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Evaluation of *Mycobacterium bovis* Isolated from Cattle in Mexico for Serum Reactivity and Antigen Production Kinetics

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Abstract

Immunodominant antigens represent a potential tool to develop serological diagnosis in bovine tuberculosis (bTB). Novel antigens from natural acquired *Mycobacterium bovis* from aerosol infected animals have been identified. However, still it is necessary to define antigen recognition directly from the cattle. Herein, we are reporting an evaluation of *M. bovis* strains isolated from cattle in Mexico using tubercle bacilli cell growth for serum reactivity and antigen production kinetics (7-21 days). During early, middle and longtime cell growth, we found that there are a set of proteins that in comparison with BCG antigens, mostly reacted toward either pooled or individual serum positive samples obtained directly from animals infected with *M. bovis*. Interestingly, we found that antigens of 45, 48, 75 and 90 KDa produced at 7 or 21 days are mostly recognized by the serum of infected cattle TST(+) *vs* uninfected (TST-). Altogether, the data reported here indicate that in addition to the cell wall factor virulence factors; the antigens mostly recognized and produced during the growth of tubercle bacilli could strengthen diagnostic tests in cattle while discarding BCG cross reactivity.

Keywords: *Mycobacterium bovis*; *M. bovis* BCG; Bovine tuberculosis; Antigens; TST; Serum antibodies; Sero-diagnostic

Introduction

Bovine tuberculosis is a zoonotic infection caused by *Mycobacterium bovis* (*M. bovis*) a member of the complex Mycobacterium tuberculosis that constitutes serious health and economic problem in livestock and agriculture worldwide [1,2]. The *RD1* locus, *RD1* (*Rv3871-Rv3879c*), a known virulence gene cluster common to pathogenic mycobacteria is deleted in *M. bovis.* Bacillus Calmette Güerin (BCG), encodes proteins that are actively secreted by pathogenic mycobacteria, including *M. tuberculosis*/*M. bovis* [3-8]. The hallmark of these proteins is their immunomodulatory properties and thus, constitute targets for further development diagnostic tests and candidates' vaccines [9]. The elegant mycobacterial secretory system, named 6 kDa early secretory antigenic target (ESAT6) protein family secretion (ESX) systems (also known as type VII secretion systems) [10-12]. Recently, there has been major progress in understanding the biogenesis, secretion and antigenicity of *ESAT-6* proteins and at least in the case of *ESAT-6* system1, in unravelling a key role of the members of the complex of *M. tuberculosis* in pathogenicity [11,13-16]. The most studied mycobacterial released antigens that have described until now are the MPB70 and MPB83, encoded as precursor proteins with typical signal peptides for export through the general secretory pathway ESX. While MPB70 is a soluble secreted protein cleaved by signal peptidase I, MPB83 is a glycosylated lipoprotein processed by signal peptidase II and located at the surface, possibly with the lipid tail coupled to the N- terminal cysteine embedded in the mycobacterial outer membrane [17]. In general, other antigens have been also reported that include several enzymes such as mucinases, lipases, proteases, RNAses and proteins that bind to fibronectin, whose role in the pathogenesis have not been further assessed [8,17,18]. Virulence factors of the genus, pathogenic mycobacteria can be released to the culture medium at early time points of the culture [14,19-21]. Previous studies in the 80's have focused on the seroreactivity of early antigen components of *M. bovis* BCG, tested against serum lepromatous leprosy patients and few reports evaluating *M. bovis* biomarkers [20,22]. A recent paper by Lyashchenko et al. identified novel antigens recognized by serum antibodies from naturally acquired *M. bovis* infected cattle [23]. Ante-mortem and post- mortem approved sero diagnosis methods in cattle have limitations either in sensibility or in precision. Therefore, it is necessary to continue searching for readability and precision sero test from *in vivo M. bovis* infected cattle. Herein, we are reporting an evaluation of *M. bovis* strains isolated from cattle in Mexico for serum reactivity using tubercle bacilli cell growth and antigen production kinetics (7-21 days). During early, middle and long time cell growth, we found that there are a set of proteins that in comparison to BCG antigens, mostly reacted either with pooled or individual serum positive samples obtained directly from animals infected with *M. bovis*. Interestingly, we found that antigens of 45, 48, 75 and 90 KDa produced at 7 or 21 days are mostly recognized by the serum of infected cattle TST(+) *vs* uninfected (TST(-). Altogether, the data reported here indicate that in addition to the cell wall factor and virulence factors, the antigens mostly recognized and produced during the growth of tubercle bacilli could strengthen diagnostic tests in cattle while discarding BCG cross reactivity [24].

