Protection efficacy of Argentinian isolates of *Mycobacterium avium* subsp. *paratuberculosis* with different genotypes and virulence in a murine model.

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Abstract

Paratuberculosis is a chronic disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*). The disease causes economic losses and, therefore, it is imperative to follow proper control strategies, which should include an effective vaccine. Several strategies have assessed the virulence and immune response of *Map* strains that could be used as a vaccine. This study evaluates the degree of virulence, immune response, and protection of Argentinian strains of *Map* with different genotype in a murine model. Four local isolates (Cattle type) with different genotypes (analyzed by MIRU-VNTR and SSRs) were selected and evaluated in a virulence assay in BALB/c mice. This assay allowed us to differentiate virulent and low-virulence *Map* strains. The less virulent strains (1543/481 and A162) failed to induce a significant production of the proinflammatory cytokine IFNg, whereas the virulent strain 6611 established infection along with a proinflammatory immune response. On the other hand, the virulent strain 1347/498 was efficient in establishing a persistent infection, but failed to promote an important Th1 response compared with 6611 at the evaluated time. We selected the low-virulence strain 1543/498 as a live vaccine and the virulent strain 6611 as a live and inactivated vaccine in a protection assay in mice. Strain 1543/481 failed to protect the animals from challenge, whereas strain 6611, in its live and inactivated form, significantly reduced the CFUs count in the infected mice, although

they had different immunological response profiles. The inactivated virulent strain 6611 is a potential vaccine candidate against paratuberculosis to be tested in cattle.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (*Map*) is the causative agent of paratuberculosis or Johne's disease, a chronic enteritis of ruminants, such as cows, goats, deer and sheep. Efforts to control the spread of the disease in domestic livestock have been largely ineffective and the disease is now recognized worldwide. In a study of *Map* prevalence in eight Latin American and Caribbean countries, Fernández-Silva et al. (2014) reported average values of 16.9 and 75.8% at the individual and herd levels, respectively. Although epidemiological data of paratuberculosis are scarce in Argentina, the disease has been well characterized in Buenos Aires province, one of the most productive regions of the country. According to the National Institute of Agricultural Technology (INTA), the seroprevalence in cattle herds from this province ranges from 7 to 19.6% (Espeschit et al., 2017). Efforts are directed towards the control of this disease because it can cause substantial economic losses in the livestock industry, particularly the dairy sector. Paratuberculosis spread occurs mainly because of its long subclinical stage, which favors an active shedding of large quantities of *Map* in feces of infected animals.

The available diagnostic tools are insufficient to make an early and specific diagnosis, especially in animals with subclinical paratuberculosis (PTB). In addition, the management measures based on the slaughter of shedding animals are not enough to eradicate this disease. Although all commercially available PTB vaccines could interfere with PTB serodiagnosis, vaccination seems to be the best strategy to deal with this disease (Bastida and Juste 2011; Garrido et al 2013; Groenendaal et al. 2015).

To date, at least three commercial paratuberculosis vaccines are available worldwide: Mycopar®, which is a killed *Mycobacterium avium* subsp. *avium* (*Maa*) strain 18, or Silirum® and Gudair®, which consist of inactivated whole cell preparations of the attenuated 316F *Map* strain. Although these vaccines reduce the detachment and elimination of *Map* by fecal matter, they fail to prevent infection (Bannantine and Talaat, 2015; Barkema et al., 2017). The advances in mycobacterial molecular biology and genome sequencing have allowed the improvement of *Map* vaccines.

For instance, live attenuated *Map* strains with potential vaccine properties have been produced by transposon mutagenesis and by allelic exchange (Park et al., 2011; Bannantine et al., 2014b; Ghosh et al., 2015). On the other hand, a study showed that a

field isolate, which was used as a heat-killed vaccine, provided better results in calves, with a significant reduction in mycobacterial colonization (Uzonna et al., 2003), than Mycopar®. In other studies, a virulent Indian strain Bison type that was used as an inactivated vaccine showed a reduction in fecal shedding of the pathogen and of the clinical disease, and improved body weight in goats (Singh et al., 2007), sheep (Singh et al., 2013) and cattle (Singh et al., 2015).

The main disadvantage of vaccination is a plausible interference with the diagnosis of tuberculosis and paratuberculosis (Bastida and Juste, 2011, Garrido et al., 2013, Groenendaal et al., 2015). However, the use of antigens of *M. tuberculosis* complex to distinguish infected from vaccinated animals (DIVA) may be useful in differentiating *Map* vaccinates from TB-infected animals (Stabel et al., 2011, Vordermeier et al., 2011). We have previously demonstrated that cocktails containing defined *M. tuberculosis* complex antigens, such as ESAT-6, CFP-10, MPB83, and FixB, can provide a sensitive and specific diagnosis of TBB (Mon et al., 2014).

Several members of the Johne's Disease Integrated Program (JDIP) have concluded that the best way to evaluate and develop *Map* vaccines is a three-phase vaccine candidate evaluation strategy: macrophages, mouse models and finally the native host (Hines et al., 2007, Bannantine et al., 2014b, Park and Yoo, 2016). Scandurra et al. (2010) were able to classify the strains according to their virulence and made a preliminary selection of vaccine candidates in a well-defined bovine monocyte-derived macrophage model. However, some studies have highlighted the limitations of the macrophage model in the initial screening of *Map* virulence and in predicting vaccine candidates (Bannantine et al., 2014b; Lamont et al., 2014). Vaccine trials in goat, sheep, deer or bovine hosts are highly costly and thus the use of small animal models such as mice facilitate comparisons between different vaccine trials. Indeed, mice are not the natural hosts of paratuberculosis and some of the clinical signs (e.g. diarrhea) that are associated with *Map* infection in ruminants are not achieved in this model. However, the low cost and easy availability of these models make them appropriate to assess *Map* vaccines (Begg and Whittington, 2008).

A better understanding of the molecular and immunological processes involved in the infection and progression to clinical paratuberculosis of local strains may help researchers to develop more efficient vaccines. The aim of this study was to analyze the degree of virulence, immune response and protection conferred by local strains of *Map*, with different genotype, in a murine model.

2. Materials and methods

2.1 Map strains

Four Argentinian *Map* isolates (Cattle-type or C-Type) from naturally infected cattle were selected from our strain collection because of their different genotypes (Table 1). All the selected strains belong to herds of Buenos Aires province, Argentina. *Map* confirmation was performed by mycobactin dependence and PCR detection of the IS*900* insertion sequence as previously described (Collins et al., 1993). The C-type were confirmed by IS*1311*-PCR REA (Marsh et al., 1999; Gioffre et al., 2015). The isolates were genotyped by Imperiale et al.(2017) with multi-locus variable number tandem repeats of mycobacterial interspersed repetitive units (MIRU-VNTR) according to Thibault et al. (2007) by assessing eight MIRU-VNTR loci (292, X3, 25, 47, 3, 7, 10 and 32). In addition, the genotyping was complemented by multi-locus short sequence repeat sequencing (SSRs) performed as Amosin et al. (2004) by using the four most variable marker loci (1, 2, 8 and 9) described by Thibault et al. (2008).

