Mycobacterial Diseases
ISSN: 2161-1068

Alfonseca-Silva et al., Mycobac Dis 2012, 2:3
DOI: 10.4172/2161-1068.1000111

Research Article Open Access

Good Protection but Excessive Pulmonary Inflammation in Balb/C Mice Vaccinated with *Mycobacterium Bovis Mce*-2A Mutant after Challenge with Homologous Strains

Edgar Alfonseca-Silva[1,3], Angel Cataldi[2], Fabiana Bigi[2] and Rogelio Hernández-Pando[3]*

[1]*Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Coyoacán, México D.F. 04510, México*
[2]*De Biotecnología, CICVyA-INTA, Los Reseros y las Cabañas, 1712 Castelar, Argentina*
[3]*De Patología Experimental, Departamento de Patología Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán", Vasco de Quiroga 15, Tlalpan, México D.F. 14000, México*

Abstract

Tuberculosis (TB) remains a major threat to public and veterinary health. Zoonotic TB (caused by *Mycobacterium bovis*) is present in wild animals and cattle in most developing countries, and *M. bovis* is also able to infect humans on a worldwide basis. Thus, the high incidence of bovine TB is a major economic problem and an additional risk to human health, being the development of new vaccines to prevent both human and bovine TB urgent and a major challenge. The aims of the present study were to characterize the pathogenicity and immunogenicity of *M. bovis mce2A* mutant in BALB/c mice, and then evaluate its potential as vaccine. Mutant *M. bovis mce2A* produced limited tissue damage (pneumonia) and lower bacilli burdens than its parental strain when administered in high dose by intratracheal inoculation, and showed limited dissemination when used as subcutaneous vaccine. Challenge experiments using low, middle and highly virulent *M. tuberculosis* or *M. bovis* strains showed similar protection conferred by mce-2 mutant than BCG. Interestingly, vaccinated animals showed low bacilli loads but high inflammatory response when were challenged with *M. bovis* strains, while vaccinated mice challenged with *M. tuberculosis* exhibited low bacilli burdens and scarce inflammation. Thus, in spite of the high genome homology between *M. tuberculosis* and *M. bovis*, it seems that there is higher antigenic recognition and in consequence extensive inflammatory response when the strain used as vaccine is homologous to the challenge strain, in this case *M. bovis*.

Keywords: Tuberculosis; *Mycobacterium bovis*; Vaccine

Introduction

Tuberculosis (TB), a chronic infectious illness caused by *Mycobacterium tuberculosis,* is still a major worldwide disease. According to the World Health Organization, TB is a considerable public health problem in Latin America, Asia and Africa. Over the last few years an increase in the incidence of TB has been observed, attributed to weak control programs, the AIDS pandemic which predisposes individuals to develop TB and to the appearance of *M. tuberculosis* strains resistant to first-line antibiotics [1]. In addition, bovine tuberculosis (bTB) caused by *Mycobacterium bovis* has increased its incidence and is thought to be due, at least in part, to a wildlife reservoir. *M. bovis* is also able to infect humans and on a worldwide basis it accounts for up to 10% of TB human cases [2,3]. Thus, the increased incidence of bTB, besides being a major economic problem, poses an additional risk to human health. Indeed, in many countries the incidence of bTB continues to rise despite the use of the tuberculin test and slaughter control policy, highlighting the need for improved control strategies. Vaccination of cattle, in combination with more specific and sensitive diagnostic tests, is suggested as the most effective strategy for bTB control. The only vaccine currently available for human and bovine TB is the live attenuated Bacille Calmette Guerin (BCG). BCG is thought to confer protection through the induction of Th1 responses against mycobacteria. However, protection against TB conferred by BCG is variable and at the present the reasons for its irregular protection are not clear. Therefore, there is a need to develop new vaccines that confer greater and more consistent protection against bTB than that afforded by BCG. Considering that BCG is currently the only available vaccine against human and bovine TB, it is likely that any new vaccine or vaccination strategy will be based on it [4,5].

The *mce* genes are conserved in all members of the *M. tuberculosis* complex, this operon is present four times in the *M. tuberculosis* genome (*mce 1-4*), being *mce-3* absent in *Mycobacterium bovis*, a subgroup of *Mycobacterium africanum*, *Mycobacterium microti* and *Mycobacterium pinnipedii.* The third gene of *mce1* operon codes for a protein that, after transformation of a non-pathogenic strain of *Escherichia coli*, confers the ability to invade macrophages and HeLa cells. Paralogous genes are present in the *mce2*, *3* and *4* operons. This gene family was termed *mceA* (*mce1A, mce2A, mce3A* and *mce4A*). Upstream of *mceA*, there are two genes (*yrbEA* and *yrbEB*) that code for integral membrane proteins, and downstream of it there are five genes (*mceA* to *mceF*) that code for proteins with signal sequences or hydrophobic stretches at the N-terminus. These features are consistent with cell surface localization and the proposed role of *mce* proteins in the invasion of host cells or in host–pathogen interactions [1,6,7,8,9,10]. In a previous study, mutants in *mce1*, *mce2*, and *mce3* operons of *M. tuberculosis* strain H37Rv were notably hypovirulent in BALB/c mice [1]. Intratracheal infection of BALB/c mice with *mce2* mutant induced lower but progressive production of IFN-γ and TNF-α, and when used

***Corresponding author:** Rogelio Hernández-Pando, Sección de Patología Experimental, Departamento de Patología Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán", Vasco de Quiroga 15, Tlalpan, México D.F. 14000, México, E-mail: rhdezpando@hotmail.com

Received March 15, 2012; **Accepted** April 16, 2012; **Published** April 19, 2012

Citation: Alfonseca-Silva E, Cataldi A, Bigi F, Hernández-Pando R (2012) Good Protection but Excessive Pulmonary Inflammation in Balb/C Mice Vaccinated with *Mycobacterium Bovis Mce*-2A Mutant after Challenge with Homologous Strains. Mycobac Dis 2:111. doi:10.4172/2161-1068.1000111

as subcutaneous vaccine induced a higher level of protection than BCG [11].

