CASE REPORT

Molecular Detection of *Cryptococcus* from Formalin-fixed Paraffin-embedded Tissue Sample - A Noncultural Approach for Diagnosis of Primary Pulmonary Cryptococcosis

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ABSTRACT

Despite pulmonary cryptococcosis showing good response to antifungal treatment, making accurate diagnosis in a timely manner remains a challenge. Tissue for fungal culture is less sensitive, nonspecific, and time-consuming to allow for prompt diagnosis. We herein report a case of 31-year-old woman with no known medical illness but presented with history of cough and haemoptysis for two months. The chest X-ray and bronchoscopy revealed left lung mass. While tuberculosis showed negative result and no growth observed in the tissue culture, the histopathological examination (HPE) finding was suggestive of fungal infection. The formalin-fixed paraffin-embedded (FFPE) tissue was sent for molecular testing, which revealed *Cryptococcus neoformans*. This report emphasises on the advantages and limitations of polymerase chain reaction (PCR) as an alternative method to confirm the diagnosis in cases of culture-negative fungal infection.

Keywords: Pulmonary cryptococcosis, Histopathological examination, Polymerase chain reaction

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INTRODUCTION

Pulmonary cryptococcosis is transmitted via inhalation of the basidiospores of the *Cryptococcus neoformans* and *Cryptococcus gattii* (1). It has potential to cause serious fungal infection and can affect both immunocompetent and immunocompromised hosts (1). The diagnosis is often missed due to nonspecific symptoms that can mimic bacterial infection and non-infective disease (1). The laboratory diagnosis of pulmonary mycoses is mainly by culture and serological methods, and histopathological examination (1–2). However, these methods have lack of sensitivity and specificity (1–2). A molecular method can reduce the turnaround time of the test result and is able to identify the fungal genus and species from the paraffin-fixed tissue specimen. Hence, it has now deemed to be a useful method in the identification of fungi-causing tissue mycoses (3–5).

CASE REPORT

A 31-year-old healthy woman presented with two months history of cough, haemoptysis, and mild shortness of breath, which were associated with poor appetite and weight loss. She otherwise denied any history of fever, night sweat, or exposure to tuberculosis patients. Upon examination, she was afebrile with normal vital signs. Examination of her respiratory system revealed reduced air entry at left lower lung. Examination of other body systems was unremarkable, and chest radiograph showed a mass at left inferior lung. At this point, clinical diagnosis of tuberculosis was made by the treating physician. However, her sputum examination for acid-fast bacilli was negative, repeatedly. The Mantoux test was also negative. A bronchoscopic examination revealed an endobronchial mass possibly a fungal ball at left lower lung lobe. Following this finding, computed tomography (CT)-guided lung biopsy was performed, and the tissue was sent for fungal culture and HPE. The tissue HPE showed numerous encapsulated budding cells that were suggestive of cryptococcosis (Figure 1).

However, the tissue culture revealed no growth after 14 days of incubation. Cryptococcal antigen test from serum sample was also negative. In view of negative microbiological test results, the tissue was further sent for panfungal PCR testing to confirm the diagnosis. DNA was isolated from the FFPE tissue samples by using QIAamp DNA FFPE tissue kit from Qiagen according to the manufacturer's recommendation. The PCR targeted the ITS2 region of ribosomal DNA using universal primer (ITS 3F, 5 ' - G C A T C G A T G A A G A A C G C A G C - 3 ' ; ITS 4R, 5'TCCTCCGCTTATTGATATGC-3') (3). *Candida albicans* was used as the positive control, while nuclease free water as the negative control in this study. The PCR product with size approximately 400bp indicates the presence of fungus DNA. The amplicons (Figure 2) were then sequenced and analysed using NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/blast) for similarity search against database. The blast result showed 97% similarity with *Cryptococcus neoformans*. The patient was tested negative for human immunodeficiency virus (HIV) infection. On further questioning, she admitted that she was exposed to pigeon's excreta for many years at home. It is known that her brother owns a pet pigeon in that particular household. She was treated with oral fluconazole 400 mg daily for six months, portraying good response by diminution of the left lower lung mass.

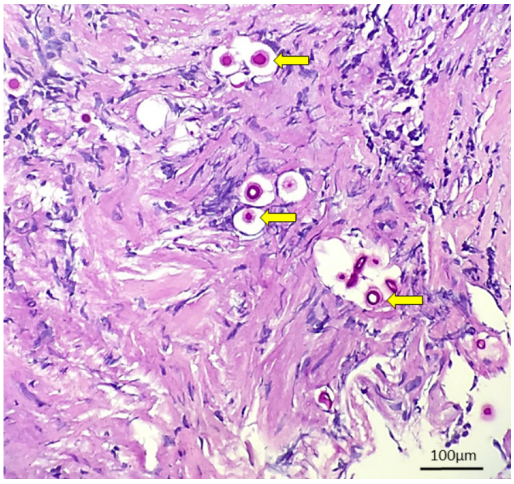

Figure 1: The thick capsule of Cryptococcus neoformans (yellow arrow) appears as magenta colour (Periodic Acid Schiff stain, original magnification x 400)

