Short Communication

# Bovine response to lipoarabinomannan vaccination and challenge with *Mycobacterium paratuberculosis*

Ana Jolly[1], Claudia Morsella[2], Laura Bass[1], María Andrea Fiorentino[2],
Fernando Alberto Paolicchi[2], Silvia Leonor Mundo[1]

[1]Cátedra de Inmunología, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires,
Buenos Aires, Argentina.
[2]Laboratorio de Bacteriología, Estación Experimental Agropecuaria Balcarce,
Instituto Nacional de Tecnología Agropecuaria, Balcarce, Argentina.

## Abstract

This study aimed to evaluate the immune response in bovines following immunization with a mycobaterial Lipoarabinomannan extract (LAMe) and the effect of Map challenge. LAMe vaccine induced specific antibody levels that diminished after the challenge and affected Map excretion at least for 100 days thereafter.

**Key words:** paratuberculosis; bovine; LAM; humoral immune response; experimental challenge.

Paratuberculosis is a chronic enteritis affecting ruminants, caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map). Current commercial vaccines, based on live or killed Map strains, feature both antibody- and cell-mediated immunity, and can cause a significant reduction in bacterial shedding conferring a partial protection against paratuberculosis (Bastida and Juste, 2011). These vaccines induce reactivity to mycobacterial antigens such as purified protein derivative (PPD) and protoplasmic antigen (PPA), which are widely used to identify infected cattle (Nielsen 2010). In countries where bovine tuberculosis is endemic, such as Argentina, vaccination against paratuberculosis is hampered by the interference with the intradermal tuberculin test.

Lipoarabinomannan (LAM) is the most immunogenic glycolipid antigen and a key virulence factor on the mycobacterial envelope (Nigou *et al.*, 2003; Welin *et al.*, 2008). Antibodies against LAM have been shown to be beneficial in passive protection experiments in tuberculosis models (Glatman-Freedman, 2006). The role of the humoral immune response on infection and protection in paratuberculosis remains controversial. We have previously shown that specific antibodies (against LAM or other Map antigens) could have beneficial effects in macrophage infection assays (Jolly *et al.*, 2011; Mundo *et al.*, 2008).

This study aimed to evaluate the kinetics of the humoral immune response in bovines following immunization with a mycobacterial LAM-enriched glycolipid extract (LAMe) and the effect of Map challenge.

Ten six-month-old Aberdeen Angus (*Bos taurus*) calves from tuberculosis-free accredited and paratuberculosis-free herds were kept in an experimental field from INTA Balcarce (Argentina) and were randomly assigned to these treatment groups: LAMe vaccinated group (LAMv, n = 5), Ma vaccinated group (Mav, n = 3) and FIA control group (C, n = 2), which were inoculated subcutaneously on days 0, 40, 90 and 170 with LAMe, *Mycobacterium avium* subsp. *avium* (Ma) or PBS, respectively, prepared as previously described (Jolly *et al.*, 2006, 2011). The experiment was approved by the "Institutional Committee for the Care and Use of Animals for Experimental Procedures" from INTA. The Map inoculum was produced by conventional culturing (Stabel, 1997) of a local strain, first isolated from feces of a dairy cow with clinical signs of paratuberculosis and positive result in PPA-ELISA, obtained from the Laboratory of Bacteriology (INTA Balcarce), and identified as "A" pattern (European type RC17) by IS*900*-PCR and Restriction Fragment Length Polymorphism (RFLP) (Moreira *et al.*, 1999).

Send correspondence to: S.L. Mundo. Cátedra de Inmunología, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Av. Chorroarín 280, C1427CWO, Buenos Aires, Argentina. E-mail: smundo@fvet.uba.ar.

Ten days after the last immunization (day 180 of the experiment), two bovines from the LAMv group, randomly selected, and the two from the C group were challenged by intraruminal inoculation for two consecutive days with a total dose of 200 mg wet weight of bacterial pellet (Hines *et al.*, 2007). Viable bacteria were retrospectively determined by serial plating onto Herrolds medium, resulting in a total inoculum of $1 \times 10^9$ cfu for each animal.

