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RESEARCH ARTICLE

MOLECULAR DIAGNOSIS OF EXTRA-PULMONARY TUBERCULOSIS FROM BLOOD SPECIMENS AMONG PATIENTS ATTENDING EL-SHAAB TEACHING HOSPITAL

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ABSTRACT

Background: Tuberculosis prevalence and mortality rates are increasing specially for the CNS infection which is life threatening and when it involves the spine it has the potential to cause serious morbidity, including permanent neurologic deficits and severe deformities and brain tuberculoma usually lead to death. Extra-pulmonary TB accounts for approximately 10% of tuberculous infections

Methods: This quantitative descriptive case study hospital based conducted at Elshaab Teaching Hospital National Center for Neurological sciences (NCNS) between August 2009 and November 2010. PCR technique was used to diagnose 121patients suspected to have Pott's disease or brain tuberculomata. A group of healthy people who received BCG vaccine were included in this study as control group.

The results: Patients suspected to have brain tuberculoma were sixty three (52.1%), Pott's disease were forty nine (40.5%) and other extrapulmonary tuberculosis were only nine (7.4%).The results of the PCR for *M. tuberculosis* from blood sample of the121 patients were positive in 60(49.6%) patients. The frequency of positive result found among Pott's disease was 23.9% (n 29) followed by tuberculoma 21.4% (n 26) & other extra-pulmonary tuberculosis 4.1% (n 5)

Recommendations: PCR for *M. tuberculosis* from blood specimen is recommended for diagnosis of patients suspected to have brain tuberculoma or Pott's disease according to their symptoms and their MRI findings.

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INTRODUCTION

Tuberculosis is a chronic, systemic infectious disease that occurs when the *M. tuberculosis* is inhaled causing pulmonary tuberculosis and can localize in alternate sites leading to extrapulmonary tuberculosis (EPTB). Tuberculosis involvement of the central nervous system (CNS) is an important and serious type of extra-pulmonary involvement [(1)]. Extra-pulmonary TB accounts for approximately 10% of tuberculous infections. While pulmonary tuberculosis is the most common presentation, extrapulmonary tuberculosis (EPTB) is also an important clinical problem1-3. The term EPTB has been used to describe isolated occurrence of tuberculosis at body sites other than the lung. However, when an extrapulmonary focus is evident in a patient with pulmonary tuberculosis, such patients have been categorized under pulmonary tuberculosis as per the guidelines of the World Health Organization (WHO). Spinal TB accounts for about 2% of cases of TB [(2)], which is prevalent in much of the world both developed and developing counties [(3)]. Polymerase chain reaction (PCR) is the most promising new approach for rapid, safe, and reproducible determination of *Mycobacterium tuberculosis* infection [(4)]; it is suitable for detection from synovial fluid, tissue sample, bone marrow aspirate and peripheral blood. CNS involvement was noted in 5 to 10% of extrapulmonary tuberculosiscases [(5)]; with more recent CDC data in 2005 indicating that 6.3% of extrapulmonary cases (1.3% of total tuberculosis cases) involve the CNS [(6)].

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METHODS

Study setting and patients

This quantitative descriptive case study is hospital based conducted at Elshaab Teaching Hospital National Center for Neurological sciences (NCNS) between August 2009 and November 2010. PCR technique was used to diagnose 121 patients suspected to have Pott's disease, brain tuberculomata, or other extrapulmonary. Approval was taken from the Ethical Review Board of Al Neelain University and written consent from the patients was obtained. Inclusion criteria includeclinical features and CT scan/MRI or X-ray suggestive of tubercular lesions in extrapulmonary sites.

Specimen's collection& processing

Sample collection: 5 ml of blood was collected in EDTA anticoagulant container for DNA extraction. Ten ml of RBCs Lysis buffer was added to 2 ml of the collected blood in Falcon tube of 15 mL, centrifuged at 6000 rpm (round per minute) for 5 min the supernatant was discharged, this step was repeated until all RBCS were washed out and clear white pellet formed. 2 ml of White cells lysis buffer was added, 1 mL of Guanidine Hydrochloride, 300 µL Ammonium Acetate and 10 µL of Proteinease K (10mg/ml) incubated

at 37ºC overnight. Pre-chilled 2 mL of Chloroform was added to the mixture then vortexed and centrifuged for 5 min at 6000 rpm. The upper layer was added to a new tube and 10 mL of cold Absolute Ethanol was added shaked and incubated for overnight at -20 ºC then centrifuged for 10 min at 6000 rpm and the supernatant was drained. The pellet was washed with 70% Ethanol, centrifuged for 10 min at 6000 rpm and supernatant was drained. Finally the pellet which is the DNA was dried from the ethanol then dissolved in 100 µl of distilled water and stored at 4 ºC for overnight incubation before running the PCR or at -20 ºC for storage.