Materials and Methods

Animals

Negative and positive bovines to the tuberculin skin test (TST).

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TST(-) to the caudal anus and the comparative cervical test was considered negative. TST(+) to both field tests, bacteriological and histopathological exams was considered as positive. TST (±) positive to field tests but negative to the laboratory exams, was considered as reactor.

Strains and cell culture

M. bovis were isolated from infected cattle (East and South regions of Zacatecas, Mexico). These isolates were verified by PCR [21,22]. Briefly, tissue homogenates in PBS were seeded on solid Middlebrook (7H10) medium enriched with OADC, (dextrose, catalase and albumin) (Gibco, Co) PANTA and THF (2- thiophenocarboxylizocidhydrazide) (2 mg/ml) (Sigma, Aldrich, Co). The plates were sealed and incubated at 37° C/5% CO2 until growth (8 days). BCG strain was grown without THF. Afterwards short, medium and long cell cultures of *M. bovis* isolates were prepared from solid culture in medium Middlebrook 7H9 and then it was transferred to a sterile flask of 500 ml with 100 ml of this medium and incubated at 37°C. After each time point, short time (8 days), 15 medium (15 days) and long (21 days) of culture, bacteria were harvested by centrifugation, and the medium was filtered through a 0.45 µm membrane. The culture filtrates as well as the pellet was stored at 20°C until use.

Antigens

A modified method by Pessolani et al. was followed [20]. Protein components of the culture filtrate and subcellular fractions of *M. bovis* BCG and *M. bovis* isolates were obtained by filtration of the culture medium. A volume of this (2 ml) was precipitated with 50% of ammonium sulphate and the rest part was precipitated with 75% of ammonium sulfate (Figure 1). The pellet was sonicated for 30 min on ice and then centrifugated (10,000 RFC/30 min). The recovered supernatant (sonicated soluble fraction) and Protein were precipitated with 50% or 75% ammonium sulphate. The recovered pellet was suspended in PBS and lysozyme (10 mg/ml). Later it was incubated for 2 h to 37°C and sonicated on ice for 15 min [25,26]. The soluble fraction was recovered and precipitated with ammonium sulphate. The protein concentration was estimated by the method of Lowry et al. keeping bovine serum albumin (BSA) as standard. The samples were stored at -20°C until use [27,28].

Bovine serum

Blood of cattle from different regions of Zacatecas, State was collected. Serum was separated by incubation at room temperature and then centrifugated at 3000 RPM/7 min. The recovered serum was stored to -20ºC until use.

Western blot and PAGE

Each fraction of short, middle and long cultures of *M. bovis* isolates (15-20 µl) was diluted by sample loading buffer (5 µl) and boiled at 100°C for 5 min. All protein components of different soluble fractions (filtrate, filtrate precipitated with ammonium sulfate and soluble fraction of sonicated extract were then separated on 12% sodium sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and electro transferred to nitrocellulose membranes using semi dry trans blot system (Bio-Rad, Corning Inc., NY, USA). Membranes were blocked with skimmed milk 6% (w/v) diluted with phosphate buffered saline (PBS) and incubated overnight at 4°C. Afterwards, membranes were then washed 4 times with PBST (PBS containing 0.05% Tween 20) and incubated with pooled (n=5) (1:30000) or individual bovine serum (1:25000) (positive or negative) diluted in 5% of skimmed milk /PBST and incubated for 3hr at room temperature. Membranes were washed 5 times with PBST and incubated at room temperature for 1 and half hour with horseradish peroxidase (HRP-conjugate anti-mouse IgG) (1:20000) (ab6789, Abcam). Final washing with PBST was followed by immune detection system Clarity western ECL substrate (Bio-Rad).

