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Detection of *Aspergillus fumigatus* by RT-PCR among patients with chronic respiratory diseases

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Abstract

The object of this study was to detect *Aspergillus fumigatus* fungus by RT-PCR test among patients with chronic respiratory diseases and to compare this result with results of chest X-Ray and CT investigations. This study was carried out on patients with chronic pulmonary diseases (asthma, TB, pulmonary cancer, chronic bronchitis) who attended to Tikrit medical teaching hospital (adult age group) during the 20th of March 2012 to the first of August 2012 in the center of Tikrit city. The sample size included (52) patients with chronic respiratory diseases. RT-PCR test was done on serum of patients to detect *Aspergillus fumigatus*. Chest X-Ray and computed tomography investigations were done to patients. Results revealed that the aspergillosis cases according to RT-PCR test were (21/52 (40%) of chronic respiratory disease patients. The high frequency of aspergillosis cases detected by RT-PCR were among patients with pulmonary cancer were (6/6) as (100%), followed by pulmonary T.B. (7/16) as (43.7%). It has been shown that 13/21 (61.9%) of aspergillosis cases which were diagnosed by RT-PCR test with abnormal chest X-Ray findings and 15/21 (71.4%) with abnormal computed tomography. It can be concluded that more than (40%) of chronic respiratory disease patients were positive by RT-PCR test.

Keywords: RT-PCR, *Aspergillus fumigatus*, Chronic respiratory disease, Iraq.

Introduction

The opportunistic mold *Aspergillus* is the etiologic agent responsible for a variety of infections and conditions referred to as aspergillosis. These manifestations include a spectrum of diseases from allergic responses to the organism (allergic broncho - pulmonary aspergillosis), to colonization with *Aspergillus* spieces. (aspergilloma or fungus ball and other superficial conditions, such as external ear colonization) and invasive infection (invasive pulmonary aspergillosis and other clinical syndromes of tissue invasion) (Patterson *et al*., 2011). Fairly recently, a new method of PCR quantification has been invented. This is called "real-time PCR" because it allows the scientist to actually view the increase in the amount of DNA as it is amplified. Several different types of real-time PCR are being marketed to the scientific community at this time, each with their advantages. (RT-PCR, 2003). Clinical features of aspergillosis may be asymptomatic or influenza like illness as fever ,cough, malaise (Livinson *et al*., 1998). The general risk factors are weakened immune system, low white blood cell level, lung cavities, asthma or cystic fibrosis, ankylosing spondylitis, long-term corticosteroid therapy, a hospital stay, genetic makeup (Mayo Clinic staff, 2011).

Materials and Methods

It was descriptive, hospital based study. This study was carried out on patients with chronic pulmonary diseases (asthma, T.B., pulmonary cancer, chronic bronchitis) who attended to Tikrit medical teaching hospital (adult age group) during the 20th of March 2012 to the first of August 2012 in the center of Tikrit city. Information regarding the epidemiological characteristics of the patients in addition to a list of investigations was obtained by questionnaire. The questionnaire was filled by the researcher by direct interviewing with the patients. The sample size included (52) patients with chronic respiratory diseases(16 T.B, 14 Asthma, 13 chronic bronchitis, 6 pulmonary cancer and 3 others patients). Blood sample from each patient and serum was isolated for RT-PCR test and chest X-ray and chest computed tomography scan were done .

Results and Discussion

The frequency of aspergillosis cases which were diagnosed by Real Time Polymerase Chain Reaction among patients sample was (21/52 (40%) (Figure 1).

Figure (2) reveals the standard curve of RT-PCR for diagnosis of aspergillosis and show the threshold

level and numbers of cycles. The curves which were above the threshold level and < 42 cycles were positive by RT-PCR (Figure 3). It has been revealed that 6/6 (100%) of pulmonary cancer, 7/16 (43.7%) of T.B., 4/13(30.8%) and 4/14(28.5%) of asthmatic patients were positive by RT-PCR (Table 1).

Frequency of aspergillosis cases diagnosed by real time- polymerase chain reaction(RT-PCR) from each type of chronic respiratory disease patients to total of aspergillosis cases were as follow: pulmonary T.B was 7/21 (33.3%), asthma 4/21as (19.1%), chronic bronchitis (4/21) as (19.1%) and pulmonary cancer (6/21) as (28.5%) (Figure 4).

It has been shown that 13/21 (61.9%) of aspergillosis cases which were diagnosed by RT-PCR test (positive) were with abnormal chest X-Ray findings (Table 2)

The distribution of aspergillosis cases (positive RT-PCR test) according to chest X- Ray findings was revealed that aspergillosis cases were 5/7(71.4%) from tuberculosis patients, ¼(25%) from each asthma and chronic bronchitis and 6/6 (100%) from pulmonary cancer patients were with abnormal chest X- Ray findings (Table 2).

