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RESEARCH ARTICLE

POSSIBLE RISK FACTORS OF PROGRESSION TO OVERT DISEASE AMONG INDIVIDUALS WITH LATENT TUBERCULOSIS INFECTION IN SUDAN

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ABSTRACT

Latent TB infections (LTBI) constitute a pool fornew TB cases, ~5% of LTBI individuals progress to overt disease during their lifetime. Simple and cheap progression risk factors are needed to triage individuals for treatment. In aprospective case control study and following informed consent, apparently healthy 98 household contacts (HHCs) and 186 community contacts (CCs) were enrolled. Tuberculin skin test (TST), whole blood stimulation/IFN-γ release assays and IL-4/IL-4δ2 mRNA copies were studied. Two hundred eighty-four volunteers were enrolled, five percent (5/98) of HHCs developed smear positive pulmonary TB compared to none in the CCs (p=0.004). The mean TST induration of the newly diagnosed TB patients was 16±2 mm at recruitment and the lapse of time to development of overt disease was 12±10 months. The majority of newly diagnosed TB patients had IFN-γ production levels below the cutoff level for LBTI diagnosis. A third of the HHCs and CCs had high IFN-γ levels, but none developed overt disease. The ratio of IL-4/IL-4δ2 mRNA copies in the newly diagnosed TB patients was significantly higher than that of the CCs (*p0.0002*). TST induration of ≥15was identified as a suitable simple and cheap test to triage individuals with LBTI for treatment.

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INTRODUCTION

Background

Tuberculosis (TB) is still a major cause of morbidity and death worldwide with grave economic consequences especially in the HIV/AIDS era. In developing countries, most overt TB cases arise because of reactivation in latently infected individuals. Most people infected with *M.tuebrculosis* remain asymptomatic as latent TB infection (LTBI), presumably due to the competence of protective immune system (Cardonaand Ruiz-Manzano, 2004). One-third of the world's population is believed to harbor latent *M.tuebrculosis* (WHO, 2010). Individuals with LTBI have a 5% chance of progressing to overt disease during their life time Richeldi (2006). Household contacts (HHC) of patients with active pulmonary TB are at a greater risk of developing *M. tuberculosis* infection. Early disease detection constitutes an important target for early preventive TB control methods (Young *et al*, 2008). Tuberculin skin test (TST) has been used extensively in epidemiological surveys and as a clinical test for the diagnosis of latent *M.tuberculosis* infections (LTBI) worldwide (Zuber *et al.*, 1997; Caylà *et al.*, 2009).TST is mainly used for the identification and treatment of individuals infected with *M.tuberculosis*, who are at a high risk of progression to active disease (Menzies *et al.*, 2011). This strategy is powerful because preventive treatment of latently infected people diminishes the risk of subsequent development of active TB by about 90% Richeldi (2006).

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Recently, IFN-γ release assays (IGRA) that use whole blood stimulation with specific recombinant antigen sand cytokines measurement have been introduced. Unfortunately, IGRA tests cannot differentiate between active and past TB infections and are fairly costly (Barth *et al.*, 2008; Pai *et al.*, 2008). In this communication the role of some possible risk factors of progression among individuals with latent tuberculosis infections were examined.

MATERIALS AND METHODS

Ethical Considerations

The study proposal was ethically approved by the ethics Committees of the Institute of Endemic Diseases, University of Khartoum and the Ethics Committee of the Federal Ministry of Health, Sudan. Volunteers were asked to sign a written informed consent. Volunteers were looked after by medically qualified personnel.

Study design and study population

This prospective and longitudinal study was conducted in the Capital of River Nile State of Northern Sudan during the period of March 2008 to February 2010. Sequential newly diagnosed, smear-positive and consenting pulmonary TB patients who reported to the TB Treatment Center, Shendi, Northern Sudan were enrolled as index cases (IC). House Hold contacts (HHCs) were traced to their localities and enrolled if they consented, were apparently healthy and were in continuous contact with the index cases for at least the last six months. Consenting age and sex-matched community contacts (CCs)

who lived in the same quarters of the HHCs who were apparently healthy and had no history of contact with individuals with chronic cough in the last six months were enrolled. HHCS and CCs were interviewed and physically examined for symptoms and signs of pulmonary TB. The sample size was contrained by the TB taboo among that closed community.

