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Efficacy of ELISA test in diagnosis of *Mycobacterium tuberculosis* infection

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ABSTRACT

Tuberculosis (TB) is a worldwide public health problem; early diagnosis is the key to prescribe an opportune treatment. Most of TB cases are diagnosed at advanced stages, being important to detect carriers TB cases to avoid its transmission. Three study groups were done. Group 1 (contacts): 77 healthy subjects in contact with sick TB individuals. Group 2 (active TB): 28 patients in course of active TB. Group 3 (healthy): 46 subjects without TB. For each group, anti-*Mycobacterium tuberculosis* (Mtb) IgM and IgG quantification was performed by ELISA, as well as, PPD test. Group 1 gave positive results as follow: 93, 40.25 and 71.4% for IgM, IgG and PPD, respectively. Group 2 had positive results as follow: 96.4, 46.42 and 100% for IgM, IgG and PPD, respectively. Group 3 got positive results as follow: 4.34, 56.52 and 43% for IgM, IgG and PPD, respectively. The IgM/IgG ratio was higher in group 1 and lower in group 3. In conclusion, this serologic test is efficient to detect anti-*Mycobacterium tuberculosis* IgM antibodies in healthy subjects who had been in contact with sick TB individuals. This means the opportunity to detect the presence of a latent infection in apparently healthy individuals. It is an advantage over PPD due to it only evaluates the activation of the Th1 lymphocytes in healthy individual which was been previously gotten in contact with *Mycobacterium tuberculosis* and not present disease, thus PPD is not an adequate test to indicate latent infection.

Keywords: Indirect ELISA, skin test, tuberculosis, *Mycobacterium tuberculosis.*

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INTRODUCTION

Tuberculosis (TB) is still a worldwide health issue and a very important cause of mortality as infectious agent preceded only by AIDS. In 2017, worldwide 1.6 million people death by TB (1.3 millions of people HIV negative and 0.3 million of people HIV positive), while in Mexico there were 2,000 deaths (1,230 of HIV negative and 770 of HIV positive) (World Health Organization, 2018). In most cases, patients infected by *Mycobacterium tuberculosis* (Mtb) remains asymptomatic and non-infectious, and this latent infection can extend during the individual's whole lifetime. However, the latent infection can be reactivated in response to alterations in the immune response, producing the active disease. HIV infection, diabetes mellitus, corticosteroid treatment, and immunosuppressant therapy increase the potential risk of reactivation of a latent infection. Although these patients do not expectorate bacteria, 5 to 10% of them will develop the active disease and will be able to transmit the infection (Ilievska-Poposka et al., 2018; Rafiei et al., 2018). Thus, there is a necessity to develop a technique to diagnose latent Mtb infection to avoid TB dissemination. The current diagnostic methods are

described below.

Microscopic identification of acid-fast bacilli (Ziehl-Neelsen stain) in sputum samples, chest X-rays and culture are the most used methods to diagnose active TB. Acid-fast stains are positives only in active and advanced TB, it has a sensitivity of 22 - 78%, while culture has 95%. However, the latter has the disadvantage that it takes 6 to 8 weeks to develop and with negative results in paucibacillary patients. Chest X-rays shows similar images to those seen in other infectious pathologies (Palma-Nicolas and Bocanegra-Garcia, 2007; Rao et al., 2009). The most used method to diagnose latent Mtb infection is the Mantoux test or purified protein derivative (PPD). It is a mixture of antigens derivate from the Bacillus Calmette-Guerin (BCG) vaccine, *M. bovis* and *M. africanum* (non-tuberculous mycobacterias). PPD has a sensitivity of 66%. Furthermore, the development of new methods such as the identification of interferon-γ in blood, gives a new perspective for the diagnosis of TB (Kang et al., 2005).