Rationally attenuated, live replicating mutants of *M. bovis* or *M. tuberculosis* are potential vaccine candidates. The advantage of using attenuated *M. bovis* strains is that they produce a large number of protective antigens, including those absent from BCG. Thus, vaccination with live attenuated *M. bovis* can induce a stronger and longer immune stimulation, conferring higher levels of protection against tuberculosis than BCG [4,12,13,14]. Here, we study the induced pathology and bacilli burdens in the lungs of BALB/c mice after intratracheal infection with the *M. bovis* mutant strain of the *mce2A* operon, comparing it with the parental strain. Then, we used this mutant as subcutaneous vaccine, comparing with BCG its ability to disseminate as indicator of attenuation and immunogenicity stimulating cell suspensions from different organs with several antigen preparations and measuring IFN-γ production by ELISA before the challenge. Finally, we tested its protective efficacy after the challenge with *M. tuberculosis* H37Rv, *M. tuberculosis* 09005186 which is a highly virulent strain, *M. bovis* AN5 and two clinical *M. bovis* strains, one isolated from a wild animal (302) and other from a cow (534), these two isolates had been shown previously to be highly virulent and attenuated respectively in BALB/c mice [15].

Materials and Methods

Growth of bacterial strains

The laboratory strain *M. tuberculosis* H37Rv was obtained from the American Type Culture Collection 25618 (Rockville, MD, USA), *M. tuberculosis* 09005186 is a member of the LAM genotype family; it is highly virulent in the BALB/c mouse model and was obtained from a wide epidemiological study performed in the south of Mexico [16]. *M. bovis* strains UK (NCTC 10772), is a bovine lymph node isolate (http://www.straininfo2.ugent.be/culture/99859/catalog;jsessionid=C2B6AA33B1E3024D2889F173B08BDBF8), AN5, 302 and 554 were donated by Dr. Ángel Cataldi from Institute of Biotechnology INTA, Castelar Argentina.

The mutant *mce2A* strain was obtained from a parental *M. bovis* UK, in which a kanamycin cassette from pUC4K was inserted into a unique Pst1 site internal to *mce-2A* gene [1]. The BCG strain used was *M. bovis* BCG Phipps. This BCG substrain was the most protective of 10 strains tested in our BALB/c model of progressive pulmonary tuberculosis [17]. Bacteria were grown in Middlebrook 7H9 broth (Difco, Detroit, MI) enriched with glycerol (*M. tuberculosis*) or without glycerol (*M. bovis*) and albumin, catalase and dextrose (Becton Dickinson. Cockysville, MD), and incubated with constant agitation at 37°C for 21 days. Growth was monitored by densitometry. As soon as the culture reached the log phase (A_{600} = 0.6-1), the bacilli were harvested, aliquoted and maintained at – 70°C until used. Before use, bacteria were counted and the concentration was adjusted to 2.5 x 10^5 viable bacilli per 100 µl of PBS as determined by diacetate of fluorescein (DAF) or SYTO 9 incorporation (LIVE/DEAD® BacLight. Bacterial Viability Kits). After animal infection, the remnant of the bacterial inoculum was plated to confirm the number of CFU administrated to the animals.

Experimental model of progressive pulmonary tuberculosis in BALB/c mice

To induce pulmonary TB infection, 8-week-old BALB/c male mice were anaesthetized with sevoflurane and inoculated intratracheally with 2.5 x 10^5 viable bacilli in 100 µl phosphate-buffered saline (PBS) from *M. bovis mce2A* mutant or *M. bovis* UK parental strain. Infected mice were kept in a vertical position until the effect of anesthesia had passed. The mice were maintained in groups of ten in cages fitted with microisolators connected to negative pressure [18]. All procedures were performed in a laminar flow cabinet in a biosafety level III facility. Animal work was performed in accordance with the Institutional Ethics Committee and the National Regulations on Animal Care and Experimentation.

Groups of 12 mice in two different experiments were sacrificed by exsanguinations at 1, 3, 7, 14, 21, 28 and 60 days after intratracheal infection. One lung, right or left, was perfused with 10% formaldehyde dissolved in PBS via the trachea and embedded in paraffin, sections stained with haematoxylin and eosin were used to follow the evolution of the infection and determine the percentage of lung surface area affected by pneumonia [18]. The other lobe was snap-frozen in liquid nitrogen and then stored at -70°C for microbiological analysis.

Comparison of dissemination and immunogenicity of BCG- and M. bovis mce2A mutant-vaccinated mice before the challenge

To study of bacilli dissemination and the production of IFNγ by cell suspensions stimulated with mycobacterial antigens as indicators of attenuation and immunogenicity respectively, two independent experiments were performed using 40 BALB/c mice for each experimental group. Three different doses (2,500, 5000 and 8000 live bacilli) of *mce2A* and BCG were compared vaccinating mice subcutaneously at the base of the tail, and the dose which induced the highest IFNγ production was used for the challenge experiments. For simplicity we present only the results obtained with the selected vaccination dose: 5000 *M. bovis mce2A* mutant and 8000 BCG.

For the bacilli dissemination experiments, groups of four mice were sacrificed at 15-, 30-, and 60-day post-vaccination. The inguinal lymph nodes, spleen, lungs, and the subcutaneous tissue at the site of vaccination (base of the tail) were immediately dissected and homogenized with a Polytron (Kinematica, Luzern, Switzerland) in sterile 3 ml tubes containing 1 ml isotonic saline. Four dilutions of each homogenate were spread onto duplicate plates containing Bacto Middlebrook 7H10 agar (Difco Lab code 0627-17-4, Difco Labs, Detroit, MI) enriched with OADC. The time for incubation and colony counting was 21 days.

Another group of four vaccinated BALB/c mice per time point was used to determine immunogenicity, by comparing the production of IFNγ by cell suspensions from lung, lymph nodes and spleen after stimulation with mycobacterial Culture Filtrate Antigens (CFA) and the immunodominant recombinant antigens ESAT-6 and Ag85. After mice sacrifice, the lung, spleen and inguinal lymph nodes were immediately removed and placed in 2 ml of RPMI medium containing 0.5 mg/ml collagenase type 2 (Worthington, NJ, USA), and incubated for 1 h at 37°C; then, passed through a 70 µm cell sieve, crushed with a syringe plunger and rinsed with the medium. Cells were centrifuged at 1500 rpm for 5 min and the supernatant was removed, red cells were eliminated with a lysis buffer. After washing, the cells were resuspended in RPMI medium supplemented with 2mM L-glutamine, 100U of penicillin per ml, 1 µg of streptomycin per ml (all from Sigma), and 10% fetal calf serum. Cultures for cytokine production (10^6 cells in 1ml of culture medium) were performed in flat-bottomed 24-well plates without and with mycobacterial antigens 5µg/ml (CFA, ESAT-6, and Ag85). After 3 days of antigenic stimulation, the cells were

centrifuged and the supernatant used for IFN-γ quantification through a commercial ELISA test kit (Pharmingen, San Diego, CA, USA).

