

Full Length Research Paper

Assessment of different samples for molecular diagnosis of extra-pulmonary tuberculosis

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Extrapulmonary tuberculosis is an important clinical problem particularly in developing countries. The aim of this study was to assess two different samples (blood and fluid) for the diagnosis of extra-pulmonary tuberculosis (abdominal tuberculosis and tuberculous lymphadenitis). The study subjects were recruited from WadMedani Teaching Hospital during 2009-2013. Seventy five ascetic fluid and blood samples were collected from each suspected patient with abdominal tuberculosis and twenty five lymphatic aspirates and blood samples were collected from each suspected tuberculous lymphadenitis patient. DNA was extracted using DNP™ kit (CinnaGenInc) and polymerase chain reaction (PCR) was done using *IS6110* gene for both samples. In abdominal tuberculosis, 20/75 (27%) were positive for tuberculosis when ascetic fluid was used and 9/75 (12%) in case of blood samples. The comparison between ascetic fluid and blood samples, showed that, there was a significant difference in both results, P-value < 0.05. In tuberculous lymphadenitis, 13/25 (52%) and 3/25 (12%) were positive to tuberculosis when lymph aspirate and blood were used respectively. This study concluded that the best sample for diagnosis of abdominal TB and lymphadenitis is ascetic fluid and LN aspirate. This study recommends that ascetic fluid and lymph aspirate samples are recommended to be used in molecular diagnostic test.

Key words: Extra-pulmonary tuberculosis, molecular diagnosis, acetic fluid, lymph aspirate samples.

INTRODUCTION

Tuberculosis (TB) is among the top ten causes of global mortality, it is estimated that approximately one-third of the world's population is infected with tuberculosis bacillus, and each year eight million people develop the tuberculosis disease which annually kills 1.8 million worldwide (Ahmed et al., 2011). In Sudan, an estimated

annual risk based on the data of the 1986 national prevalence survey of TB is 1.8% which gives an incidence of 90/100,000 smear positive cases, and puts Sudan among the high prevalence countries for TB in the eastern Mediterranean region (Crofton, 2000).

The World Health Organization estimated that Sudan

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ranked twenty-third in the list of countries with the greatest number of estimated incident of TB cases (78 030), with an estimated rate, half again as high as the estimate for the whole world (220 vs.140 per 100 000 population) (El Sony et al., 2007). According to the Sudan annual health report 2007, the incidence of TB rate was 58 per 100,000, where Khartoum State reported the highest number of TB patients admission of 4878 cases, then Gezira State, 3105 cases, North Kordofan with 1853 cases, at the end of the list, North States became the fewest prevalence state of TB with 820 cases (Annual Health Statistical Report, 2007). In Gezira State, the incidence of TB cases from 2003 to 2008 was between 2706 - 3259, (WHO TB Program in Gezira State). The prevalence of TB in Sudan is 209 cases per 100,000 of the population and 50,000 incident cases during 2009 (Sharaf Eldin et al., 2011). Most of the cases of TB are pulmonary TB which is about 75%, the other 25% cases are extra-pulmonary TB (EPTB), (CDC, 2003).

The incidence of extra-pulmonary forms of tuberculosis varies from country to another, such that on the average between 1964 and 1989, 5 to 10% of the approximately seven million new cases each year in the developing countries were extra-pulmonary. This distribution also can be affected by origin of the individuals within a country (Talavera et al., 2001).

The diagnosis of extra-pulmonary tuberculosis is still now challenging for diagnostic routine laboratories. The aim of this study is to assess two different specimens (blood and acetic fluid for abdominal TB, blood or lymph aspirate for tuberculous lymphadenitis) using polymerase chain reaction (PCR) to diagnose abdominal TB and tuberculous lymphadenitis in patients attending Wadmedani Teaching Hospital during the period of 2009-2012.