Humoral response does not play an important role in Mtb infection; however, antibodies detection has been used as diagnostic method for TB by Tiwari et al. (2014). One of these techniques detect serum immunoglobulin IgA, IgG, and IgM antibodies raised against the Mtb A60 antigen for the diagnosis of TB (Ben-selma et al., 2010). Other technique that uses two antigens isolated from the H37Ra strain had a sensitivity of 66% in patients with meningeal TB who had negative cultures (Krishnan and Mathai, 1991). A third technique is a serodiagnosis test that uses 38-kDa, Ag16 and Ag85B as antigenic complex to detect IgG antibodies and had a sensitivity of 76% (Imaz et al., 2008). In 2000, a comparative list of 7 diagnostic tests was published and all of them showed different sensitivities (Pottumarthy et al., 2005). There was reported a test that detect IgG, IgA, and IgM antibodies using a 38 kDa protein from an H37Rv strain as antigen. This technique showed sensitivity and specificity of 68 and 96%, respectively for IgG and IgA; while for IgM its sensitivity was 71% and the specificity 90% (Uma Devi et al., 2001). An immune chromatography test that uses recombinant antigens of 6, 16 and 38 kDa that detects IgG and IgM antibodies in human serum or plasma and can distinguish between active TB and other pulmonary diseases (Ben-Selma et al., 2011). Also, IgA was studied; it was postulated as a marker for acute TB using three mycobacterial antigens: glycolipid, recombinant protein and crude antigen from Mtb. These antigens constitute a powerful tool for the diagnosis and monitoring pulmonary TB (Bezerra et al., 2009). Laal et al. (1997) studied the immune dominance of the Mtb antigens that induce the most important production of antibodies in response to infection and found that the antigens with the highest molecular mass were the most frequent. Min et al. (2011) characterized the antibody production against 10 purified recombinant antigens and old tuberculin in rhesus monkeys experimentally infected with Mtb. They found antibodies production between the 4th and 6th week against the CFP10, CFP10-ESAT-6, U1, MPT64, and Ag85b antigens. CFP10 and ESAT-6 are not present in the BCG vaccine. These antigens are secreted early by Mtb and other mycobacteria (*M. kansasii*, *M. marinum*, *M. szulgai* and *M. flavescens*) and are absent in *M. bovis* BCG, *M. avium*, and non-tuberculous mycobacteria. That's way CFP10 and ESAT-6 are useful to distinguish between BCG vaccine individuals and those infected by Mtb (Min et al., 2013).

IgM presence is an indicator of recent or current infection, while IgG appears later, and its levels can remain high as immunologic memory; that way IgM and IgG identification is used as indicator of active or latent Mtb infection, respectively. Thus, the objective of this study was to determine the presence of IgM and IgG antibodies in sera of healthy individuals who are exposed to patients with active tuberculosis, through the standardization of an ELISA technique that uses a cocktail of purified extracellular proteins as antigen, including CFP-10 and ESAT-6 proteins from Mtb H37Rv, and then propose this technique as opportune serologic diagnostic test of latent tuberculosis in a population from the Northeast of Mexico.

MATERIALS AND METHODS

Studied population

The studied population was 151 individuals; they were divided into 3 groups: 1) 77 healthy person who were in contact with patients who coursed active TB; this group (contacts), was in close, daily contact (at least 6 hours per day) with patients who had active TB. 2) 28 had pulmonary TB; this group, were patients with active TB who were diagnosed through a positive Löwenstein-Jensen culture and were taking treatment to TB over 2 months. 3) 46 healthy individuals. This group, were healthy individuals that had not been exposed to patients with active TB.

Ethical approval

Research was approval by ethical committee (protocol No. IN13-004) and all subjects signed an informed consent to be included in the study and had been vaccinated with the BCG vaccine at the birth.

Samples

Five milliliter serum samples were obtained from 10 ml of peripheral venous blood from each patient, then placed in plane tubes without anticoagulant. The sera samples were stored at -20°C until their use.