Results

Antigen production kinetics of *Mycobacterium bovis* isolated from cattle in Mexico

Sero-diagnostic tests of bovine tuberculosis have been improved in sensibility using a method from naturally acquired *M. bovis*

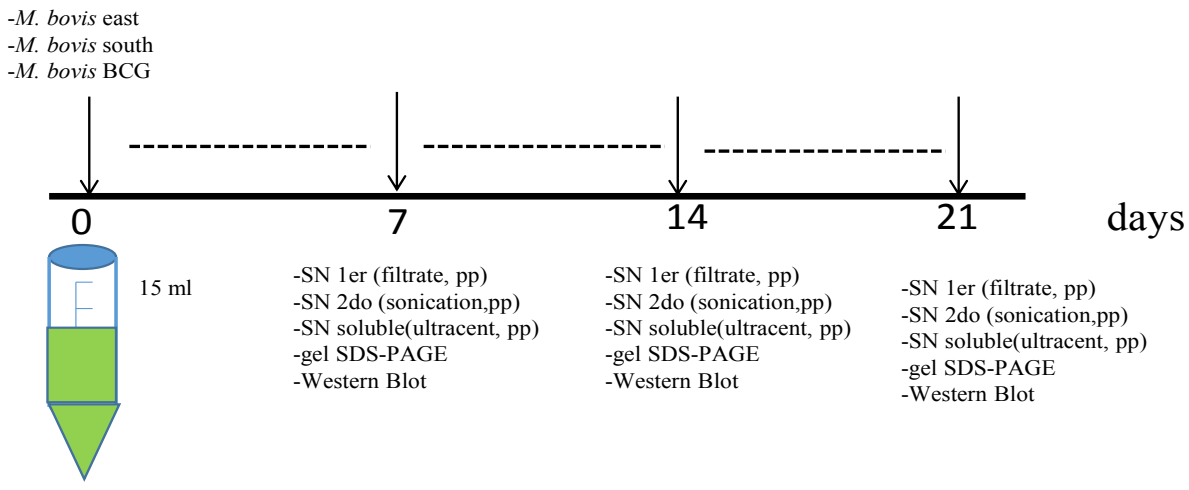

Figure 1: Protocol designed for this study. Tubercle bacilli cell growth kinetics was made. Briefly, *M. bovis* isolates were grown in solid culture medium (Middlebrook 7H9/THF), followed by culture in liquid culture (Middlebrook 7H10/THF) as described in Materials and Methods. Cellular and subcellular fraccionation were made: filtration through 0.22 µm; ammonium sulphate precipitation; sonication and ultrancentrifugation. These fractions were analyzed in SDS-PAGE 12% and stained with blue Coomassie.

