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Histoplasmosis in Pulmonary Infection Patients from Hospitals in Hanoi, Vietnam

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Abstract

Histoplasmosis, a fungal infection acquired from soil contaminated with birth/bad or chicken droppings, caused by *Histoplasma capsulatum* (*H. capsulatum*), has a worldwide distribution, but is not well studied in Vietnam. While infections are usually asymptomatic, the symptoms of acute or epidemic histoplasmosis are high fever, cough, and asthenia. Immunocompromised patients are at highest risk groups of infection (e.g., persons with cancer, transplant recipients, persons with HIV infection). In this study histoplasmosis has been identified from patients with pulmonary infections using an indirect ELISA (HistoplasmaDxSelect™ kit-USA). *Histoplasma capsulatum* antibodies were detected in 18% (26/144) of patients. Among these, 57.7% (15/26) were HIV positive and 65.4% (17/26) were chronic pulmonary infections. *Histoplasma* could not be isolated from patient samples, however, nine cases were detected from clinical samples using nested PCR and sequencing methods. This is the first report of the presence of *H.capsulatum* in Vietnam. Further studies, therefore, need to be conducted to determine the actual prevalence of histoplasmosis in Vietnam and to map out endemic areas of the disease in Asia.

Keywords: Histoplasmosis; Pulmonary infection patient; ELISA; PCR; Hanoi

Introduction

Histoplasmosis is a fungal infection caused by *Histoplasma capsulatum* (*H. capsulatum*) a dimorphic fungus that, can affect both humans and animals [1-3]. Most *Histoplasma* infections are subclinical, however, occasionally disease occurs linked in to the host immune response [4]. The disease is the most common pulmonary mycosis in North America and Central America, but the organism is known to exist in many areas around the world [5-9]. Histoplasmosis usually presents as in immune-compromised individuals with serious underlying disease, including AIDS [10,11]. The occurrence of histoplasmosis in Asia has been recently reviewed with cases of histoplasmosis from India, Malaysia, Singapore, Indonesia, Thailand, South Vietnam and Japan, but the autochthonous nature of the cases reported has not been established unequivocally [8,12,13]. Recently, *H. capsulatum* was recovered from environmental sources contaminated with bat guano and chicken droppings in Thailand [14]. In Vietnam, two histoplasmosis cases were reported from Southern Vietnam, however, the endemicity of the disease has not been studied. Histoplasmosis is difficult to distinguish from diseases with similar clinical features, such as tuberculosis and bacterial pneumonia. In the laboratory, diagnosis of histoplasmosis is also problematic because the culture-positive rate in patients is usually low, and several weeks are required for *H. capsulatum* to grow on solid media. In addition, *H. capsulatum* is a biosafety level 3 pathogen requiring specialist facilities for culturing. Serological methods are faster than culture, but can sometimes generate false positives *via* cross reaction with other fungal pathogens such as Coccidioidomycosis [3]. Moreover, in cases in which a histopathological diagnosis of histoplasmosis has been made, confusion can occur with other pathogens such as *Cryptococcus spp.*, or *Penicillium marneffei*, which can have similar pathologies. Molecular methods such as nested PCR and sequencing are alternatives for rapid detection of *Histoplasma*.

Here, we aimed to determine the prevalence of histoplasmosis among pulmonary infection patients in Hanoi, Vietnam using different diagnostic methods.

Materials and Methods

Sample collection and preparation for culture

Between August 2012 and January 2013, 206 serum and 158 bronchial washing samples were collected from pulmonary infection patients at Bach Mai and Military 103 hospitals in Hanoi. Criteria for enrolment of patients were at least one of the followings: i) abnormal chest shadow and clinical symptoms such as cough, sputum (haemptylis), or dyspnea; ii) negative mycobacterial examination (smear negative or culture negative). Each patient was asked to provide blood and bronchoalveolar lavage fluid (BALF) sample. Aliquots of the BALF were centrifuged and the supernatant was removed. Sterile PBS

was added to the sediments, which were thoroughly mixed before being used to inoculate fungal cultures. Serum was obtained by centrifuging at 2000 g (or rpm) for 10 min at room temperature and was stored at -300°C until analysed.

