Egypt. Acad. J. biolog. Sci., 3(1): 27-32 (2011) G. Microbiology
Email: egyptianacademic@yahoo.com ISSN: 2090-0872
Received: 20/5/2011 www.eajbs.eg.net

Validity of Antimycolic Acids Antibodies in the Diagnosis of Pulmonary Tuberculosis in TB/HIV Co-infected Patients in Khartoum State, Sudan

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ABSTRACT

This study aimed to determine antimycolic acid antibodies [IgG and IgM] among TB/HIV Co-infected patients in Khartoum State.

Sputum and blood specimens were collected from patients attended Alsha'ab Teaching Hospital, Tuberculosis Reference Laboratory, Ibrahim Malik Hospital and Abu Anga Hospital; patients were all informed and consented.

Direct smears from sputum samples of 90 suspected patients showed that 17 (18.9%) were acid fast bacilli positive while 73 (81.1%) were negative. The ninety sputum specimens were subjected to PCR to amplify IS6110 region specific for *Mycobacterium tuberculosis* complex. The result showed that 79 (87.8%) were positive for IS6110 while 11 (12.2%) were negative.

The 90 serum samples were investigated for HIV using dot plot technique, 9 samples (10%) were found HIV positive and they were all TB positive by PCR.

80 Serum samples were analyzed by ELISA, 16 (20%) gave positive result for antimycolic acid IgG while 64(80%) were negative and 55 (68.8%) were positive for antimycolic IgM, while 25 (31.3%) were negative.

When HIV infection was correlated with specific antibodies for antimycolic acids, in 6 HIV positive samples, one (17 %) was positive and 5 (83 %) were negative for IgG, while 2 (33.3 %) were positive and 4 (67 %) were negative for IgM.

This study concluded that patients with TB/HIV Co-infection have less capability of producing antimycolic acids antibodies, however, IgM antibodies could be of more serodiagnostic value.

Key words: Mycolic acids, antibodies, HIV, tuberculosis, Sudan

INTRODUCTION

Tuberculosis remains a major global public health problem. The actual global prevalence of *Mycobacterium tuberculosis* infection is 32% corresponding to approximately 1.9 billion people. According to the World Health Organization (WHO), there were 8.8 million estimated new cases (Case rate, 140/100,000) of pulmonary tuberculosis, including 3.9 million smear-positive cases. In 2003, an estimated 1.9 million people died of tuberculosis, including patients co-infected with human immunodeficiency virus (HIV; 229,000) (Patrick *et al*., 2007).

Immunological mechanisms against *M. tuberculosis* have mainly been investigated in murine models, often a low dose of bacilli are introduced

through an aerosol, to resemble human infection. The immune resistance to *M. tuberculosis* relies mainly on cellular immunity, which is stimulated by proteins excreted by the bacilli. However, an appreciable number of B cells have been found in granulomas of TB infected mice. Little is known about the natural evaluation of antibiotics against *M.tuberculosis* in mouse models. *Mycobacteria* produce a wide variety of glycolipids that are located in the outermost layers of the cell. Several of these compounds (cord – factor (CF), diacetyl trehaloses (DAT), phenoglycolipids (PCL), sulpholipids (CL-1), and Lipoarabinomannan (LAM) have been studied as target molecules for serology in diagnosis of TB. These molecules also interact with host immune cells and contribute to pathogenesis. Their structures are based on very characteristics methyl-branched long-chain acids and alcohols (Minnikin *et al*., 2002).

The first myocolic acid was isolated from the tubercle bacilli in 1929 by Anderson (Anderson, 1929). The name mycolic acid was proposed in 1935 for a portion of the lipid fraction from *M. tuberculosis* by Stodola. Alex Lusuk and Anderson (Stodola *et al*., 1938) structural characteristic was followed in 1950 by Asselineau (Asselineau and Lederer, 1950). Mycolic acids are the major constituents of the inner leaflet of cell wall, where they form an effective impermeable barrier to protect the mycobacteria from antimicrobial agents (Stodola *et al*., 1938).