Protection against M. tuberculosis H37Rv and M. tuberculosis hypervirulent strain and against M. bovis AN5, 302 and 534 strains in BALB/c mice vaccinated with M. bovis mce2A mutant or BCG

Two different experiments were performed using 40 mice for each of five experimental groups; mice were vaccinated as described above and at 60-day post-vaccination, 20 mice of each group were challenged through the intra-tracheal route with 2.5×10^5 CFU of *M. tuberculosis* H37Rv, *M. tuberculosis* 09005186 and *M. bovis* strains AN5, 302, 534. We selected these strains because they have a different level of virulence in this animal model [15,16]. Levels of protection were determined at 60 day post-challenge by the quantification of CFUs in lung homogenates, following the same procedure described above, and by automated morphometry, measuring the lung surface affected by pneumonia (alveolar spaces occupied by inflammatory cells and protein exudates). After the determination of the total surface area of the lung by automated morphometry (QWin Leica 500 W equipment from Leica Imaging Systems, Cambridge UK), the surface occupied by pneumonia was quantified. Then, the percentage of lung surface affected by pneumonia was calculated. Ten more animals per group were left untouched and deaths were recorded to construct survival curves.

The effect of vaccination conferring long immunological memory was assessed by challenge mice after 6 months of vaccination. Three groups of 20 BALB/c mice each were used. The first group was vaccinated s.c with 5000 live *M. bovis mce2A* bacilli in the base of the tail. The second group received 8000 live BCG bacilli by the same route, and the control group was injected only with the vehicle solution (isotonic saline). Animals were challenged 6 months after vaccination with 2.5 x 10^5 viable bacilli of *M. bovis* AN5. At 60-day post-challenge, levels of protection were determined by the quantification of CFUs in lung homogenates and measuring the lung surface affected by pneumonia. Another group of 10 animals was left untouched, recording mortality to construct survival curves.

Statistical analysis

Statistical analysis for survival curves was performed using Kaplan–Meier plots and Log Rank tests. Student's *t* test was used to determine statistical significance of CFU and histopathology. $P < 0.05$ was considered as significant.

Results

Comparative pulmonary pathology and bacilli burdens in mice infected by intratracheal route with *M. bovis* mutant mce-2 strain and its parental strain

As an indicator of attenuation, BALB/c mice were infected by the intratracheal route with a high dose of *M. bovis UK* strain and its *mce2A* mutant. This dose induces progressive pulmonary tuberculosis [15]. Animals infected with either mutant *mce2* or parental strain showed similar bacilli burdens during the first week of infection. Then, lower bacilli loads were determined in animals infected with the mutant strain, being significant at day 60 when two-fold higher bacilli loads were seen in mice infected with the parental strain. This result correlated with tissue damage (pneumonia), which was lesser in mice infected with the mutant strain (Figure 1).

Comparative dissemination and immunogenicity before challenge in mice vaccinated with *M. bovis* mce2A mutant or BCG

As an indicator of the level of attenuation, bacilli dissemination after vaccination was determined by quantification of bacilli burdens in different organs. For simplicity purposes, we present only the results obtained with the selected vaccination dose: 5000 *M. bovis mce2A* mutant and 8000 BCG. Two weeks after inoculation both strains BCG and *M. bovis mce2A* mutant showed similar bacilli loads at the site

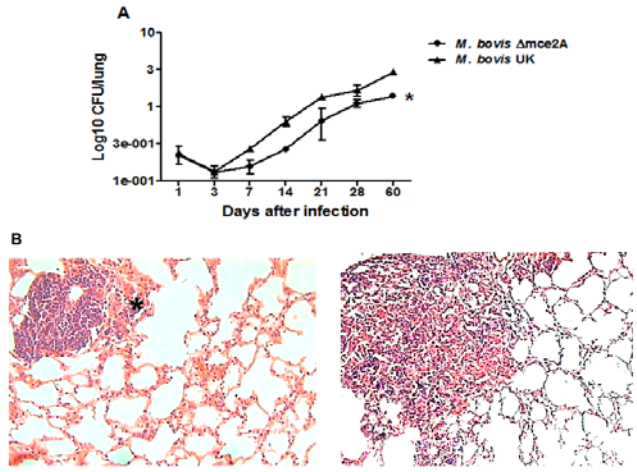

Figure1: Bacilli burdens and representative histopathology in the lungs of BALB/c mice after intratracheal high dose inoculation of *M. bovis mce-2A* mutant or its parental strain: (A) Mice were sacrificed at the indicated days post infection and their lungs used to determine colony forming units. Infected animals with the mutant strain showed lower bacilli loads. Asterisk represents statistical significance ($p<0.05$) (B) Left figure shows smaller area of pneumonia (asterisk) in a mouse infected after 60 days with the mutant strain, than the lung of mouse infected with the parental strain at the same time point (right panel). (HE staining, 200x magnification).

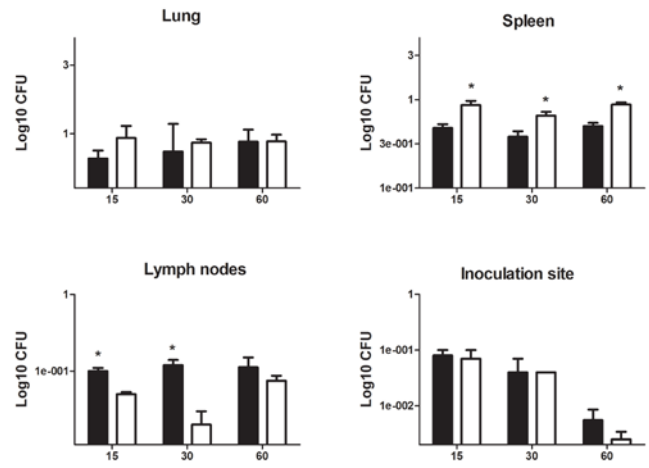

Figure 2: Bacillary loads at different tissues collected from vaccinated BALB/c mice before challenge: At the indicated time points after vaccination with BCG (white bars) or *mce-2A* mutant (black bars) and before challenge, animals were sacrificed and the indicated organs were immediately removed, homogenized and plated for CFU quantification. Bars represent the means and standard deviations for four different animals at each time point in two separate experiments. Asterisks represent statistical significance ($P< 0.05$).

of vaccination (subcutaneous tissue from the base of the tail), with similar progressive decrease trend until day 60. In the lungs at day 15, BCG vaccinated mice showed higher bacilli loads but not significant than mice vaccinated with the mutant strain. In the spleen of BALB/c mice vaccinated with BCG there were a significant higher bacterial burdens than in the same organ from animals vaccinated with the mutant *mce2A*, whereas in lymph nodes near to the site of vaccination (inguinal) *mce2A* vaccinated animals showed significant higher bacilli loads at days 15, 30 and 60 post-vaccination (Figure 2).