It has been shown that 15/21 (71.4%) of Aspergillosis cases which were diagnosed by RT-PCR test (positive) were with abnormal chest CT scan findings (Table 3).

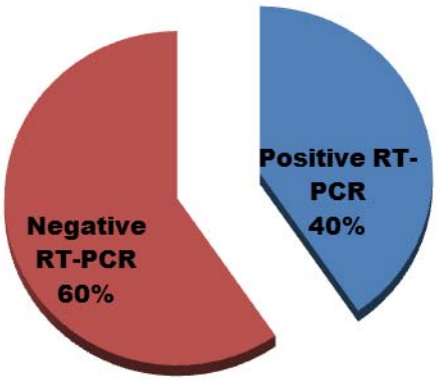

Figure (1): Percentage of Aspergillosis cases (detected by RT-PCR) among Chronic Respiratory Disease patients.

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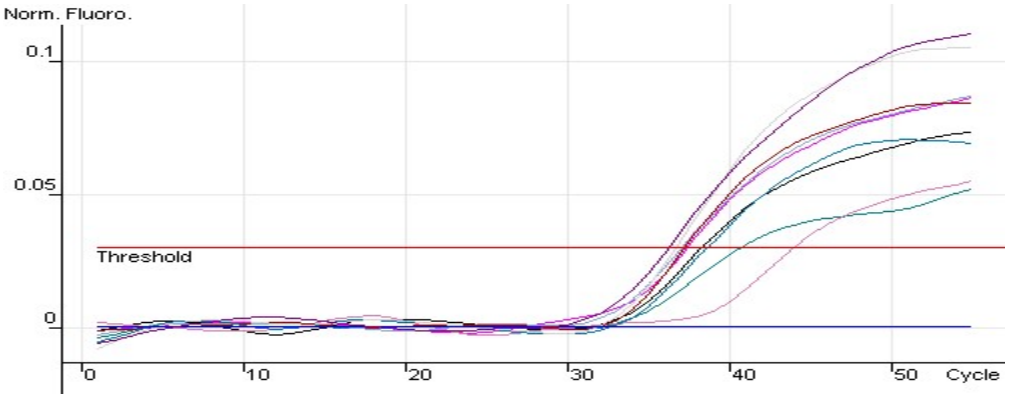

Figure (2): Real time–polymerase chain reaction standard curve for aspergilosis.

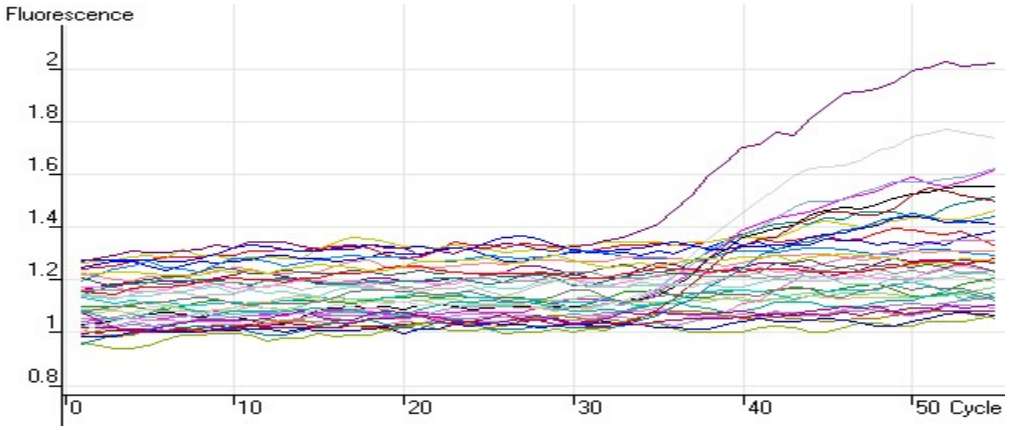

Figure (3): The curve of aspergillosis cases above threshold detected by real time – polymerase chain reaction results.

Table (1): Distribution of aspergillosis cases among chronic respiratory disease type according to RT-PCR results.

Disease type	Positive RT-PCR	Negative RT-PCR	Total
Pulmonary TB	7	9	16
%	43.7%	56.3%	100%
Asthma	4	10	14
%	28.5%	71.5%	100%
Chronic bronchitis	4	9	13
%	30.8%	69.2%	100%
Pulmonary cancer	6	0	6
%	100%	0%	100%
Others	0	3	3
%	0%	100%	100%
Total	21	31	52
%	40.4%	59.6%	100%