Antigen preparation

Mycobacterium tuberculosis H37Rv colonies were cultured in Löwenstein Jensen media (Bioxon S.A. de C.V. Lot 08B2333I) and were suspended in Proskauer-Beck broth modified by Youmans (PBY). The broth was incubated for 12 weeks at 37°C, then

autoclaved at 121°C for 15 min, and later the extracellular proteins were obtained through filtration to eliminate bacterial mass. Then, extracellular filtrate was concentrated using a concentrator Eppendorf 5301 at 1,400 rpm to the vacuum by 5 h. Extracellular proteins were purified by precipitation with saturated ammonium sulfate at 50% in constant shaking at 4°C. The precipitate was resuspended in 20 ml phosphate buffer pH 7.2 and dialyzed against distilled water at 4°C for 72 h until sulfate ions were eliminated. Protein concentration was determined by Bradford method. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 18% was carried out to confirm the presence of proteins include ESAT-6 and CFP-10 (Min et al., 2013).

ELISA tests

Indirect ELISA tests were carried out according the patent No. 285260 called "Proceso de detección de tuberculosis" developed in the Immunology Department of the Medical School in the UANL (Arce-Mendoza and Rosas-Taraco, 2011). 1 µg/well of the antigen EPPES6CF10 diluted in acetate buffer pH 7.2 - 7.4 was placed in 96-well costar plates. The plates were incubated overnight at 4°C, then supernatants were discarded and the plates were blocked with 200 µL of 5% diluted skim milk in phosphate buffer. Samples were diluted at 1:50 in 1% skim milk. Peroxidase-conjugated anti-human IgM and IgG antibodies were diluted at 1:10,000. The plates were read in a spectrophotometer (Bio-Rad iMark™) at $\lambda = 490$ nm.

PPD assay (tuberculin skin test)

To carry out the PPD test, 0.1 ml of PPD (5TU Tuberculin Purified Protein Derivative, Tubersol, Sanofi Pasteur) were injected intradermal in each patient's forearm, following asepsis with ethanol. Indurations were read 48 h later (≥ 10 mm was considered positive).

Specificity and sensitivity

These parameters were estimated by the following formulas: sensibility = TP/(TP+FN) and specificity = TN/(TN + FP); where TP = True Positive, FN = False Negative, TN = True Negative and FP = False Positive (Alvarez et al., 2012).

Statistical analysis

Comparative studies of each group were done through a student's t test using the GraphPad Prism 5.0 software.

RESULTS

Antigen preparation

The cocktail of secretory proteins obtained from culture filtrate of Mtb included proteins from 6 to 85 kDa as shown in Figure 1.

ELISA tests

IgM and IgG presence was evaluated in the 3 groups. IgM detection was statistically significant higher in two groups (contact and active TB) respect to the group of healthy patients ($p < 0.001$) (Figure 2). Cut-off value for IgM was established at 2 SD (standard deviations) (SD = 0.41) above the mean (0.40), its value being 1.22. Results of IgM were positive for 2 healthy individuals (group 3 = 4.34%), 72 contacts (group 1 = 93.50%), and 27 patients with pulmonary TB (group 2 = 96.42%). The results for IgG were more disperse than IgM (Figure 3) however, there was a statistically significant difference between contacts and healthy individuals (group 1 and 3, p <0.001). We could not establish a cut-off value since the values from the 3 groups were very far apart; however, considering only the mean of the absorbance, we can observe that 26 healthy individuals (group 3 = 56.52%), 30 contacts (group 1 = 38.96%), and 13 patients with pulmonary TB (group 2 = 46.42%), have values above the mean. For the IgM/IgG ratio, we observed a statistically significant difference between contacts and active TB respect to the healthy individuals ($p < 0.001$) (Figure 4). The sensibility and specificity of the test to detect IgM in group 1 were 93.5 and 95.6%, respectively; while in group 2 were 96.4 and 95.6%, respectively (Tables 1 and 2).