In order to compare the efficiency of cellular immunity activation induced by *M. bovis mce2A* mutant and BCG vaccination before the challenge, cell suspensions from the lungs, spleen and inguinal lymph nodes were stimulated with mycobacterial antigens at different time points after vaccination, and the concentration of IFNγ in the supernatants was quantified through ELISA. Figure 3 shows that lung and lymph node cells stimulated with CFA from animals vaccinated with *mce2A* mutant produced more IFNγ than BCG-vaccinated mice, being significant only at day 60. Because BCG lacks the ESAT-6 gene, animals vaccinated with BCG secreted insignificant amount of IFN-γ after ESAT-6 stimulation, whereas vaccination with *M. bovis mce2A* mutant induced high IFN-γ production after stimulation with the same antigen. Lung and lymph node cells stimulated with Ag85 from mice vaccinated with *mce2A* mutant produced significant more IFN-γ than those from BCG-vaccinated mice at days 15 and 60 post-vaccination. Similar trend was observed in spleen cells, but only were significant at day 30 (Figure 3). Thus, in spite of vaccinate with a lower dose, *M. bovis mce2A* mutant is more efficient to induce the production of IFN-γ than BCG in lung, regional lymph nodes and spleen after subcutaneous vaccination.

Comparative protection against diverse *M. tuberculosis* and *M. bovis* strains in BALB/c mice vaccinated with *M. bovis mce2A* mutant or BCG

Since, it has been demonstrated that the virulence level of the bacilli used for challenge is a significant factor that determine the level of protection; vaccinated animals were challenged with diverse strains of *M. bovis* and *M. tuberculosis* that showed different degrees of virulence in the BALB/c mouse model [12,15,16].

Two separate experiments were performed using 20 mice for each of 5 experimental groups, using a dose of 5000 *M. bovis mce2A* mutant bacilli or 8000 BCG live bacilli sub-strain Phipps, which were the doses that induced the highest IFNγ production. At 60-day post-vaccination, all mice were challenged intra-tracheally with 2.5×10^5 CFU of *M. tuberculosis* H37Rv (group 1), *M. tuberculosis* 09005186 hypervirulent-strain (group 2), highly virulent *M. bovis* strains AN5 (group 3) and 302 (group 4) and highly attenuated *M. bovis* strain 534 strain (group 5). Ten mice were then sacrificed at 60-day post-challenge. Levels of protection were determined by survival rates, quantification of CFU recovered from the lungs, and the extension of tissue damage evaluating the percentage of the lung surface affected by pneumonia.

After 4 months post challenge with *M. tuberculosis* H37Rv, all the mice vaccinated with the *mce2A* mutant survived, while 90% and 70% survival was showed by BCG vaccinated and non-vaccinated control animals respectively (Figure 4).

Vaccinated mice with the mutant strain and challenged with *M. tuberculosis* highly virulent strain showed total survival, while 90% of the BCG vaccinated and 50% of the non-vaccinated animals survived at the end of the experiment (Figure 4). These results correlated with lung bacillary loads and histopathology, showing significantly fewer CFU and lesser pneumonia in mice vaccinated with the *mce2A* mutant or BCG than control non-vaccinated animals (Figure 4).

Regarding to the vaccinated animals challenged with *M. bovis* strains, all the control non-vaccinated mice infected with *M. bovis* AN5 died after 5 weeks. In contrast, mice vaccinated with *mce2A* mutant and BCG started to die at 5 weeks post-infection, and all had died by 14 and 16 weeks, respectively. Interestingly, vaccinated animals showed extensive pneumonia, similar than observed in control non-vaccinated mice but with a significant lower number of CFU in the lungs of vaccinated animals, being the lowest in BCG vaccinated mice (Figure 5). Thus, the vaccination with attenuated *M. bovis* and challenged with *M. bovis* induced good control of bacilli growth but

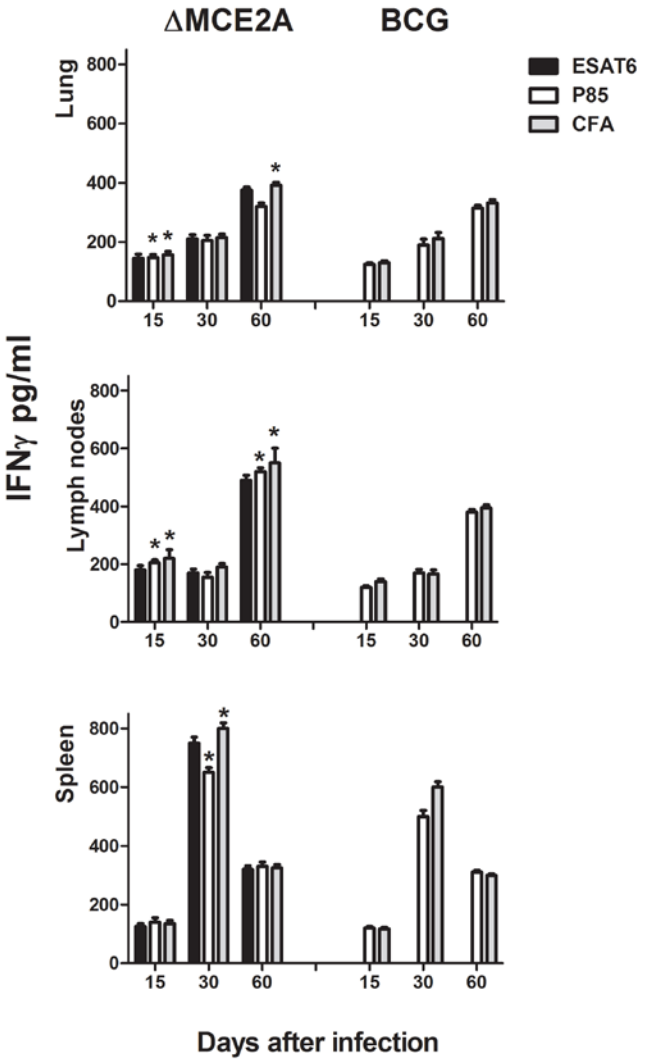

Figure3: Production of IFN-γ by cell suspensions from different organs stimulated with different mycobacterial antigens after vaccination and before challenge: Mice were vaccinated with the mutant *mce-2A* or BCG bacteria and after 15, 30 and 60 days the indicated organs were used to get cell suspensions which were stimulated with whole culture filtrate antigens (CFA) or the immunodominant antigens ESAT-6 or Ag85, IFNγ was quantified by ELISA in the supernatants. Bars represent the means and standard deviations of four different animals at each time point. Asterisks represent statistical significance ($P< 0.05$) among the indicated groups.

