Microarray detection of fungal infection in pulmonary tuberculosis

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Abstract  Background: Fungal pulmonary infection can be acquired in tuberculosis, immunodeficiency patients, and other chronic diseases. Many physicians missed fungal pulmonary infection because it does not show specific clinical manifestations. The aim was to identify the presence of overlapping fungal infections in tuberculosis patients, using high multiplexing capacity of DNA microarray.

Methods: The present study was conducted on 50 tuberculosis patients who were subdivided into: Group I consisted of 30 cases of multidrug resistance tuberculosis, Group II consisted of 10 fresh cases and Group III consisted of 10 relapse cases. Morning sputum samples were examined by DNA microarray.

Results: Aspergillus spp., was the only fungus detected in 24% of cases, Group I showed the highest percentage (26.6%) with statistically significant difference compared to Group II and III (20%) for each. Aspergillus fumigatus was the predominant spp. identified followed by Aspergillus niger and Aspergillus flavus. Mixed infection was identified in 4 cases in Group I. A statistical significant association between fungal detection and MDR-TB, diabetic patients, smoker, being male, presence of haemoptysis and toxic manifestations, presence of cavitary lesion or abscess and severity of X-ray finding.
Introduction

Fungal pulmonary infection has been emerging recently due to widely used broad spectrum antibiotics and steroids [1]. It can be acquired primarily or secondarily in tuberculosis (TB), immunodeficiency patients, other chronic diseases such as diabetes mellitus or malignancy, and may worsen the primary disease [2–6]. TB is principally a disease of poverty, with 95% of cases and 98% of deaths occurring in developing countries [7]. Globally, about 3% of all newly diagnosed patients have multidrug resistance tuberculosis (MDR-TB) [8]. Egypt is ranked among the mid level incidence countries in tuberculosis. In 2007 the annual risk of infection was calculated mathematically by WHO it was (24/1,00,000) [9]. In (2010), Egypt is estimated to have 15,000 TB cases and a rate of 18 cases per 1,00,000 of population [10]. The percentage of mycotic infections increase in pulmonary tuberculosis patients. Mainly four types of fungi, i.e. Aspergillus niger, A. fumigatus, Histoplasma capsulatum and Cryptococcus neoformans were recorded, which causes severe infection in lungs in patients suffering from pulmonary tuberculosis [11]. Many physicians missed fungal pulmonary infection because it does not show specific clinical manifestations and usually hindered by other diseases and cause high rates of morbidity and mortality [12]. There is an increasing awareness amongst clinicians and microbiologists pertaining to importance of infection caused by opportunistic fungi [13–15]. Therefore there is an acute need for proper diagnosis of the opportunistic fungal pathogen especially in tuberculosis patients. The conventional identification of pathogenic fungi based on phenotypic features and physiological tests is time-consuming and, therefore, often imperfect for the early initiation of an antifungal therapy [16]. DNA microarrays were introduced for the rapid and simultaneous identification of different fungal species at the same time. Based on pan fungal internal transcribed spacer (ITS) primers directed at the conserved regions between the 18S and 28S rRNA, which were shown to correlate well with culture results [17]. Aim of the work: Is to identify the presence of overlapping fungal infections in tuberculosis patients, using high multiplexing capacity of DNA microarray which may help in correct diagnosis of these diseases that may increase the cure rate.

Patients and method

Subjects

The present study was conducted on 50 tuberculosis patients, randomly selected amongst the diagnosed pulmonary tuberculosis cases admitted in El Abbasia Chest Hospital, They were divided into the following groups:

- Group I consisted of 30 cases diagnosed as MDR-TB.
- Group II consisted of 10 cases diagnosed as new cases (fresh cases).
- Group III consisted of 10 cases diagnosed as relapse cases.

Case definition by previous anti TB treatment [9].

New case

A patient who has never had treatment for TB or who has taken anti-TB drugs for less than 4 weeks.

Relapse

A patient who has been declared cured of any form of TB in the past by a physician after one full course of chemotherapy, and has become sputum smear-positive.

MDR-TB

It is defined as resistance to any combination of anti-TB drugs that include INH and rifampicin.

Cases were radiologically classified based on chest X-ray (CXR) findings according to National Tuberculosis Association of USA (1961) [18] into: minimal, moderately advanced, and far advanced cases.

Samples collection

Informed consent was obtained from each participant prior to specimen collection. Early morning sputum was collected in a sterile dry wide-necked, leak-proof container from each case and transported to the laboratory.

