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Letter to the Editor

Mycobacterium bovis deleted in *mce2* and *phoP loci* protects C57BL/6 mice against tuberculosis

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Introduction

Mycobacterium bovis (*Mb*), the causative agent of bovine tuberculosis (bTB), infects cattle and other animals, including humans. In this study we found that a *M. bovis* knockout strain in *phoP* and *mce2* operons protected C57BL/6 mice against virulent *M. bovis* challenge. The double mutant was significantly more attenuated than the parental *M. bovis* Δmce2 strain in immunodeficient mice.

Bovine tuberculosis is a chronic infectious disease caused by *Mycobacterium bovis* affecting cattle and other mammals, including humans. Infection in humans occurs when unpasteurized milk (or derivatives) is consumed or when people are in contact with infected cattle. Zoonotic TB is particularly relevant in many developing countries in which the bTB is highly prevalent and human populations usually live in close contact with domestic and production animals [1-3]. Therefore, bTB is a factor that undermines the development of the dairy and meat industry and also a threat for public health.

Given that the elimination of TB-cattle is economically not affordable in developing countries, cattle vaccination may represent an attractive intervention strategy to reduce the impact of bTB on livestock. To date, however, there is no available vaccine against bTB.

Bacillus Calmette–Guérin (BCG) is the vaccine against human tuberculosis. It is a live *M. bovis* that has lost its virulence and has been widely used since 1945. However, the efficacy of BCG to protect against tuberculosis in humans has shown to be highly variable and dependent on different factors, such as the genetic of the subject population, environmental factors and the BCG substrain diversity [4]. In cattle, vaccination with BCG has demonstrated reductions in disease severity after experimental challenge with virulent *M. bovis* strains but the protection induced was, in general, not complete [5]. Apart of BCG, very few attenuated *M. bovis* strains have been used as experimental vaccine against bTB and some of them have shown to confer protection against *M. bovis* challenge in animal models [6,7]. In previous studies we reported the development of a *M. bovis* strain deleted in the virulence genes *mce2A-B*, the strain was called MbΔmce2. This strain was superior to BCG to protect cattle against bTB [9] and was unable to produce tuberculosis in cattle [10], which suggests that in cattle this is a safety vaccine. However, the Geneva consensus criteria [11] have established that TB vaccine must be based on two independent stable deletion mutations without antibiotic resistance markers. Therefore, to fulfil part of the requirements for a live TB candidate vaccine, we deleted the *phoP* virulence gene from MbΔmce2 [12]. The election of *phoP* as gene target for knockout was based on the fact that a *Mycobacterium tuberculosis* mutant in the two-component system PhoP-PhoR have shown successful results and is the first live attenuated *M. tuberculosis* vaccine to enter in clinical evaluation [13]. We then reported that the double knockout in *phoP-phoR* and *mce2*A/B *loci* of *M. bovis* conferred protection against virulent *M. bovis* challenge in BALB/c mice [12]. In order to better characterize this double *M. bovis* mutant as bTB vaccine, here we evaluated its protection capacity in C57BL/6 mice and we compared its attenuation to that of the parental strain, *M. bovis* Δmce2, in immunodeficient BALB/c mice.

Results and discussion

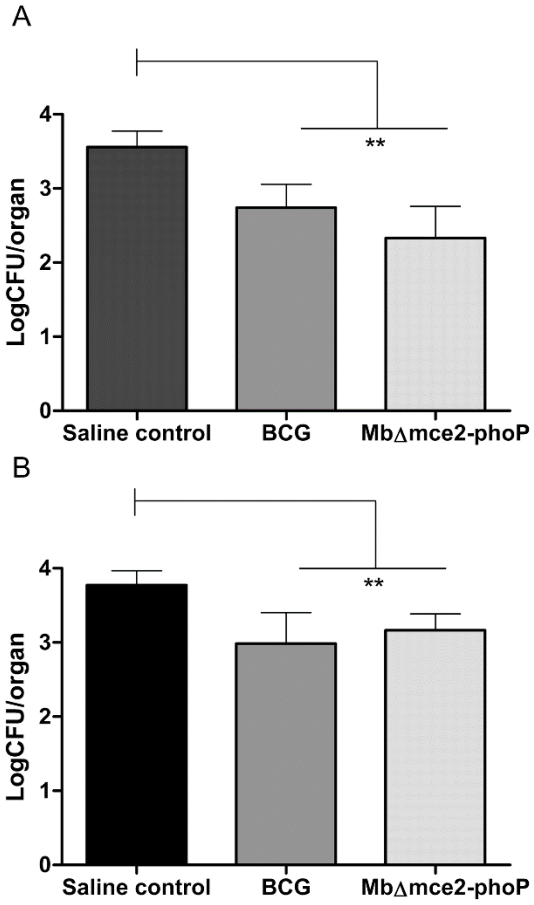
The *phoP* gene (Mb0780), *mce2A* (Mb0604) and *mce2B* (Mb0605) have been knocked out in *M. bovis* NCTC10772 genome, resulting in single (*phoP* or *mce2*) and double (*phoP* and *mce2*) *M. bovis* mutants [9,10,. When tested in BALB/c mice all vaccine strains, including BCG, have showed a reduced bacterial burden in organs after *M. bovis* challenge, as compared to non-vaccinated mice [12]. However, *Mb*Δmce2 has also been assessed as experimental vaccine in cattle, showing better protection than BCG [9]. In this study we evaluated the vaccine properties of the double *Mb*Δmce2-phoP mutant in C57BL/6 mice.