The strains were originally isolated in Herrold´s Egg York medium. Then they were grown for 12 to 16 weeks in Middlebrook 7H9 medium (Difco) with 0.05 % Tween 80, supplemented with 0.5 % albumin, 0.4 % dextrose, 0.5 % glycerol and 2 mg/mL mycobactin J (Allied Monitor Inc, Fayette,USA). The strains were stored in glycerol at −80 °C until use. The strains were propagated in supplemented Middlebrook 7H9 broth medium before each experiment (all strains had low-passage-number). Tubes with 50 mL of the *Map* cultures were centrifuged, washed and suspended in sterile phosphate buffer saline (PBS). Clumps were eliminated by sonication in a water ultrasonic bath and with 20 passages through a syringe with a 25G needle. The inocula were estimated by optical density (OD), following the formula OD_{600} $0.3=10^9$ colony-forming units (CFU)/mL (Janagama et al., 2006), and then confirmed by CFU count on supplemented Middlebrook 7H10 medium (Difco).

2.2 Mice.

BALB/c female mice (n=62, for the virulence assay; n=75, for the protection assay) of 18 to 21 grams and 5 to 6 weeks of age were adapted for a period of one week. They were acquired from the animal facility of the School of Veterinary Sciences, National University of La Plata, Buenos Aires, Argentina. The infections were performed inside the biosafety level 2 (BSL2) facilities and the mice were kept in cages fitted with microisolators connected to negative pressure. All animal experiments were performed according to the protocols approved by the Institutional Animal Care and

Use Committee and under the regulations of the Ethical Committee of the National Institute of Agricultural Technology (INTA).

2.3 Virulence assay

The degree of virulence and immune response of the local strains were analyzed in four groups of 14 animals infected with different *Map* strains (1347/498, 6611, 1543/481 and A162). A control group of six animals was inoculated with sterile PBS. Bacillary suspensions were adjusted to $5x10^6$ viable bacteria/mL in 1x sterile PBS. Each animal was intraperitoneally inoculated with 0.2 mL of one of the inocula. The inoculum doses were determined by CFU counting on 7H10 plates supplemented with mycobactin J. The mice were euthanized at 3, 20 and 60 days post-infection (dpi) and the spleen and liver were removed and weighed. Blood was collected at 60 dpi for antibody detection. The initial colonization was estimated by the CFU recovered from the spleens of four mice per group at 3 dpi. Five mice per infected group were euthanized at 20 and 60 dpi and each spleen was removed and homogenized in 1 mL of RPMI 1640 without antibiotics (Figure 1). Serial dilutions of each homogenate were spread onto duplicate plates. Colony forming units were determined 6 to 8 weeks later. The remaining cell suspensions were used for cytokine quantification. The cytokine profile induced by each of the four strains was determined at 60 dpi in the cell suspension of splenocytes stimulated with *Mycobacterium avium* purified protein derivative (PPDa). The measurement of cytokines, total IgG, IgG1 and IgG2a isotypes as well as the histopathological analysis of the livers and spleens are described below.

2.4 Protection assay

The objective of the protection trial was to evaluate if the assessed local strains could be potential vaccine candidates. The low virulent strain 1543/481 and the virulent strain 6611 were selected to analyze the immune response and efficacy as *Map* vaccines based on the virulence assay. Their safety and protective effect against *Map* infection were assessed in mice. The time scheme for the vaccination trial and the immunization and challenge doses were adapted with minor modifications from previous studies (Ghosh et al. 2015; Bannantine et al. 2014a; Stabel et al. 2012). Three groups of 20 animals were subcutaneously vaccinated with two doses (0.2 mL of 3×10^5 bacterium strain /mouse) separated by 17 days. The analyzed groups were the following: *Group 1*- live vaccine with the low-virulence strain (1543/481) in PBS, *Group 2*- live vaccine with the virulent strain (6611) in PBS and *Group 3*- heat inactivated (80°C for 30 min) vaccine with the virulent strain (6611) formulated with incomplete Freund's adjuvant. In

addition, 15 mice were inoculated with sterile PBS, control group (unvaccinated). At 4 and 14 weeks post-vaccination (wpv), five mice per vaccinated group were euthanized (without challenge) to evaluate colonization of the spleen by the live vaccines. At 4, 9 and 14 wpv, blood samples were collected for antibody detection. At 14 wpv, five mice of each group were euthanized to evaluate cytokine production. The remaining animals (n=10 per group) were intraperitoneally inoculated with 0.2 mL suspension of the virulent *Map* strain 1347/498 (3.3×10^7 viable bacteria/mouse) at 4 wpv. The inoculum doses were determined by CFU counting on 7H10 plates supplemented with mycobactin J. At 5 and 10 weeks post-challenge (wpc), five animals per group were euthanized and their spleens and livers were removed and weighed (Figure 1). The spleens were homogenized and serial dilutions of each homogenate were spread onto duplicate plates for CFU counting, whereas the livers were reserved for histopathological analysis.

2.5 Measurement of total IgG, IgG1 and IgG2a

Total IgG, IgG1 and IgG2a were measured by indirect ELISA in collected sera using flat bottom 96-well immuno-plates Nunc™ Maxisorp™ (Sigma-Aldrich Co.) coated with Paratuberculosis Protoplasmic Antigen (PPA-3 Allied Monitor Inc.). Serum dilutions were incubated for 1 h at 37 °C in plates coated with PPA-3. Then, the plates were washed and subsequently incubated with the secondary antibody, mouse anti-IgG conjugated to peroxidase diluted 1:2,500 (KPL, Kirkegaard & Perry Laboratories Inc., Washington, USA), mouse anti-IgG1 or IgG2a biotinylated 1:3,000 (KPL Inc.), for 1 h at 37 °C. Finally, they were incubated with streptavidin-peroxidase diluted 1:50,000 for 1 h at 37 °C, washed three times with PBS and then ABST-citrate buffer pH 5 was added to the plates. The optical density reading at 405 nm (OD_{405nm}) was taken after 20 min.