MATERIALS AND METHODS

Study subjects

All clinically suspected patients with abdominal TB or tuberculous lymphadenitis patients attending Wadmedani Teaching Hospital during the period of study from 2009 - 2012 were recruited for this study.

Inclusion criteria of subjects

Patients with symptoms of guarding and free fluid (ascetic), abdominal pain and mass, weight loss, fever sweating, diarrhea and vomiting, and patients with past history of pulmonary TB disease or/and history of TB contact were included. Patients presented with lymphadenopathy in different body regions including cervical, inguinal and auxiliary lymph node were included in the study.

Exclusion criteria of subjects

Patients with HIV infection, Chron's disease, malignancy, cirrhosis,

ulcerative colitis, chronic diseases were excluded from this study.

Sample collection

Ascetic fluid or lymph nodes aspirate and 2 ml of venous EDTA blood from each patient were collected after a written informed consent. Ethical approval for this study was given from National Cancer Institute Research Ethical Committee (NCI-REC).

Molecular methods

DNA was extracted from lymph node aspirate and ascetic fluid blood using DNP™ kit (cinna GenInc, Cat. No.DN115C form Iran). The kit was designed to isolate double stranded DNA from human and animal sources. Lymph node aspirate and ascetic fluid samples were treated with 4% sodium hydroxide, then they were centrifuged, homogenized, supernatant discarded, and the rest used for DNA extraction as described by manufacturer.

Polymerase chain reaction (PCR)

Identification detection of *Mycobacterium tuberculosis* was done by using a specific pair of primers (the sequence of these primers, T4 and T5, are: 5'-CCT GCG AGC GTA GGC GTC GG 3' and 5' CTC GTC CAG CGC CGC TTC GG 3', respectively) designed to amplify an insertion sequence *IS6110* gene in the *M. tuberculosis* complex, the expected band size was about 123 bp. The total volume of was 25 µl for each reaction (positive and negative control was done for each PCR run). All the amplicons were checked run using agarose gel electrophoresis. The presence of 123 bp for *IS6110* gene fragments indicated a positive test for *M. tuberculosis*.

Statistical analysis

A statistical analysis was performed with SPSS statistical package version 16.0. Descriptive analysis was done and correlations between different variables were calculated using Pearson Chi-square test.

RESULTS

Hundred suspected extra-pulmonary TB cases were recruited from WadMedani Teaching Hospital during the period of September 2009 to September 2012. Seventy five out of hundred (75%) of the cases were suspected with abdominal TB while 25/100 (25%) were suspected to have lymphadenitis TB. The mean of ages of suspected abdominal subjects was 46.7 ± 18.2 years with range between (min 3 - max 80 years old). The mean age of suspected tuberculous lymphadenitis patients was 34.7 ± 20.7 years with range between (min 2 - max 80 years) as shown in Figure 1. The description of the study subjects showed that in suspected abdominal TB and TB lymphadenitis, males were 45 (60%), 10/25 (40%) respectively as shown in Table 1. The majority of the study subjects were from rural areas: 56/75 (74.7%) and 18/25 (72%) respectively as shown in Table 2. Regarding suspected abdominal TB patients, ascites was the common presenting symptom in all study subjects and

