MULTIPLEX PCR DIAGNOSIS OF ASPERGILLUS PARASITICUS AND ASPERGILLUS FLAVUS IN RESPIRATORY SAMPLES IN TUBERCULOSIS-SUSPICIOUS BRONCHOALVEOLAR LAVAGE (BAL)

Nahid Pedram1, Mohammad Hassan Shahhosseiny2-3, Mansour Bayat4, Saeed Bokaei5 and Mohammad Ghahri6

1 Ph. D. Student, Department of Mycology, Faculty of Specialized Veterinary Sciences, Science and Research Branch, Islamic Azad University (IAU), Tehran, Iran
2 Department of Microbiology, Shahr-e Qods Branch, Islamic Azad University, Tehran, Iran
3 Institute of Iranian Gene Fanavar (IGF), Tehran, Iran
4 Department of Mycology, Faculty of Specialized Veterinary Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran
5 Department of Food Hygiene, Faculty of Veterinary, University of Tehran, Tehran, Iran
6 Department of Biology, Faculty of Science and Engineering, Imam Hossein University, Tehran, Iran

e mail: nahidpedram@yahoo.com

(Accepted 30 September 2014)

ABSTRACT: Aspergillosis is induced by the inhalation of fungal spores, which leads to invasive diseases in organs. Also, a relationship has been found between aspergillosis and tuberculosis. This study aimed to investigate fungal contamination of Aspergillus parasiticus and A. flavus in tuberculosis-suspicious bronchoalveolar lavage cells that are TB negative. In the optimized PCR test, 343bp and 413bp products of Aspergillus parasiticus and A. flavus were respectively amplified. Fifty tuberculosis-negative BAL samples were directly tested and cultured using multiplex PCR method. Eighteen samples were positive, 17 only by M-PCR and 1 by both direct testing and culturing and M-PCR. Results of McNemar’s test demonstrated lack of agreement among the three tests (p<0.001). M-PCR test was more successful than traditional methods. Molecular methods such as M-PCR are more appropriate techniques for diagnosing fungal factors than direct testing and culturing methods in tuberculosis-suspicious samples.

Key words: Aspergillus parasiticus, Aspergillus flavus, multiplex PCR, tuberculosis.

INTRODUCTION

Aspergillus is one of the fungi with a very broad spread in nature. Aspergillus species cause a wide range of diseases such as allergy, superficial infections, and invasive infections in humans. Invasive aspergillosis is a fatal disease that often occurs in people with immune deficiencies. Based on the results of studies conducted in the past decade, invasive aspergillosis is a fungal infection with high mortality rate in patients hospitalized in intensive care unit (Hedayati Mohammad Taghi et al, 2009). Aspergilloma or fungal ball happens due to Secondary colonization hole and already existing in the lung (consequent to tuberculosis, sarcoidosis, bronchiectasis, and ankylosing spondylitis) and contains interwoven hyphae, fibrin, formless pieces, and few inflammatory cells. Chronic necrotizing aspergillosis or semi-invasive aspergillosis happens in patients with chronic pulmonary problems; but, invasive aspergillosis occurs in those with immune deficiency (Symoens et al, 1993, Soubani and Chandrasekar, 2002). Allergic aspergillosis is not accompanied by lung cavities. Aspergilloma has been known as fungus ball and shows the growth of Aspergillus fumigatus (Perfect et al, 2001). Aspergilloma has been reported in a cavity in the lung which has been caused by tuberculosis (1970). In the areas in which there is endemic tuberculosis, it is still the most prevalent predisposing factor for developing aspergilloma (Lejay et al, 2011).

Invasive pulmonary aspergillosis has been reported among patients with HIV virus who usually include patients with neutropenia or corticosteroid drug users (Mylonakis et al, 1998). Invasive pulmonary aspergillosis is developed as follows: First, a nodule is formed in the chest which has sensitive and polished sides (Blum et al, 1994). After the appearance of this nodule, a cavity is formed during one or two weeks, which is often observed in patients with neutropenia who have recovered (Horger et al, 2005). However, by prolonged hospitalization these patients, in critical conditions in intensive care unit and using immunosuppressive drugs in them leads to increased prevalence of invasive aspergillosis (Pasmans et al, 1992). Delayed definitive diagnosis, lack of timely and appropriate treatment, and existence of various neutropenia and...
prediposing diseases are among the reasons for high mortality of this disease. Despite recent advances in the diagnosis and treatment of this disease, emergence of invasive aspergillosis in ICUs is usually due to the lack of correct prediction and diagnosis of diseases (Curtis et al., 1979). Also, in some cases, differentiating this disease from tuberculosis (TB) only based on clinical symptoms seems to be suspicious and needs conducting supplementary and precise experiments (Gefter et al., 1985).