excessive inflammatory responseNon-vaccinated control animals infected with the clinical isolate *M. bovis* 302 started to die after 4 weeks of the intra-tracheal challenge and after 7 weeks 80% had died (Figure 5). In contrast, after 4 months of challenge, mice vaccinated with *mce2A* mutant and BCG showed a significant 100% and 90% survival respectively (Figure 5). These survival results were in agreement with lung CFU determinations. Mice vaccinated with *mce2A* mutant and BCG showed significantly fewer CFU than non-vaccinated mice at day 60 after the intra-tracheal challenge. In contrast, vaccinated and non-vaccinated animals showed a high percentage of lung area affected by pneumonia at 60 days after challenge.

Considering that animals vaccinated with attenuated *M. bovis* and challenged with *M. bovis* induced good protection in terms of survival and bacilli loads but with extensive inflammation, another group of animals was vaccinated and challenged with an attenuated *M. bovis strain*. All the animals vaccinated and non-vaccinated challenged with *M. bovis* 534 strain survived, showing low bacilli burdens and limited lung consolidation after 4 months of infection (Figure 5). Thus, when the infecting *M. bovis* is attenuated, vaccinated mice with BCG or mutant *M. bovis mce2A* efficiently control bacilli growth without excessive inflammatory response.

In order to determine and compare long lasting protection by vaccination, mice were vaccinated with either *mceA2* mutant or BCG and 6 months later were challenged with *M. bovis* AN5. All non-vaccinated mice died after 11 weeks, while mice vaccinated with BCG and *mce2A* mutant survived 50% and 40% respectively, with very low and similar pulmonary bacilli burdens, but with high inflammatory response exhibiting similar lung consolidation than control animals at 60 days of infection (Figure 6).

Discussion

Both, *M. tuberculosis* or *M. bovis* can enter and replicate in macrophages and non-professional phagocytic cells, such as bronchial epithelial cells or endothelium. The mycobacterial invasion into non-professional phagocytic cells is apparently important in their dissemination and is mediated by specific bacterial proteins such as the *mce* proteins [1,6,10,19].

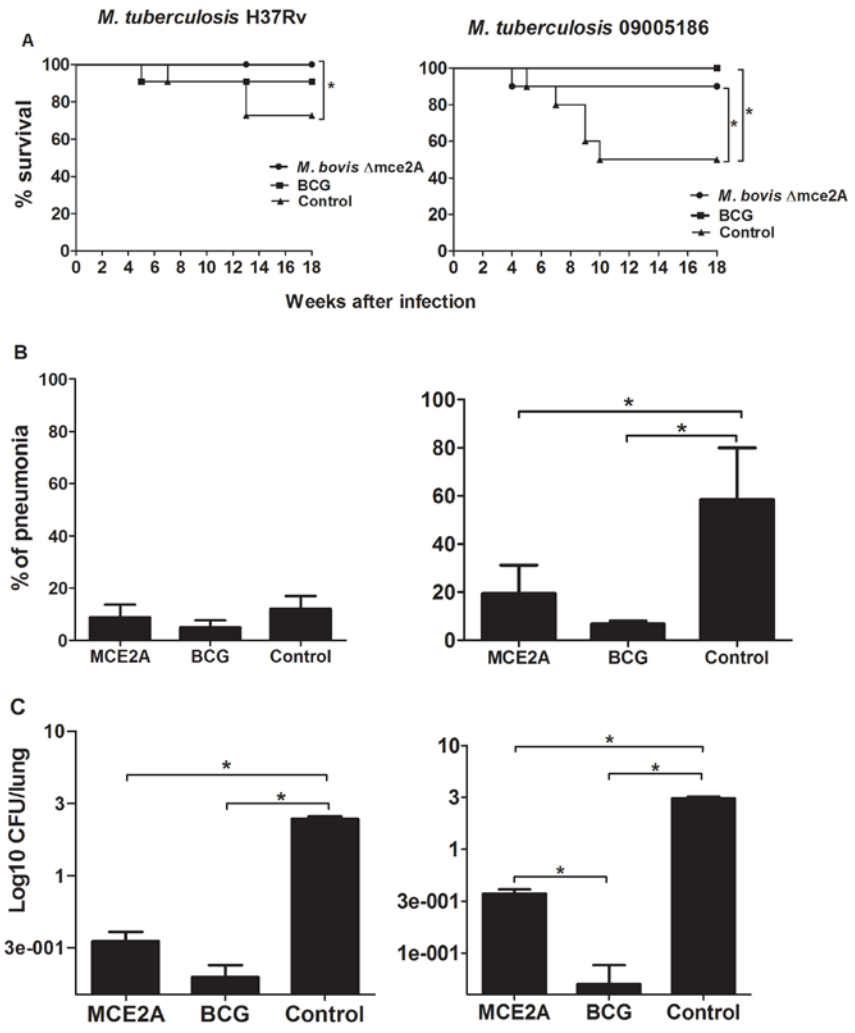

Figure 4: Survival, histomorphometry and lung bacillary loads after intratracheal challenge with *M. tuberculosis* H37Rv or *M. tuberculosis* 09005186 hypervirulent strain: BALB/c mice were vaccinated with the *mce2A* mutant or BCG and compared with control non-vaccinated animals. (A) Survival of BALB/c mice (20 mice per strain). (B) Percentage of lung surface affected by pneumonia determined by automated morphometry and (C) lung bacillary loads, both determinations after 60 days of challenge. Asterisks represent statistical significance ($P < 0.05$).