2.6 Quantification of cytokines

At 60 dpi (virulence assay) or 14 wpv (protection assay), the rest of the cell suspensions of homogenized splenocytes in RPMI 1640 were treated with ammonium chloride for erythrocyte lysis and subsequently concentrated. Splenocyte viability was determined by Trypan Blue staining using a Neubauer chamber. Splenocytes (1×10^6/well; in RPMI 1640 supplemented with 10% fetal calf serum, 100 U of penicillin/mL and 100 µg of streptomycin/mL) were incubated in 96-well round-bottom plates at 37 °C in a 5 % CO_2 chamber and stimulated with 10 µg/mL of PPDa (BOVIGAM®, Thermo Fisher Scientific Inc., Massachusetts, USA) or 2 µg/well of Concanavalin A for 72 h. Cytokines IL-2, IL-4, IL-6, IFNg, TNF, IL-17A and IL-10

were quantified using the BD Cytometric Beads Array Mouse Th1/Th2/Th17 Cytokine® kit (BD Bioscience Co.) in a BD FACS Calibur flow cytometer, following the manufacturer's instructions.

2.7 Histopathology

The organs were fixed in buffered 10% formalin solution and then embedded in paraffin following routine procedures. The 3 µm thick sections were stained with Hematoxylin-Eosin (H/E) and Ziehl Neelsen modified by Ellis and Zabrowarny (ZN). For the virulence assay, we performed a descriptive histopathology of the liver and spleen. The microscopic changes in the livers from the protection assay were determined with scores ranging from 0 to 12 . This score is based on the presence, distribution, number, size and type of lesions (sections stained with H/E) and on the presence of acid-fast bacilli (AFB) and percentage of lesions with AFB (sections stained with ZN).

2.8 Statistical analysis

The Kruskal-Wallis and Dunn post-test with multiple comparisons were used in the virulence assay to analyze the weight of the animals and organs, the CFU in the spleens and the levels of cytokines and antibodies. A p-value below 0.05 was considered significant. The One-Way ANOVA and Bonferroni multiple comparison test with a p-value of 0.05 were used in the protection assay Data and graphical representation were based on Graph Pad Prism version 6.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1 Virulence assay

a) CFU counting in the spleens

The four strains of *Map* with different genotype (6611, 1347/498, A162, 1543/481) selected for this assay were able to colonize the spleens, with an average of 10^4 CFU/mL at 3 dpi. The strains 1347/498 and 6611 were able to replicate at 60 dpi and thus were the most virulent strains, whereas A162 and 1543/481 displayed the lowest survival rates after two months of infection (Figure 2).

b) Immune response induced by different Map strains

Total IgG along with the IgG1 and IgG2a isotypes were evaluated at 60 dpi. The mice inoculated with the most virulent strains (1347/498 and 6611) displayed a significantly higher production of total IgG (Figure 3A) and both isotypes (Figure 3B) than the control group (PBS), with higher values of IgG2a over IgG1 (IgG2a/IgG1>1)

(Figure 3C). The animals inoculated with strain A162 elicited the lowest levels of IgG (Figure 3).

With respect to the cytokines evaluated at 60 dpi, only the mice inoculated with strain 6611 showed significant production of IL-2 (9.25 ± 3.029 pg/mL; $p<0.01$). This group also displayed significantly higher levels of IFNg ($p<0.001$) and IL-17 ($p<0.05$) than the control group. The mice infected with both virulent strains, 1347/498 and 6611, showed a significant increase in TNF production ($p<0.05$). The group 1543/481, which showed low CFU counting in the spleens of mice, only elicited a significant increase in IL-10 ($p<0.05$). The group inoculated with the other low virulent strain A162 displayed non-significant higher levels of IL-17A ($p=0.0716$) than the control group (Figure 4).

c) Histopathological examination of the liver and spleen

All the livers from the infected animals displayed histological changes compatible with *Map* infection at 20 and 60 dpi, whereas the spleens showed no apparent structural or histologic anomalies. At 20 dpi, the predominant inflammatory exudates were lymphocytic and lymphohistiocytic in all the infected animals. The animals infected with A162 displayed limited histological changes, whereas the animals infected with 6611 had a typical organization of granuloma. At 60 dpi, all the infected animals presented granuloma organization, whereas those infected with 1347/498 showed more granulomatous foci.

At 20 dpi, only the animals infected with the virulent strains (6611 and 1347/498) had AFB in the liver, whereas at 60 dpi all the infected animals had the bacilli. The highest and lowest percentages of foci with AFB were in the groups infected with 6611 and 1543/481, respectively. Furthermore, AFB were present at 60 dpi in spleens from animals infected with 1347/498 and 6611. These changes are consistent with the results obtained with the CFU analysis.

3.2 Protection assay

a) Vaccine immune response

Based on the results of the virulence assay, we decided to use the 1543/481 and 6611 strains as candidate vaccines and the most virulent strain (1347/498) as the challenging strain. The 1543/481 strain was selected as a live vaccine for its low virulence and its production of an anti-inflammatory response, whereas the virulent strain 6611 was evaluated as a live and inactivated vaccine, because it induced the strongest proinflammatory immune response. The vaccinated group with the inactivated

strain 6611 was the only one that induced significant production of total IgG (Figure 5), with a predominance of the IgG1 isotype ($p<0.05$, data not shown).

Figure 6 displays the analysis of different cytokine production in vaccinated groups after 16 and 72 h of stimulation with PPDa. Whereas vaccination with the low virulence strain (1543/481) induced no significant increase in the levels of cytokines at the evaluated times, vaccination with 6611 strain, either live or inactivated, stimulated the production of many different cytokines. After 16 h, the stimulation with PPDa of splenocytes from mice vaccinated with the live strain 6611 induced a significant production of IFNg, TNF, IL-17A and IL-6. After 72 h, this stimulation induced a significant production of IL-6 and TNF. This group also displayed the highest values of IFNγ. However, no significant difference with the unvaccinated group were detected at 72 h. On the other hand, the group vaccinated with the inactivated strain 6611 showed a significant IL-2 production after 16 h of stimulation. The production of this cytokine was maintained at 72 h; however, this production was not significantly different from that of the unvaccinated group. This group also displayed a significant production of IL-10 after 72 h of stimulation.

b) Histopathology

Splenomegaly without microscopic lesions was evident in all challenged animals. AFB were detectable in spleens at 5 wpc in all groups except for the group vaccinated with the inactivated strain 6611 (Figure 7). The degree of liver lesions (score) at 10 wpc was significantly lower in the group inoculated with the inactivated strain 6611 than that of the unvaccinated group. At 5 wpc this group also had the lowest score but the differences with the unvaccinated group were not significant ($p=0.0706$; Figure 8 and Supplemental figure).

c) Spleen CFU count

At 10 wpc a significant reduction in the CFU counts was evident in the animals vaccinated with the virulent strain 6611, both live and inactivated, when we compared it with the CFU counts from the unvaccinated group and from the group vaccinated with the less virulent strain 1543/498 (Figure 9).