It is essential to utilize diagnostic techniques such as PCR and galactomannan and D-glucan antigen evaluations by serological methods in different samples in addition to traditional diagnostic methods in order to obtain a better image of the patients at the risk of invasive aspergillosis and perform required planning for better health and preventive measures (Sider and Davis, 1987).

Although immunological diagnostic methods such as antibody and antigen detection are faster, they lack the required specificity and accuracy and are sometimes impossible in patients with deficiency in the immune system which have problem in antibody production (Gerson et al., 1985). Today, molecular methods such as M-PCR which have sufficient sensitivity and appropriate velocity are very helpful. In this research, BAL samples which were shown to be TB-negative using culturing, direct, and molecular tests were experimented using M-PCR method for diagnosing fungal contamination including Aspergillus parasiticus and Aspergillus flavus. Finally, to compare with traditional methods, these samples were cultured and directly tested. Major tendency of this study was to investigate fungal contaminations such as Aspergillus parasiticus and Aspergillus flavus in TB-suspicious BAL samples using molecular method (Reichenberger et al., 2002).

**MATERIALS AND METHODS**

**Preparing strains of Aspergillus flavus and parasiticus and culturing method**: First, lyophilized and standard strains of Aspergillus flavus and parasiticus belonging to Iranian Industrial Bacteria and Fungi Collection (Persian Type Culture Collection, PTCC) with code 5004 (IR 111) were cultured at GYEP liquid medium.

After the organism growth, 500 µl of the liquid medium was removed and centrifuged at 12000 rpm for 5 min. The supernatant was discarded and resulting precipitation was deionized in 100 µl of sterile double-distilled water and suspended; then, its DNA was extracted using phenol-chloroform methods (Sambrook and Russell, 2006).

**Extracting DNA from standard strain**: Phenol-chloroform method was used for DNA extraction (Sambrook and Russell, 2006).

100 µl of the cultured strain in the liquid medium was removed; then, at the first stage, 500 µl of the lysis solution or buffer lysis (Proteinase K = 250 µg/ml, Tris-Hcl = 50 Mm, SDS = %10) was added.

At the second stage, 10 µl protease was added and shaken for 10 sec.

At the third stage, it was put in a 65° heater block for 20 min. After isochoronic removal of the solution inside the tube, phenol-chloroform solution was added and, after 10 times of inversion, it was centrifuged for 5 min. Afterward, the tube was placed on ice for 30 min. After removing from ice, the supernatant was transferred to a new tube and some isopropanol with the same volume of the solution was added to the tube. After 10 times of inversion, it was put in a -20° C freezer for 10 min. Once taken out of the freezer, it was centrifuged for 10 min at 12000 rpm and the supernatant was discarded; because isopropanol always causes DNA precipitation, the supernatant lacks any DNA. Afterwards, 1000 µl of alcohol 70% was poured on it and, after 10 times of inversion, centrifuged for 10 min at 12000 rpm (alcohol separates isopropanol from DNA). Finally, the supernatant was decanted (discarded), the test tube was placed in the 65° heater block, and 100 µl distilled water was added.

**Specific primers of Aspergillus flavus and A. parasiticus**: Two specific primers of Aspergillus flavus (AFLA-F and AFLA-R) and Aspergillus parasiticus (APA1 and APA2) are as shown in Table 1 (Hue et al., 2013).

**PCR reaction**: Every reaction contained 5 µl DNA extracted from the sample or strain, 2.5 µl 10x PCR Buffer, 0.5 µl (0.2 µm) forward primer, 0.5 µl (0.2 µm) 

<table>
<thead>
<tr>
<th>Size of product</th>
<th>Sequence of primer</th>
<th>Name of primer</th>
</tr>
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<tbody>
<tr>
<td>AFLA-F</td>
<td>5'-GGT GGT GAA GAA GTC TAT CTA AAGG-3'</td>
<td>413bp</td>
</tr>
<tr>
<td>AFLA-R</td>
<td>5'-AAG GCA TAA AAGG GTG TGGAG -3'</td>
<td></td>
</tr>
<tr>
<td>APA1</td>
<td>5'-GGA TTC GTG AGT GTC TTT AGG G -3'</td>
<td>343bp</td>
</tr>
<tr>
<td>APA2</td>
<td>5'-GGT AAA TGC TCC GCA CAG TC -3'</td>
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Multiplex PCR diagnosis of *Aspergillus* in respiratory samples