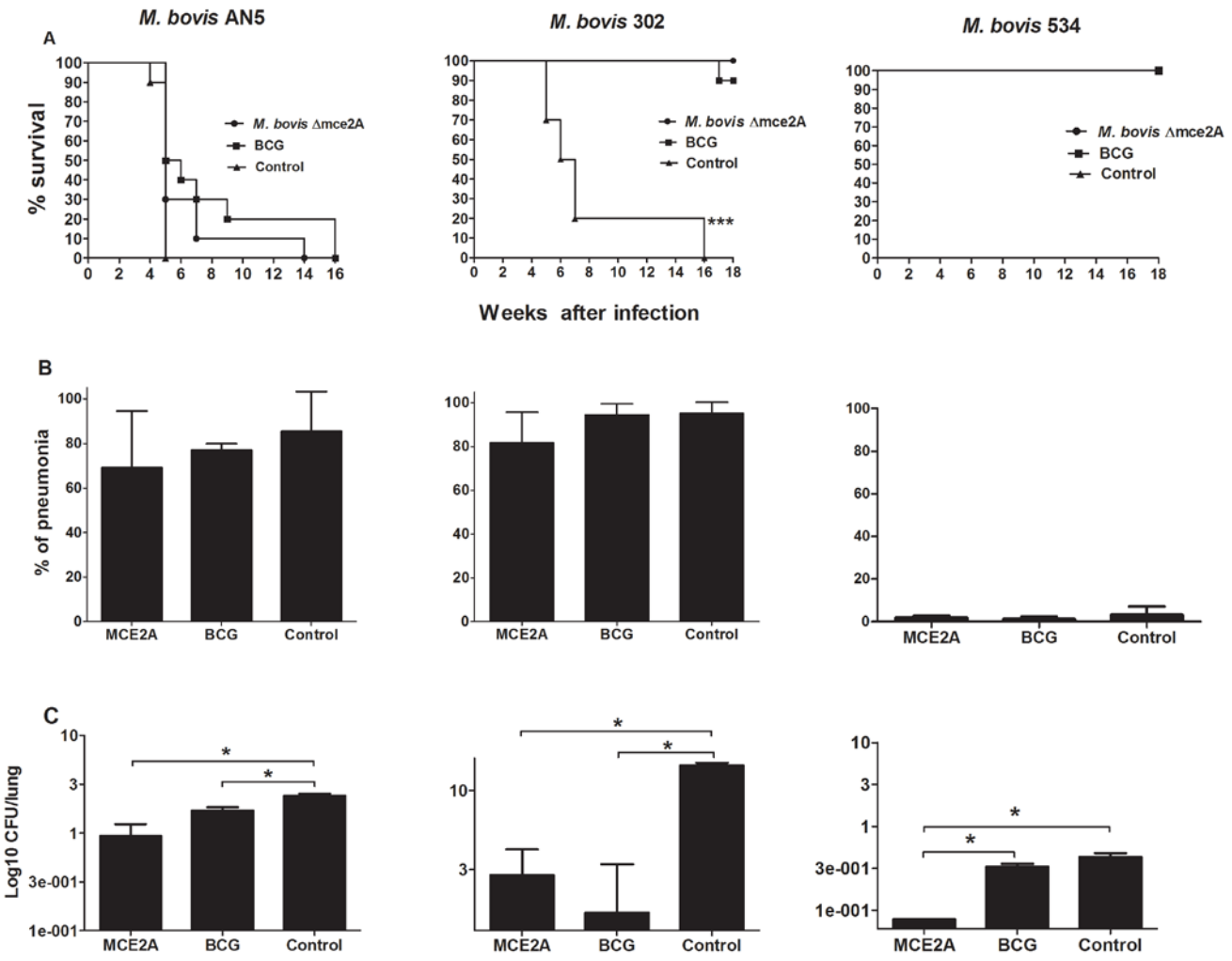

Figure 5: Survival, histomorphometry and pulmonary bacillary loads after intratracheal challenge with *M. bovis*: (A) Strains AN5 and 302 (hypervirulent strains) and 534 (low virulence) were used to challenge BALB/c mice vaccinated with *mce2A* mutant, BCG or non-vaccinated control animals and their survival was recorded during four months (20 mice per strain). (B) Percentage of lung surface affected by pneumonia determined by automated morphometry. (C) Lung bacillary loads, both determinations were done after 60 days of challenge. Asterisks represent statistical significance ($P < 0.05$).

The *mce* gene family has been found in all the *M. tuberculosis* complex strains, and homologous sequences have been demonstrated in members of the *M. avium* complex and other saprophytic strains. This wide *mce* operons distribution in pathogenic and non-pathogenic mycobacteria suggests that these genes are not an indicator of the bacilli virulence; but instead it seems that their participation in the bacterial virulence might be determined by the regulation of their expression [8,9,10]. We previously demonstrated that *M. tuberculosis mce2* mutant was attenuated and it can be a useful vaccine, conferring higher protection than BCG in the BALB/c mice model [1,11]. In the present study we determined the pathogenicity and immunogenicity induced by the same mutation but in *Mycobacterium bovis*, with the aim to get a novel vaccine candidate for bTB, with the hypothesis that *M. bovis* is better adapted to cows. Thus, we first determined the induced pathology and immunogenicity of this mutant in BALB/c mice, and then the level of protection evoked by the vaccination with this mutant compared with BCG in BALB/c mice challenged with diverse *M. tuberculosis* and *M. bovis* strains.

Our results demonstrated that in comparison with the parental strain, *M. bovis mce2A* mutant produces limited pulmonary pathology and its growth is efficiently controlled in BALB/c mice. After vaccination, both *mce2A* mutant and BCG showed similar dissemination and growth in the lungs, but mutant strain showed higher growth than BCG in lymph nodes and the inverse was seen in spleen. Interestingly, despite the observation that *M. bovis mce2A* mutant vaccinated mice had lower bacterial burden in lungs and spleen, a higher expression of IFN-γ was seen after 15 days of vaccination when compared with BCG, suggesting that *M. bovis mce2A* mutant elicits a rapid and strong immune response.