PPD assay

PPD results were read 48 h after the test was done. Results were positive for 55 contacts (group 1 = 71.4%), 28 active TB (group 2 = 100%) and 20 healthy (group 3 = 43%). A comparison between PPD and ELISA test is shown in Table 3.

DISCUSSION

There are several commercial tests to diagnostic active TB that use different antigens; however, their accuracy is not 100% to detect Mtb infection. Most of these kits are designed to detect either all antibodies or only IgG and they have a sensibility < 70% (Pottumarthy et al., 2000). Today, the development of new immunoenzymatic techniques that use as antigens specific Mtb proteins, such as CFP-10 and ESAT-6, seems very logic and attractive, since these are the first proteins that the immune system recognizes during the infection. IgM is the first immunoglobulin to be produced in the primary immune response to Mtb infection and it disappears when the disease is resolved; thus, the presence of IgM antibodies against Mtb can be indicative of a present infection with the bacillus (Min et al., 2011).

In group 1, 93 and 71.4% were positive for IgM and PPD tests, respectively. It demonstrated that our ELISA method was more useful that PPD to detected latent infection; thus, this ELISA test can be used as predictor of latent infection. In patients with active TB (group 2), 96.4 and 100% were positives for IgM and PPD tests, respectively. It suggests that our new ELISA method can

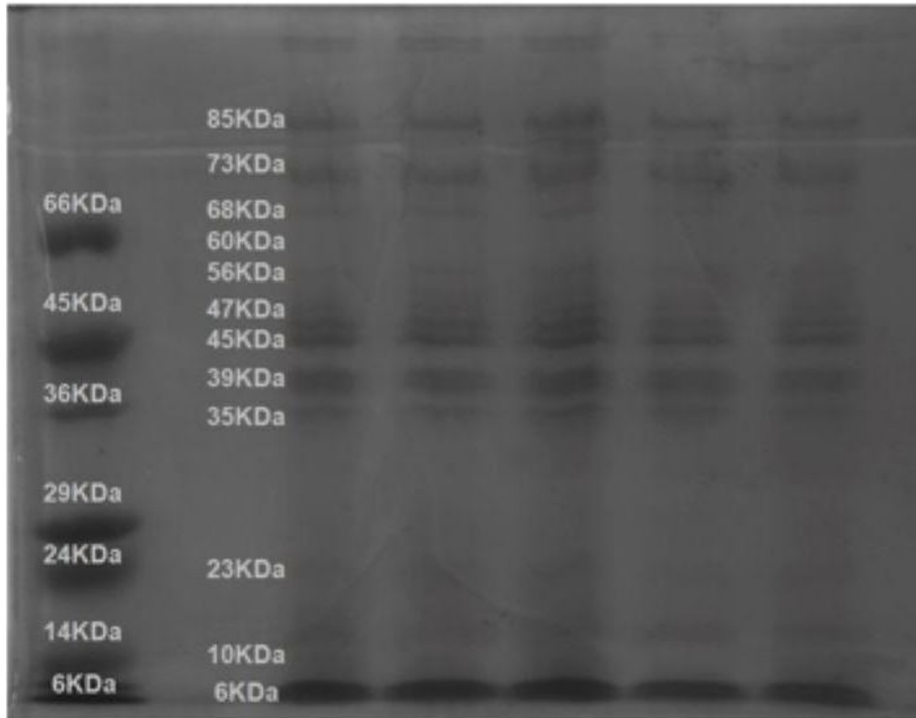

Figure 1. SDS polyacrylamide gel electrophoresis 18%. Lane 1: Molecular weight marker DALTON MARK VII-L for SDS Gel Electrophoresis (Molecular Weight Range: (6,000 - 70,000). Lane 2: None. Lane 3 - 7: Extracellular protein complex that includes 85 kDa, 73 kDa, 68 kDa, 60 kDa, 56 dDa, 47 kDa, 45 kDa, 39 kDa, 35 kDa, 23 kDa, 10 kDa, and 6 kDa proteins.