Tuberculosis is a fatal disease. Direct microscopic detection by direct smear from clinical specimens gives quick results but with specificity not more than 33% and culture on LJ media is time consuming, therefore, there has been strong need for the development of reliable, rapid and less costly diagnostic methods for detection of pulmonary tuberculosis. On the other hand, HIV emerged as an important infection that co-exist with tuberculosis and may affect the immune response as well as efficiency of TB treatment. TB is a prominent pulmonary opportunistic infection in HIV co-infected patients with an importance by far super passing other opportunistic pathogens (Stodola *et al*., 1938).

This study aims to determine the antibodies levels of antimycolic acids surrogates (anti IgG and anti IgM) among TB/HIV co-infected patients by using ELISA.

MATERIALS AND METHODS

Ninety patients with symptoms of pulmonary tuberculosis attending different medical centers in Khartoum State were included in this study. Data were collected using a questionnaire with informed consent. Sputum and venous blood samples were collected from all study subjects. ZN stain was used to detect acid fast bacilli in the sputum samples.

Molecular Identification

Polymerase chain reaction (PCR) was used to confirm the results of conventional method. DNA was extracted from sputum using isopropanol extraction method; briefly, 100 µl of sputum was put into eppendorff tube and 400 µl of Lysis buffer were added and vortexed, then 300 µl of Isopropanol was added and mixed gently then centrifuged at 1200 rpm for 10 minutes, supernatant was decanted by gently inverting the tubes then the pellet was washed 3 times using 70% ethanol (by adding 1ml, mixing gently and centrifuging at 12000 rpm for 5 min each time). Finally, ethanol was poured completely and the tubes were inverted on tissue papers to dry then the pellet was resuspended in 50 µl TE buffer and kept in -20°C till use.

Amplification of insertion sequence *IS6110* (123 bp) was performed with a set of primers having the following sequence:

Forward: 5' (CCTGCGAGCGTAGGCGTCGG)3'
Reverse: 3'(CTCGTCCAGCGCCGCTTCGG) 5'

PCR

A master mix reagent was prepared for 25 reactions which contained:

82.5 µl PCR buffer (Promega), 25 mM $MgCl_2$ (Promega), 15 µl each dNTP (100mM) (Promega), 1 µl of each primer (Inqaba Biotec), 3 µl Taq polymerase (5U/µl) (Promega), 480 µl distilled water and 3µl template DNA. *M. tuberculosis* H37R strain was used as the positive control. The negative control contained reaction mixture without template DNA.

A PCR program was conducted with an initial 5 minutes denaturation step at 94°C for one cycle followed by a repeating cycles of denaturation (45 seconds at 94°C), annealing (30 seconds at 68°C) and extension (30 seconds at 72°C) for 25 cycles, followed by 5 minutes final elongation step at 72°C. PCR product was visualized on 1.5% agarose gel stained with 5 µl of ethidium bromide on UV transilluminator.

Monitoring of Antimycolic Acid Antibodies

Indirect enzyme-linked immunosorbent assay (ELISA) was to detect antimycolic acid profiles. Mycolic acids originating from *Mycobacterium tuberculosis* were isolated as described by Minnikin *et al*. (1988) and were used as the antigen for this assay. To prepare the coating solutions, the antigen was heated in PBS buffer for 20 min at 85°C. The solution was kept at the -80°C till used. The wells of flat-bottom ELISA plates were coated overnight at 4°C with 100 µl/well of antigen solution. The coating solution was flicked out of the plates and replaced with 100 µl/well blocking buffer for 2 hours at room temperature. The blocking solution was aspirated and 100 µl/well from sera diluted 1:50 in dilution buffer were introduced in duplicates. The plates were incubated at room temperature for 1 hour. The sera were flicked out and the wells were washed three times by washing buffer. 100 µl/well of Peroxidase-conjugated anti-human IgG was added and the plates were incubated for one hour at room temperature. After removal of the conjugate, the wells were washed three times with the washing buffer and then emptied by aspiration, 100 µl/ well of substrate solution (TMP) were added and the plates were kept in a dark place for 30 minutes at room temperature and the color development was monitored, 100 µl/well of stop solution (20% H_2SO_4) were added and the results were read at 450 nm. Anti-IgM antibodies were detected in a similar way but anti-human IgM was used instead of anti-human IgG.