Vaccination with the virulent strain 6611 (live or heat-inactivated) resulted highly immunogenic (predominantly Th1/Th17 or humoral immune response, respectively) and protective, thus reducing the bacterial burden in the murine model. The live strain 6611 vaccination, however, was not safe because the vaccine strain was recovered in two animals at the pre-challenge time (146 ± 68 CFU/spleen) and from one

animal at 14 wpv (80 CFU/spleen). In contrast, the inactivated vaccine was safe and reduced the tissue pathology (score) generated by the challenge with a local virulent strain of *Map*.

4. Discussion

Several immunization studies have suggested positive effects of the administration of inactivated vaccines. Indeed, the positive effects include decrease on *Map* fecal shedding after challenge, delayed onset of clinical disease and increase on mean daily milk yield (Patton, 2011; Knust et al., 2013; Stringer et al., 2013; Windsor et al., 2014). On the other hand, field isolates could be better as vaccines against paratuberculosis than commercial vaccines (Uzonna et al., 2003; Singh et al., 2007, 2013 and 2015). Accordingly, for the rational design of a novel vaccine, researchers need to better understand the virulence and immune profile induced by different field isolates.

With this in mind, in the present study, we first evaluated the virulence of local *Map* strains (Argentinian strains) with different genotypes in a mouse model as well as the immune response elicited after infection. In the virulence assay, the recovery of the CFU from the spleens of the infected mice demonstrated that 6611 and 1347/498 replicate in the mouse model throughout the evaluated period, whereas A162 and 1543/481 were eliminated after two months of infection.

After the initial exposure to *Map,* naturally infected cattle display a predominant T-cell response characterized by the release of cytokines such as IFNg, IL-6, IL-2 and IL-17, which are related with a Th1/Th17 response. At the end of the subclinical phase of *Map* infection, the proinflammatory Th1-like response often switches to a Th2 and regulatory T-cell mediated response, which is characterized by the production of cytokines such as IL-4 and IL-10 along with IgG1 antibodies. Progression to clinical disease is often associated with this change of the immune response (Begg et al., 2011). However, there is no evidence of interference between Th1 and Th2 immunity and, furthermore, the immunological links of protection against development towards the disease remains unclear (Ganusov et al., 2015). B-cells and an humoral immunity may be required for mounting an effective immune response through effects on the presentation of antigens and the development of T-cell memory in *Map* (de Silva et al., 2015) and BCG (Bacillus Calmette-Guérin) vaccination (Kazakiewicz et al., 2013). Unfortunately, this potentially required response is not completely understood yet.

In the present study, we evaluated the immune parameters of the Th1, Th17 and Th2 responses in mice infected with field isolates of *Map* and the ability of these isolates to cause injury *in vivo*. The strategies used to identify and evaluate *Map* strains allowed us to differentiate between strains with low and high virulence. *Map* strains with low virulence, according to CFU counts (1543/481 and A162), showed an impaired ability to stimulate the production of IFNg and low levels of IgG. This result is in accordance with bacterial clearance. The strain 1543/481 may have failed to induce an effective Th1 response because of the significant production of the anti-inflammatory cytokine IL-10. Limiting the Th1 effector response could enhance the pathology associated with mycobacterial infections, but may also ameliorate immunopathological tissue damage and reduce the severity of the disease (Subharat et al., 2012).

One of the most virulent strains (6611) elicited a Th1/Th17 immune response (with production of IL-2, IFNg, TNF and IL-17A) and a high IgG2a/IgG1ratio. When we analyzed the histopathology of the liver in the animals infected with 6611, we detected more lesions and granulomas at 20 dpi. The other virulent strain 1347/498 efficiently established a persistent infection (with the highest CFU and more lesions in the liver at 60 dpi), but, in contrast to 6611, failed to promote a strong Th1 response at the evaluated times.

The most virulent local strain (1347/498) was selected for the challenge in the protection assay, instead of the reference strain K10. To evaluate the protective efficacy of the local strains, we decided to use strain 1543/498 (low virulence) as a live vaccine and the virulent strain 6611 as a live and inactivated vaccine in same doses.

Several researchers have evaluated live attenuated mutant strains of *Map* for the development of better vaccines (Faisal et al., 2013; Hines II et al., 2014; Bannantine et al., 2014b). Vaccines formulated with live attenuated strains of *Map* have been considered to control the infection, since they induce an immune response in the host that controls the infection. (Park et al., 2011; Settles et al., 2014; Ghosh et al., 2013, 2014 and 2015). However, the studies on the efficiency of live attenuated vaccines compared to inactivated vaccines in the murine model are scarce and they used lower doses of the inactivated vaccine than the live vaccine (Bannantine et al., 2014a; Ghosh et al., 2015).

Despite the protection obtained with live attenuated strains in other studies, the low-virulence strain (1543/481) used as a live vaccine failed to protect the animals. Indeed, the group vaccinated with this vaccine resulted in a CFU count in spleen and

lesions in liver after challenge that were similar to those of unvaccinated animals. Moreover, this vaccine did not significantly induce the production of antibodies or cytokines.

On the other hand, vaccination with 6611 virulent strain, both live or inactivated, significantly reduced the CFU counts, although the immunological response elicited in mice had different profiles. The degree of injury in animals vaccinated with this live strain was higher 5 wpc, likely because of a higher pro-inflammatory response in this group. This is probably not a proper vaccination strategy because of the biological risk involved in manipulating and applying a live virulent strain to the animals. Moreover, the 6611 strain, as a live vaccine, was recovered in the spleen of two animals 4 wpv and one mouse 14 wpv.

In contrast, the vaccination with the inactivated strain 6611 triggered predominantly a humoral response with significant IgG production. In terms of histopathology, at 10 wpc, this group achieved a significantly lower lesion score than the unvaccinated group; which coincides with the significant reduction of the CFU in the spleen.

Altogether, the local virulent *Map* strain 6611 displayed different immune profile responses when we administered as a live vaccine or inactivated but both forms managed to significantly protect the infected animals in the murine model. Therefore, 6611 is a potential vaccine candidate against paratuberculosis that must be further study in the mouse model and eventually in its host, cattle.

These results suggest that there is probably not a single type of immune response associated with protection and thus further studies are necessary to understand protection. In future studies, we will evaluate 6611 as an inactivated vaccine candidate in a mouse and cattle model in comparison with the commercial vaccine Silirum®.

5. Conflict of interest

All authors declare that they have approved the submission of this manuscript for publication in Research in Veterinary Science. This manuscript contains unpublished original work that is not under consideration for publication to any other journals and all authors have no conflict of interests.