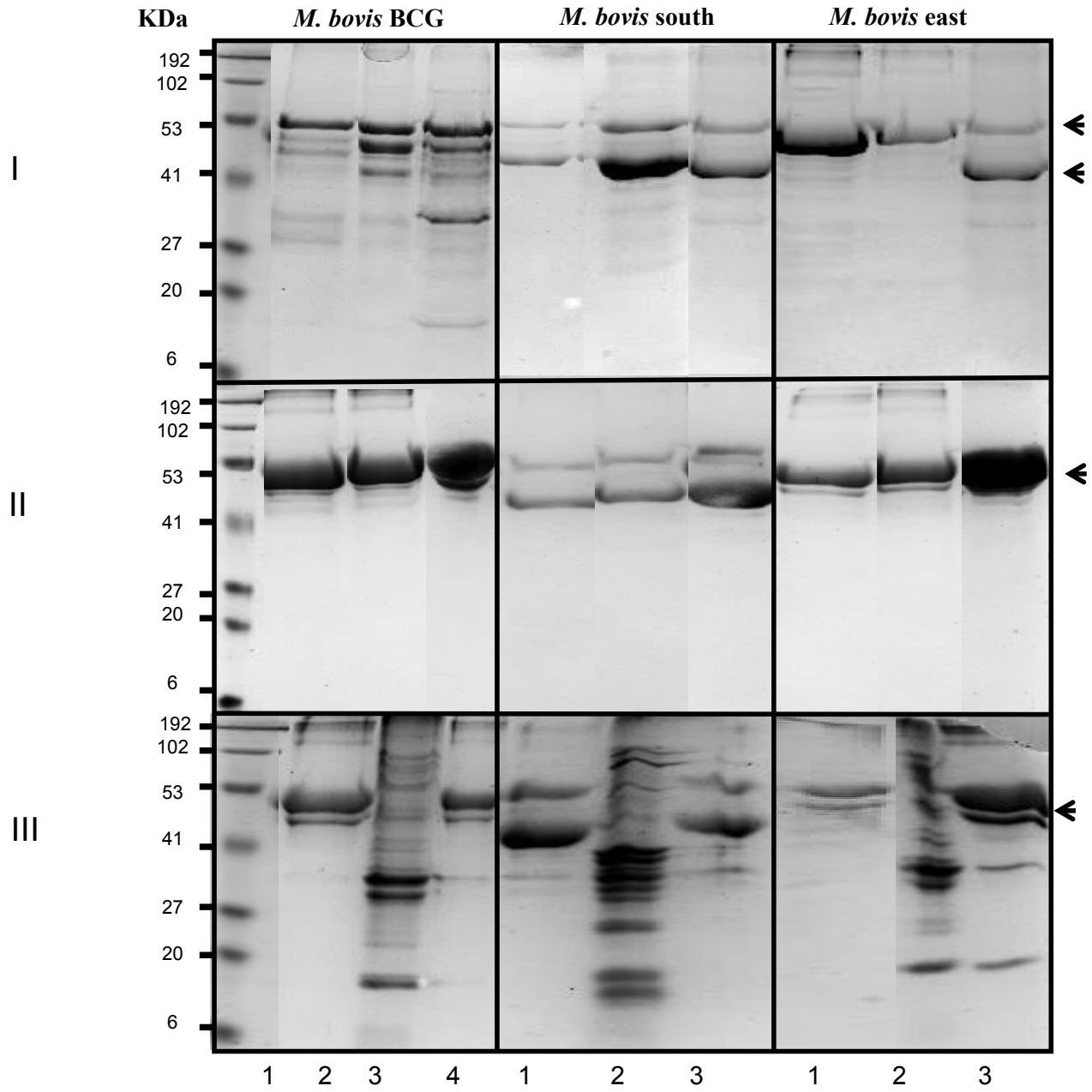

Figure 2: Antigen production kinetics of Mexican *M. bovis* isolates. Protein components of kinetics were analyzed in 12% of SDS-PAGE 12% and stained with blue Coomassie. Filtrate (lane 2 and lane 1), ammonium sulphate precipitate filtrate (lane 3, lane 2) as well as soluble sonicated extract and ammonium sulphate (lane 4, and lane 3). In each lane, 14 µg of protein fraction was applied. Molecular Weight Markers (lane 1)(Bio-Rad). A representative gel is shown from two independent experiments.

infected cattle [23]. However, it might be desirable to optimize this type of test directly from the blood samples of infected cattle. Thus, in the present work we pursued to evaluate tubercle bacilli cell growth antigen production and serum reactivity kinetics of *M. bovis* isolated from Zacatecas, Mexico (confirmed by PCR) (data not shown) (Figure 1) [23]. During the bacilli early cell growth, we found that different protein components of low to high molecular weight (6, 41 to > 90 KDa) were produced by *M. bovis* isolates (Figures 2I-2III, lanes 2-4 and 2-I, lanes 2-4). From these set, 4 proteins of 41, 43, 48 and 60 KDa were present in the filtrate medium and enriched with 75% ammonium sulphate precipitation (Figure 2-I, lanes 2). In the soluble fraction of the sonicated extract, precipitated with ammonium sulphate (50%) two proteins of 41, 48 KDa were found (Figure 2-I lane 4). At mid-term bacilli growth (15 days), two predominant protein components of 48 and 53 KDa were produced (Figures 2II and 2III, lanes 2-4). At long term cell growth (21 days), two main components (antigens) were mostly precipitated in the soluble fraction of sonicated extract of 48 and 75 KDa proteins (Figure 2III, lanes 4). The soluble fraction of the cell wall was obtained and currently studies are going on in the Guerrero et al., preparation in 2019.