HIV Detection

HIV was detected by ICT. The test device was removed from the foil pouch, and placed on a flat, dry surface and blood specimen, serum or plasma (0.2 ml) was added into the sample well, 4 drops (about 0.12 ml) of assay diluents were added to the sample well. As the test begins to work, purple color across the result window in the center of the test device, will indicate a positive result.

RESULTS AND DISCUSSION

Direct smear showed acid fast bacilli positive in 17/90 while PCR results showed that 79/90 were positive for IS 6110 (Fig.1). ICT Screening showed that 9 (10%) were HIV positive.

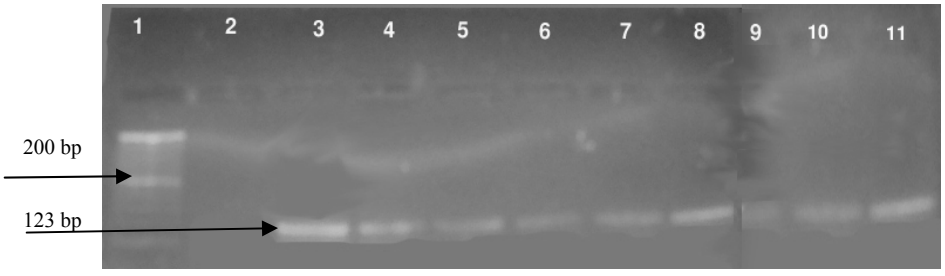

Fig. 1: The amplicon of *M. tuberculosis* after first run of PCR on 1% agarose gel; 1= DNA marker; 2 negative control; 3 = control positive; 4-11 = patients samples = 123 bp bands of *M. tuberculosis.*

When antimycolic IgG and IgM among TB/HIV Co-infected patients was monitored, the results showed that only one patient was positive for IgG. (Fig. 2). and two patients were positive for IgM (Fig. 3), while the rest were negative.

Regarding direct smear in this study, it reflected similar results to those reported by Aftab *et al*., (2008) and Sharma *et al*., 2011, who reported the low sensitivity of the direct smear in detecting *M tuberculosis* infection, this may be due to poor reagents in ZN stain or poor decolorization.