Tuberculin Skin Testing

Tuberculin skin testing was performed by injecting 5 tuberculin units (TU) in 0.1 mL intra-dermally on the volar surface of the forearm. The mean of the largest two transverse indurations was calculated after 72 hours using the ball-point pen technique. A reaction of ≥10 mm was considered as reactive *(Aggarwaland Dutta, 1995;* Shakak, 2012*).*

IFN-γ release assays

One ml of blood was taken in each of the three heparinized tubes: **1.***M. tuberculosis*-specific antigens: ESAT-6/CFP-10/TB7.7 (Cellestis Ltd., Victoria, Australia) **2.**Phytohemagglutinin (PHA) as positive control **3.**No antigen added(negative control). The tubes were incubated for 24 hours at 37º C in a Cellestis incubator (Cellestis Ltd., Victoria, Australia), and plasmas were collected after centrifugation and stored at -20ºC until assayed. The amount of IFN-γ in the plasma was measured by ELISA as per manufacturer' instructions (Cellestis Ltd., Victoria, Australia and Komabiotech, South Korea). The manufacturers' recommended cut-off of 0.35 IU/mL for IFN-γrelease assay positivity was adopted, Shakak (2012*).*

Erythrocytes Sedimentation Rate (ESR)

Two and a half mls of EDTA blood were taken for ESR estimation and total mRNA extraction.

IL-4 and IL-4δ2mRNA extraction for qRT-PCR

Trizol reagent (TRI reagent from Sigma-Aldrich, Corporation St. Louis, USA) was used for total RNA extraction as per manufacturer's instructions. Briefly: Take 0.2 ml of EDTA-whole blood and lyse with 0.75 ml of Trizol reagent supplemented with 20 µl 5 N Acetic Acid with shaking and standing at room temperature for 5 minutes. Add 0.2 mls of chloroform with shaking and standing at room temperature for 2-5 minutes. Centrifuge at 12,000 Xg for 15 minutes at 4º C remove the aqueous phase into a fresh tube. Add 0.5 ml of ethanol toprecipitate the RNA. Mix and shake and stand at room temperature for 5-10 minutes then centrifuge at 12,000 Xg for 8 minutes at 4-25º C. Remove the supernatant wash the RNA pellet by adding 1 ml of 75% ethanol. Vortex then centrifuge at 7,500 Xg for 5 minutes at 2-8ºC. Remove the ethanol and solubilized the mRNA in 50 µl RNase-free water.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Using one stepq RT-PCR kit (Promega Corporation, Madison, USA) and the Esco, Swift™ Spectrum 48 Real Time Thermal Cycler (Esco, Singapore), with 20 µl of reaction mix and 5 µl of the sample. Standard curves of quantifies PCR product, and at least eight positive controls per 48 plates in PCR runs were prepared. The following primers were used:

IL4: (X)(5-iso-dC-)5 '-AGGCTAAAGACC AGTTG TG TTCTT-3' (5-iso-dC-)5 'GTTGACCGTAA CAGAC ATC G TCTT-3'

IL4δ2: (X)(5-iso-dC-)5 '-AGTTGT GTTCT TC TGCTCTGCTT-3' (5-iso-dC-)5 'ACAGACATCG TCTTT AGCC TT TCC-3'.

Cycling conditions of an initial activation step of 15 minutes at 95°C followed by 45 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 1 minute at 72°Cwas used for each primer pair. Cytokine message was normalized against human β-actin house-keeping gene.

Data collection

Data was collected using a specially designed case record form (CRF) with sections for demographic, clinical and laboratory data.

Statistical analysis

Statistical analyses were performed using Epidemiological Information (Epi Info) software version 3.5.3. The levels of cytokines (IFN-γand IL-4 mRNA / IL-4δ2 mRNA copies) and TST induration between the CCs, HHCs and those who progressed to overt pulmonary TB were compared using student t-test and Chi-squire. Pearson correlation test was used to correlate TST and IFN- γ levels. *P* levels of <0.05 were considered significant, Shakak (2012).

RESULTS

Two hundred eighty-four volunteers were enrolled in this study, 98 were house-hold contacts (HHCs) and 186 were community contacts (CCs).The mean ages of the CCs and the HHCs were comparable, with females dominating both groups (M:F ratio of 1:4). Five percent (5/98) of the HHCs developed smear positive pulmonary TB compared to none in the CCs (p=0.004). All the individuals who developed smear-positive TB were females with a mean age of 28 ±15 years (range 14-50 years). The lapse of time to development of overt disease was 12±10 months (range 3-26 months). The means of ESR at D0 (55±39 mm/first hour) and D730 (60±8 mm/first hour) in the newly diagnosed TB patients, HHCs who did not develop pulmonary TB and CCs were not significantly different (p0.08). The mean TST induration was significantly higher in the newly diagnosed TB patients compared to other HHCs and CCs at enrollment (*p=0.000*) (Tables 1and 2), Shakak (2012*).* The mean IFN-γ production inall HHCs was significantly higher (0.5±0.7 IU/ml) compared to that in CCs and the newly diagnosed TB patients (0.2±0.2IU/ml) (*p=0.000*). The majority (4/5; 80%) of the newly diagnosed TB patientshad IFN-γ production levels below the cutoff point of 0.35 IU/ml andwere labeled as not LTBI by the IFN-γ release assay test.Thirty per cent of the HHCs and CCs had high IFN-γ levels between 1.1-2.0 IU/ml, none of those developed overt disease within the two years of follow up. On the other hand, fifth of twenty five per cent of HHC with TST induration of ≥11 mm developed overt disease during follow up (Tables 1 and 2). No correlation between TST induration size and IFN-γ production levels in HHCs and CCs could be detected (r=0.4), Shakak (2012*).* The mean IL-4 copies were higher in HHCs and the newly diagnosed TB patients compared to CCs, while the mean of IL-4δ2 mRNA copies were higher in CCs compared to HHCs. The ratio of IL-4/IL-4δ2 mRNA copies in the HHCs and newly diagnosed TB patients (1.9 and 1.8 respectively) was significantly higher than that of the CCs (1.4) (*p0.0002*). One patient (# 5) had very high IL-4 mRNA copies, high IFN-γ production levels and a TST induration of 15 mm (Tables 1, 2 and 3).