Serum ELISA reactivity to LAMe, Map and PPA was assessed on days 0, 40, 90, 180 and 280, following previously described methodology (Fernández *et al.*, 2012; Jolly *et al.*, 2011). All immunized calves developed specific antibodies against LAMe and Map after vaccination (Figure 1A and B), whereas the control group did not show positive results (Figure 1C). A single dose of LAMe vaccine induced the highest level of specific antibodies detected, independently of the antigen evaluated (LAMe or Map). The second dose of LAMe did not increase these levels (Figure 1A). These findings seem to be consistent with the glycolipid nature of this antigen and the way in which this kind of antigens stimulate an immune response (Abbas and Lichtman, 2004). In the case of the Mav group, we found a different kinetics of the humoral immune response against the evaluated antigens. Animals in this group required at least two doses to stimulate the production of high levels of specific antibodies (Figure 1B). This apparent delay in the antibody peak after vaccination has been already described by other authors for Map proteic antigens (Muskens *et al.*, 2002; Stabel *et al.*, 2011). The differences in reactivity against LAMe detected between the LAMv and the Mav groups could be related to the thermolability of LAM during heat inactivation of bacteria (Kang *et al.*, 2005) or to the influence of protein compounds present in the whole bacterial vaccine. Both experimental vaccines (LAMe and Ma) induced PPA reactivity with the first dose. Nevertheless the LAMv group maintained low reactivity levels with successive doses, whereas the Mav group continued increasing them until the final time point. This temporary reactivity detected against PPA in the LAMv group could be related to the presence of protein traces in LAMe or of small amounts of LAM in PPA.

IFNγ production of whole blood cells stimulated with PPDa and PPDb was assessed on days 0, 90 and 180 (prior to challenge) (Paolicchi *et al.*, 2003) using the Duoset Elisa Development Kit (R&D Systems Inc., MI, USA). Basal levels of IFNγ were detected in the three groups on days 0 and 90 (values lower than 230 pg/mL for PPDa and than 200 pg/mL for PPDb estimulated cells). On day 180, we could detect a significant increase in IFNγ levels produced in Mav group when whole blood cells were stimulated with both PPDa ($838 \pm 167$ pg/mL) and PPDb ($481 \pm 80$ pg/mL), as compared with the levels detected in the other groups, that remained basal ($p < 0.05$, ANOVA and Tukey's test). Immunization with LAMe did not induce the specific secretion of this cytokine in any of the evaluated time points,
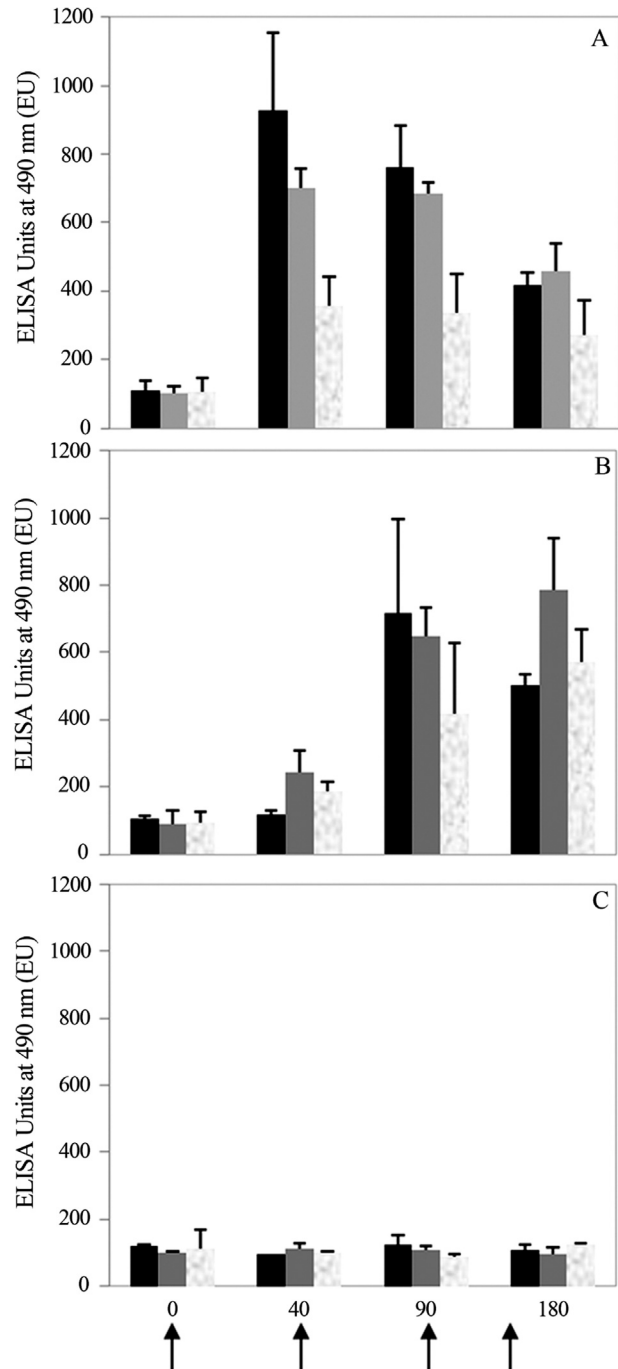


**Figure 1** - Kinetics of specific antibody response from day 0 to 180 (prior to challenge) against: LAMe (black bars), Map (gray bars) and PPA (spotted bars). Mean ELISA Units (EU) ± standard deviation of three experiments are represented for each group A) LAMv, B) Mav, C) C. EU were estimated as the mean OD value for each serum 100/ mean OD value for a pool of sera from five healthy bovines. Numbers under bars indicate days after first vaccination and arrows indicate vaccination days.

while immunization with the whole mycobacteria did interfere with this diagnostic technique, as previously reported (Muskens *et al.*, 2002; Stabel *et al.*, 2011).