Chi square = 12.629. It is significant association at $p<0.05$

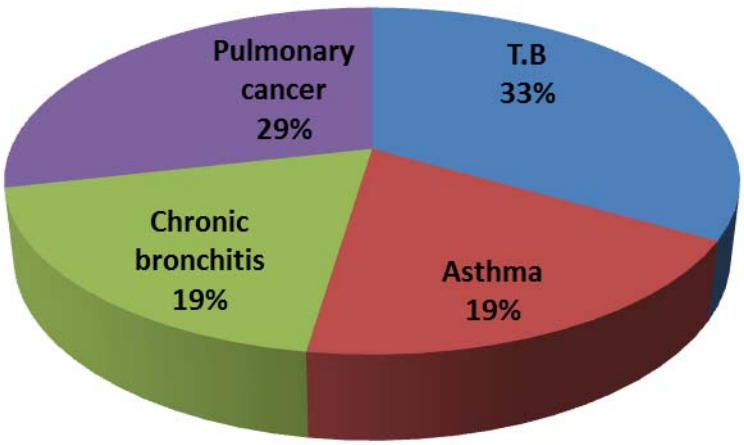

Figure (4): Frequency of aspergillosis cases from each chronic respiratory disease type (diagnosed by RT-PCR) according to total aspergillosis cases.

Table (2): Distribution of aspergillosis cases (diagnosed by RT-PCR test) among chronic respiratory patients according to chest X-ray findings.

Aspergillosis diagnosed by RT-PCR test	Abnormal chest X-ray findings	Normal chest X-Ray findings	Total
Pulmonary T.B.	5	2	7
%	71.4%	28.6%	100%
Asthma	1	3	4
%	25%	75%	100%
Chronic bronchitis	1	3	4
%	25%	75%	100%
Pulmonary cancer	6	0	6
%	100%	0%	100%
Total	13	8	21
%	61.9%	38.1%	100%

Chi square=9.536. Significant association at p<0.05

Table (3): Distribution of aspergillosis cases (diagnosed by RT-PCR test) among chronic respiratory patients according to chest CT scan findings.

Aspergillosis (by RT-PCR test	Abnormal chest CT scan findings	Normal chest CT scan findings	Total
Pulmonary T.B.	6	1	7
%	85.7%	14.3%	100%
Asthma	2	2	4
%	50%	50%	100%
Chronic bronchitis	1	3	4
%	25%	75%	100%
Pulmonary cancer	6	0	6
%	100%	0%	100%
Total	15	6	21
%	71,4%	28.6%	100%

Chi square =5.208. It is no significant association at p<0.05

The distribution of aspergillosis cases (positive RT-PCR test) according to chest CT scan findings was revealed that aspergillosis cases were 6/7(85.7%) from tuberculosis patients, 2/4(50%) from asthma, ¼(25%) from chronic bronchitis and 6/6 (100%) from pulmonary cancer patients were with abnormal chest CT scan findings (Table 3).

Humans and animals constantly inhale numerous conidia of this fungus. *A. fumigatus* is the most common etiologic agent, being responsible for approximately 90% of human infections (Latge *et al*., 1997; Schaffner, 1992). The most important risk factors of aspergillosis are weakened immune system, low white blood cell level, lung cavities, asthma or cystic fibrosis, ankylosing spondylitis, long-term corticosteroid therapy, a hospital stay and Genetic makeup (Mayo Clinic staff, 2011).

A. fumigatus DNA was amplified by real-time PCR (RT-PCR) with a Thermocycler/ABI Prism 7300 sequence detector (Applied Biosystems). The target was a 67-bp DNA fragment specific to the multicopy gene encoding the 28S rRNA of *A. fumigatus* (Challier *et al*., 2004).

The sensitivity of RT-PCR (expressed as the mean minimum number of cycles necessary to detect *A. fumigatus* DNA) was 34.3 cycles (1 copy). A sample was considered positive only when the crossing point value was ≤ 41 cycles (Challier *et al*., 2004).

According to this study, more than (40%) of patients samples were diagnosed as pulmonary aspergillosis . This result agree with that reported by Patterson *et al*. (2000) and Denning (1998). This result also was higher than that reported by Jombo *et al*. (2010) in a study done in Nigeria (28%) and it

was higher than that reported in Paris by Suarez *et al*. (2008) (28.9%).

This result was also higher than that reported by Verweij *et al*. (2005) that only (11% of patients with proven or probable invasive aspergillosis were PCR positive, and higher to that reported in Mdrid (14.4%) (Verweij *et al*., 2005). These differences the results may be a consequence of the varying degrees of invasion (Verweij, 2005), the DNA which not detected during exponential growth and was only released after hyphal autolysis (Costa *et al.*, 2001).

In other hand, Yamakami *et al*. (1996) reported a sensitivity of 70% and Einsele *et al*. (1997) reported excellent sensitivity (100%).