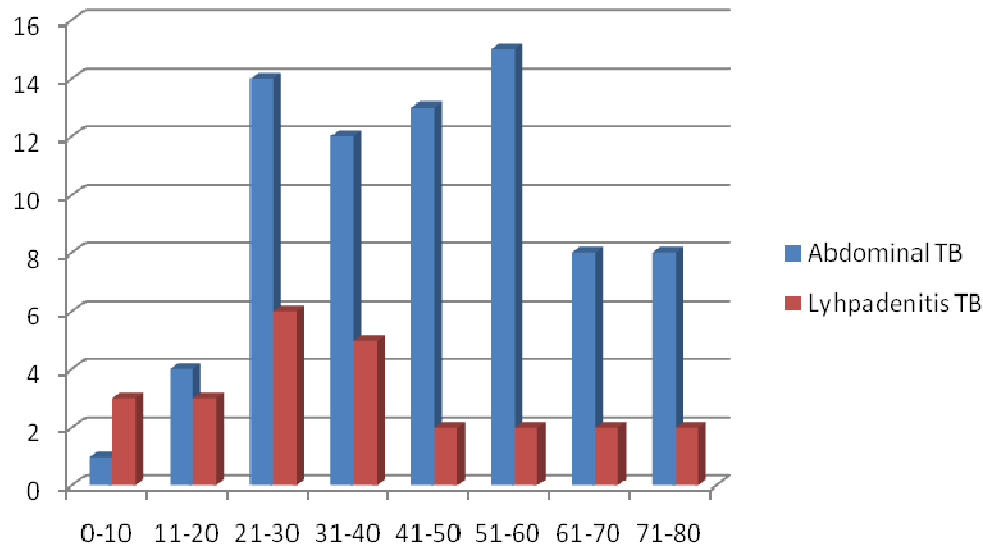


Figure 1. Description of study subjects by age groups.

Table 1. Description of study subjects by gender.

Gender	Abdominal TB (%)	Lymphadenitis TB (%)	total no. of subjects
Male	45 (60.0)	10(40.0)	55
Female	30 (40.0)	15(60.0)	45
Total	75	25	100

Table 2. Residence description of study subjects.

Sex description	Abdominal TB (%)	Lymphadenitis TB (%)	Total no. of subjects
Urban	19(25.3)	7 (28.0)	26
Rural	56(74.7)	18 (72.0)	74
Total	75	25	100

weight loss was 58/75 (77.3%), while lymphadenopathy was common presenting for TB lymphadenitis and night sweating, 10/25 (40%) were the common presenting symptoms in the suspected TB lymphadenitis study subjects.

Molecular detection of *M. tuberculosis* in abdominal TB study subjects

The *IS6110* gene is a multi-copy gene found only in *M. tuberculosis* complex. Most of the PCR studies have targeted *IS6110* gene sequence of *M. tuberculosis*

genome because of the presence of repetitive sequence of *IS6110* gene, this characteristic helps to increase the sensitivity of PCR over that obtained in the amplification of single DNA sequence. PCR was done for the ascetic fluid samples and blood for seventy five suspected abdominal TB, PCR resulted to 20/75 (26.7%) of ascetic fluid and 9/75 (12%) of blood samples were positive for *M. tuberculosis* as shown in Figure 2, PCR results indicate *M. tuberculosis* DNA with length 123 bp as in Appendix 1.

The comparison between ascetic fluid samples blood samples results, showed that there was a significant difference, between the two samples using person Chi-

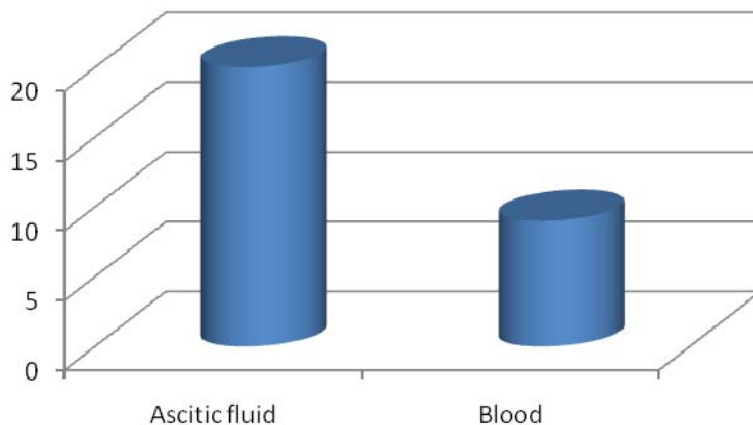


Figure 2. The positive PCR results in ascetic fluid and blood samples of study subjects. N=75.

