Eur J Clin Microbiol Infect Dis (2015) 34:1759–1765
DOI 10.1007/s10096-015-2409-7

ARTICLE

Prevalence of chronic pulmonary aspergillosis in patients with tuberculosis from Iran

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Received: 31 January 2015 / Accepted: 10 May 2015 / Published online: 24 May 2015

Abstract In patients with preexisting lung disease, especially a cavity, *Aspergillus* can infect the surface of the cavity, causing chronic cavitary pulmonary aspergillosis (CCPA), and may form an aspergilloma, collectively called chronic pulmonary aspergillosis (CPA). In the present study, we assessed tuberculosis (TB) patients for CPA based on culture and serological methods. During a period of 1 year (from March 2013 to March 2014), we studied 124 patients with TB (94 with current TB and 30 with previous TB) at Masih Daneshvari Hospital in Tehran, Iran. Sputum specimens were analyzed by direct microscopic examination (DME) and fungal culture. The clinical and radiological features of all patients were recorded, to categorize the patients into CCPA and aspergilloma. All patients were screened for serum-specific IgG against *A. fumigatus*, by enzyme-linked immunosorbent assay (ELISA). Out of 124 patients with TB (66 male, age range: 10–91 years), 48 patients (38.7 %) exhibited residual cavities. Eighteen (14.5 %) patients had cavities with pleural thickening. A round-shaped mass lesion was detected in six patients (6.8 %). DME was positive in ten patients for septate fungal hyphae. *A. fumigatus* was grown from 14 samples. Fifty-five (44.3 %) cases were positive for serum-specific IgG against *A. fumigatus*. Of 124 patients with TB, 3 (2.4 %) met criteria for aspergilloma and 14 (11.3 %) for CCPA. CPA is a common clinical presentation in individuals with healed TB in Iran, as reported by previous studies from other countries.

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Introduction

Tuberculosis (TB) is a widespread, and, in many cases, fatal, infectious disease and remains a health problem worldwide. The number of incident cases for TB in individuals who are human immunodeficiency virus (HIV)-negative has increased from 5.0 million (4.8 million to 5.1 million) in 1990 to 7.1 million (6.9 million to 7.3 million) in 2013 [1]. Deaths from TB in HIV-negative individuals were 1.3 million in 2013 [1]. In Iran, the incidence and deaths of TB in HIV-negative individuals were 16,485 and 1,402 in 2013, respectively [1]. It has been reported that patients with TB have defective macrophages, monocytes, and T cells function, as well as chemotaxis defects that predispose them to opportunistic fungal infections [2, 3]. On the other hand, healed pulmonary TB can result in a pulmonary cavity. These preexisting cavities become infected with *Aspergillus* and an aspergilloma may then form after months or years of infection [4]. Among the many different underlying conditions, TB is the single most common primary underlying condition in the development of chronic pulmonary aspergillosis (CPA) [5–8].

Aspergillosis is a fungal infection resulting from infection or allergy to fungi in the *Aspergillus* genus [9]. Among the different species of *Aspergillus*, *A. fumigatus* is the most prevalent species involved in aspergillosis [9]. Pulmonary aspergillosis is the usual clinical entity, which includes: CPA (including single pulmonary aspergilloma), allergic bronchopulmonary aspergillosis, subacute invasive (chronic necrotizing pulmonary), and acute invasive aspergillosis. Immunodeficiency and/or underlying lung disease are major factors preceding aspergillosis.

There are a few reports of aspergillosis complicating TB from different countries, diagnosed with different diagnostic methods [5–8, 10–15]. Prior studies from developed countries of aspergillosis complicating TB, in particular those with old TB cavities, show high rates of *Aspergillus* antibody detection and aspergilloma development in up to 25 % [10–14]. The prevalence of CPA after TB has not been studied in the Middle East or Iran.

This is the first study with established diagnostic methods to evaluate CPA in patients with pulmonary TB (PTB) in Masih Daneshvari Hospital in Tehran, the capital city of Iran, as a specialized center for TB patients.

Materials and methods

Patients

During a period of 1 year (from March 2013 to March 2014), 124 consecutive patients with PTB (94 with current TB and 30 with previous TB) in Masih Daneshvari Hospital were included in the study. The patients with current TB were treated with standard combination therapy including isoniazid, rifampicin, ethambutol, pyrazinamide, and streptomycin as needed during hospitalization. The patients gave written consent to participate in this research and it was approved by the ethical committee of Mazandaran University of Medical Sciences. The history of all included patients was recorded by a specialist in lung diseases. All included patients had chest X-rays (68 patients) and/or computed tomography (CT) (56 patients). Patients with HIV were excluded from the study.