After challenge, our results show that the levels of serum-specific antibodies significantly decreased in bovines

of the LAMv group, as compared with the levels detected before the challenge in the same group (p < 0.005, paired T test) (Figure 2A). We hypothesize that the circulating antibodies induced by LAMe vaccination, priorly described as predominantly composed of IgG1 (Jolly *et al.*, 2011), could be consumed during the intestinal replication of Map, while this isotype is the most relevant in the intestinal tract of cattle (Butler and Kehrli, 2005). Specific antibodies in control challenged bovines remained low after challenge (Figure 2A). Taking into account the relatively short experimental period and the generally delayed humoral immune response induced by Map infection, this result is not unexpected. In fact, similar results were published by other authors, detecting no antibody response during a post-challenge period of 134 (Waters *et al.*, 2003), 150 (Munjal *et al.*, 2007) or 210 days (Koo *et al.*, 2004).

On days 210 and 280 (30 and 100 days postchallenge, respectively), Map was isolated from the fecal samples of the two control bovines, but not from those of challenged bovines of the LAMv group (Figure 2B). The identity of Map isolates was verified by IS*900*-PCR and the RFLP pattern confirmed to be the same used for the challenge. To our knowledge, this paper proposes for the first time the intraruminal route for experimental infection with paratuberculosis and is the first report of challenge with a native Map strain in bovines in Argentina. It has been described that passive fecal shedding of Map occurs as early as 12 hours after oral inoculation in challenged bovines. Positive results in fecal culture from 14 days after inoculation should be considered shedding due to infection (Hines *et al.*, 2007). In our study, the isolation of Map from feces of control challenged bovines on days 30 and 100 after the challenge demonstrated multiplication of bacteria in nonvaccinated animals. Remarkably, we were able to attempt

bacterial replication in control challenged bovines, aged approximately 1 year at challenge. Generally, newborn calves are considered the most susceptible category for contracting the infection, with resistance increasing with age (Windsor and Witthington, 2010). Even so, it has been recently demonstrated that age-resistance to infection can be overcome by pressure of the infection, achieving Map infection in one- and two-year-old cattle exposed to heavily contaminated pastures (Fecteau *et al.*, 2010).

Our results show that LAMe vaccine induces a systemic humoral immune response and affects Map excretion in challenged bovines at least until 100 days after challenge. It would have been interesting to see if different degrees of lesions develop if these bovines had been kept longer. However, this was beyond the scope of this work. Further experiments should be conducted, using more animals, the natural route of infection, and a longer postchallenge period, and including more evaluation techniques. These studies would provide useful data for the development of new strategies in paratuberculosis prevention.

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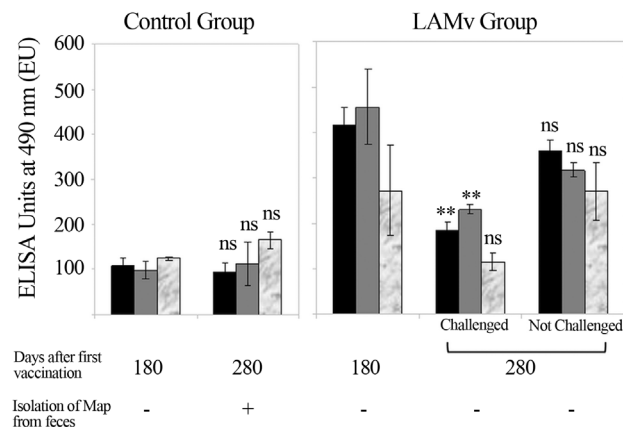


**Figure 2** - Effects of Map challenge. On antibody levels. Mean ELISA Units (EU) ± standard deviation of three measurements obtained for each group are shown. Black bars represent reactivity against LAMe, gray bars against Map, and spotted bars against PPA. Text above bars indicates the group analyzed, and numbers under bars indicate days after first vaccination. ** means p < 0.005 and ns means no significant differences with p > 0.05, between 180 and 280 days (paired T test). On Map isolation from feces.

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