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**Fig. 1**: Optimized M-PCR test (amplicons of 343 bp *Aspergillus parasiticus* and 413 bp *Aspergillus flavus*) for diagnosing *Aspergillus parasiticus* and *flavus*. **M**: Size of 100bp DNA Ladder fermentas marker, **Line 1**: Amplified part of 413 bp *Aspergillus flavus* and 343 bp *Aspergillus parasiticus*, **Line 2**: Negative control.

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**Fig. 2**: PCR product of *Aspergillus parasiticus* and *flavus* cloned in plasmid. **M**: Size of 100bp DNA Ladder (vivantis) fermentas marker, **Line 1**: Cloned product of 343 bp *Aspergillus parasiticus*, **Line 2**: Cloned product of 413 bp *Aspergillus flavus*, **Line 3**: Negative control.

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**Fig. 3**: Results of sensitivity test. **M**: Size of 100bp DNA Ladder fermentas marker. **C+**: Positive control. **Line 1**: Dilution of 10-1 equivalent to 1,000,000 copies of DNA, *Aspergillus flavus*, *Aspergillus parasiticus*. **Line 2**: Dilution of 10-2 equivalent to 100,000 copies of DNA, *Aspergillus flavus*, *Aspergillus parasiticus*. **Line 3**: Dilution of 10-3 equivalent to 10,000 copies of DNA, *Aspergillus flavus*, *A. parasiticus*. **Line 4**: Dilution of 10-4 equivalent to 1000 copies of DNA, *Aspergillus flavus*, *Aspergillus parasiticus*. **Line 5**: Dilution of 10-5 equivalent to 100 copies of DNA, *A. flavus*, *A. parasiticus*. **Line 6**: Dilution of 10-6 equivalent to 10 copies of DNA, *A. flavus*, *A. parasiticus*. **Line 7**: Dilution of 10-7 equivalent to 1 copy of DNA, *A. flavus*, *A. parasiticus*. **C-**: Negative control.

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**Fig. 4**: Specificity test. **M**: Size of 1Kb DNA Ladder fermentas marker. **Line 1**: DNA of *Aspergillus flavus* and *parasiticus*. **Lines 2 to 8**: DNAs related to *cryptococcus neoformance, Fusarium spp, Fusarium solani, Candida albicans, Escherichia coli* (E.Coli), *Hepatitis B virus* (HBV), Human. **Line 9**: Negative control.

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reverse primer, 0.75 ¼l MgCl2 (50 mM), 0.5 ¼l dNTP 10mM, 0.3 ¼l Taq DNA polymerase 5u/¼l enzyme, and 15 ¼l sterile double-distilled water in the final volume of 25 ¼l. Thermal protocol was as initial denaturation at 94°C for 30 sec, annealing temperature at 66°C for 30 sec, and polymerization (extension) stage at 72°C for 30 sec in 35 cycles. Final polymerization was at 94°C for 5 min (Shah Hosseini). Thus, thermal profile was used for optimizing monoplex PCR tests for diagnosing *Aspergillus flavus* and *parasiticus* and also M-PCR test.

**Observing PCR product**: Reaction product in 1.5% agarose gel containing CYBR Green (SINA Gene, Cat.No.: MR7730C) was electrophoresed in TBE 0.5 x buffer.
Cloning PCR product as positive control: After purifying PCR product, it was cloned using fermentas T/A cloning kit (cat: K1214) and Vector pTZ57/R.

Determining sensitivity and specificity of PCR test: To investigate test sensitivity, different dilutions of fungal DNA from a million DNA copies (10-1 concentration) to one DNA copy (10-6 concentration) were prepared. This test is used to measure sensitivity of primers and indicates the delusions of fungus that can be identified by these primers.

Specificity test: DNA of different micro-organisms such as Cryptococcus neoformans, a species of Fusarium, Fusarium solani, Candida albicans, E. coli, hepatitis B virus, and human DNA along with fungal DNA of Aspergillus flavus and specific primers of the fungus were put in different tubes and underwent PCR test. Specificity test was separately performed for Aspergillus parasiticus.

Sample preparation: Fifty TB-suspicious BAL samples with negative TB were collected and their DNA (100 μl per each sample) was extracted according to the above-mentioned method.

