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ABSTRACT

Objective: **To determine whether *Mycobacterium tuberculosis* infection spreads through the blood to different lymph-node groups in patients with tuberculous lymphadenitis.**

Design: **Prospective analytical study.**

Setting: **The patients were recruited, managed and followed at the lymphodenopathy clinic, Central Police Hospital, Burr, Khartoum, Sudan.**

Subjects: **Fifty two sequential patients were enrolled. Thirty patients with FNAC diagnosis of tuberculous lymphadenitis and positive PCR for *M. tuberculosis* complex had a mean age of 26.9±11.2 years and similar male, female affection. Nine patients with FNAC tuberculous lymphadenitis, but negative PCR had a slightly higher mean age (32.6±18.2 years) with similar male : female proportions. Patients with reactive lymphadenopathy (9/52) were older than patients with tuberculous lymphadenitis with a mean age of 45±24.6 years.**

Results: **None of the patients were positive for HIV or had clinical or radiological evidence of pulmonary tuberculosis. *M.tuberculosis* DNA was detected in the blood samples of 30/39 (77%) patients with tuberculous lymphadenitis, but in none of the cases with reactive or malignant lymphadenopathy. The presence of *M.tuberculosis* DNA correlated strongly to multiple lymph-node involvement [OR (odds ratio) = 96.7, 95% confidence interval (CI) 9.0 – 1,039] and to caseating-granulomatous and predominantly necrotic cytomorphological categories [OR = 70, 95% confidence interval (CI) 7.0 – 703].**

Conclusion: ***M.tuberculosis*** **most probably disseminates through the blood from one node group to the other in patients with tuberculous lymphadenitis.**

INTRODUCTION

Tuberculosis remains one of the major health problems worldwide with 8.7 million new cases every year and an estimated 1.7 million annual deaths (1). Tuberculosis is a systemic disease affecting almost all organs with two clinical forms; pulmonary and extra-pulmonary. The incidence of extra-pulmonary tuberculosis is dramatically rising, largely because of HIV/AIDS pandemic (2). Tuberculous lymphadenitis is the most common type of extra-pulmonary tuberculosis (3-6). Lymph-

nodes are usually involved as a component of a primary complex. Less often lymphadenitis is seen in secondary tuberculosis but the nodes are usually smaller and firmer than in primary disease. Tuberculous lymphadenitis may involve a single discrete lymph-node, multiple lymph-nodes or multiple sites of lymph-nodes and could even be generalised. Nevertheless, the most commonly affected lymph-nodes are those of the cervical region (7,8). Since tuberculous lymphadenitis is predominantly a primary disease, the question of how the infection spreads from the primary lymph-node to other nodes arises. Using blood culture techniques, circulating *M. tuberculosis* has been found to be one of the frequent causes of bloodstream infections (BSI) among febrile adults with advanced HIV infection in sub-Saharan Africa. These patients have radiological, microbiological or clinical evidence for active pulmonary disease. Recently, non-tuberculous mycobacteria was detected by culture technique in immunocompetent individuals (9-13).

In this communication we provide evidence to the probable route of *M. tuberculosis* spread in peripheral tuberculous lymphadenitis by demonstrating circulating *M. tuberculosis* DNA in immune-competent Sudanese patients who had no signs of pulmonary disease, using a highly sensitive molecular technique (PCR).

MATERIALS AND METHODS

The study proposal was scientifically and ethically reviewed by the Ethical Committee of the Institute of Endemic Diseases, University of Khartoum. Fifty two sequential patients with lymphadenopathy were enrolled in this prospective study following informed consent. FNAC was performed on all patients as a routine diagnostic procedure. Following thorough clinical examination; blood cell count, ESR, Mantoux test, HIV ELISA test and a chest X-ray were performed. Five milliliters of EDTA-blood were collected from all patients and DNA was extracted from the mononuclear blood cells (PBMCs) using the phenol-chloroform iso-amyl alcohol (PCI) method. Peripheral blood mononuclear cells (PMBCs) were collected using density gradient centrifugation with FicolHypaque. The quality of the extracted DNA was checked by 1.2% agarose gel electrophoress.