Then sample was homogenized and liquefied using NALC–Na OH method that involves use of N-acetyl-L-cysteine (NALC) according to Colle et al. [19]. DNA extraction was done using Qiamp DNA Mini Kit (Qiagen, Izasa, Madrid, Spain), the yield of total DNA obtained was determined spectrophotometrically. Universal fungal primers were used for amplification of the ITS1 and ITS2 regions. The sequence of primers is ITS1: 5'-TCCGTAAGGTGAAACCTGCGG-3' (position 36–54) and ITS 4: 5'-TCCTCCTGTTATTGATATG-3' (position 601–620), as described by White et al. [20]. The sequence of the forward primer ITS1 is complementary to a conserved region at the end of the 18S rRNA gene, and the sequence of the reverse primer ITS4 binds to a conserved region at the beginning of the 28S rRNA gene [21]. DNA amplification was performed in parallel with positive and negative controls. The positive control strain was isolated from clinical sample. The clinical isolate was identified by standard methods according to Colle et al. [19]. The negative control consisted of an equal volume of water replacing the DNA template. A total
reaction volume of 50 μl was prepared for PCR. The mixture contained 5 μl of 10× reaction buffer (100 mM Tris, 500 mM Kcl; pH 8.3), 3 μl of 25 mM MgCl2, 1 μl of 10 mM PCR Nucleotide mix, 2.5 μl of each primer (20 μM), 0.2 μl of Taq DNA Polymerase (5 unit/μl) Biogenet – Korea, 500 ng of template DNA and DEPC treated water. The amplification was performed in a Master cycler gradient (Eppendorf, Hamburg, Germany). An initial denaturation step (94°C for 5 min) was followed by 35 cycles (with each cycle consisting of DNA denaturation at 94°C for 30 s, primer annealing at 57°C for 30 s, and elongation at 72°C for 1 min) and a final extension step at 72°C for 7 min [21]. Amplified DNA products were separated by electrophoresis in a 1% agarose gel containing ethidium bromide (0.5 mg/ml); the running buffer was TAE buffer. DNA bands were visualized by UV transillumination. DNA ladder was used as a molecular size marker (Promega – USA). DNA bands were visualized by UV transillumination. Then, DNA was denaturated for 5 min at 95°C – USA). DNA bands were visualized by UV transillumination. A 100-bp DNA ladder was used as a molecular size marker (Promega – USA). DNA bands were visualized by UV transillumination. After hybridization, the slides were washed with 2× SSC (1× is 0.15 M NaCl plus 0.1% sodium dodecyl sulfate, then 2× SSC, and finally, 0.2× SSC for 10 min each time. The washing procedure was performed at room temperature with shaking in a glass container [21]. Staining of microarray slides was done using ParagonT DNA Microarray QC Stain Kit with SYBR R 555 Stain and Control Slide (Cat P32930 – Invetrogen, USA, according to manufacturer’s instructions. Microarray slides were placed into a staining tube containing the prepared staining solution (27 ml of stain buffer was added to 30 μl SYBR Green nucleic acid stain concentrate). The slides were incubated in the staining solution for 5 min at room temperature. After the incubation, the staining solution was poured off. Tubes (with slides inside) were centrifuged at 3000 rpm for 1 min with the tube lid off. Any remaining stain solution was decanted. Twenty-seven milliliter of fresh wash buffer were added and the slides were washed for 5 min with mild shaking. The working wash buffer was poured off and the slides were centrifuged at 3000 rpm for 1 min with the tube lid off. Then the remaining wash buffer was poured off, and centrifuged again for an additional 5 min with the tube lid off. Microarray slides were then read to be scanned. Each microarray slide was imaged by Carl Zeis camera. Image analysis was performed by the Fluorescent image analyzer, Image Pro-Plus to determine the optical density of each spot in addition to the positive and negative controls.

Data analysis

Analysis was performed using statistical software (SPSS version 17; SPSS, Inc., Chicago, IL). Percentages, Chi square test ($\chi^2$) and correlation coefficient test were used, with significant alpha level less than (0.05).

Results

The present study was conducted on 50 tuberculosis patients admitted to El Abbasia Chest Hospital. Their age was ranging from (25 to 65) years. The patients were divided into three groups: Group I consisted of 30 cases diagnosed as MDR-TB (60%) (20 males and 10 females). Group II consisted of 10 cases fresh diagnosed TB (20%) (8 males and 2 females). Group III consisted of 10 cases with relapse of TB (20%) (8 males and 2 females). The presence of fungal infection in pulmonary tuberculosis patients was detected by DNA microarray which allowed rapid and simultaneous identification of many fungal species at the same time. Aspergillus spp., was the only type detected in this study, while other fungal species as H. capsulatum or C. neoformans were not identified. Twelve out of 50 cases (24%) were positive for Aspergillus spp., 8 cases from Group I (26.6%), 2 cases from Group II (20%) and 2 cases from Group III (20%). Comparison between the studied groups shows statistically significant difference between Group I and both Groups II and III, while no statistically significant difference was found between Group II and III as shown in Table 2.