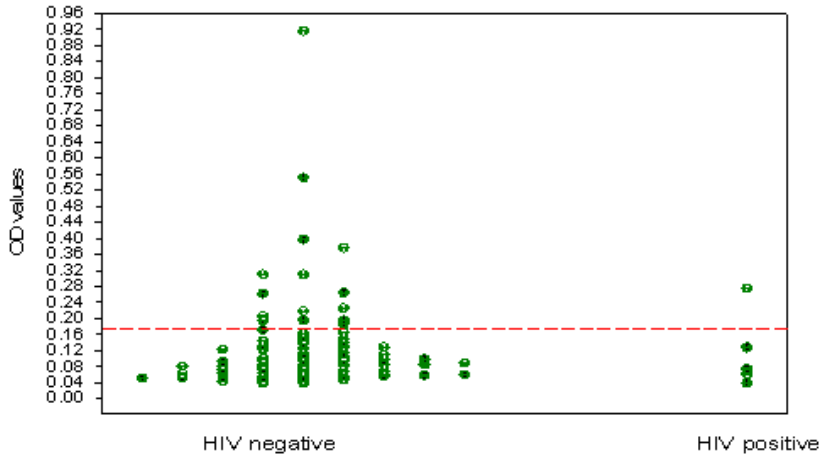

Fig.2: ntimycolic IgG for HIV positive and HIV negative individuals

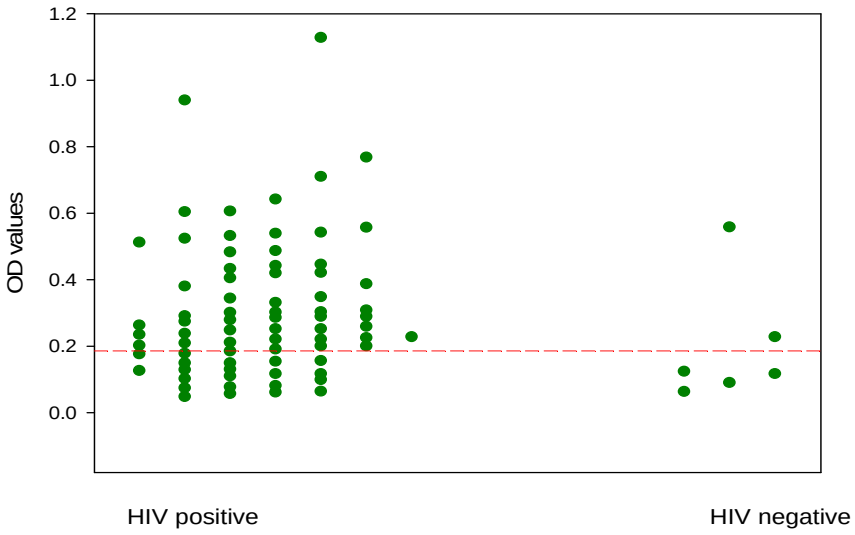

Fig. 3: Antimycolic acid IgM for HIV positive and HIV negative individuals.

In this study, an attempt was made to show if anti-MA antibodies can be used as a marker for the diagnosis of active tuberculosis infection, especially in HIV endemic areas.

The results of PCR technique targeting IS6110 (87.8%) reflect that there was a high prevalence rate of TB among the total number of patients, as well as the higher sensitivity of PCR over

direct smear detection. Similar findings were observed by Noord *et al*., (1994).

Patients with active pulmonary tuberculosis have elevated levels of specific antibodies to *M. tuberculosis* mycolic acids compared to negative controls as detected by ELISA.

Among TB/HIV co-infected patients, more than 80% of HIV positive showed no antibodies in their sera due to immunodeficiency. These findings were in agreement with those reported by Thanyani, (2008).

Antibodies to mycolic acids (MA) antigens can be detected as a marker of active tuberculosis with ELISA. In this technique, the lipid antigen is not encapsulated, but was directly adsorbed to the well bottoms of the microtiter plates. Thus, it doesn't yield the required sensitivity and specificity for accurate diagnosis of TB. One reason for this is the cross reactivity of natural anti-cholestrol antibodies with anti-mycolic acid antibodies (Benadie *et al*., 2008).

In conclusion, the fact that anti-MA antibodies still recognize MA in HIV seropositive patients with active pulmonary TB warrants further investigation into the use of anti MA antibodies as a possible serodiagnostic test for TB.

ACKNOWLEDGEMENT

The authors express their sincere gratitude to Professor Jan A. Verschoor from the department of Biochemistry, University of Pretoria, South Africa, for his kind help in establishment of antigen preparation method.

REFERENCES

Aftab, R. ; Farzana Amjad, Rukhshan Khurshid, Nazifa Ahmed (2008) Detection of Mycobacterium tuberculosis in clinical samples by smear and culture Medical College, Lahore

Anderson RJ (1929). The chemistry of the lipoids of the tubercle bacilli. IX. The occurrence of hexacosanic acid in the unsaponifiable wax. *J. Biol. Chem.,* 85: 351-354.

Asselineau J and Lederer E (1950). Structure of the mycolic acid of mycobacteria. *Nature.* 166: 782-783

Benadie Y, Deysel M, Siko DG, Roberts VV, Van Wyngaardt S, Thanyani ST, Sekanka G, Ten Bokum AM, Collett LA, Grooten J, Baird MS, Verschoor JA (2008). Cholesteroid nature of free mycolic acids from *M. tuberculosis*. *Chem Phys Lipids*. 152(2): 95-103.

Minnikin D.E., Kremer L., Dover L.G.and Besra G.S. (2002). The Methyl-branched fortification of Mycobacterium tuberculosis. *Chem Biol*., 9: 545-553.

Patrick R.M., Jo E.B., James H.J.L ouise M.L., and Michael A.N. (2007) – Manual of Clinical microbiology, 9th edition, Volume 1. American Society for Microbiology. Washington.