Chronic cavitary pulmonary aspergillosis (CCPA) was diagnosed as non-immunocompromised patients according to the following criteria [5]: (1) chronic pulmonary or systemic symptoms compatible with CPA, for a minimum of 3 months, including at least one of the following symptoms: weight loss, productive cough, or hemoptysis; (2) radiologic findings showing cavitary pulmonary lesion(s) with evidence of paracavitary infiltrates and adjacent pleural thickening with/without a fungal ball; (3) positive anti-*Aspergillus* immunoglobulin G antibodies (precipitins) in blood and/or a positive *Aspergillus* culture or sputum microscopy; (4) exclusion of similar presentations caused by active TB, other mycoses, neoplasm, abscess, Wegener's granulomatosis, etc.

A single aspergilloma was diagnosed in patients with a single pulmonary cavity containing a fungal ball, with serological or microbiological evidence implicating *Aspergillus* spp. in a non-immunocompromised patient with minor or no symptoms and no radiological progression over at least 3 months of observation [8].

CCPA was distinguished from single aspergilloma on three criteria: progressive symptoms, multicavity disease (including bilateral disease), and the presence of an aspergilloma was not required for diagnosis.

Laboratory diagnosis

Sputum and blood samples were collected from all patients who were included in the study. Each collected sputum sample was mixed in an equal volume of pancreatin 0.5 % and centrifuged for 10 min at 3,000 rpm. The supernatant was discarded and the sediment was vortexed for 30 s. The sediment was then divided into two samples; one for fungal culture and the other for direct microscopic examination. The sample for fungal culture was inoculated into Sabouraud's glucose agar (Difco) supplemented with chloramphenicol (0.5 mg/mL) (SC) and incubated at 27–30 °C for 4 weeks. The remaining part of the sediment was mounted with 20 % potassium hydroxide (KOH).

The *Aspergillus* species were identified by subculture onto Czapek Dox agar medium and described according to macroscopic and microscopic characteristics of each colony. These were then identified to the complex level using keys by Raper and Fennell [16]. According to these keys, species identification is based on colony characteristics, including color of surface and reverse, rate of growth (colony diameter), texture, and diffusing pigment, as well as the morphology of conidial head, conidiophore, vesicle, conidiogenous cell, and conidia, but also on the characteristics of the cleistothecia, sclerotia, and Hülle cells, if they were present. The discovery of many cryptic species among the aspergilli has limited conventional identification to the complex level.

Yeasts identification was done by subculture on corn meal agar (CMA) + tween 80 medium and, also, the germ tube test.

Specific IgG against *Aspergillus fumigatus* measurement

All patients were screened for IgG antibody against *A. fumigatus*, using an enzyme-linked immunosorbent assay (ELISA) kit (Omega kit, distributed by Genesis Diagnostics Ltd., Cambridgeshire, UK). In brief, the patient's serum sample was diluted to 1:200 in diluted sample diluents. One hundred microliters of the standard and diluted samples were added to the microwells, respectively. Each sample was analyzed in duplicate. After incubation for 30 min at room temperature, the microwells' contents were emptied and washed with an automated washer (SCO Diagnostics washer MPW1,

Germany). Afterwards, 100 μl of conjugated solution was added to all microwells and incubated for 30 min at room temperature and washed. One hundred microliters of tetramethylbenzidine (TMB) was immediately added to the microwells and incubated for 10 min at room temperature. Reactions were stopped with 100 μl of stop solution, and optical densities (ODs) at 450 were read by an ELISA reader (Bio-Rad, Model 680, Japan). The results are expressed in U/mL. A serum-specific IgG more than 12 U/ml was considered as elevated based on the kit manufacturer's instructions.

Statistical analysis

A Chi-square test was performed using SPSS software (version 18.0) and differences were considered significant at a *p*-value of <0.05.

Results

Out of 124 patients with TB, 66 (53.2 %) were men. The age range was between 10 and 91 years. The median age was 48 years. Table 1 shows the details of the patient characteristics. Most of the patients were aged between 21 and 30 years. TB had been treated 1 to 7 years prior to inclusion. Figure 1 shows the evaluation time and *Aspergillus* IgG positivity after TB. The rate of positivity to *Aspergillus* IgG was proportionately more often positive the more time had elapsed between the end of anti-tuberculous therapy and evaluation ($p<0.0005$).