Table 1. Demographic characteristics, TST, whole blood IFN-γ production and IL-4 mRNA/IL-4δ2 mRNA copies ratio of House-Hold Contacts who developed pulmonary TB.

Variables	Developed pulmonary Tb (n=5)
Age (mean ±SD)	28±15
Male: Female	0:5
TST (mean ±SD)	
Day 0	16±02
Day 730	15±05
ESR mm/first hour (mean ±SD)	
Day 0	55±39
Day 730	60±8
IFN-γ IU/ml (mean±SD)	0.2±0.1
IL-4 mRNA copies/ IL-4δ2 mRNA copies Ratio	
Mean	1.8
Median	1.0
Time to development of TB (months)	
Mean ± SD	12±10
Median	13
Range	3-26

Continuous variable are expressed as means ±SD

Table 2. Possible risk factors of progression to overt disease in the study population at recruitment (D0)

Variables	House-Hold Contacts *(n=98)*	Community Contacts *(n=186)*	*p value*
TST *(mean ±SD)*	5 ±6	2±3	*0.000**
ESR mm/first hour *(mean ±SD)*	35 ±22	36 ±23	*0.7*
IFN-γ Mean level IU/ml: *(with recombinant antigens) Mean ±SD*0.	5±0.7	0.2±0.2	0.000*
IL-4 mRNA/IL-4δ2 mRNA Copies Ratio *(mean ratio)*	1.9	1.4	*0.0003**

Continuous variable are expressed as means ±SD.
*Significant difference (p<0.05.)

the infection. Such dichotomy of the immune response has been reported before in other human intra-cellular infections (Khalil *et al.*, 2005; Khalil *et al.*, 2011). In the absence of accurate markers for LBTI progression to overt disease, we present TST induration as a possible marker of disease progression that will have a great impact on case detection rates in developing countries. In addition, this presents a cheaper option for screening, prediction and triaging of individuals, since not all LBTI individuals will progress to overt disease as was clearly shown by our data. We are aware that the follow up period was short, but the figure reported was in agreement with the fact that LBTI individuals have 5-10% chance of progression during their lifetime. Based on our findings we can reason ably assume that TST is suitable as a simple marker that can help triage

Table 3. TST induration, IFN-γ production, IL-4 mRNA/IL-4δ2 mRNA ratio & time to development of overt disease among HHCs who developed pulmonary TB (n=5)

Patient ID	Age/years	Time to Tb development/ Months	TST induration	ESR mm/ First hour	IFN-γ IU/ml	IL-4/IL-4δ2 ratio
			D0 – D730	D0 – D730		
1	39	03	- 15	- 120	0.25	0.9
2	14	13	16 15	35 60	0.24	3.2
3	18	26	18 22	20 50	0.16	01
4	20	15	20 11	45 70	0.1	0.6
5	50	03	15 13	55 60	0.45	10

*§There was a low correlation between IFN-γ level >0.35 IU/ml and TST ≥10 mm (r =0.4).*D0=day 0 of follow up; D400=day 400 of follow up

DISCUSSION

Risk factors of disease progression among individuals with LTBI are not well characterized. Despite TST low specificity, it is still a useful tool for TB surveys and for diagnosis of overt as well as LTBI. It is a cheap and a simple test that needs little expertise and can be carried out at primary healthcare settings. Treatment of all individuals with LTBI based on TST or the use of tests like IGRA could prove costly for some developing countries. In this study, four tests were evaluated as possible risk factors of progression to overt TB: ESR, TST, IFN-γ and IL-4/IL-4δ2 mRNA copies ratio. The study aimed to triage individuals with LTBI into treatment and no-treatment groups to initiate early treatment for individuals at risk of progression to overt disease. TST lack of specificity has always been blamed on exposureto non-tuberculous *mycobacteria and* interference with BCG vaccination. Exposure to non-tuberculous *mycobacteria* with effects on the immune system is difficult to prove. Secondly, the size of the TST induration following BCG depends on a number of factors that include age at BCG vaccination, quality and strain of the BCG mycobacteria, number of doses of the BCG and the immunological status of individuals. There is no evidence to suggest that TST induration following BCG is expected to be >10 mm. There is limited data to determine how the length of time following BCG vaccination affects TST induration. A large study from Turkey reported that the change in induration would stay for up to six years of age if BCG was given at 0-3 months.