Multiplex PCR on samples: A 25 μl M-PCR test was performed as follows:

- 14 μl sterile double-distilled deionized water
- 0.5 μl from each 4 primers (Aspergillus parasiticus and flavus)
- 2.5 μl 10x PCR buffer
- 0.75 μl MgCl2 (50mM)
- 0.5 μl dNTP10mM
- 0.3 μl Taq DNA polymerase 5u/μl enzyme
- 5 μl DNA extracted from the samples

Furthermore, each M-PCR test was accompanied by a negative and a positive control tube.

Direct testing and culturing on the samples: First, sabouraud dextrose agar containing 50 mg/lit chloramphenicol (SC) was prepared and then the BAL samples were cultured after homogenization from their precipitations using either transplanting 1 or point planting methods at the mentioned medium. In the case of the growth of mold colonies, slide culturing was done for their identification.

RESULTS

Optimized multiplex PCR product with DNA extracted from standard strains was loaded on the 5.1% agarose gel. Size of the obtained parts was 343 bp using specific primers of Aspergillus parasiticus and 413 bp by Aspergillus flavus (Figure 1).

The PCR product related to Aspergillus flavus and parasiticus was separately cloned to make positive control and determine sequences in pTZ57/R plasmid. After isolating single white clones, DNA was extracted and confirmed by PCR method (Figure 2).

Sensitivity of M-PCR test with specific primers for Aspergillus flavus and parasiticus could identify at least 100 copies of DNA related to each fungus of Aspergillus flavus and parasiticus (Figure 3).

Results of M-PCR test on 50 tested TB-suspicious BAL samples showed 18 positive samples, which included 17 samples containing Aspergillus flavus alone and only one sample containing Aspergillus flavus and parasiticus (Figure 5).

Results of M-PCR test demonstrated that, out of 50 BAL samples, 32 samples were negative and 18 were positive. All the 32 negative samples were again negative in microscopic culturing observation. Out of the 18 positive samples using M-PCR test, 17 were negative and 1 was positive in microscopic culturing and observation.

Results of these tests are presented in the following table. McNemar’s test demonstrated lack of agreement between two tests between results of direct test and culturing on the one hand and M-PCR result on the other (P <0.001). It seems that M-PCR test was more successful in terms of BAL samples (Table 2).

DISCUSSION

Aspergillosis is an infection which is caused by various species of Aspergillus fungi mostly in people with
pulmonary diseases such as TB, immune system disorders, transplanted organ, or pulmonary and respiratory problems (Denning et al, 2013). Different forms of Aspergillosis have been observed: 1- Pulmonary aspergillosis (Allergic type): is an allergic reaction to fungus and is usually expanded in people with pulmonary problems (such as asthma (Smith and Denning, 2011, Guinea et al, 2010); 2- Aspergilloma: includes mass-like fungal growth in an area which has been formerly involved in pulmonary diseases (such as TB); (Chen et al, 2013, Garcia-Vidal et al, 2008) 3- Pulmonary aspergillosis (invasive type): is dispersed in other parts of the body. This type is induced in people with weak immune system, because they cannot prevent the spread of fungal spores in their bodies and these spores easily enter their blood flow, could spread throughout their body, and contaminate important organs such as the heart and kidneys; 4- Chronic pulmonary aspergillosis: which is very prevalent in patients with pulmonary TB who have undergone treatment (Nam et al, 2010; Ross et al, 2010). In the patients with treated pulmonary TB, it can lead to complications such as progressive loss of pulmonary function, acute and continuous respiratory symptoms, and finally chronic pulmonary aspergillosis (Jewkes et al, 1983). In 1960, by the Research Committee of British Thoracic Society and Tuberculosis, chronic pulmonary aspergillosis incidence of tuberculosis in patients treated with holes about 2.5 cm in their lungs caused by tuberculosis, were reported (1968).

According to WHO’s clinical data, most cases of chronic pulmonary aspergillosis are probably related to people with treated pulmonary TB who have residual cavities in their lungs (Denning et al, 2013).