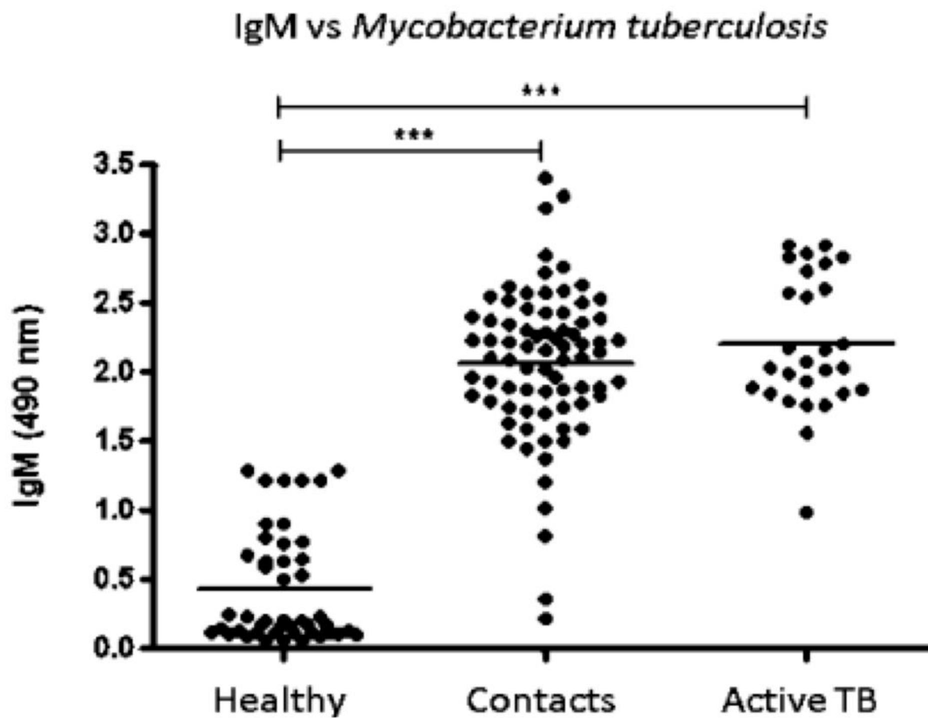

Figure 2. Detected IgM against *Mycobacterium tuberculosis*. Graph shows a significantly high concentration of IgM in the contact and active TB groups of patients respect to the healthy group. P values were calculated through student's t test *** ($p < 0.001$).