Of 124 patients with TB, 40.3 % exhibited underlying diseases, including diabetes, hepatitis, transplant recipients, and coronary thrombosis.

Out of 124 patients with TB, 48 (38.7 %) exhibited residual cavities, including 31 in the right lobe, ten in the left lobe, and seven in both lobes (Table 2). Thirty-nine (81.2 %) patients had a single cavity and 9 (18.8 %) had more than one cavity. Of these patients, 33 (68.7 %) had elevated IgG against *A. fumigatus*. Eighteen (14.5 %) patients had cavities with pleural thickening; 14 of these (77.8 %) patients showed both cavities with pleural thickening and positivity for IgG. A round-shaped mass lesion (diameter: 25–64 mm) was detected in six patients (6.8 %) (Fig. 2).

Direct microscopic examination on sputum samples revealed septate hyphae in 10 (8.1 %) patients. Sputum samples from 16 (12.9 %) patients were positive for *Aspergillus* spp. by culture. Of the *Aspergillus* spp. isolated, 10 (62.5 %) were *A. fumigatus* complex. Table 2 exhibits more details of the radiographic, mycological, and serological findings from TB patients. Fifty-five (44.3 %) patients were positive for specific IgG against *A. fumigatus* using the 12 U/mL cut-off; all titers being higher than 16 U/mL. The Chi-square test showed a significant relationship between positive culture, direct microscopic examination, and serum IgG profile level ($p<0.0001$).

According to the criteria used in this study, of 124 patients with TB, 3 (2.4 %) met criteria for single aspergilloma and 14 (11.3 %) for CCPA. Among patients with aspergilloma, one suffered from hepatitis, and two from diabetes. Table 3 summarizes the clinical characteristics and history of those patients diagnosed with CCPA. The most common clinical symptoms of CPA were chronic productive cough (100 %), fatigue (64.3 %), and weight loss (57.1 %). Although 55 patients had *Aspergillus* antibody detectable during or after TB, only 17 (31 %) met current criteria for CPA including aspergilloma. Follow-up to determine whether the later development of CPA occurs or antibody becomes negative would be desirable.

Discussion

CPA is one of the clinical presentations of aspergillosis which affects patients with underlying pulmonary conditions [5].

Table 1 Characteristics of the studied patients ($n=124$)

Characteristics	No. (%)
First episode of TB	94 (75.8)
Prior TB	30 (24.2)
Sex	Male: 66 (53.2)
TB smears positive at time of TB diagnosis	115 (92.7)
TB culture positive at time of TB diagnosis	9 (7.3)
Smear and culture negative at time of TB diagnosis	6 (4.8)
Resistant strains of *Mycobacterium tuberculosis*	9 (7.2)
Pulmonary diseases	Asthma: 8 (6.4), viral pneumonia: 2 (1.6), bronchiectasis: 1 (0.8)
Any other underlying factors	Hepatitis: 4 (3.2), kidney transplantation: 2 (1.6), diabetes: 13 (10.5), coronary thrombosis: 10 (8.1)
Treated with corticosteroids	12 (9.7)

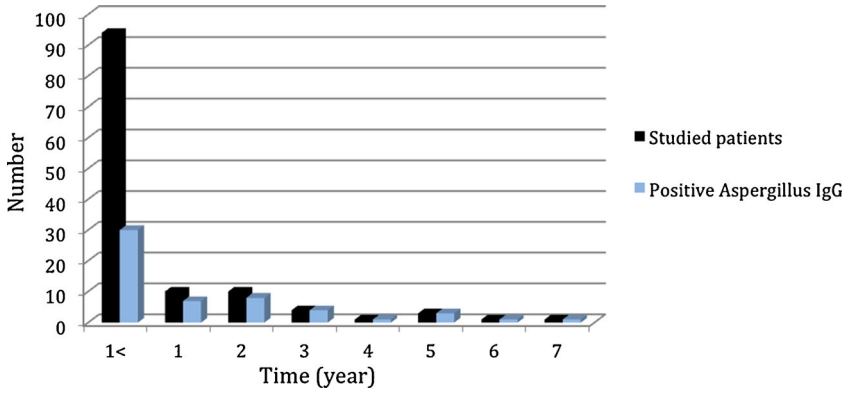

Fig. 1 Timing of positive *Aspergillus* serology after pulmonary tuberculosis (PTB) in the studied patients