The Turkish study reported marginal difference in TST induration between children with scar and those without (Bozaykut *et al.*, 2002). Although, there is a huge body of data that supports the introduction of IGRA for the diagnosis of LBTI. We failed to see any response or political will in our country to introduce IGRA tests to increase TB case detection rates. The cost and the complexity of the IGRA tests could be a factor. Despite suggested low specificity and the need to come back for result reading, which is not unique to TST, it is our belief that TST could be a useful test for LTBI diagnosis and a good marker for triaging individuals to early TB treatment. In addition, our findings present TST as a cheap and simple predictor for TB progression in individuals with LTBI in developing countries. The individuals who progressed to overt disease, although strongly reactive in TST were mostly passed as normal in the IGRA tests. One patient who progressed to overt TB had IFN-γ level of >0.35 IU/ml, strongly reactive TST and a high IL-4/IL-4δ2 mRNA copies, probably indicating a mixed Th1/Th2 immune status. Individuals showing this mixed response will either develop disease or overcome LBTI individuals into treatment and no-treatment groups. In this study IFN-γ release assay could not identify individuals who progressed to overt disease. This insensitivity of the IGRA tests in detecting individuals with strongly reacting TST (induration ≥15) has been previously reported (Shakak, 2012; Arend *et al.*, 2007). ESR was not different at D0 and D730 of follow up making it of little value in picking up disease progression in individuals with LTBI. We opted to measure mRNA copies of IL-4 and its antagonist IL-4δ2, because IL-4 has proven difficult to detect as a protein, being both labile and present in very low concentrations. IL-4δ2 is an alternative spliced IL-4 variant that acts as an effective IL-4 antagonist that has been shown to be associated with control of *M. tuberculosis* infection (Vasiliev *et al*, 2003; Demissie *et al*, 2004). High IL-4δ2 levelswere reported in individuals with LTBI who do not progress to active disease (Dheda *et al*, 2005).

We noted that the mean of IL-4 mRNA copies were higher in HHCs compared to CCs (i.e. Th2 immune response), while the mean IL-4δ2 mRNA copies was significantly higher in CCs compared to HHCs (increased ability to fight *M.tuberculosis*). Similar results were reported from Ethiopia (Demissie *et al.*, 2004; Demissie *et al.*, 2006). Our finding that high mean of IL-4/IL-4δ2 ratios seen among HHC scompared to CCs is in agreement with recent reports from Ethiopia (Wassie *et al.*, 2008).The high expression of IL-4 mRNA in HHCs who developed TB is concordant with reports by Wassie and colleagues from Ethiopia. This is probably explainable by the fact that these individuals were in a Th2 immune state where they are more prone to intra-cellular pathogens like *M. tuberculosis* (Wassie *et al.*, 2008).The median IL-4/IL-4δ2 ratio in HHCs that developed active TB was 1.0 which is similar to results reported by Dheda and colleagues in 2005 (Dheda *et al.*, 2005).We realize that the measurement IL-4/IL-4δ2 is beyond the reach of many developing economies, but it is hoped that with time the technology could be affordable sometime in the future. The majority of individuals who progressed to pulmonary TB had IFN-γ level <0.35 IU/ml (cut-off level), this is in agreement with previous reports that showed that IFN-γ negative contacts are more likely to progress to active TB (i.e. in naïve/Th2 immune response) (Yoshiyama *et al.*, 2010). These findings are in disagreement with reports that suggested an increased risk of progression to overt disease among IFN-γ responsive healthy HHCs (Doherty *et al.*, 2002; Diel *et al.*, 2008).One of our TB converters who was 50 years of age had a positive IFN-γ release assay. The age and the increased IFN-γ levels (0.45 IU/ml) may have increased her chance of progression to overt disease (Katsenos *et al.*, 2011). All individuals who converted to open pulmonary TB were

from the HHCs group, which is in agreement with previous reports that showed that HHCs of active pulmonary TB are at a greater risk of developing *M. tuberculosis* infection (Young *et al*., 2008).

Conclusion

A strongly reactive TST (induration ≥15) is a simple and sensitive risk marker for progression to overt TB among individuals with LTBI.TST can help triage patients for anti-tuberculous treatment in developing countries. A longitudinal study with a larger sample size and a longer duration is needed to confirm these findings.

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Conflict of interest: Non.

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