Figure 3A

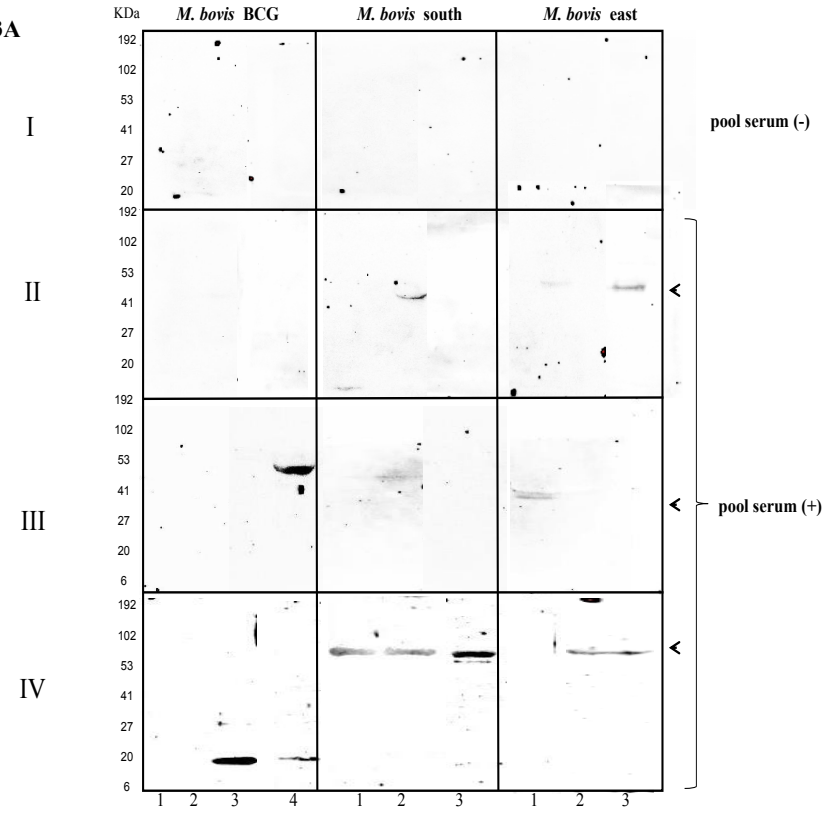

Figure 3B

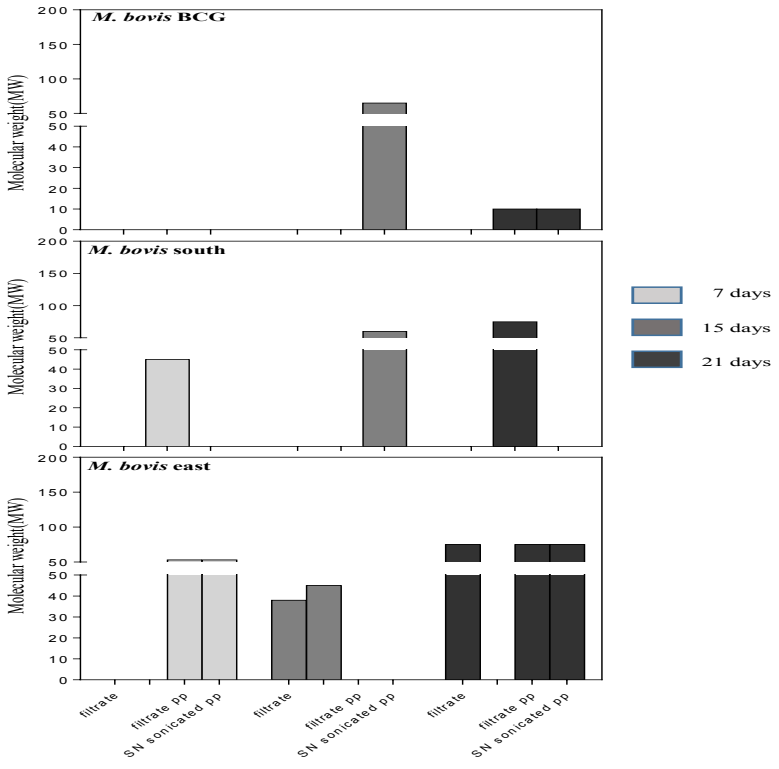

Figure 3: Recognition of *M. bovis* antigens of pooled serum samples from infected Mexican cattle. Protein components of tubercle bacilli cell growth kinetics that were fractionated accordingly to a modified protocol of (20) as described in materials and methods. Briefly, these components were transferred to nitrocellulose membranes and were blocked with low fat milk in PBS Tween 0.05% overnight. Transferred components of each time point reacted with pooled serum (n=5) [3A (II-IV)]. Bar representation of serum reactivity kinetics to pooled serum samples of infected cattle (3B).