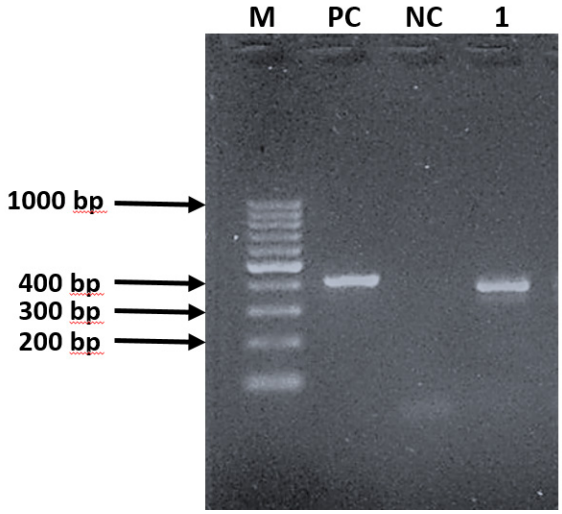

Figure 2: Agarose gel electrophoresis of PCR product. Lane M, 100 bp DNA; lane PC, positive (Candida albicans); lane NC, negative control (nuclease free water); lane 1, patient's sample. Positive ITS 2 is indicated by the presence of band with approximate PCR amplicon 400bp

DISCUSSION

Microbiological culture of clinical specimens is the gold standard. However, this method has low sensitivity and long incubation growth time, as well as requires high yield of viable pathogen (1). A cryptococcal antigen test is reported to be more sensitive in immunocompromised patient but less useful in the case of isolated pulmonary cryptococcosis in immunocompetent host (1). On the other hand, tissue HPE is rapid, reproducible, and cheap, though it is less specific (2). Histologically, *Cryptococci* has a thick polysaccharide capsule that appears as a clear space around the cell. However, some strains may express lesser amount of polysaccharide, hence, giving the appearance of other yeast-like or dimorphic fungi, such as *Candida* spp., *Histoplasma capsulatum*, conidia of *Aspergillus* and endospores of *Coccidioides* (2).

Molecular method is a non-culture approach for fungal diagnosis (3–5). There are many types of PCR tests, such as conventional panfungal with sequencing, semi-nested PCR, and real-time PCR (3–5). In this case, conventional panfungal with sequencing was used as the diagnostic method. This method targeted the hypervariable region, namely, internal transcribed spacer (ITS) region; which has a different sequence to that of all other fungi. (3–4). The hypervariability of the ITS region made panfungal PCR with analysis of the sequence as one of the accurate methods to speciate the pathogenic fungal from the FFPE tissue samples. Several studies have documented the utility and performance of panfungal PCR for FFPE samples (3–5).

The panfungal PCR is able to reduce the turnaround time of test result with accurate diagnosis, even if samples are contaminated with human DNA (3–5). However, the sensitivity of the test could be compromised for FFPE sample (68%), as compared with that for fresh tissue sample (97%) (3). This is possible because of the DNA degradation due to storage and formalin cross-linking that will result in shortening of basepair length (3,5). To increase thedetection rate, shorter fragments, such as ITS1 and ITS2, were used for amplification purpose (3). A study by Babouee et al. revealed that ITS2 PCR (310 ± 67 base pairs) produces higher sensitivity result (53.8%) compared with the entire ITS1-2 (~600 base pairs) fragment (sensitivity 38%) (3).

In molecular investigation, DNA quality and quantity play a major role in determining the successful rate of detection. Insufficient amount of DNA in the tissue sample results in poor detection rate, as reported in the previous study by Anna et al. (4). As FFPE sample used formaldehyde and paraffin for preservation, this may affect the quality of DNA. In addition, presence of inhibitors in human tissue will affect the PCR amplification. Thus, many commercialize FFPE DNA extraction kits available in the market such as QIAamp have been optimized for extraction procedure that allow better quality and quantity of DNA to be obtained.

Another factor to be considered is the false positive result due to cross and carry-over contaminations during PCR process or contamination by saprophytic fungi that grew during the storage of the FFPE samples (5). Therefore, the PCR result needs to be carefully interpreted by correlating it with adequate clinical and HPE findings. As illustrated in this case, using shorter ITS2 region primer had successfully amplified the cryptococcal DNA. Although microbiological testing was negative, the HPE findings were found consistent with PCR result.

CONCLUSION

Pulmonary cryptococcosis is deep-tissue mycoses with high fatality rate. The diagnosis of the disease poses a great challenge as the symptom can mimic other diseases. A molecular method is more sensitive and specific than culture method in diagnosing pulmonary cryptococcosis, thus allowing for early diagnosis and appropriate therapy. However, it must be carefully interpreted to prevent misdiagnosis.

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