Journal of Kerman University of Medical Sciences

JKMU

Journal of Kerman University of Medical Sciences, 2017; 24(4): 312-319

Molecular Identification of Mycobacterium Tuberculosis Complex in Formalin-Fixed, Paraffin-Embedded Tissue Blocks of Extra Pulmonary Speciemens using Genomics Extraction

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Received: 17 October, 2016 Accepted: 13 June, 2017

ARTICLEINFO

Article type:
Original article

Keywords:
Extra pulmonary Tuberculosis
Paraffin-embedded tissue blocks
Epidemiology

Abstract

Background: Tuberculosis has been detected in some extra pulmonary ecological niches. Although extra pulmonary tuberculosis (EPTB) is less frequent than Pulmonary Tuberculosis (PTB), its incidence has increased worldwide. The aim of this study was to investigate the presence of EPTB and MDR-EXPT in Formalin-fixed, paraffin-embedded tissue blocks among different samples in Kermanshah, Iran.

Methods: Among all the suspected tuberculosis cases referred to the pathology laboratories, 116 patients were included based on the diagnosis of the infectious disease specialist .DNA were extracted from formalin-fixed, paraffin-embedded tissue blocks using genomics extraction G-spin Total DNA Extraction kit and the presence of EPTB was examined by TB Resistant Modual Isoniazid, Rifampicin kit.

Results: The frequency of EPTB in the paraffin-embedded block samples was 32% (37 cases), and lymph nodes had the highest frequency (44.5%) followed by Pleural fluid, breast and CSF with the frequencies of 34.6%, 33% and 27% respectively.

Conclusions: In the present study we found a high and increasing frequency of EPTB in paraffin-embedded blocks of the participants and it is one of the highest frequencies reported around the world. Since EPTB cases constitute a large number of TB burden, serious attention of public health organizations is recommended.

Citation: Izadi B. Jalilian S, Madani S.H, Mohajeri P. Molecular Identification of Mycobacterium Tuberculosis Complex in Formalin-Fixed, Paraffin-Embedded Tissue Blocks of Extra Pulmonary Speciemens using Genomics Extraction. Journal of Kerman University of Medical Sciences, 2017; 24(4): 312-319.

Introduction

Despite the widespread use of bacillus calmette Guerin (BCG) vaccination and anti-tuberculosis drugs, tuberculosis (TB) has remained a serious global public health problem (1, 2). TB is the second leading cause of death in the world following HIV (3). According to the World Health Organization (WHO), over 9 million new cases of TB occur each year, resulting in approximately 2 million deaths worldwide (4).

TB is a multisystem disease which usually affects the lungs; however, it can disseminate through hematogenous or lymphogenous circulation and may also affect other parts of the body, such as brain, intestine, kidney, bone marrow, or spine which is then called Extra Pulmonary Tuberculosis (EPTB) (5, 6). Thus, a variety of clinical specimens other than lung specimens might be submitted for examination when EPTB is suspected (7).

Pulmonary and extra-pulmonary forms of this disease have respectively shown 58% and 15% frequency(8). About 95% of TB cases happen in developing countries(9). Despite vigorous control of EPTB and routine implementation of directly observed treatments (DOT), 14,000 new cases have been reported annually in Iran located in west of Asia (10).

Drug resistance in TB is a major public health concern. Multi-drug resistant (MDR) TB is defined as *M. tuberculosis* that is resistant to at least rifampicin (RIF) and isoniazid (INH) antibiotics (11).

EPTB diagnosis is difficult mostly because of various clinical manifestations and aggressive procedures needed for its diagnosis (12).

There are several methods available for detecting EPTB or PTB. Fresh clinical samples are desired for laboratory diagnosis of mycobacterial infections that due to being reduced in patients with EPTB, it cannot be cultured. Diagnosis of EPTB is often based on tissue material with few bacilli, so it is difficult to confirm the disease with standard microbiological techniques and in such cases; formalin-fixed paraffin-embedded tissue samples are used. So, histopathology screening and molecular methods are valid diagnostic methods for detection of EPTB (12, 13).

On the other hand, PCR is a feasible, rapid and easy technique that can detect Mycobacterium DNA in EPTB specimens that may not represent disease activity and with choosing one or more appropriate target (s), it can be a great way to achieve a high sensitivity (14, 15).

Early diagnosis and treatment are important to control TB and EPTB (9). Considering the importance of EPTB as an infectious disease and different prevalence rates around the world, the objective of the present study was to investigate the presence of EPTB and MDR-EXPT in Formalin-fixed, paraffin-embedded tissue blocks among different samples in Kermanshah, Iran.

Material and methods

Among all the suspected tuberculosis cases referred to Pathology laboratories in Kermanshah, Iran, in 2015, 116 patients were selected based on the diagnosis of the infectious disease specialist. . Inclusion criteria were the presence of bone fistula, lymphoadenopathy, erythematous skin and caseating granuloma, which are the clinical and/or pathological findings compatible with tuberculosis.