A. fumigatus was the predominant Aspergillus spp. identified in all the patients followed by A. niger and A. flavus. Distribution of the isolated fungal spp. among the studied groups was as follows:

A. fumigatus was identified in 2 cases of Group I, 2 cases of Group II and another 2 cases of Group III. While A. niger was identified in 2 cases of Group I only. Mixed infection was identified in 4 cases in Group I only (two of them were A. fumigatus with A. niger and the other two were A. fumigatus with A. flavus) as shown in Table 3.

In the present study 22 patients were smokers, fungal infection was detected in 10 of them (45.5%). Out of 50 cases 6 were suffering from DM, fungal infection was detected in 5 of them (83.3%). Twenty-two patients were presented with haemoptysis and toxic manifestation, fungal infection was detected in 10

| Table 1 Oligonucleotide probes sequence for identification of selected fungal species. |
|----------------------------------------|-----------------|--------|
| Probes sequences                        | Name            | Position |
| ACA AGA GAC GAC GGT AGC TTC ACG        | H. capsulatum   | 663–686 |
| GAACCCACCGCCCTTC                      | C. neoformans   | 540–565 |
| GGAGACACCCAGCAGACTCTGT                | A. flavus       | 175–194 |
| CCAACAGGACACCTGTCTGA                  | A. niger        | 114–133 |
| CCGACACCCAACTTTATTT                  | A. fumigatus    | 502–520 |
of them (45.5%), their distribution among the studied groups is shown in Table 4.

Based on chest X-ray findings cases were radiologically classified into:

- Minimal extent cases (6/50), moderately advanced cases (20/50), fungal infection was detected in 4 of them (20%), far advanced cases (24/50); fungal infection was detected in 8 of them (33.3%).
- Ten patients presented with cavitary lesions or abscess, fungal infection was detected in 6 of them (60%) as shown in Tables 5 and 6.

Table 7 showed that there was a statistically significant correlation between presence of fungal infection and male sex, smoking, DM, presence of haemoptysis and toxic manifestations, presence of cavitary lesion or abscess and severity of X-ray finding. While no statistically significant correlation was found between presence of fungal infection and age and female sex.

**Discussion**

Fungal infections remain a leading cause of infectious morbidity and mortality in heavily immunosuppressed patients [23–28]. For diagnosis of fungal infection, establishing cultures from blood and bronchoalveolar lavage (BAL) samples is often unsuccessful due to the low yields of CFU, and in the case of immuno-compromised high-risk patients who are febrile, pulmonary tuberculosis, neutropenic, thrombocytopenic, and often seriously ill, tissue biopsy specimens, in general, are not available [29]. Early initiation of effective antifungal therapy and reversal of underlying host defects remain the cornerstones of treatment for pulmonary fungal infections [30]. More sensitive and rapid detection assays of mycotic infections in pulmonary tuberculosis patients have been established by use of the PCR method. However, traditional methods in molecular biology generally work on a “one gene in one experiment” basis. Recently, DNA microarray has attracted tremendous interests among biologists as it promises to monitor the whole genome on a single chip. An experiment with a single DNA chip can provide researchers information on thousands of genes simultaneously [21].

The current study was carried upon 50 pulmonary tuberculosis patients; the presence of fungal infection in pulmonary tuberculosis patients was detected by DNA microarray. *Aspergillus* spp., was the only type detected in this study in (24%) of cases, while other fungal species as *H. capsulatum* or *C. neoformans* were not identified. Njunda et al. [31] found that the prevalence of *Aspergillus* spp. in the sputum of patients suspected of pulmonary tuberculosis was 15%, also, Kurhade et al. [32] reported that the prevalence of *Aspergillus* spp. was (16.26%). However Ekkena et al. [33] noticed that the most common fungal isolates were *Aspergillus* spp. (42.9%).
The observed discrepancy between these results might be attributed to the limited number of patients enrolled in the present study and different geographical distribution. Patients were positive for both pulmonary Mycobacterium tuberculosis and Aspergillus sp. mainly, this established relationship was also observed by Sahoo et al. [34] and a possible justification for this is the fact that tuberculosis remains the most important cause of sub-acute and chronic respiratory morbidity which most often leaves behind a scarred pulmonary parenchyma vulnerable to fungal colonization.