Figure 4A

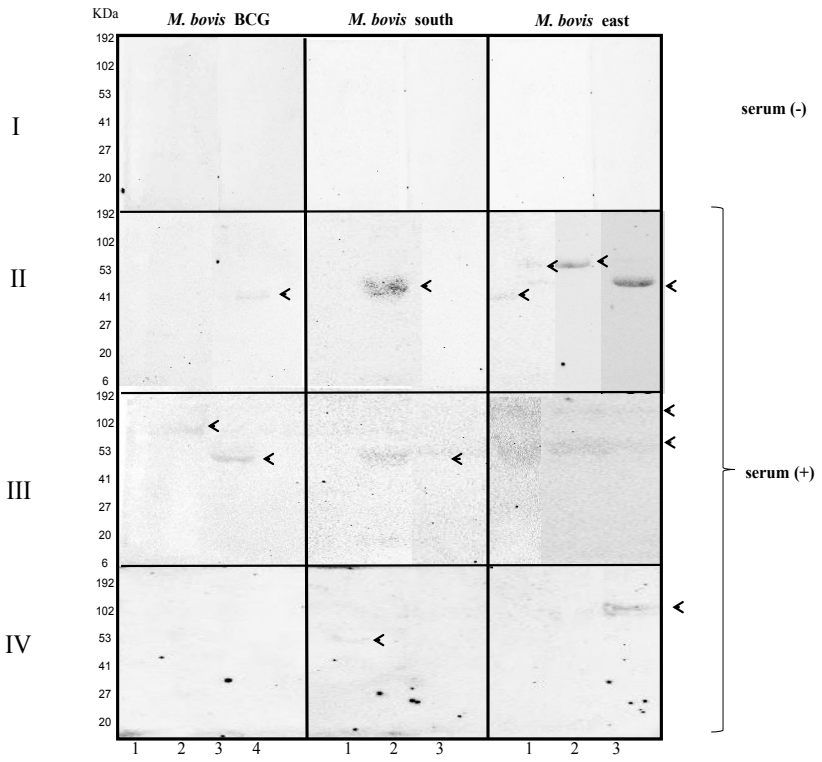

Figure 4B

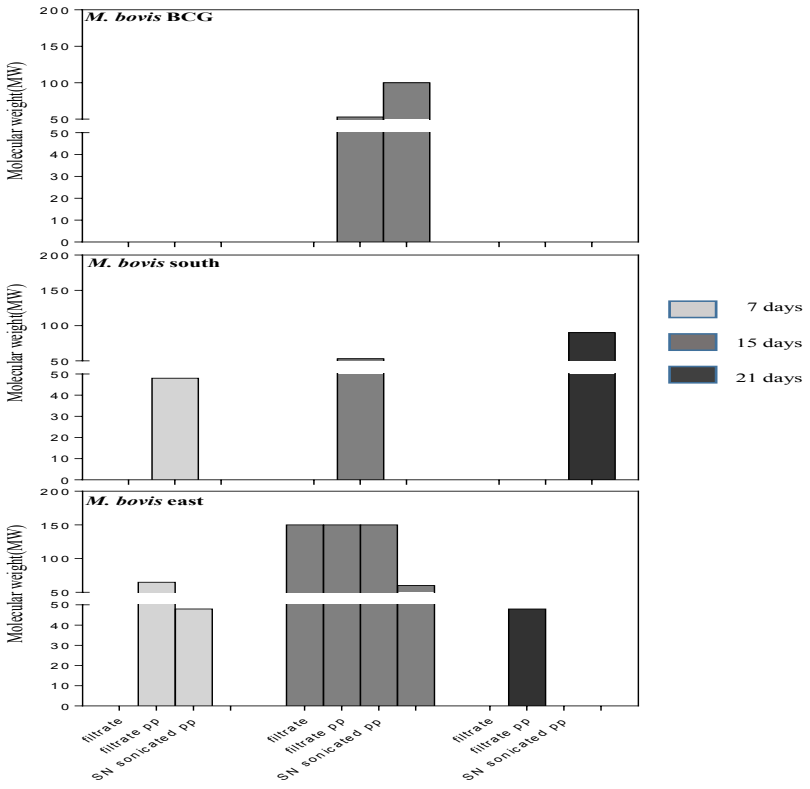

Figure 4: Sero-recognition of a set of proteins of Mexican *M. bovis* isolates. Protein components of tubercle bacilli cell growth kinetics that were fractionated accordingly to a modified protocol of (20) as described in materials and methods. Briefly, these components were transferred to nitrocellulose membranes and were blocked with low fat milk in PBS Tween 0.05% overnight. Band of proteins of mostly 48,7590 Kda were reacted with individual positive serum of infected cattle [4A (II-IV)]. Bar representation of sero-reactivity of proteins t individual serum of infected cattle with *M. bovis* (4B).

Sero-reactivity kinetics of *M. bovis* antigens toward pooled serum samples from infected cattle

As a first screening of *M. bovis,* antigen kinetics sero-reactivity components obtained after cellular fractionation were analyzed against pooled negative or positive pooled bovine serum (n = 5) (3A-3B). After 7 days *M. bovis* antigens of 45 KDa (filtrate pp with 75%) and 53 KDa (filtrate and pp soluble sonicated extract fraction) reacted against a pooled positive serum sample of infected cattle) while non recognition of these serum samples toward *M. bovis* BCG antigens (Figure 3II, lanes 2-3). However, 65 KDa (pp soluble fraction of sonicated extract) of *M. bovis* BCG was recognized by these serum samples (Figures 3III, lane 3) (Figure 3B). At this time, *M. bovis* antigens of 15 days, of 60 (filtrate pp), a doublet of 38-45KDa (filtrate) were also recognized (Figures 