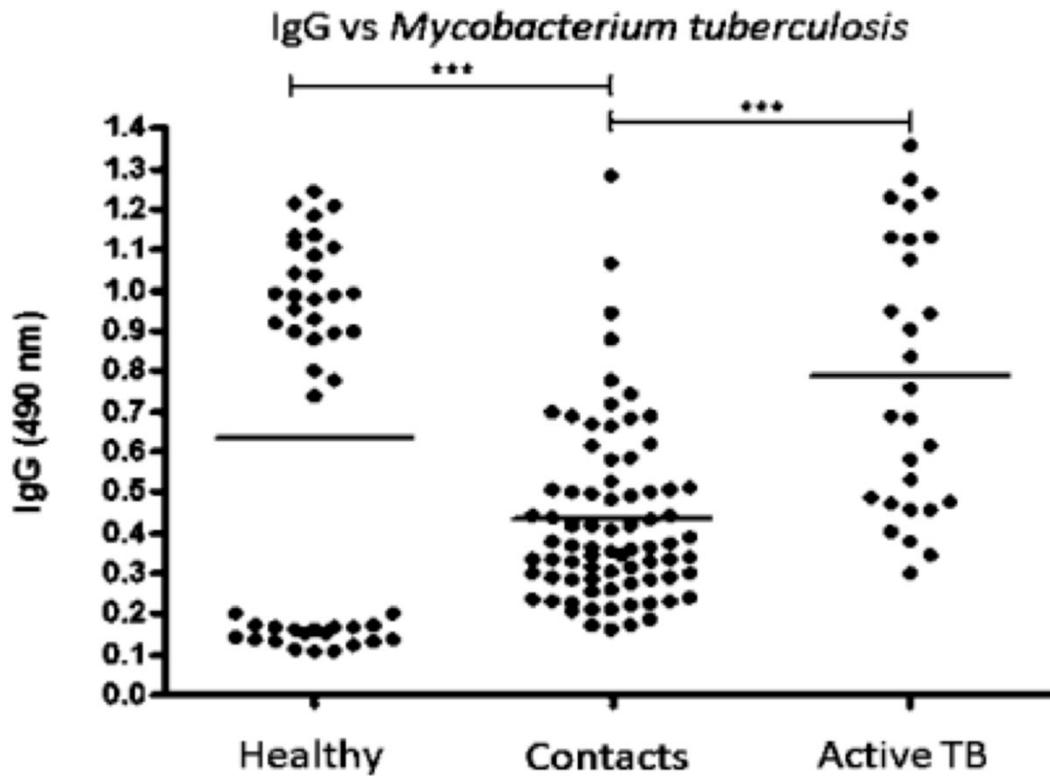

Figure 3. Detected IgG against *Mycobacterium tuberculosis*. Considering the arithmetic mean of the absorbance, the group of healthy individuals show a higher proportion of positive cases for IgG (56.52%) compared to contacts (38.96%) and patients with active pulmonary TB (46.42%). P values were calculated through student's t test *** ($p < 0.001$).

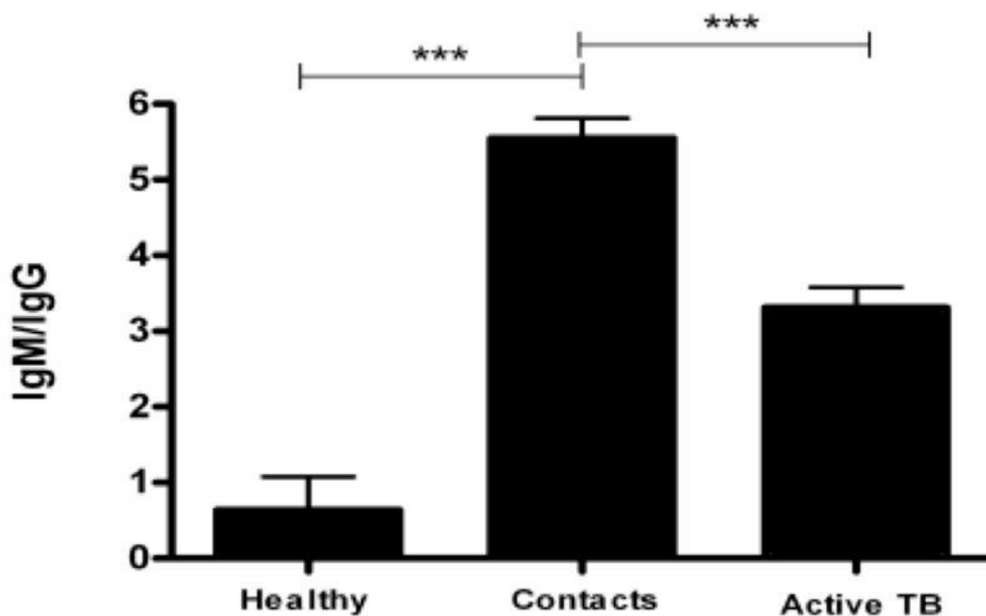

Figure 4. IgM/IgG ratio. IgM/IgG ratio is higher in contacts and in active TB respect to healthy individuals. Student's t test *** ($p < 0.001$).

Table 1. Subjects who had positive IgM and IgG test.