In the present study, the detected fungal species were in higher percentage in MDR Group I (26.6%), than Group II (20%) and Group III (20%). Comparison between the studied groups shows statistically significant difference between Group I and both Groups II and III, while no statistically significant difference was found between Group II and III. Meawed et al. [35] found that the prevalence of co-association between retreatment of pulmonary TB and pulmonary fungal infection was 30.8%. Study by Jain et al. [36] and Kurhade et al. [32] also reported that in treated group, the fungal infection was found in higher percentage than in the fresh group. They explained that patients at the greatest risk for fungal infection are those with prolonged periods of pulmonary tuberculosis. As common use of broad spectrum antibiotics in the pulmonary disease for long duration, for example in pulmonary tuberculosis stimulate the growth and virulence of infecting fungus by destruction of competing bacterial flora. Another explanation for high susceptibility of MDR-TB patients to fungal infection could be clarified by understanding the immunological changes associated with multi-drug resistant tuberculosis. It was confirmed that the most important host defenses against fungi are neutrophils and alveolar macrophages. IFN-γ produced by the T lymphocytes increases the production of nitric oxide and other nitrogen and oxygen-reactive radicals of macrophages [37]. It was observed that patients with MDR-TB show low IFN-γ production when compared with patients with non-resistant tuberculosis before and after treatment [38]. A recent study evaluating the immune response of MDR-TB patients revealed that the CD4+ cells of these patients have a weaker response to stimulation with higher IL-4 production and lower IFN-γ response than fresh cases and normal individuals. This observation suggesting that the T CD4+ cells of MDRT patients polarize to Th2 response. Furthermore, those patients had increased IL-10 and TGF-β cytokine production that promote an environment, in which the recently recruited cells of the immune system become refractory to stimulation by the immunological activation signals [39].

The predominant Aspergillus species detected from sputum in the present study was A. fumigatus detected in 10 cases, followed by A. niger detected in 4 patients, while A. flavus was detected from 2 patients. Among the collected samples 2 had mixed infection with A. fumigatus and A. niger, and 2 had mixed A. fumigatus and A. flavus. These results were in accordance with Kurhade et al. [32] who reported that A. fumigatus was the commonest species isolated: followed by A. niger and A. flavus. Also Njunda et al. [31] reported that A. fumigatus (5%) was the predominant Aspergillus species isolated from sputum in their study followed by A. flavus (3%). However Razmpa et al. [40], reported higher prevalence for A. flavus (30%). This wide variation in the incidence and frequency of isolation of various Aspergillus species may be due to geographical differences [41], different technologies used for identification or number of patients enrolled in the different studies.

It was noticed that the MDR group (Group I) carry the risk of higher percentage of fungal infections and it was the only group which was harboring mixed species. As treatment of MDR group necessitate the use of different antibiotics and anti-metabolites for long duration which may influence the incidence of fungal infection.
The present study showed that, there was a statistically significant correlation between presence of fungal infection and male sex, smoking, DM, presence of haemoptysis and toxic manifestations, presence of cavitary lesion or abscess and severity of X-ray finding. While no statistically significant correlation was found between presence of fungal infection and age and female sex.

Men are more vulnerable to infection than females due to their greater exposure to the surrounding. These results were in agreement with that of Bansod and Rai [11], who observed their greater exposure to the surrounding. These results were age and female sex.

A relation was found between presence of fungal infection and severity of X-ray finding. While no statistically significant correlation between fungal infection and severe toxemia and haemoptysis.

This study showed that there was a statistically significant correlation between presence of fungal infection, smoking and DM. Smoking and DM act as a co-factor in accelerating and amplifying the immuno-suppression state [42,43]. The predominance of fungal infection in diabetic patients has been reported by Jain et al. [36]. Khanna et al. [33] reported that factors mainly responsible for prevalence of fungal super infection in pulmonary tuberculosis are presence of resistant strain, chronicity of diseases, and associated disease such as diabetes mellitus. The present study showed that, there was a statistically significant correlation between presence of fungal infection and presence of cavitary lesion or abscess and severity of X-ray finding. In the present study fungal infection was higher (60%) in cavitary group than in the non cavitary group (15%). This was in agreement with Jain et al. [36] who reported that, fungus positivity was higher (59%) in cavitary group than in the non cavitary group. Higher positivity was found according to the extent of lesion but this difference was not statistically significant ($P > 0.05$).

Conclusion

Microarray detection of mycotic infection represents a rapid diagnostic tool helping early diagnosis of fungal co-infection and pulmonary TB. MDR-TB patients carry the risk of higher percentage of fungal infections and more liable for acquiring mixed fungal pathogens. Presence of male sex, smoking, DM and far extent of lesion must attract physicians’ attention for fungal co-infection with pulmonary tuberculosis.

References

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