Specimens were obtained from paraffin-embedded tissue blocks from different sites. 4µm serial sections from each block were cut using a microtome.

DNA extraction and PCR amplification

Briefly, paraffin blocks were deparaffinized by xylene and 100% ethanol and total DNA was extracted by standard proteinase K digestion and column based method.

DNA was extracted from formalin-fixed, paraffin-embedded tissue blocks using genomics extraction G-spin Total DNA Extraction kit (Intron biotechnology, Seongnam,

Korea) and according to the instructions of the manufacturer. Mycobacterium tuberculosis complex was identified by TB Resistant Modual Isoniazid, Rifampicin kit (Autoimmun Diagnostika GmbH, Strassberg, Germany) which is based on a multiplex PCR followed by reverse-hybridization using sequence-specific oligonucleotide probes and enables accurate diagnosis.

Briefly, PCR was carried out in a total volume of 25µl containing 15 µL working master mix, 2.5 µL 10xbuffer, 2.5mM $MgCl_2$, 1 unit Taq DNA polymerase and 100 ng of DNA sample. The PCR reactions were carried out by Initial denaturation at 95°C - 5 min and 14 cycles consisting 95°C – 30s and 60°C – 2min and 26 cycles consisting of denaturation at 95°C - 10 sec, annealing at 55°C – 30s and extension at 72°C for 30 s. The final step was extended to 8 min at 72°C. To analyze the PCR products, 5 µL of each PCR product was run in a 2% agarose gel and was visualized under UV gel documentation (16).

Data were analyzed through SPSS software version 16.0 using Pearson and Chi-square test for assessing the relationship of TB with sex, age group and paraffin blocks. $P < 0.05$ was considered as significant.

Results

In this study, the frequency of EPTB in 116 suspected samples was 32% (Figure1). Mean age of patients was 43 years (ranged from 1 to 80 years). From all, 63 ones (54.5%) were female and 53 ones (45.5%) were male. The age group of 39-49 years had the highest rate of TB infected patients. We retrieved different number of samples from formalin-fixed, paraffin-embedded tissue blocks of different sites including breast (21), Pleural fluid (26), lymph node (18), CSF (15), colon, testis and stomach (5 from each), bone marrow and the intestine (4 from each), Intervertebral disk, kidney and blood (3 from each), skin and abdominal fluid (2 from each). All of the patients' specimens were sensitive to INH and RIF.

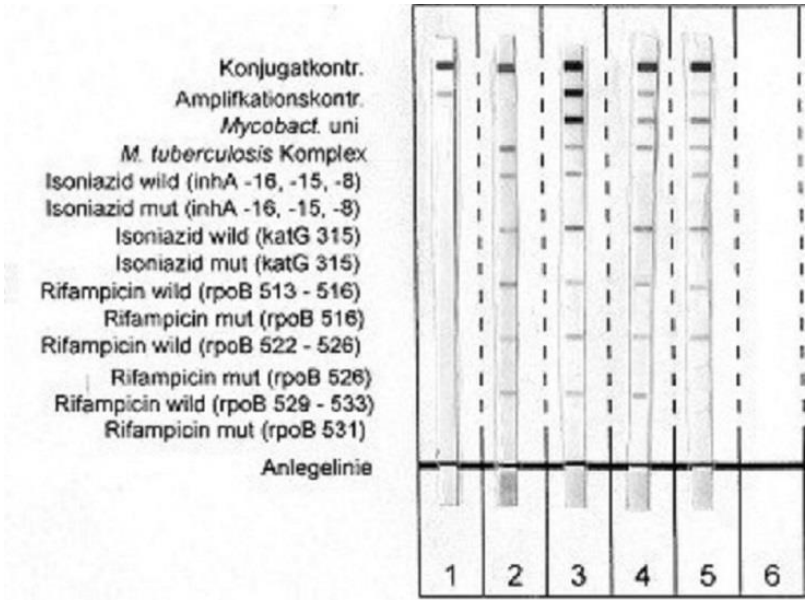

Fig1. Examples of EXPT results obtained using the Linear Array TB resistance module INH and RIF kit. (Autoimmun Diagnostika GmbH, Strassberg, Germany). 1. negative sample, 2.positive sample extracted from bacteria medium, 3, 4, 5: positive samples extracted from patients.

Among EPTB specimens, lymph nodes were the most frequent to be TB predominant positive (44.5%), followed by Pleural fluid, Breast and CSF with the frequency rates of 34.6%, 33% and27% respectively (Table 1). The frequency of TB positive was high in blood, testis and intestinal specimens, but the samples were few. Statistical analysis showed no significant association between EPTB paraffin blocks, age groups and TB.