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Strain	MIRU-VNTR pattern and TRs at loci (292-X3-25-47-3-7-10-32)	SSRs			
		1[a]	2[a]	8[b]	9[b]
6611	INMV1 (42332428)	7	>11	4	4
1347/498	INMV2 (32332228)	7	11	4	4
A162	INMV16 (32332528)	7	>11	4	4
1543/481	INMV2 (32332228)	7	>11	4	4

Table 1: ***Selected strains from our collection***. These strains were genotyped by multi-locus variable number tandem repeats of mycobacterial interspersed repetitive units (MIRU-VNTR) and short sequence repeats (SSRs). Eight loci (292, X3, 25, 47, 3, 7, 10 and 32) for MIRU-VNTR were PCR amplified and the INMV pattern was obtained as described by Thibault et al. (2007). Other four loci (1, 2, 8 and 9) were PCR amplified for SSRs analysis.
TRs = Tandem repeats.
[a]=Numbers of G mononucleotides present (>11, more than 11).
[b]=Number of copies of the trinucleotides repeat (loci 8: GGT; loci 9: TGC).

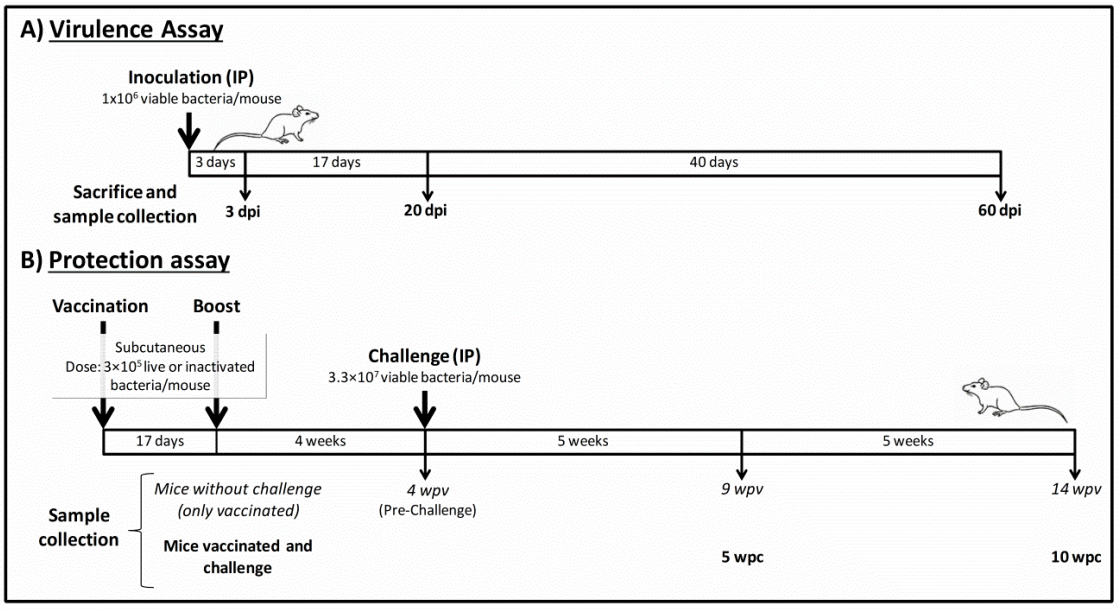


Figure 1: ***Schematic outline for the virulence and protection assay*****. A) Virulence assay**: Intraperitoneal inoculation of the local *Map* strains 1347/498, 6611, 1543/481 or A162 (1x10^6 viable bacteria/mouse). The mice were euthanized at 3, 20 and 60 days post-infection (dpi) and the spleen and liver were removed. Blood samples were collected for antibody detection at 60 dpi. **B) Protection assay**: The animals were subcutaneously vaccinated with two doses (separated by 17 days) of the 1543/481 or 6611 *Map* strain (3×10^5 live or inactivated bacteria). Four weeks later the animals were challenged with the virulent strain 1347/498 (3.3×10^7 viable bacteria/mouse). Non-challenged mice were euthanized at 4 and 14 weeks post-vaccination (wpv) to evaluate vaccine safety and serum was collected at 4, 9 and 14 wpv. Vaccinated and control group with challenge were euthanized at 5 and 10 weeks post-challenge (wpc).

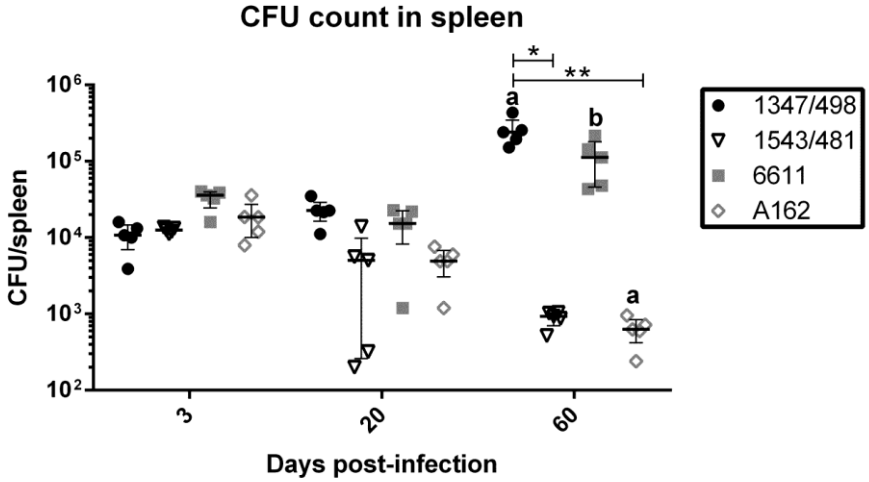


Figure 2: ***Survival of Map strains in the spleens of mice by CFU counting*.** The results are shown as the Median (Log_{10} CFU/spleen) ± interquartile range. The statistical analysis was performed using Kruskal-Wallis Test and Dunn Test (significant difference between 1347/498 and 1543/481 *p<0.05 and significant difference between 1347/481 and A162 **p<0.01 at 60 dpi).
a Significant difference between 3 dpi and 60 dpi values of the 1347/498 or A162 groups (p<0.01).
b Significant difference between 20 and 60 dpi values of the 6611 group (p<0.01).