Oligonucleotide primers: A single set of oligonucleotide primers was used (MOL BIOL, Berlin, Germany®). The lyophilised primers were reconstituted as described by the manufacturer. The target for PCR amplification was IS6110 (size 123bp), an insertion-like element found in *Mycobacterium tuberculosis* complex strains. The oligonucleotides primers used were:

E1 (20 mer)(5'-CCTGCGAGCGTAGGCGTCGG-3')
E2 (5' -CTCGTCCAGCGCCGCTTCGG-3')

DNA amplification: Following strict Standard Operating Procedures, DNA extraction and PCR were carried in separate rooms to reduce cross contamination. PCR for the specimens was performed in a total volume of 50μl of the reaction mixture containing 10X PCR buffer, 2.25μM. Magnesium Chloride, dNTPs mixture (dATP, dGTP, dCTP & dTTP) 100 each (Boehringer Mannheim, Germany), E1 and E2 oligonucleotide primers 0.2μM each and 2 U/μL of *Taq* polymerase (Finnzmes®, Vienna, Austria). The reaction mixture was overlaid with mineral oil. The tubes were then subjected to 40 thermal cycles in a programmable heat block (Biometra®, Göttingen, Germany). The cycle was as follows: denaturation at 95°C for five minutes, annealing at 65°C for one minute, extension at 72°C for one minute and final extension at 72°C for ten minutes.

Detection of amplified DNA: The PCR products were analysed by ethidium bromide-stained agarose gel electrophoresis on 1.5% agarose gel. Positive, negative controls and a 100 bp DNA marker were included with every electrophoresis run.

Statistical analysis: Odds ratio were computed on AcaStat statistical software. If any of the four values in the contingency table were zero, one is added to all values before calculating the odds ratio.

RESULTS

Baseline characteristics, laboratory findings, FNAC and PCR results were summarised in Table I. Cervical lymph nodes were the most commonly affected group and were seen in 91% of patients with lymphadenopathy. The cytological diagnosis was; tuberculous lymphadenitis in 39 cases (75%), reactive in nine (17.3%) and malignant in four cases

Table 1

Baseline characterisitics, cytomorphological patterns and circulating mycobacterial DNA of the study patients

Cytomorphological type of lymphadenopathy	M: F	Mean age	Mean ESR mm	Mean TBCs	Mean mantoux mm
PCR positive (n = 30)	1:1.5	26.9 ± 11.2	87 ± 31	5.3 ± 1.6	22 ± 6
Necrotising Tb (n = 6)	1:5	23 ± 8.6	69 ± 24.9	6.8 ± 2.3	19.2 ± 5.5
Granulomatous Tb (n = 3)	1:2	43.7 ± 15.1	113 ± 25.1	4.6 ± 0.7	36.3 ± 7.6
Necro/Granul.Tb (n = 21)	3:4	25.1 ± 9.2	69.3 ± 32.4	4.6 ± 0.6	21.4 ± 6
PCR negative (n = 22)	2:1	34.6 ± 21.6	77 ± 30	5.9 ± 3.2	13 ± 10.8
TB lymphadenitis (n=9)	2:1	32 ± 18.2	90.4 ± 27.8	7.3 ± 4.5	19.3 ± 8.2
Reactive (n = 9)	2:1	45 ± 24.6	76 ± 35.1	5.3 ± 1.7	5.3 ± 9.2
Malignancy (n = 4)	1:0	26.8 ± 25.5	56.7 ± 23.1	4.3 ± 0.6	8.5 ± 6.6

Continuous variables are expressed as means ±SD

(7.7%). Based on the cytological findings, cases of tuberculous lymphadenitis were categorised into three groups; necrotising-granulomatous (21/39; 54%), predominantly necrotic (6/39; 15.3%) and granulomatous (12/39; 30.7%). Multiple lymph-nodes were seen in 28 cases, but a single discrete node was seen in 11 cases of tuberculous lymphadenitis. All cases in the caseating-granulomatous (21/21) and the predominantly necrotic (6/6) categories had multiple lymphnodes. Single lymph-node presentation was mainly associated with granulomatous lesion (11/12). An ear, nose, throat examination revealed no abnormalities.

None of the patients were HIV positive or had radiological or clinical evidence of pulmonary or miliary disease.

The ESR was high in the tuberculous group especially the granulomatous type, while it was variable in patients with reactive and malignant groups. A significant Mantoux reactivity (> 1 5 mm) was seen in all patients with tuberculous lymphadenitis, while it was < 10 mm in the reactive and the malignant groups.