It has been demonstrated in experimental models that BCG protection depends also on the virulence of the infecting organism [12,20]. For this reason, besides the laboratory strain H37Rv, we used a *M. tuberculosis* clinical isolate which in the BALB/c model is highly virulent [16], and *M. bovis* strains with different degree of virulence and isolated from different hosts [15]. Neither *M. bovis mce2A* mutant nor BCG prevented the infection, as was indicated by the pathological

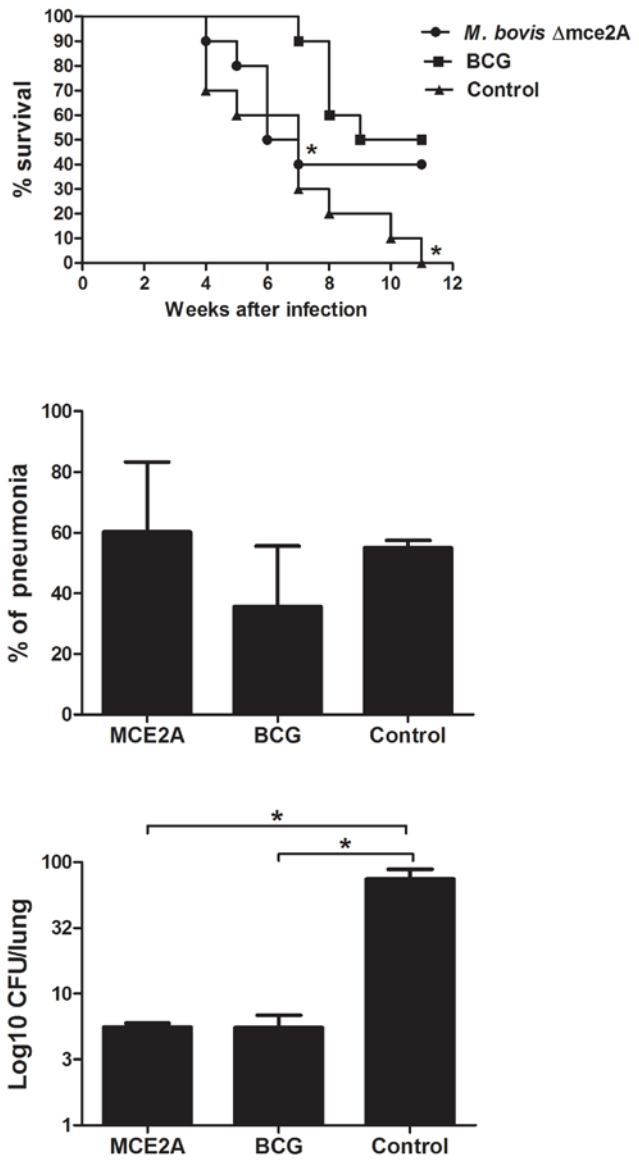

Figure 6: Survival, histomorphometry and lung bacillary loads after long term vaccination: (A) *M. bovis* strain AN5 was used to challenge after six months of vaccination with *mce2A* mutant or BCG and the survival compared with nonvaccinated control animals (20 mice per strain). (B) Percentage of lung surface affected by pneumonia, determined by automated morphometry and lung bacillary loads (C), both determinations after 60 days of challenge. Asterisks represent statistical significance ($P < 0.05$).

changes and CFU determinations. Mutant *mce2A* showed similar level of protection than BCG in mice challenged with *M. tuberculosis* strains, being significantly higher than control non-vaccinated mice challenged with the same strains. In contrast, previous results showed that *mce2A M. tuberculosis* mutant was better conferring protection than BCG [11].

Protection results in vaccinated mice with *M. bovis mce2A* mutant or BCG and challenged with *M. bovis* strains also showed differences depending of the bacterial virulence and genotype. *M. bovis* strain AN5 was selected for the challenge experiments because it has been used worldwide for bovine purified protein derivative production, due to its high bacterial mass yield in glycerinated media. This phenotype was selected by repeated subcultures in laboratory growth medium following a procedure similar to that used to generate BCG [21]. Interestingly, our results showed that even after many subculture passages, strain AN5 is not attenuated, and the level of protection in vaccinated mice with BCG or *mce2A* mutant was similar and mild but significantly higher when compared with the non-vaccinated control group. The most striking feature was the paradox low bacilli loads and extensive tissue damage (pneumonia) in vaccinated animals. In fact, the percentage of lung surface affected by pneumonia in control non-vaccinated animals was similar than measured in vaccinated mice, more than 80%.

The other highly virulent *M. bovis* strain was isolated from a wild boar. Several wildlife species are infected naturally with *M. bovis* and the wild boar is a major reservoir in some regions in Europe, such as the south central area of Spain, where the prevalence of macroscopic tuberculosis lesions observed in these animals reached 100% [22]. Thus, although there is host genetic variability [23], wild boars seem to be infected easily by *M. bovis*. Using the same BALB/c mouse model, we observed that isolates from these animals were highly virulent [15]. Both mutant and BCG were equally efficient inducing significant protection after challenge with this strain, evoking high survival with low bacilli loads but again with very high percentage of pneumonia when compared with non-vaccinated animals. Moreover, when vaccinated mice were challenged with attenuated *M. bovis*, the bacilli growth was efficiently controlled and lung inflammation was low but similar than exhibited by non-vaccinated animals. Interestingly, this kind of inflammatory response was not observed in vaccinated mice challenged with *M. tuberculosis* strains, suggesting that *M. tuberculosis* and *M. bovis* have different immunogenicity. Thus, although the DNA sequence homology between *M. bovis* and *M. tuberculosis* is very high (99.95%), significant differences in gene expression and regulation during infection could be related to these differences. In comparison with *M. tuberculosis*, the bovine strain has a smaller genome, lacking several regions called RDs (regions of difference) [11]. Indeed, the greatest degree of sequence variation between the human and bovine bacillus occurs in genes encoding cell wall compounds or secreted proteins [1,15,21,24,25], which should contribute to the observed inflammatory responses. However, the different vaccination dose and growth rate of *mce2A* mutant and BCG could also contribute to the observed differences in the evoked inflammation.

In conclusion, *M bovis mce2A* mutant confers similar protection than BCG substrain Phipps in BALB/c mice challenged with different *M. tuberculosis* or *M. bovis* strains, but when challenge is done with *M.*

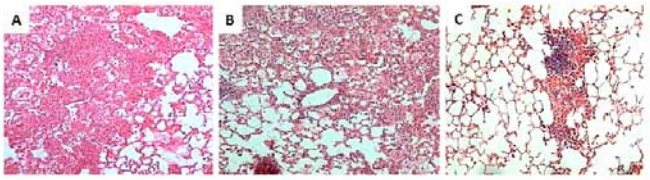

Figure 7: Representative lung histopathology in non-vaccinated and vaccinated mice after 60 days of challenge with *M. tuberculosis* or *M. bovis* strains: A) Extensive areas of alveolar spaces are occupied by inflammatory cells corresponding to pneumonia in non-vaccinated animal infected by the intratracheal route with *M. bovis* AN5. B) Extensive lung consolidation is also observed in mutant *mce-2A* vaccinated mouse challenged with *M. bovis* AN5. C) In contrast, medium size patch of pneumonia is seen in *mce-2A* mutant vaccinated mouse challenged with *M. tuberculosis* H37Rv. (All figs 100x magnification, hematoxylin/eosine staining).

bovis strains there is high inflammatory response which could produce significant tissue damage (Figure 7).