3III, lane 1) (Figure 3B). Moreover, *M. bovis* (either from the south or east) antigen of MW of 75 KDa present in all the fractions (Figure 3-IV, lanes 1-3) were recognized by pooled positive serum samples, whereas, bands of MW higher than 10 KDa produced by *M. bovis* BCG were also recognized by these serum samples Figure 3-IV, lanes 3-4) (Figure 3B).

Sero-reactivity kinetics of *M. bovis* antigens toward individual serum samples from cattle

Serum kinetics reactivity toward individual animals were further evaluated. To this end, protein profiles of *M. bovis* isolates (south or east) were used (Figure 1). From the western blot analysis, we found that at early stage (7 days), bands of 48 KDa (produced by south or east M. bovis strains) and 65 KDa (east) were recognized by individual positive serum of infected animal (Figure 4B), whereas a band of around 41 KDa produced by M. bovis BCG strain was also recognized. At 15 days, bands of 60 and 150 KDa (*M. bovis* east) (M. bovis east) (Figure 4III, lanes 1-3) were recognized (Figure 4B) by the serum of infected cattle while a band of around 53 KDa produced either by BCG or *M. bovis* south were recognized (Figures 4III-lane 3, 4III-lane 2 and 4B). Moreover, a band of 100 KDa produced by *M. bovis* BCG was recognized (Figures 4III-lane 2 and 4B). At 21 days, a band of 48 KDa (*M. bovis* east), a band of 90 KDa were mostly recognized by individual positive serum samples (Figures 4A-lane 1, 4IV-lane 3) (Figure 4B). No reactivity toward BCG antigens produced at this last point of bacilli cell growth (Figures 4A-4B).