The CPA definition has been inconsistent in the literature because of a wide range of clinical, radiologic, and anatomopathological manifestations. Recently, it has been accepted that CPA is an umbrella term covering mainly CCPA and single aspergilloma [5]. Some patients with what was called chronic necrotizing pulmonary aspergillosis have CCPA, while others have subacute invasive pulmonary aspergillosis [17]. The morbidity and mortality of CPA remains high, even with treatment [5, 18]. Apart from those with single aspergillomas, which can be resected, ill patients require long-term maintenance antifungal therapy to improve symptoms and prevent hemoptysis and relapse, but, despite this, many continue to have some disability; for example, fatigue and intermittent secondary infections of remaining pulmonary cavities [5, 19]. As TB may proceed to CPA, the present study evaluated patients with current and post-TB for CPA in Iran for the first time.

Table 2 Radiographic, mycological, and serological findings in the studied patients (n=124)

	No. (%)
Radiographic/CT features	
A. Cavity lesion in lungs: cavitation	48 (38.7 %)
Single	39 (31.4)
Multiple	9 (7.2)
Right lobe	31 (25.0)
Left lobe	10 (8.1)
Bilateral	7 (5.6)
B. Consolidation	105 (84.7)
C. Pleural thickening	52 (41.9)
D. Cavity with pleural thickening	18 (14.5)
E. Mass presentation	6 (4.8)
F. Fungus ball	3 (2.4)
Microbiologic testing (sputum of patients)	
A. Direct examination: hyphae	7 (5.6)
Yeast	15 (12.1)
Mixed (hyphae + yeast)	3 (2.4)
B. Culture:	
Aspergillus spp.	4 (3.2)
A. fumigatus	10 (8.1)
Candida spp.	10 (8.1)
C. albicans	5 (4.0)
Aspergillus spp. + *Candida* spp.	2 (1.6)
Serologic testing (ELISA kit)	
IgG titer:	
>12 U/ml: positive	55 (44.3)
8–12 U/ml: equivocal	9 (7.3)
<8 U/ml: negative	60 (48.4)

In the present study, out of 124 patients with TB, 12.9 % were culture-positive for *Aspergillus* spp. and 44.3 % demonstrated specific IgG against *A. fumigatus*. Though *Aspergillus* species constitute one of many fungi found in respiratory samples, their relative proportion and species distribution show considerable geographical variation. In contrast to the study of Biswas et al. [20], in which *A. flavus* was reported as the prevalent species of *Aspergillus* from tubercular sequelae sputum specimens, in the present study, *A. fumigatus* was the most prevalent species among *Aspergillus* spp. in sputum samples from patients with TB. Sivasankari et al. [21] studied fungal cultures and found 32.5 % of cases of TB to be positive in sputum samples. Among the 26 isolates, only 8 (30.8 %) were found to be *Aspergillus* spp. In contrast to our study, Sivasankari et al. [21] reported *A. flavus* and *A. niger* as the most common species in the *Aspergillus* genus. Our findings are consistent with the results of Kurhade et al. [11] and Shahid et al. [12], who reported *A. fumigatus* as the prevalent species of *Aspergillus* in chronic pulmonary infections including pulmonary TB. All mentioned studies [11, 12, 20, 21] were from different parts of India with different climate conditions, underscoring the importance of geographical differences in fungal recovery. Several studies have shown that *Aspergillus* species, especially *A. fumigatus*, can colonize and infect the respiratory tract in different underlying conditions, including TB [5–8]. Shirai et al. [22] suggested, that although sputum bacteria represent only "colonization" of the respiratory tract in TB patients, they are associated with a poor prognosis, suggesting that they often cause infection.