ELISA

Serum samples were analysed using HistoplasmaDxSelect™ kit (USA) following the manufacturer's instructions. ELISA results were compared with the reference cut-off OD value reading using an ELISA reader. An index value of >1.10 was used to indicate the presence of antibodies to *Histoplasma*. An index value of <0.90 indicated that antibodies to *Histoplasma* were not detected.

Nested PCR

DNA extraction from patient's samples was performed using QIAamp® DNA mini kits in a 50 µl elution volume following the manufacturer's instructions. Five microliters of extracted DNA was used for the nested PCR assay. Primers used in the assay to detect *H. capsulatum* specifically amplified a sequence of DNA coding for a specific portion of the *H. capsulatum* M antigen gene (Msp1F/Msp2R and Msp2F/Msp3R) (see detail in Table 1) [15].

Primer	Primer sequence	Product size (bp)
Msp1F	5-aca aga gac gac ggt agc ttc acg-3	318
Msp2R	5-acc agc ggc cat aag gac gtc-3	
Msp2F	5-cgg gcc gcg ttt aac agc gcc-3	269
Msp3R	5-ata agg acg tca cga agg gc-3	

Table 1: *Histoplasma capsulatum* PCR specific primers.

Sequencing

The second nested PCR amplicons were purified using the QIA quick PCR purification kit (Qiagen).The purified products were sequenced directly in both directions using a Big Dye Terminator v3.1 Cycle sequencing kit (Life Technologies) in an Applied Bio system 3130 genetic Analyser (Life Technologies). Nucleotide sequences for the 269 bp products were verified using the National Centre for Biotechnology Information, Basic Local Alignment Search Tool.

Culture

One hundred microliter aliquots of each PCR-positive samples were cultured onto Brain Heart Infusion agar (Merck) with 1% glucose. Plates were incubated at 28-30°C for up to 8 weeks. Colonies were identified by microscopic morphology.

Ethical review

The study was approved by the ethics committee of the National Institute of Hygiene and Epidemiology, Hanoi, Vietnam (No. 01 IRB, dated January 6, 2012).

Results and Discussion

Histoplasmosis is a community acquired infection, most often presenting as pneumonia [1]. Although histoplasmosis is not a common cause of lung disease in the tropics, it is probably underdiagnosed due to the technical difficulties of microbiological diagnosis and the likelihood of confusion with tuberculosis. Histoplasmosis diagnosis is usually based on visualization of the fungus in organic fluids (sputum, blood and liquor) or tissues (histopathological evaluation) and by the culture of biological samples. The present study used HistoplasmaDxSelect™ ELISA kit, which utilizes inactivated purified *Histoplasma* antigen for the qualitative detection of antibodies to *Histoplasma capsulatum* var. *capsulatum* in human serum. In this study, a total of 206 serum and 158 bronchial lavage samples were collected from patients with acute or chronic pulmonary infections during 2012-2013 (Table 2). The acute pulmonary group (90/206) was hospitalized with typical symptoms as dry or non-productive cough, fever, chest pain and fatigue. Distinct patterns were occasionally seen on a chest X-ray. The chronic pulmonary patients (116/206) were commonly in older men who had the clinical manifestations include fatigue, mild fever, night sweats, chronic cough, sputum production and weight loss. Of the 206 patient serum samples tested for antibody reactivity by ELISA, *Histoplasma* antibodies were detected in 13.1% (27/206) which is high compared with studies form Japan and Thailand [1,5]. Positive samples were not significantly linked to occupational group with 16/206 (7.8%) from farmers, 7/206 (3.4%) from workers and 4/206 (1.9%) from other groups, respectively (Table 3). Presence of *Histoplasma* antibodies was high in chronic pulmonary patients compared to the acute pulmonary group 15.5% (17/116) and 10% (9/90) respectively. Of the HIV-infected patients 42.8% (15/35) were identified positive with *Histoplasma* antibodies (Table 3). The higher prevalence of antibodies in farmers might be explained by their close contact to soil and material contaminated with bird/chicken dropping. In Thailand, Pirron et al. showed 90.6% of histoplasmosis patients were Acquired Immunodeficiency Syndrome (AIDS) [5]. However, those patients were positive with *H. capsulatum* by culture while our patients were cases that diagnosed by clinical signs. In addition, we found 8.74% of chronic pulmonary patients were sero-positive, compared with 4.36% with acute pulmonary patients. This could be due to the development of anti-*Histoplasma* antibodies occurring two to six week's post-exposure [6]. In acute case where *Histoplasma* infections is suspected, a second serum, sample should be collected 7-14 days later for testing to avoid false negative results [6]. The serological should be only used as a screening test for evaluation potential exposure to, or infection with, *Histoplasma* and confirmatory are required.