Conflict of Interest

None

Acknowledgements

This work was supported by the Argentinian-Mexican agreement of bilateral scientific collaboration CONICET/CONACyT, contract: J000.345. EAS was supported by a PhD scholarship DGAPA from the National University of México.

References

1. Gioffre A, Infante E, Aguilar D, Santangelo MP, Klepp L, et al. (2005) Mutation in *mce* operons attenuates *Mycobacterium tuberculosis* virulence. Microbes Infect 7: 325-334.
2. Cosivi O, Grange JM, Daborn CJ, Raviglione MC, Fujikura T, et al. (1998) Zoonotic tuberculosis due to Mycobacterium bovis in developing countries. Emerg Infect Dis 4: 59-70.
3. Thoen C, Lobue P, de Kantor I (2006) The importance of Mycobacterium bovis as a zoonosis. Vet Microbiol 112: 339-345.
4. Young DB(2003) Building a better tuberculosis vaccine. Nat Med 9: 503-504.
5. Hope JC, Villarreal-Ramos B (2008) Bovine TB and the development of new vaccines. Comp Immunol Microbiol Infect Dis 31: 77-100.
6. Arruda S, Bomfim G, Knights R, Huima-Byron T, Riley LW (1993) Cloning of an M. tuberculosis DNA fragment associated with entry and survival inside cells. Science 261: 1454-1457.
7. Haile Y, Caugant DA, Bjune G, Wiker HG (2002) Mycobacterium tuberculosis mammalian cell entry operon (mce) homologs in Mycobacterium other than tuberculosis (MOTT). FEMS Immunol Med Microbiol 33: 125-132.
8. Kumar A, Bose M, Brahmachari V (2003) Analysis of expression profile of mammalian cell entry (mce) operons of Mycobacterium tuberculosis. Infect Immun 71: 6083-6087.
9. Kumar A, Chandolia A, Chaudhry U, Brahmachari V, Bose M (2005) Comparison of mammalian cell entry operons of mycobacteria: in silico analysis and expression profiling. FEMS Immunol Med Microbiol 43: 185-195.
10. Casali N, Riley LW (2007) A phylogenomic analysis of the Actinomycetales mce operons. BMC Genomics. 8: 60.
11. Aguilar LD, Infante E, Bianco MV, Cataldi A, Bigi F, et al. (2006) Immunogenicity and protection induced by Mycobacterium tuberculosis mce-2 and mce-3 mutants in a Balb/c mouse model of progressive pulmonary tuberculosis. Vaccine 24: 2333-2342.
12. López B, Aguilar D, Orozco H, Burger M, Espitia C, et al. (2003) A marked difference in pathogenesis and immune response induced by different *Mycobacterium tuberculosis* genotypes. Clin Exp Immunol 133: 30-37.
13. Martín C (2005) The dream of a vaccine against tuberculosis; new vaccines improving or replacing BCG? Eur Respir J 26: 162-167.
14. Parida SK, Kaufmann SH (2010) Novel tuberculosis vaccines on the horizon. Curr Opin Immunol 22: 374-384.
15. Aguilar León D, Zumárraga MJ, Jiménez Oropeza R, Gioffré AK, Bernardelli A, et al. (2009) *Mycobacterium bovis* with different genotypes and from different hosts induce dissimilar immunopathological lesions in a mouse model of tuberculosis. Clin Exp Immunol 157: 139-147.
16. Marquina-Castillo B, García-García L, Ponce-de-León A, Jimenez-Corona ME, Bobadilla-Del Valle M, et al. (2009) Virulence, immunopathology and transmissibility of selected strains of Mycobacterium tuberculosis in a murine model. Immunology 128: 123-133.
17. Castillo-Rodal AI, Castañón-Arreola M, Hernández-Pando R, Calva JJ, Sada-Díaz E, et al. (2006) *Mycobacterium bovis* BCG substrains confer different levels of protection against *Mycobacterium tuberculosis* infection in a BALB/c model of progressive pulmonary tuberculosis. Infect Immun 74: 1718-1724.
18. Hernández-Pando R, Orozcoe H, Sampieri A, Pavón L, Velasquillo C, et al. (1996) Correlation between the kinetics of Th1,Th2 cells and pathology in a murine model of experimental pulmonary tuberculosis. Immunology 89: 26-33.
19. Flesselles B, Anand NN, Remani J, Loosmore SM, Klein MH (1999) Disruption of the mycobacterial cell entry gene of *Mycobacterium bovis* BCG results in a mutant that exhibits a reduced invasiveness for epithelial cells. FEMS Microbiol Lett 177: 237-242.
20. Medina E, Ryan L, LaCourse R, North RJ (2006) Superior virulence of Mycobacterium bovis over Mycobacterium tuberculosis (Mtb) for Mtb-resistant and Mtb-susceptible mice is manifested as an ability to cause extrapulmonary disease. Tuberculosis 86: 20-27.
21. Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, et al. (2002) A new evolutionary scenario for the Mycobacterium tuberculosis complex. Proc Natl Acad Sci USA 99: 3684-3689.
22. Gortazar C, Vicente J, Gavier-Widén D (2003) Pathology of bovine tuberculosis in the European wild boar (Sus scrofa). Vet Rec 152: 779-780.
23. Acevedo-Whitehouse K, Vicente J, Gortazar C, Höfle U, Fernández-de-Mera IG, et al. (2005) Genetic resistance to bovine tuberculosis in the Iberian wild boar. Mol Ecol 14: 3209-3217.
24. Clarke KA, Fitzgerald SD, Zwick LS, Church SV, Kaneene JB, et al. (2007) Experimental inoculation of meadow voles (Microtus pennsylvanicus), house mice (Mus musculus), and Norway rats (Rattus norvegicus) with Mycobacterium bovis. J Wildl Dis 43: 353-365.
25. Waters WR, Palmer MV, Thacker TC, Davis WC, Sreevatsan S, et al. (2011) Tuberculosis immunity: opportunities from studies with cattle. Clin Dev Immunol. .