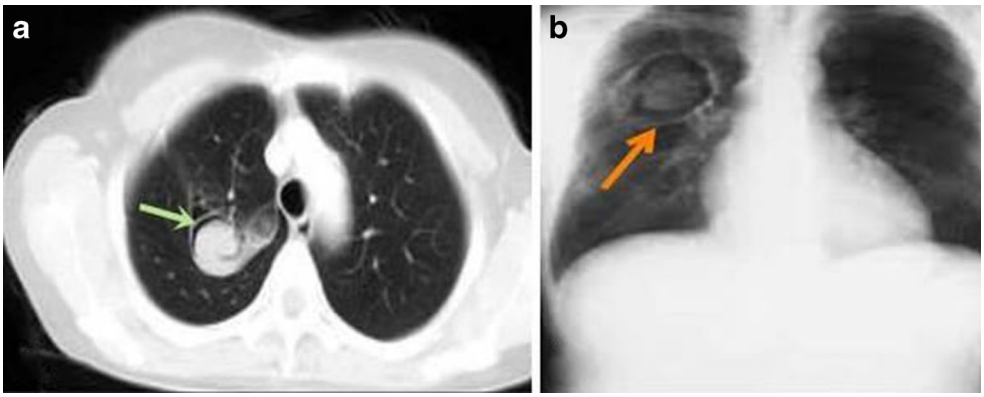

Fig. 2 Radiographic findings show cavities lesions in the lungs of a patient with aspergillomas. **a** The computed tomography (CT) scan shows a mass representing a crescent of air (*arrow*). **b** Chest radiograph showing a mass in the right upper lung capped by a crescent of air (*arrow*)

In the present study, 44.3 % of patients had elevated specific IgG against *A. fumigatus*, including 3 and 14 patients who met criteria for single aspergilloma and CCPA, respectively. In a previous study from Iran on TB patients who were still on anti-TB therapy, 50 % of patients showed positive antibodies against *Aspergillus* by indirect hemagglutination (IHA) [23]. In Kurhade et al. [11], 23.58 % of patients with chronic pulmonary infections, including PTB, showed a positive reaction with *A. fumigatus* antigen by the agar gel double diffusion test. In the study of Shahid et al. [12], 25 % cases showed anti-*Aspergillus* antibodies by ELISA. Iwata et al. [15] applied complement fixation (CF), IHA, and counter immunoelectrophoresis (CIE) to detect serum antibody against *A. fumigatus* in patients with active and cured pulmonary TB. Antibodies against *Aspergillus* by CF and IHA were positive in 8.6, 2.9, and 19.5 % of sera. Nakamoto et al. [24], using a CF test, found that 75 % of patients with previous TB were positive for serum antibody to *A. fumigatus*. Comparing our study and those of Kurhade et al. [11] and Shahid et al. [12], it is clear that the incidence of seropositivity in our patients was high. The study authored by Iwata et al. [15] shows that different methods have different sensitivities for antibody against *A. fumigatus*. We used a standard ELISA kit, whereas the other two studies applied a laboratory-prepared procedure to detect antibody against *Aspergillus*. We limited our patient population to only those with TB, whereas the other two aforementioned studies included patients with TB and different underlying conditions. In our study, the rate of positivity to *Aspergillus* IgG showed a strong relationship with elapsed time since TB ($p<0.0005$). However, only those with symptoms similar to TB were evaluated and most patients cured of TB remain well. Furthermore, it should be noted that the highest rate of positivity of 68.7 % was in patients with residual cavities.

These results strongly suggest that a residual cavity after TB facilitates *Aspergillus* infection with a concurrent rise in IgG against *A. fumigatus* in serum. Those with positive *A. fumigatus* IgG, without symptoms, may have silent disease or have had a sufficient immune response to resolve local *Aspergillus* cavity infection, with the *Aspergillus* IgG reflecting past infection. Other means of distinguishing these scenarios are required. Our results revealed a significant relationship between positive culture, direct microscopic examination, and serum IgG profile level. Similar results were also reported by Shahid et al. [12] and Kurhade et al. [11]. It should be noted that the ELISA method for the detection of IgG against *A. fumigatus* is useful in culture-negative cases. In our study, culture positivity for *Aspergillus* was only 12.9 versus 44.3 % for the detection of IgG against *A. fumigatus*. Several studies have also shown much greater sensitivity and usefulness for serologic tests for *A. fumigatus* IgG antibodies in the diagnosis of CPA compared with culture [5, 14, 24, 25]. Uffredi et al. [26] suggested that the analysis of predisposing factors, in conjunction with immunological tests and examination of bronchoscopic specimens, is helpful in distinguishing between colonization and infection due to *A. fumigatus*, as well as for differentiating between pulmonary aspergilloma and chronic necrotizing pulmonary aspergillosis in non-granulocytopenic patients, including those with TB. *Aspergillus* polymerase chain reaction (PCR) and/or antigen in sputum or bronchoscopy may be helpful [27, 28].