Table 3. Comparison between ascetic fluid and blood.

		PCR results for blood		Total
		Positive	Negative	
PCR results for fluid	Positive	9	11	20
	Negative	0	55	55
Total		9	66	75

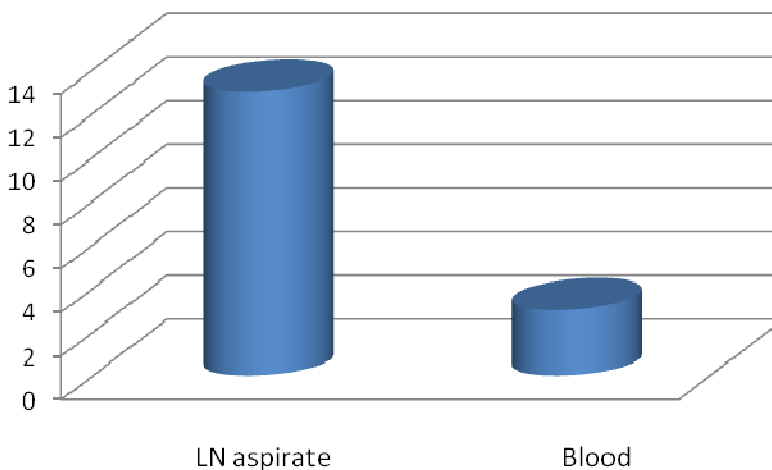


Figure 3. The positive PCR results in LN aspirate and blood samples by *IS6110* in suspected LN TB patients. N=25.

Square test = 28.1, P- value = 0.000 as shown in Table 3.

Detection of *M. tuberculosis* in tuberculous lymphadenitis study subjects

PCR was done using *IS6110* gene for 25 lymph node

aspirate samples, 13/25 (52%) of the study subjects were positive for *M. tuberculosis* indicating bands with 123 bp. For the blood samples 3/25 (12%) of the study subjects were positive for PCR as shown in Figure 3. In comparison between the lymph node aspirate samples and blood samples, there was a significant difference, Person Chi-square test = 28.1, P- value = 0.000 as

Table 4. Comparison between LN and blood samples results.

		PCR results for blood		Total
		Positive	Negative	
PCR results for LN aspiration	Positive	3	10	13
	Negative	0	12	12
Total		3	22	25

shown in Table 4.

DISCUSSION

Extra-pulmonary tuberculosis (EPTB) is considered as an important clinical problem, it can occur in isolation or along with a pulmonary focus as in disseminated tuberculosis. It has been observed that EPTB constituted about 15 to 20% of all cases of TB (Sharma and Mohan, 2004). Abdominal TB involve the gastrointestinal tract, peritoneum, lymph nodes or solid viscera, constitutes up to 12% of extra-pulmonary TB and 1-3% of the total TB cases. The disease can mimic many conditions, including inflammatory bowel disease, malignancy and other infectious diseases, thus diagnosis is therefore often delayed (Uzunkoy et al., 2004). In this study, in 100 suspected EPTB, 75 were abdominal TB, 20/75 (26.7%) of them were positive. Tuberculosis of the lymph node (tuberculous lymphadenitis) is the most common form of extrapulmonary tuberculosis. In developed countries, tuberculosis is implicated in as few as 1.6% of patients with lymphadenopathy (Sarwar et al., 2004). But in developing countries almost two third of the cases of lymphadenopathy are due to tuberculosis (Sarwar et al., 2004).

The mean age of the abdominal TB cases in this study was 47.8 ±18.2 years, while the age range was found to be between 21-70 years which was similar to study done by Uzunkoy et al. (2004) in Turkey; they found that the mean age was 39 years (range 18-65). In Taiwan, Huan-Lin et al. (2009) found that patients with abdominal TB, had age range from 22 to 88 years, with a mean age of 50 ± 18[9]. In the tuberculous lymphadenitis patients, the mean ages was 36.4±16.6 years and the age range was 21-40 years, this was different from study done by in Sudan that showed found that a mean age of thirty patients was 26.9±11.2 years (Sharaf-eldin et al., 2002).