In the current study the frequency of aspergillosis cases were very clearly associated with pulmonary cancer (100%) and T.B. patients (more than 43%). The more frequent cases of pulmonary aspergillosis diagnosed by RT-PCR were among pulmonary cancer patients followed by T. B. patients (cavitary lung diseases) who were more liable to be affected by fungal infection as a result of sever immune system suppression (Innes *et al*., 2006; Shahhosseini *et al*., 2011).

It has been reported that fungal growth within a cavitating carcinoma of lung and necrotic carcinoma cell clusters intermingled with hyphae (McGregor *et al*., 1989). On the other hand, complication of noncavitating lung cancer by aspergillosis has been described (Yoshitomi *et al.,* 2000)

The association between *Mycobacterium tuberculosis* and *Aspergillus species* is not new. The latter is well known to colonize pre-existing lung cavities and produce fungal balls or aspergilloma. The majority of these cavities are a sequelae of primary tuberculosis (Caidi *et al*., 2006; Regnard *et al*., 2000).

In the current study the frequency of aspergillosis cases diagnosed by RT-PCR cases among asthmatic patients was 28.5% which were nearly similar to that reported by Agarwal *et al*. (2009) (29%) and lower to that documented by Maurya *et al*. (2005) and higher to that done by Jomo *et al*. (2010) which was (15.4%).

Regarding pulmonary cancer patients, it has been obvious that all the cases were positive by RT-PCR for presence of aspergillosis. This result is higher than that reported by Malik *et al*. (2003) in which they found that only (14.2%) of pulmonary cancer cases were affected by aspergillosis. This might be due to the difference in sample size and severity of cases.

PCR sensitivity and specificity have also been reported as 100% and 92%, respectively, in serum samples(Hizel *et al*., 2004; Buchheidt *et al*., 2001; Loeffler *et al*., 2002; Halliday *et al*., 2006).

In the current study the underlying conditions of aspergillosis were different. It has been revealed that tuberculosis represented the main underlying cause (33.3%) of all detected aspergillosis cases follwed by pulmonary cancer (28.5%) and (19.1%) for both asthmatic and chronic bronchitis diseases. This result is different from result reported by Smith *et al*. (2011); Camuset *et al*. (2007); Sugino *et al*. (2008) in which the underlying conditions of pulmonary aspergillosis were as follow T.B. represented (30.2%), chronic obstructed diseases (33.3%), Asthma 10.3% and pulmonary cancer (9.5%) of underlying cause.

Studies done in each of Taiwan, South Korea and India in which it has been documented that tuberculosis represented (93%) of underlying cause of Aspergillosis. (Chen *et al*., 1997; Shah *et al*., 2008; Nam *et al*., 2010).

Tuberculosis represented the first preexisting condition for occurring pulmonary aspergillosis (Babatasi *et al*., 2000)

These discrepancies could be due to the different technical approaches used and sampling methods and sample size. Indeed, a major difference was the type of PCR method used in these studies, i.e., nested PCR (*Buchheidt et al*., 2004; Halliday *et al*., 2006), PCR–enzyme-linked immunosorbent assay (Florent *et al.,* 2006), or RT-PCR (Jordanides *et al*., 2005; Kawazu *et al*., 2004). These different types of PCR are not equivalent in terms of contamination with previously amplified products; the nested PCR dramatically increases the risk of "false-positive results." A second major difference was the type of blood sample used for the molecular detection of DNA, that different studies used whole blood (Buchheidt *et al*., 2004; Halliday *et al*., 2006), plasma (Kawazu *et al*., 2004), or serum (Florent *et al*., 2006).

In the current study it has documented that chest X-Ray abnormal findings were about (62%) of aspergillosis cases detected by RT-PCR. This result was higher to that reported by Levison *et al.* (1998) in which the percentage was (50%). This result differ from the result of abnormal chest X-Ray findings among aspergillosis cases detected by galactomannan test (about 70%) because the numbers of aspergillosis cases detected by RT-PCR were more than that detected by galactomannan. These cases of aspergillosis that showed abnormal chest X-Ray abnormality seemed to be nearly similar in both tests.

The abnormal chest X-Ray findings were more prominent among aspergillosis cases from pulmonary cancer and T.B patients because of

presence of either cavitation or consolidation or pleural thickening((Binder *et al*., 1982).

The frequency of cases of aspergillosis which were diagnosed by RT-PCR with abnormal chest CT scan findings were (71.4%). This result is higher than that reported in a study done in Japan by Masahiro *et al*. (2001) in Japan in which RT-PCR method was used for the diagnosis of pulmonary aspergillosis. They found that only (34.6%) of aspergillosis cases showed abnormal CT findings and it is higher than this reported by Heussel *et al*. (1999) when the percentage is (67%), while it is lower than this reported by Greene *et al*. (2005) when the percentage was (94%).

These differences may be attributed to sample size and selection of patients group. The frequency was more prominent among aspergillosis cases from pulmonary cancer and T.B. patients.

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