In the present study, 2.4 and 11.3 % of patients showed aspergilloma and CCPA, respectively. No large population study to evaluate CPA incidence or prevalence to determine the real incidence of aspergilloma and CPA after TB has been done so far. Most existing reports are case reports [10, 13, 14, 29] or case series on previously diagnosed CPA patients [5, 8, 24, 25, 30, 31]. However, Denning et al. [32], in a study on the global burden of CPA as a sequel to pulmonary TB, have suggested that, in 2007, 7.7 million cases of PTB occurred globally, and, of them, an estimated 372,000 developed

Table 3 The clinical characteristics of patients with chronic cavitary pulmonary aspergillosis (CCPA; $n=14$)

Clinical presentation	Weight loss	Fever	Hemoptysis	Cough	Fatigue	Sputum production
Patients (%)	8 (57.1)	6 (42.8)	5 (35.7)	14 (100)	9 (64.3)	13 (92.8)

CPA. They have also shown that the prevalence rate of CPA in TB patients ranged from <1 case per 100,000 population in large western European countries and the United States of America to 42.9 per 100,000 in both the Democratic Republic of the Congo and Nigeria. China and India had intermediate 5-year-period prevalence rates of 16.2 and 23.1 per 100,000, respectively. These estimates were based on two reports from the Research Committee of the British Tuberculosis Association in 1968 and 1970 [33, 34] on 544 patients from 55 chest clinics in Great Britain with post-tuberculous cavities. Evidence of "colonization" with *Aspergillus* species was sought using precipitins to *Aspergillus* in the serum (25 %), and aspergilloma was present on chest X-rays (14 %).

The most common clinical symptoms of CPA in our patients were chronic productive cough, fatigue, and weight loss, followed less frequently by fever and hemoptysis. Our data are consistent with some previous studies [5, 35, 36]. As these suggested, the acute clinical presentation with accompanying fever are more often due to CNPA [5, 9, 35, 36] or coexistent bacterial infection. Hemoptysis is a major contributor to CPA morbidity and mortality [37, 38].

The existing data have also shown that TB is usually the most common primary underlying condition in the development of CPA [5, 8, 24, 25]. The diagnosis of non-invasive pulmonary aspergillosis can be challenging, except for cases whose chest X-ray findings show a typical fungus ball and an *Aspergillus* antibody test provides the diagnosis of CPA. As Smith and Denning [8] suggested, in addition to pulmonary cavitation on chest X-ray, even the presence of fungus ball and hyphae, and also mass in a pulmonary cavity, with a positive culture (preferably positive direct examination), the presence of *Aspergillus* IgG antibodies must be definitively demonstrated for the diagnosis of aspergilloma or CPA. Often, the diagnosis of CPA is difficult because of its overlapping clinical and radiological characteristics with TB; hence, the importance of microbiological and serological testing to diagnose CPA early. Untreated CPA is progressive and may be fatal; yet, it responds to antifungal therapy [19, 39–41]. Treatments of CPA with a triazole, such as voriconazole or itraconazole, reduce morbidity and mortality and may reduce the apparent mortality for TB across the world.

In the present study, there were some limitations, including the absence of CT images for all patients, the limited time of follow-up, and the absence of any spirometry-based evaluation of the patients. We may have slightly underestimated point prevalence, as the antibody assay used is not 100 % sensitive, and probably will not identify the uncommon case of CPA caused by other species of *Aspergillus*.

Conclusion

In Iran, our data revealed that chronic pulmonary aspergillosis (CPA) is a common clinical presentation in individuals with healed tuberculosis (TB), as reported by previous studies from different countries. Colonization or surface infection with *Aspergillus* in lung cavities produced by TB should be considered as a risk factor for aspergilloma or CPA. In accord with previous studies, we emphasize the importance of measuring *Aspergillus* antibody to diagnose CPA.

Acknowledgments The participating patients are thanked for their kind cooperation, which was essential for the completion of the study. This study was supported by the Invasive Fungi Research Center, Mazandaran University of Medical Sciences, Sari, Iran.

Conflict of interest The authors declare that there is no conflict of interests. David Denning is President of the Global Action Fund for Fungal Infections (http://www.gaffi.org/), which aims to improve the outcome of patients with serious fungal infections across the world, including chronic pulmonary aspergillosis after TB.

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