Master Mix Preparation

Samples and reagents were brought out from the freezer to room temperature except the Taq polymerase enzyme which was always kept in frozen cryo-rack. The master mix was prepared in a 0.5ml PCR tube and a worksheet was prepared to record the reagents, date, the label of the tested DNA and finally after the PCR the result was also recorded on this sheet(A notebook was used for the worksheet so all the reaction are kept together). The reagents were 10 x PCR buffer (10mM tris-Hcl, Ph 8.3, 50 mM KCL, 2.0mM $Mgcl_2$), $MgCl_2$ with final concentration 50mM, dNTPs 10 mM, the primers (20 pmol each), Taq polymerase, and the tested DNA.

Primers

The primers used for the amplification were originally designed from sequences which are repeated several (10 to 16) times in the chromosome of M. tuberculosis (6). The sequences of the primers amplify a 123-bp fragment of the repetitive sequence (6) were (5' to 3')

	5'CCTGCGAGCGTAGGCGTCGG3'
	5'CTCGTCCAGCGC CGCTITCGG3'

Primer preparation

Each of the upstream and downstream primers were prepared as follows: 10µl of each stock primer (100 µM) were added to 90µl distilled water and aliquoted in 0.5 ml PCR polypropylene tube to yield a concentration of 10µM, and the solution was mixed carefully to ensure the homogeneity.

Deoxynucleotide (dNTPs) preparation

All four deoxynucleotide with 100 mM concentration were prepared by adding 10µl of each nucleotides (total volume 40µl), in 60 µl of sterile distilled water to a final concentration 10 mM in a PCR tube, mixed by vortex mixer to collect any dNTPs from the tube surface in the button of the tube and store at – 20 ºC.

PCR amplification

Samples and reagents were brought to room temperature to make a reaction mixture of 25µl. 1n 0.5ml PCR tube 2.5 µl of 10 x PCR buffer (10mM tris-Hcl, Ph 8.3, 50 mM KCL, 2.0mM $Mgcl_2$), 1µl $MgCl_2$ with final concentration 50mM, 1 µl of 10 mMdNTPs, from the primers 1 µl of each (20 pmol each), 3 µl DNA, 0.3 µl of 1 U Taq polymerase, and 3 µl from the DNA then the volume was completed to 25 µl by the distilled water . The DNA was denatured for 4 min at 94°C; 35 amplification cycles were performed with an automated thermal cycler (TC300). Each cycle consisted of denaturation at 94°C for 50 sec, annealing of primers at 68°C for 40 sec, and primer extension at 72°C for 2 min finally holding temperature 4°C.

Gel Electrophoresis

A 2% agarose gel prepared by adding 35 ml of the buffer (7ml of 5X Tris Borate EDTA (TBE) with 28 ml of distilled water) to 0.7g of agarose powder. The mixture was homogenized by the microwave, cooled to 50 °C then 0.7 µl of Ethidium Bromide (final concentration is 2% in the agarose gel) was added to visualize the DNA bands, finally the gel was poured on the tray with combs to make the wells. Loading of 1.5 µl of a 100-bp ladder at the first well of each row, followed by positive control then the patients samples mixture that contains 5 µl of the amplified product with 3 µl of the loading dye Bromophenol blue. Before loading the running buffer of 1% TBE buffer was poured in the tray. The power supply was adjusted to run at 20 Amber, 120 Volt for 1 hour. Then the DNA was photographed on a UV transilluminator (Syngene). The presence of the 123-bp amplification product is positive result for the presence of the DNA of *M. tuberculosis* in the patient's blood.

