial thrombosis and tissue ischaemia (25-26).

Twelve FFPE specimens gave overlapping double bands from PCR results indicating the presence of possible DNAs belonging to pathogenic fungi that had been fragmented with insufficient quantity for sequencing (14). The second bands that belonged to saprophytic fungi in the discordant cases were identified as *Aspergillus tardicrescens* and *Penicillium species*. These fungi were documented as xerophilic saprophytic fungal

in the literature (27-28). The high contamination rate during the storage of specimen gives better amplification of saprophytic fungi than the pathogenic ones that cause tissue mycoses.

CONCLUSION

Conventional pan fungal-PCR with sequencing can be used as a method for detection of fungus in FFPE specimens. However, the quality of specimens is an important factor that determine the success of PCR and sequencing result. Faint PCR band will most likely exhibit poor result due to poor quantity of fungal DNAs. FFPE samples need to be stored properly to prevent saprophytic fungal contamination. Both pathogenic and saprophytic fungi can be amplified from FFPE sample; therefore, result interpretation from panfungal PCR needs to be carefully correlated with adequate clinical information and the HPE findings.

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