Circulating *M. tuberculosis* DNA was detected in 30 out of 39 cases (77%) with tuberculous lymph nodes, but in none of the cases with reactive or malignant nodes. The PCR positive rates varied among tuberculous patients with different smear finding, while it was 100% (27/27 cases) in the caseating-granulomatous and the predominantly necrotic categories; the positivity rate was 25% (3/12 cases) among patients with granulomatous lesions. The computed odds ratio was 70 with a 95% confidence interval CI between 7.0 and 703. Circulating *M. tuberculosis* DNA was detected in all of the 28 patients (100%) with multiple lymph nodes, but in 2 out of 11 cases (18%) with single node involvement. The odds ratio was 96.7 with a 95% confidence interval CI between 9.0 and 1,039.

DISCUSSION

Tuberculous lymphadenitis is characterised by painless enlargement of lymph nodes and occasional constitution symptoms like nocturnal fever and sweating. The cervical group is the most commonly affected group. Peripheral tuberculous lymphadenitis usually develops as a part of a primary complex i.e. a primary focus and regional glands. Involvement of lymph-nodes as a manifestation of a generalised tuberculous infection is rather rare (14). For tuberculous lymphadenitis of the neck, the primary focus would be mainly in the tonsils and mouth, however recent or previous seeding of the lymph nodes from an occult site cannot be ruled out. None of our cases had shown clinical or radiological evidence of another primary complex or military disease and the lymph-nodes were large and soft. These findings probably support the primary nature of the disease in our cases. Therefore, the demonstration of circulating mycobacteria in such cases would suggest spread by blood stream. Patients with multiple nodes were considered as having bulky disease.

For the detection of circulating *M. tuberculosis*, a highly sensitive molecular technique (PCR) was used in this study. PCR has proven to be more sensitive compared to the conventional microbiological methods (LJ culture and ZN staining for acid fast bacilli in smears) in the identification of mycobacterium (15-19). However, PCR is known to be associated with high false positive results, with rates ranging from 3 – 20%, mainly due to cross-contamination (20, 21). To prevent cross-contamination we followed strictly the manufacturer instructions, DNA extraction and amplification were carried out in different rooms and the sequences of the process were adequately monitored. Positive and negative controls were included as extra quality assurance measure with every run. We have recently shown that, over 96% of cases of tuberculous lymphadenitis in Sudan are caused by *M. tuberculosis* (18). This made the use of a single primers set that amplifies a 123 bp sequence common to all *M. tuberculosis* more appropriate for this study. Circulating *M. tuberculosis* DNA could be demonstrated in most patients with tuberculous lymphadenitis in this study. The highest positive rate was reported among cases with caseating-granulomatous or predominantly necrotic changes. The positive rate among patients with granulomatous lesion was low. This agrees well with previous studies demonstrating lower mycobacterial load in granulomatous lesions which was reflected in scantier *mycobacteria* in ZN smears and lower positive rates in LJ culture (22,23). Although circulating *M. tuberculosis* DNA was seen in patients with multiple lymph-node involvement or showing caseating-granuloma tous or predominantly necrotic cytological pictures, the confidence intervals were wide probably indicating weak association. The granulomatous pattern is associated with single node involvement (91.6%) and a low positive rate (25%) for circulating *M. tuberculosis* DNA. Many studies showed that bloodstream infections (BSI) by *M. tuberculosis* do occur. However, it affects mainly immuno-compromised adults with advanced HIV infection with radiological, microbiological and/ or clinical evidence of pulmonary tuberculosis. The demonstration of circulating *M. tuberculosis* DNA in immuno-competent patients, mainly in association with multiple lymph node involvement probably suggests haematogenous spread. Lack of multiple organ involvement by the disease in-spite of haematogenous spread can be explained by compartmentalisation of mycobacterial infection. Garcia de Viedma *et al* (24), demonstrated that infection by more than one mycobacterial strains is very rare and when occurs, the co-infecting strains are not equally distributed at pulmonary and extra-pulmonary sites. Circulating *M. tuberculosis* DNA in our patients could explain the nocturnal fever that was reported by more than 80% of our patients (unpublished data). The pattern of the fever could be explained by episodic release of the mycobacteria and its antigens into the blood stream. Demonstration of circulating *M. tuberculosis* DNA was previously shown by Mirza *et al* (25).

It has long being claimed that the mantoux test can help to differentiate between tuberculous and non-tuberculous lymphadenitis (26,27). All our patients with tuberculous lymphadenitis had strongly positive mantoux test (induration >15mm).

We conclude that the presence of circulating *M. tuberculosis* DNA in the blood of most patients with tuberculous lymphadenitis, especially those with multiple lymph node involvement indicates that heamatogenous spread is the probable route of mycobacterial dissemination in tuberculous lymphadenitis.

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