Discussion

In the present work, we evaluated *M. bovis* isolates from cattle in Zacatecas, Zac, Mexico for serum reactivity and antigen production kinetics. We found that during the early, middle and late bacilli growth, there are protein profiles (45, 48, 75 and 90 KDa) produced either at 7 or 21 days that were immuno dominant toward either pooled or individual serum of infected cattle while did not cross react with *M. bovis* BCG antigens. These properties could be used to potentiate classical diagnostic test in bTB. It is known the pivotal role of the released mycobacterial antigens and the ESX-1 secretory system in the interaction of the host-pathogen [29-31]. In the decades of 80's and 90's, several seminal issues in immune diagnostics were focused in the development and improvements of sero-diagnosis based mainly virulence factor released to the culture medium of mycobacteria [3,19,20]. Unconventional techniques like proteomics and deep next generation sequencing are emerging as a powerful tool for the identification of putative virulence determinants [21,32,33]. A recent work by Lynshchenko et al. identified a set of novel antigens that reacted against positive serum, obtained from aerosol *M. bovis* infected cattle [23]. However, still it is necessary to make direct approach from the herds that enable to integrate all the information (*in vitro*, aerosol) and *in vivo* (this work), for the development of a more precise and sensible diagnostic test available worldwide. Here we have found that a set of proteins (38/45(doublet) 48, 75 and 90 KDa), (7 and 21 days) produced during tubercle bacilli growth kinetic reacted either with pooled positive serum samples or with individual serum samples (Figures 2I and 2III, 3A-II; 3B and 4A-II;4B). These in agreement with those reports from the literature that released proteins as well as components of the cell wall play a key role in the *Mycobactterium tuberculosis* complex pathogenicity [8-11,14,15]. Thus, secretory machinery used by pathogenic mycobacteria to release proteins to counter act the host immune response [11,34-38]. Therefore, our data suggest that the set of protein released to the culture medium and recognized by the serum antibodies of infected cattle could potentially be involved in the primary (7 days) and late (21 days) step of the evasion mechanism of *M. bovis*. However, since virulence factor of cell wall play also a role in this process, more work on going. This could rise more straightforward role of these components, particularly taking in account that active bTB versus latent bTB is still not very defined as it is in human tuberculosis [10,14]. Moreover, *M. leprae*, that also produced cytoplasmic soluble factors are recognized by leprosy patient's serum *vs* healthy patients. Thus, it is tempting to think that during the bacilli growth of *M. bovis*, antigenic components that are recognized by serum of infected cattle (TST+) constitute potential candidates that might be participating in the pathogenesis of *M. bovis*. On the other hand, it has been described that protein bands of around 45/47 KDa antigen of *M. tuberculosis* complex, released to the culture medium might be useful for sero-diagnosis of tuberculosis. In another study, it was reported that a diagnostic test based on reagents, peptides and protein cocktails (ESAT-6; MPB64; MPB70: MPB83), has been designed to discriminate between *M. bovis* infection and BCG vaccination. Also, a very recent work by Lynshchenko et al. identified a set of novel antigens that reacted against positive serum, obtained from aerosol *M. bovis* infected cattle, thus providing a step forward in the development of a more readable and precise test for bTB. Remarkably all these data from the literature and the one that is reporting here indicates that serological response is still an alternative in diagnosis, since most of the studies of bovine tuberculosis have been focused in molecular epidemiology and in the development (Multiplex PCR) for molecular detection of *M. bovis* and/or *M. tuberculosis* either in bovines or in humans. We think that this study is a first approach to use serum antibodies for rapid screening test *in vivo* of bTB in endemic regions.

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