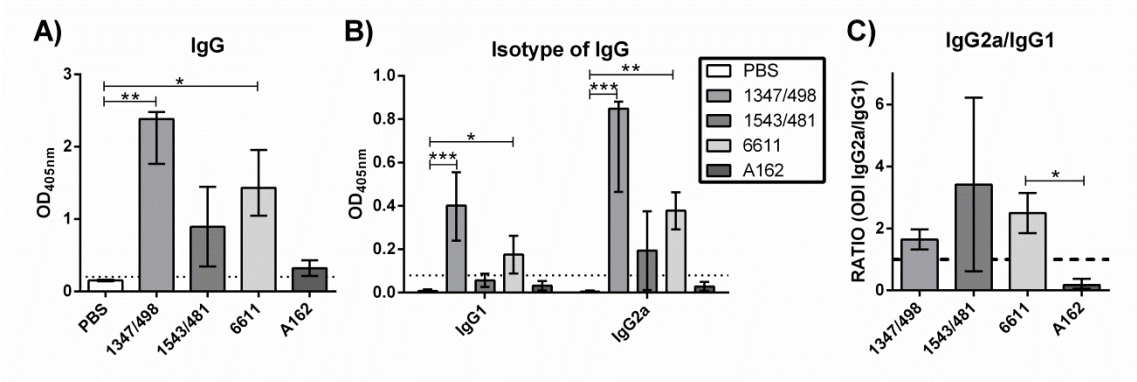


Figure 3: ***A) Optical density (OD) of total IgG*** by indirect ELISA-PPA3 in sera collected at 60 dpi. The cutoff point for positive determination is an OD_{405nm} of 0.2 (dotted line). ***B) OD of the isotypes IgG2a and IgG1*** by indirect ELISA-PPA3 in sera collected at 60 dpi. The cutoff point for positive determination is an OD_{405nm} of 0.08 (dotted line). ***C) Relationship between isotypes IgG2a and IgG1 (OD ratio).*** The dotted line represents the OD ratio=1.
The data are shown as the Median (OD or OD ratio) ± interquartile range. The statistical analysis was performed by using Kruskal-Wallis Test and Dunn Test and comparing the infected groups with the PBS group (* p< 0.05, ** p< 0.01, *** p< 0.001).

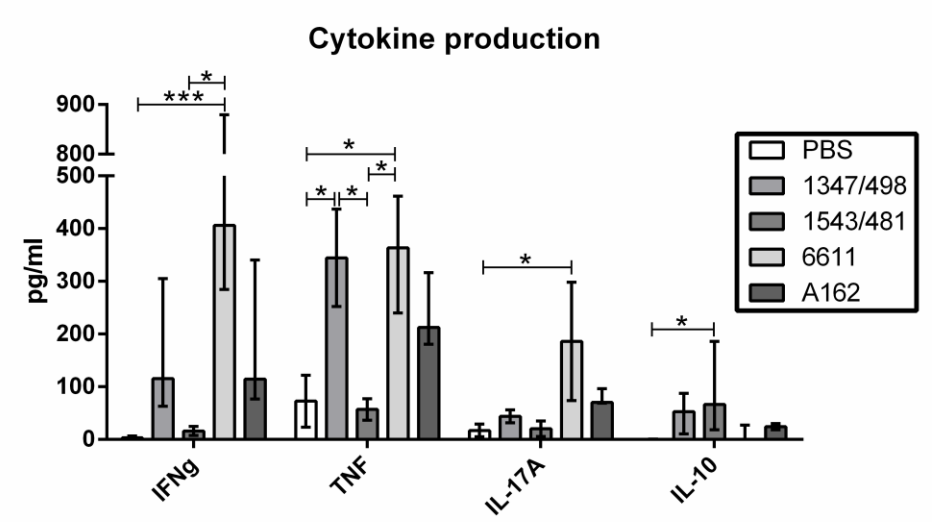


Figure 4: ***Cytokine production induced by different strains of Mycobacterium avium subsp. paratuberculosis in mice.*** The splenocytes from BALB/c mice inoculated with 1x10^6 CFU of 6611, 1347/498, 1543/481, A162 and PBS were stimulated with protein purified derivative of *M. avium* (PPDa). The production of specific cytokine proteins was measured by flow cytometry. The data were analyzed with FCAP Array™ v3.0 Software. The data are shown as the Median (pg/mL) ± interquartile range. The statistical analysis was performed using Kruskal-Wallis Test and Dunn Test (* $p< 0.05$, *** $p< 0.001$).
a Significant difference in IFNg production between 1543/481 and 6611 group ($p<0.05$)
b Significant difference in TNF production between 1543/481 group with 1347/498 and 6611 groups ($p<0.05$).

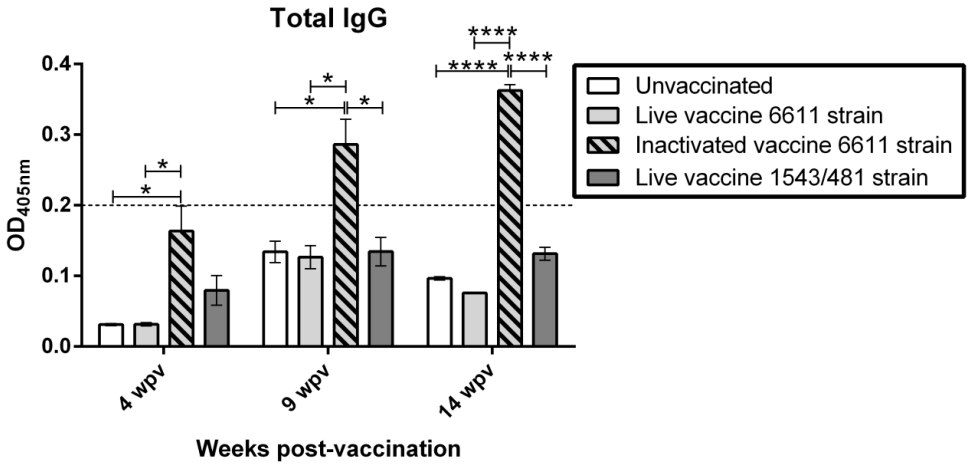


Figure 5. ***Graphic of the total IgG at 4, 9 and 14 wpv***. Expressed as Mean (OD_{405nm}) ± Standard Error of the Mean (SEM). The cutoff point for positive determination was an OD_{405nm} of 0.2 (dotted line). Statistical analysis of variance with One-Way ANOVA. Post-test Bonferroni multiple comparison of vaccinated groups with the unvaccinated group (*p <0.05, ****p <0.0001).