Sharma K, Sharma A, Sharma SK, Sen RK, Dhillon MS, Sharma M (2011). Does multiplex Polymerase Chain Reaction increase the diagnostic percentage in osteoarticular tuberculosis? A prospective evaluation of 80 cases. *Int Orthop*. [Published ahead of print (pubmed)]

Stodola, F.H., Lesuk, A., and Anderson, R.J.(1938). The chemistry of the lipids of Tubercle bacilli LIV. The isolation and properties of mycolic acid. *J. Biol. Chem.* 126:505-513

Thanyani, S T., Vanessa Roberts, D. Gilbert R. Siko, Pieter Vrey and Jan A. Verschoor (2008). A novel application of affinity biosensor technology to detect antibodies to mycolic acid in tuberculosis patients *Journal of Immunological Methods* 332 (1-20): 61-72.

ARABIC SUMMARY

صحة استخدام الأجسام المضادة لأحماض المايكوليك في تشخيص السل الرئوي في المرضى المصابين بالسل و فيروس نقص المناعة البشرية في ولاية الخرطوم ، السودان.

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هدفت هذه الدراسة إلى تحديد أضداد حمض الميكوليك [الغلوبولين المناعي M والغلوبولين المناعي G] بين المرضى المصابين بالسل مع فيروس نقص المناعة البشرية في ولاية الخرطوم.

تم جمع عينات الدم والبلغم من المرضى بمستشفى الشعب التعليمي و المختبر المرجعي للسل، مستشفى إبراهيم مالك و مستشفى أبوعنجة ؛ تم تنوير المرضى بموضوع الدراسة وأخذ موافقتهم.

أظهرت المسحات المباشرة من عينات البلغم ل ٩٠ مريضا يشتبه في اصابتهم بالسل أن ١٧ (١٨.٩ ٪) كانت ايجابية لصبغة الحمض السريعة للعصيات في حين أن ٧٣ (٨١.١ ٪) كانت سلبية. عندما أخضعت عينات البلغم التسعون للتفاعل السلسلي البلمري لتضخيم منطقة *IS 6110* المحددة لمجموعة المتفطرة السلية. أظهرت النتيجة أن ٧٩ (٨٧.٨ ٪) كانت ايجابية للـ *IS 6110* في حين أن ١١ (١٢.٢ ٪) كانت سلبية.

كذلك تم فحص عينات المصل التسعين لمعرفة الاصابة بفيروس نقص المناعة البشرية باستخدام تقنية ot blot ، وجدت ٩ عينات (١٠ ٪) موجبة للاصابة بالفيروس وكانت جميعها ايجابية من قبل للاصابة بالسل كما دلت على ذلك تقنية التفاعل السلسلي البلمري.

تم تحليل ٨٠ من عينات المصل بواسطة تقنية الُ ELISA و أظهرت النتائج أن 20 (16٪) كانت إيجابية للغلوبولين المناعي G ضد حمض الميكوليك في حين أن ٦٤ (٨٠ ٪) كانت سلبية و ٥٥ (٦٨.٨ ٪) كانت ايجابية للغلوبولين المناعي M ضد حمض الميكوليك ، في حين أن ٢٥ (٣١.٣ ٪) كانت سلبية.

عندما تم ربط الإصابة بفيروس نقص المناعة مع وجود الأجسام المضادة لأحماض الميكوليك، و في ٦ عينات إيجابية لفيروس نقص المناعة البشرية ، كانت واحدة (١٧ ٪) إيجابية و٥ (٨٣ ٪) كانت سلبية بالنسبة للغلوبولين المناعي G ، في حين أن ٢ (٣٣.٣ ٪) كانت ايجابية و ٤ (٦٧ ٪) كانت سلبية بالنسبة للغلوبولين المناعي M.

خلصت هذه الدراسة إلى أن المرضى الذين يعانون من عدوى السل مع فيروس نقص المناعة البشرية لديهم قدرة أقل على انتاج اجسام مضادة لأحماض الميكوليك ومع ذلك ، يمكن لأضداد للغلوبولين المناعي M (IgM) أن تكون ذات قيمة مصلية تشخيصية أكبر