Extra-pulmonary tuberculosis, which remained without bacteriologic or histological confirmation, and the diagnosis based on recognition of signs and symptoms is raised from involvement of particular organ or system. Molecular technique using nucleic acid amplification methods to detect *M. tuberculosis* in clinical specimens are increasingly in use as a tool for TB diagnosis. *IS6110* gene is used as a target by the majority of the

investigators performing PCR based diagnosis of TB (Kulkarni et al., 2006). The principal reason for using *IS6110* gene is because it is considered to be a good target for amplification as this insertion sequence is found in almost all members in high copy number in most strains of the *M. tuberculosis* complex, which was thought to confer higher sensitivity (Kulkarni et al., 2006; Sharma et al., 2013).

The aim of this study was to compare between different samples molecular diagnosis of extra pulmonary TB by using *IS6110* gene. In comparison between ascetic fluid and blood samples, the study showed that there was a significant difference ($P<0.05$) between the two different samples, this means that ascetic fluid had a high positivity than blood and it may be due to the presence of the microorganism at the location of the lesion while low level of was found in the blood. This is consistent with a study done in Pakistan using ascetic fluid DNA sample and *IS6110* specific primer sequence for *M. tuberculosis*, 50/55 (90.9%) patients were positive (Phulpoto et al., 2010). Several studies had the same results with high positivity from ascetic fluid (Uzunkoy et al., 2004; Protopapas et al., 2003). Thus, ascetic fluid is a suitable sample for diagnosis of abdominal TB. Therefore, detecting *M. tuberculosis* from blood samples was less likely (Chhuttani et al., 1986).

Comparison between lymph node aspiration and blood samples for patient with lymphadenitis, showed that lymph node aspirate samples 13/25 (52%) were positive when compared with only 3/25 (12%) in blood samples, and there was a significant difference ($P<0.05$), this is consistent with a study conducted in lymphadenopathy clinic, Central Police Hospital, Burri, Khartoum, Sudan, showing that circulating *M. tuberculosis* DNA was detected in the aspirate fluid of all the 28 patients (100%) with multiple lymph nodes (Sharaf-eldin, 2002). Another study detected *M. tuberculosis* from blood, in 191 suspected patients with extrapulmonary TB, they found a low rate of detection (Kolk et al., 1998). Same result was also found in a study done on sputum-smear positive patients with *M. tuberculosis* (Aguado et al., 1996).

Conclusion

For PCR diagnosis of abdominal TB and tuberculous

lymphadenitis, the best samples are ascetic fluid and lymph nodes aspirate, respectively.

Recommendation

Ascetic fluid and lymph aspirate are recommended to be used as sample for diagnosis of extra-pulmonary tuberculosis.

Conflict of Interests

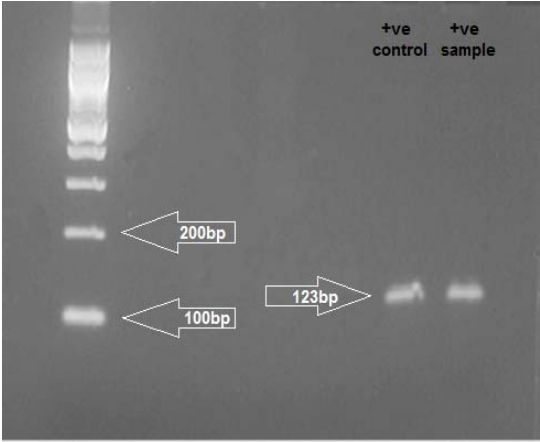
The author(s) have not declared any conflict of interests.

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Appendix 1. A band of 123 bp for positive sample using *IS6110*.