Several reports suggested that PCRs might improve the accuracy of identification of *H. capsulatum* in clinical samples such as tissues, body fluids. In this study, we used a nested PCR with the specific primers for detection of *H. capsulatum* in both type of clinical samples. Detail of the primers were previously published but were modified by Ohno H et al. to increase the sensitivity and specificity of PCR allowing for detection of at least 10 fg *H. capsulatum* DNA [15]. In the present work, 9/206 BALF samples were positive with the nested PCR (Figure 1) and sequence analysis of these PCR products showed approximately 100% similarity to the *H. capsulatum* M antigen gene. Among these PCR positive samples, 3 of them correlated with a positive ELISA results. While this provided strong evidence that those patients were infected with *H. capsulatum*, isolation of *H. capsulatum* from PCR-positive samples by culture was negative after 8 weeks of the incubation period. This could be due to deterioration of the samples during long-term storage or that patients had undergone long-term antibiotic therapy. Therefore, knowledge of clinical syndromes and the patient travel history may lead the physicians to decide sending sample on time and avoid inappropriate treatment regime.

Our results provide evidence that histoplasmosis should be considered in patients with pulmonary infections in Hanoi, Vietnam and that such infections may be under-reported. Physicians should therefore include histoplasmosis in the differential diagnosis of diseases thought to be tuberculosis or community-acquired pneumonia. In addition, diagnosis of histoplasmosis should include culture to allow validation of the non-culture diagnostic technique used in this study in the Vietnam setting. Adaptation and validation of the *Histoplasma* antigen tests for use in low resource setting could assist with recognition of patients with the infection, allowing early intervention with an appropriate treatment regime.

Sex		Age (yr)		Occupational			Clinical manifestations		
Male	Female	20-40	40-60	Farmer	Worker*	Others**	Acute pulmonary	Chronic pulmonary	HIV***
119	87	66	140	107	77	22	90	116	35

*Worker:

**Other groups include teacher, house worker, officer

***HIV: including acute and chronic pulmonary patients

Table 2: Characteristics of patients included in the study.

Patients group (n)	Patients positive by ELISA/total number (%)	Patients positive by ELISA/total number of group (%)
Acute pulmonary	9/206 (4.37%)	9/90 (10%)
Chronic pulmonary	18/206 (8.76%)	18/116 (15.5%)
HIV positive	15/206 (7.28%)	15/35 (42.8%)

Table 3: Comparison of ELISA results in the patients group.

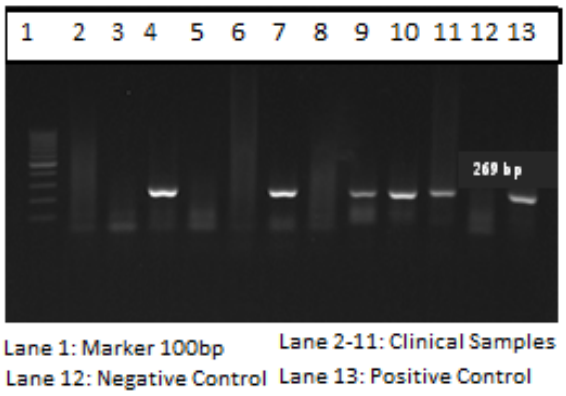

Figure 1: Nested PCR results in pulmonary infection patients.

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