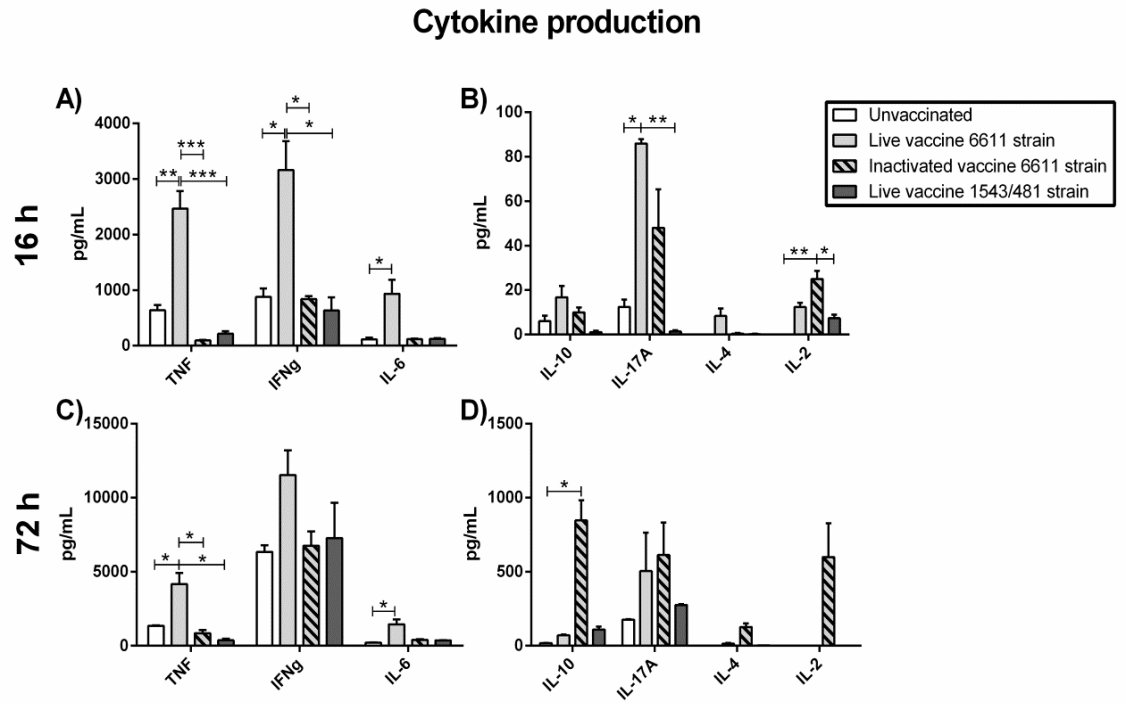


Figure 6: ***Cytokine quantification at 14 wpv of the culture of splenocytes stimulated with 10µg of PPDa for 16 (A-B) and 72 hours (C-D)***. Expressed as the Mean (pg/mL) ± SEM. Statistical analysis of variance with One-Way ANOVA. Post-test Bonferroni for multiple comparison (* p <0.05, ** p <0.01, *** p <0.001).

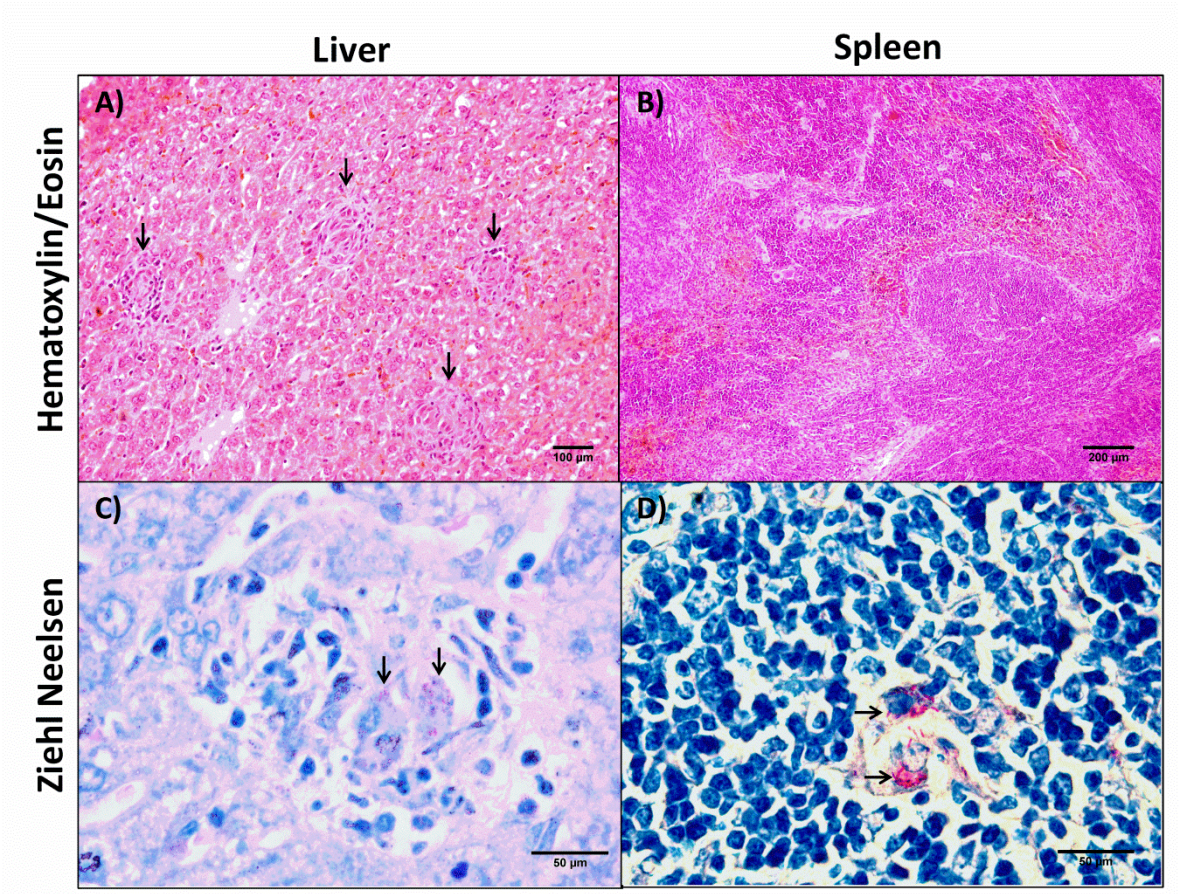


Figure 7: ***Photographs of histological sections of the liver and spleen of the unvaccinated group at 5 wpc*. A)** Granulomatous lesions (arrows) in the liver (H/E 20x). **B)** No lesions in the spleen (H/E 10x). **C)** Granulomatous lesion in the liver containing AFB (arrows) (ZN 100x). **D)** Spleen with macrophages containing AFB (arrows) (ZN 100x).