Individuals	IgM (+)	IgG (+)	Total
Group 1 (contacts)	72	31	77
Group 2 (active TB)	27	13	28
Group 3 (healthy)	2	26	46
Total	101	70	151

Table 2. Sensibility and specificity to detect IgM in groups 1 and 2. Results showed a sensibility of 93.5 and 96.4% in contact and active TB patients, respectively and a specificity of 95.6% in both groups.

Group 1	Group 3	
TP 72	FP 2	Sensibility = $\frac{TP}{TP + FN} \times 100 = \frac{72}{72 + 5} \times 100 = 93.5\%$
FN 5	TN 44	Specificity = $\frac{TN}{TN + FP} \times 100 = \frac{44}{44 + 2} \times 100 = 95.6\%$
Group 2	**Group 3**	
TP 27	FP 2	Sensibility = $\frac{TP}{TP + FN} \times 100 = \frac{27}{27 + 1} \times 100 = 96.4\%$
FN 1	TN 44	Specificity = $\frac{TN}{TN + FP} \times 100 = \frac{44}{44 + 2} \times 100 = 95.6\%$

TP = True Positive, FP = False Positive, FN = False Negative and TN = True Negative.

Table 3. Positive results of PPD and ELISA assays from the three studied groups.

Group 1 (contacts)	**77**
PPD	71.4% (55)
ELISA IgM+	93% (72)
ELISA IgG+	40.25% (31)
Group 2 (active TB)	**28**
PPD	100% (28)
ELISA IgM+	96.4% (27)
ELISA IgG+	46.42% (13)
Group 3 (healthy)	**46**
PPD	43% (20)
ELISA IgM+	4.34% (2)
ELISA IgG+	56.52% (26)

be used to diagnose active TB with a sensibility and specificity of 96.4 and 95.6% respectively (Tables 2 and 3). Immunoglobulin IgG is produced after IgM. IgG concentration is directly proportional to the stimulus, that's way tests that detect only IgG have low sensibility (Uma Devi et al., 2001; Ben-Selma et al., 2011). IgG can remain elevated as immunologic memory after the disease has resolved. It is observed in group 3, where 26 of 46 subjects were IgG positive. Only 2 healthy subjects had positive IgM result, it suggests that they could be infected since they were also positive for PPD test. Follow-up is needed in these patients. IgG cut-off value could not be established because antibody concentration was scattered in the 3 groups. It could be due to geographic region at the study was carried out is endemic for TB, suggesting that healthy individuals could have been in contact with the bacillus at some point of their lives and trigger immunologic memory. Another explanation could be due to groups 1 and 2 (contacts and active TB), switch to IgG antibody production.

IgM/IgG ratio in contacts (group 1) was the higher, suggesting presence of the active bacillus presence; thus, latent Mtb infection is very probable. While in healthy subjects (group 3), this ratio was the lowest, since IgM concentration decreases respect to IgG. In the other hand, PPD test was positive in 71.4% of contacts (group 1). PPD is the test most used to detect Mtb infection; however, it detects immunologic memory, as well as, BCG vaccine; thus, it does not necessarily detect a present infection. That's way PPD test is not very useful to diagnostic present infection. Its use and interpretation should be carried out with reservation.

We showed a high accuracy test (Table 2), that uses specific Mtb antigens (ESAT-6 and CFP-10) and a cocktail of extracellular proteins to detect antibodies against Mtb. We propose this method as a serologic test to tell apart latent and active Mtb infection (Figure 4), it would enable us to diminish the infection risk by providing prophylactic treatment to those individuals who are in contact with sick TB subjects.

Conclusion

This serologic test is efficient to detect anti-Mtb IgM antibodies in healthy subjects who had been in contact with sick TB individuals. This means the opportunity to detect the presence of a latent infection in apparently healthy individuals. It is an advantage over PPD due to it only evaluates the activation of the Th1 lymphocytes in healthy individual which was been previously gotten in contact with Mtb and not present disease, thus PPD is not an adequate test to indicate latent infection.

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