In a study Seyed Hossein Mirhendi et al, 428 BAL samples were collected in a 19 month period from the patients referring to pulmonary department of Shariati Hospital, Tehran, Iran, for bronchoscopy and also from the patients referring to the laboratory of Faculty of Health of the Mazandaran University. In order to investigate fungal contamination of these samples, after preparing BAL samples, 20% potash was added to 150 µl of it to perform direct microscopic test and 150 µl was inoculated at 4% sabouraud dextrose agar. Additionally, nested-PCR and real-time PCR molecular methods were used to examine the mentioned samples in terms of different fungi. Then, these methods were compared for diagnosing fungal contamination in the samples. Results were as follows: 21 (5%) cases out of 428 samples had positive direct test and 61 (14.2%) had positive culturing (35 cases of Aspergillus flavus, 7 cases of Aspergillus niger, 6 cases of Aspergillus fumigatus, 6 cases of Aspergillus fumigatus/Aspergillus niger (mixed), 1 case of Aspergillus terreus, 3 cases of penicillium, 2 cases of rhizopus, 1 case of Fusarium, and 73 cases (17%) of real-time PCR were positive, and 193 (45%) of nested-PCR were positive. Considering the direct test cases as standard, sensitivity of culturing, nested-PCR, and real-time PCR were estimated as 91%, 94%, and 39%, respectively. Results of this study were different from those of the present work in positive cases and also dominant diagnosed species. This study only aimed to diagnose two species of Aspergillus flavus and Aspergillus parasiticus fungi in TB-suspicious BAL samples, while Seyed Hossein Mirhendi’s investigation worked on fungal contamination of several different species in patients with different respiratory infections and problems (Seyed Hossein Mirhendi et al, 2012).

In Spain, Guinea et al used secretions in lower respiratory tract of 175 patients using real-time PCR to search for invasive aspergillosis. The studied population consisted of patients with cancer, cirrhosis, corticosteroid therapy, positive HIV, TB, and transplanted heart, kidney, and liver and found that 17.7% of patients were positive. In this study, 15 out of 31 positive patients were diagnosed to have the invasive disease.

Maximum isolated cases from the patients were related to Aspergillus fumigatus. Time needed for culturing was 3 days and PCR test lasted for 4 h. In addition to reducing diagnosis time using molecular tests, this method was able to diagnose invasive cases of the
disease compared to culturing. Results of this study were different from those of the present work in terms of positive cases and dominant diagnosed species, which can be attributed to the difference in selecting patients and methodology; but, they were similar in general conclusions, in which molecular techniques were superior to culturing (Guinea et al., 2013).

Hoeing collected 78 cases of pulmonary invasive aspergillosis-suspected bronchoalveolar (BAL) from two university hospitals in Austria and Germany. After conducting 4 experimental methods of beta-D glucan (BDG), LFD, PCR, and galactomannan (GM) in the mentioned samples, diagnosis of pulmonary invasive aspergillosis was declared as follows: 3, 14, and 17 people were definitely, suspiciously, and probably suffering from it and the rest were healthy. They declared sensitivity of three tests of beta-D glucan, LFD, and PCR from 70 to 80% and combined sensitivity of two tests of galactomannan (GM) and LFD was about 94%, while the combination of PCR and galactomannan (GM) methods had sensitivity of 100%. Thus, three experimental methods of LFD, PCR, and galactomannan (GM) are important in the diagnosis of invasive pulmonary aspergillosis; but, in the absence of PCR method, it is better to use LFD. Hoeing’s work aimed to provide the best diagnosis method of invasive pulmonary aspergillosis among the experimental methods regardless of the type of respiratory disease; however, in the present study, patients with suspected or treated TB were included (Hoenigl et al., 2014).

In an epidemiological study on the relationship of chronic pulmonary aspergillosis and treated pulmonary TB, David et al reported that annually at least 372,385 treated TB patients suffered from chronic pulmonary aspergillosis. According to the statistics presented by WHO, new cases of chronic pulmonary aspergillosis in treated TB patients were estimated as 42011 cases in Europe, 61520 in Eastern Mediterranean region, 61012 in Americas, 55198 in Africa, 81583 in west region of Pacific Ocean, and 372,145 in south-east of Asia (Denning et al., 2013).

Boz et al (2009) studied an 11 year old girl, who was suffering from pulmonary allergic aspergillosis after treating pulmonary TB. They reported that different species of aspergillosis are cloned in the residual lesions of the lungs of treated TB patients which could cause different types of aspergillosis (Boz et al, 2009).

CONCLUSION

Results showed that, for diagnosing Aspergillus flavus and parasiticus, M-PCR test was far more sensitive than culturing and direct testing. Also, in Iran, Aspergillus flavus was more prevalent than Aspergillus parasiticus. Because contamination with these fungi was very high in treated TB or TB-suspicious patients, physicians should pay particular attention to this issue and avoid unnecessary treatments without definitive diagnosis.

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