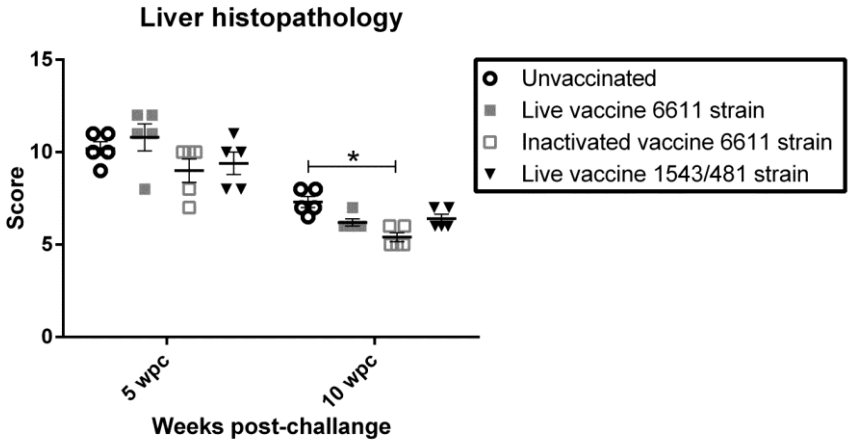


Figure 8: ***Degree of liver lesion at 5 and 10 wpc.*** Expressed as the Mean (Score) ± SEM. Statistical analysis of variance with One-Way ANOVA. Post-test Bonferroni multiple comparison (* $p < 0.05$).

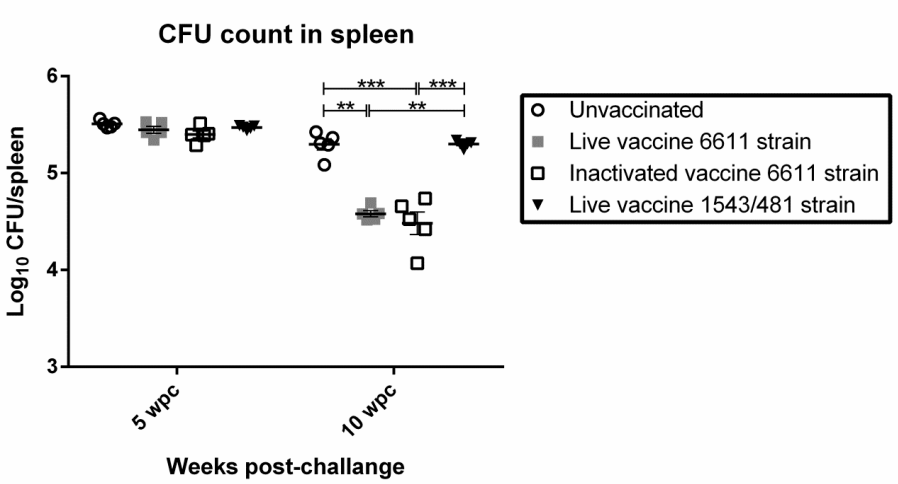
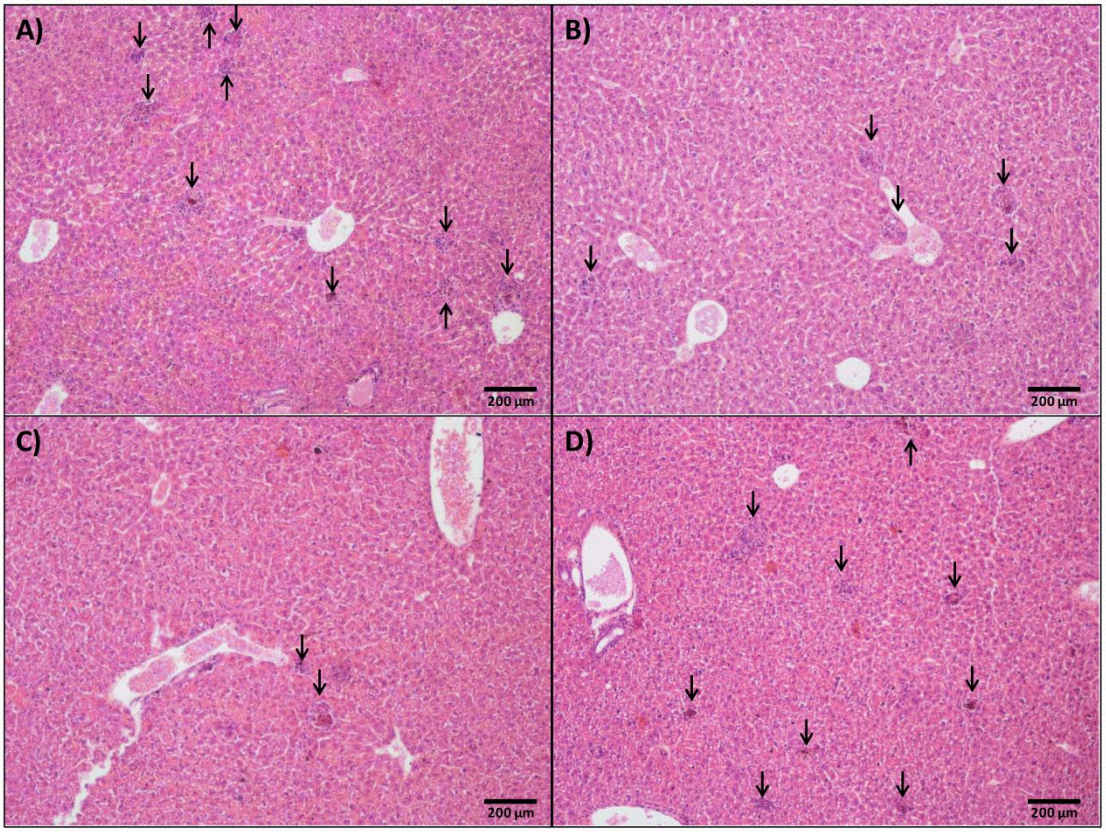


Figure 9: ***Colony forming units (CFU) in the spleen at 10 wpc.*** Expressed as the Mean (Log_{10} CFU / spleen) ± SEM. Statistical analysis of variance with one-way ANOVA. Post-test Bonferroni multiple comparison of groups vaccinated with the 6611 strain (live or inactivated) with the unvaccinated group and the group vaccinated with the live 1543/498 strain (** p <0.01, *** p<0.001).



Supplemental figure: Histological sections of the liver at 10 wpc. **A) Unvaccinated group. B) Live vaccine 6611 strain. C) Inactivated vaccine 6611 strain. D) Live vaccine 1543/481 strain.** Granulomatous lesions (arrows) in the liver (H/E 10x).

Highlights for the original article "Protection efficacy of local isolates of *Mycobacterium avium* subsp. *paratuberculosis* with different genotypes and virulence in a murine model"

- The different *Map* strains studied here showed diverse immune responses and virulence profiles.
- The mouse model allowed us to select strains to test vaccine candidates.
- The less virulent strain (1543/481) induced a low immune response and failed to protect mice.
- The virulent strain (6611) protects mice when used as a live vaccine or as an inactivated vaccine.