THE RESULTS

A total of 121 patients attended (NCNS) suspected to have extrapulmonary TB according to their symptoms and their MRI findings were test by PCR for *M. tuberculosis* from blood specimen. As indicated in table (1), about 36% (n 43) of the study group were between ages of 41-60 year followed by age group of 21-41 year 31.4% (n 38) where as the frequency of age group of less than 20 years & more than 60 years were 10.7% & 23.3% respectively. In Table (2) blood PCR for *M. tuberculosis* for 121 patients revealed that Pott'sdisaess was23.9% (n 29) followed by tuberculoma 21.4% (n 26) & other extrapulmonary tuberculosis 4.1% (n 5)

Table 1. The distribution of the study group according to their Age ((NCNS) 2009-2010, n: 121)

Age group Year	Frequency	
	n	%
<20	13	10.7
21-40	38	31.4
41-60	43	35.6
>60	27	23.3
Total	121	100

Table 2. The frequency of blood PCR results among the study group (NCNS) 2009-2010, n: 121)

Extra-pulmonary TB	Blood PCR for *M. tuberculosis*		
	Positive n (%)	Negative n (%)	Total n (%)
Tuberculoma	26 (21.4)	37 (30.5)	63(52.0)
Potts	29 (23.9)	20 (16.5)	49 (40.4)
Other Extra PTB	05 (04.1)	04 (03.3)	9 (07.4)
Total	60 (49.5)	61 (50.5)	121 (100)

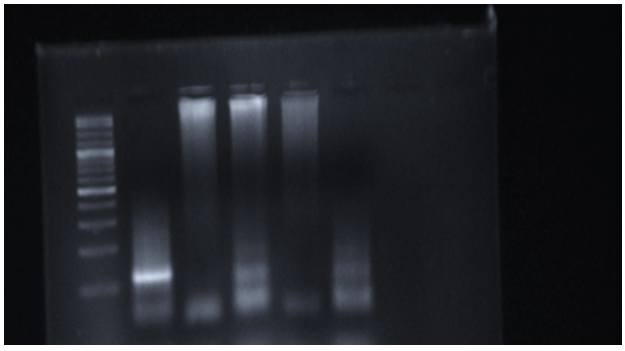

Fig. 1. shows gel electrophoresis product of the 123 bp amplification of *M.tuberculosis* by PCR with a 100 bp ladder from left to right:

Lane 1: represents the positive control DNA (*M.tuberculosis* strain from culture)
Lane 3 and 5: shows positive blood samples from patient.
Lane 2 and 4: represent negative samples
Lane6: is negative control

DISCUSSION

To our Knowledge this is one of few studies in Sudan concerning about molecular diagnosis of CNS tuberculosis by PCR technique for *M.tuberculosis* from blood specimen. Our data support the value of the PCR as a promising tool for the detection of *M. tuberculosis* from blood specimen in CNS tuberculosis. In our data we found the results of the PCR for *M.tuberculosis* from blood sample of the121 patients were positive in 60(49.6%) patients and negative in 61(50.4%) patients which agrees with the findings of Amin *et al.* (2011) found (46.5%) of the samples were positive by PCR for MTB[8], Dil-Afroze *et al* (2007) in India[9] studied 47 patients with CNS tuberculosis and the positive were 21/27 with the variation in the sample size but they both found PCR more sensitive than any other conventional method in the diagnosis. In the patients suspected to have tuberculoma twenty six were positive PCR while thirty seven were negative. They were followed up after treatment and surgery, 10 of them had surgery, 4 patients with positive PCR TB was confirmed by histopathology, 1 patient negative PCR but by histopathology was positive for TB and 5 were negative PCR and confirmed by histopathology because lesions found to be brain tumors. For Pott's disease we found twenty nine (59%) had positive PCR and twenty were negative, this findings go with Kumar *et al.* (2011) who found out of 62 suspected Pott's cases PCR detected MTBC in 33 (53.2%) cases [10] and Anil, *et al.* (2008) in India [11] who found the PCR was positive in 49 (98%) cases but here the positive percent is higher may be the Prevalence of Pott's disease in that area is higher than here. The diagnosis of Pott's disease in Sudan used to depend on the clinical features and the MRI, in late stages it is easier but the early stages symptoms may confuse with the tumors and MRI cannot discriminate the type of lesion or mass, so biopsy or surgery or CSF is done to confirm the diagnosis before starting anti-tuberculous. Also if not diagnosed early deformity (hunched back, paralysis) occurs, then very expensive fixation is needed. The outcome from using PCR to diagnose Pott's can reduce the need of surgery (including biopsy) by 59% that agree with Kotil *et al.* (2007) in Turkey [12].

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