Table 1. The frequencyof TB positive/negative in extra pulmonary paraffin- embedded block speciemens

Block type		breast	CSF	Lymph node	plural	Colon	Testis	skin	intestinal	stomach	BMA	blood	interverteb ral disc	Ascites	kidney
TB	Positive N (%)	7 (33)	4(27)	8(44.5)	9(35)	2(40)	2(40)	0	2(50)	1(20)	1(25)	1(33.3)	0	0	0
	Negative N (%)	14(67)	11(73)	10(55)	17(65)	3(60)	3(60)	2(100)	2(50)	4(80)	3(75)	2(66.7)	3(100)	2(100)	3(100)
Sum		21	15	18	26	5	5	2	4	5	4	3	3	2	3

Discussion

TB is widely accepted as an important health problem worldwide but there is little published data regarding EPTB. It has been approved that TB can infest and affect every organ of the body which is referred to as EPTB (17). Since there is no particular sign of infection, reactivation of the disease may not be recognized clinically. Diagnosis of EPTB may be difficult, as its symptoms are nonspecific and lack of a sensitive and specific method for the diagnosis of EPTB delays therapeutic interventions (18). Many reports have confirmed that PCR amplification in formalin- fixed tissues detects TB even with few DNA genomes (19-21).

In 1984, EPTB was a major health problem in Australia, where 24.3% of all new cases were extra pulmonary. Some studies have also showed that the proportion of EPTB is increasing (22, 23). For instance, comparing the two studies of 1991-1996 and2003- 2008, shows EPTB increasing rate from 30.6% to 37.6% (23).

In this study, TB was most frequently found in the age group of 39-49 years old and the frequency of EPTB was estimated as32% which is surprising. The prevalence of TB in EPTB samples has been previously reported between 13 to 47% in Iran (24). In a previous study in Kermanshah, the prevalence of EPTB had been reported 28.6% (25) and the results of the present study in this region (32%) shows an increasing rate of the proportion of TB in EPTB samples. Iran is surrounded by countries that have very high incidence and prevalence of TB such as Afghanistan, Pakistan, Tajikistan and Iraq and it can be one of the reasons for underestimating the high frequencies in this region and its border towns. EPTB prevalence in Asian countries such as Japan and Pakistan has been estimated to be 23% and 18% respectively. Moreover, TB prevalence has been reported 15- 20% in India (26).

The prevalence of EPTB shows wide variation among different countries, such as the USA (21%), Italy (32%), Cameroon (26.6%), Australia (39%) and Europe (4% to 48%) (27-29). Although the frequency of EPTB in the present study is higher compared to some developed countries and Asian countries, it is approximately similar to that in Australia and Europe.

The reasons for these differences could be related to different epidemiological patterns and to the late recognition and/or lack of diagnosed EPTB and/or differences of methodological, specificity and sensitivity of the primers used (30).

In the present study, the most frequent EPTB sites were respectively lymph nodes (44.5%), pleural fluid (34.6%), breast (33%) and CSF (27%). Whereas, in the previous report from Kermanshah, the prevalence of TB among EPTB samples had been20.5%, 12.9% and 5% in Lymph nodes, Spinal cord and pleural fluid respectively (25). Both studies show high involvement of lymph nodes in this region. It is in accordance with retrospective studies on TB patients in France and the USA (31, 32). For instance, in the USA, , EPTB frequency has been 18.1% during 1993 to 2006 and the frequency rates for the involvement of lymph nodes and pleural fluid, as the common sites of EPTB involvement, have been40.4% and 19.8% respectively (32) that are less than the rates found in the current study. In contrast, there are reports that have mentioned pleural fluid as the most frequent site of EPTB, followed by the lymph nodes (33, 34). Others studies have reported different results (34, 35). However, in a study on Turkish patients, the most commonly seen types of EPTB were genitourinary (27.2%) and meningeal TB (19.4%) (34).

In the present study, TB Resistant Modual Isoniazid, Rifampicin kit caused accurate and rapid modular detection of Mycobacterium tuberculosis complex and made it possible to identify resistance to INH and RIF. In the present study, all of the specimens were sensitive to INH and RIF and MDR obtained zero. While, the prevalence rates of MDR- EPTB have been respectively 12.5% and10% in Nepal and India (36, 37). Although drug resistance in EPTB is not as common as that in pulmonary tuberculosis, MDR in EXPT cannot be overlooked.

Differences in frequency of EPTB might be explained by differences in methodological and geographical differences, risk factors for EPTB, lack of early diagnosis, failure to control and treat the disease and the emergence of multi-drug resistant disease strains which make the diagnosis challenging (38). It seems that more analytically sensitive and specific method, as a non-invasive test, is needed for TB detection in EPTB samples for epidemiological and clinical settings.

Although it is not usual, but EPTB might spread to the lungs and become contagious and eventually is converted into a threat to public health (39). This study demonstrated the high prevalence of TB among EPTB cases in Kermanshah. Therefore, according to the information which is mentioned above and results of the current study, EPTB shows an increasing trend in Iran. Thus it is necessary that public health organizations pay serious attentions in diagnostic facilities for early recognition and more efficient therapeutic interventions.

Acknowledgement

The authors would like to acknowledge Vice-Chancellor for Research and Technology, Kermanshah University of Medical Sciences; the study was financially supported by the Kermanshah University of Medical Sciences (grant 94255).

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