Female C57BL/6 mice (5-6 weeks old) were immunized subcutaneously with 1.10^5 colony forming units (CFU) in a single dose with BCG or *Mb*Δmce2-phoP mutant. A group was inoculated with saline solution as unvaccinated control. After 60 days post immunization the animals were infected with 5.10^4 CFU of virulent *M. bovis* NCTC 10772 by intratracheal instillation as previously described [12]. Thirty days after challenge all mice were sacrificed and CFU were determined in lungs and spleen.

As showed in Figure 1, all immunized mice were protected compared to the saline control ($p < 0.01$). The protection conferred by the *Mb*Δmce2-phoP mutant was equivalent to that of BCG in both spleen (Figure 1A) and lungs (Figure 1B). The fact that *Mb*Δmce2 has shown to be more effective than BCG to protect cattle [10] but not mice [12] illustrates the limitation of using mice to test anti-tuberculosis vaccine candidates. However, the mouse is a widely accepted model of TB because reproduces the disease in the lungs, is cheaper than other alternative models and a large number of knock out strains and immunological reagents are available for investigation. It has been demonstrated that C57BL/6 mice control the TB infection better than BALB/c mice [14]. Therefore, the findings of this study reinforce the protective attributes of *Mb*Δmce2-phoP in a bTB-restrictive environment.

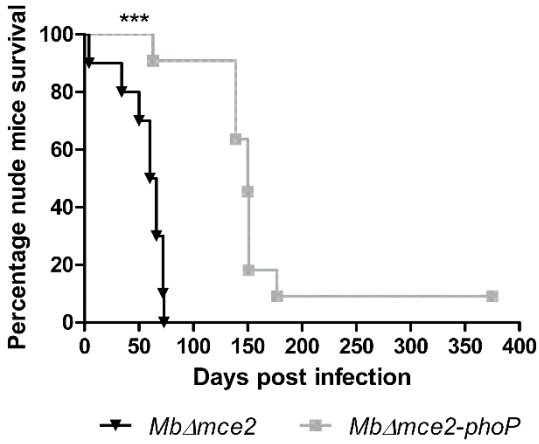
We next compared the virulence of the double *Mb*Δmce2-phoP mutant to that of the single *Mb*Δmce2 mutant in order to know whether or not the elimination of the virulence *phoP* improves the safety properties of the candidate vaccine. For this purpose groups of female nude (N:NIH (S)- *Foxn1*^{nu}) BALB/c mice of 6–8 weeks of age were infected with 50,000 CFUs of *M. bovis* strains and the survival was subsequently assessed. The median survival of *Mb*Δmce2-phoP (150 days) was statistically different ($p < 0.001$) to that of the *Mb*Δmce2-infected animals (63 days) (Figure 2). This result demonstrates that *Mb*Δmce2-phoP is safer than

Figure 1. Protection assays of *Mb*Δmce2-phoP in C57BL/6 mice.



Groups of 10 mice were vaccinated with 1.10^5 CFU of BCG or mutant strain, and compared with control nonvaccinated animals. 30 days post-vaccination, mice were challenged by intratracheal route with 50,000 CFU of *M. bovis* and one month later the bacterial burden in spleens (A) and lungs (B) was determined. Columns show the mean values and error bars of CFU/organ. Data were analysed using one-way ANOVA analysis and Bonferroni's post-test. (**$p < 0.01$).

Figure 2. Virulence assays in nude mice.



Survival of nude mice after intratracheal inoculation *M. bovis* strains (5×10^5). Statistical analysis was performed using Mantel-Cox test. dpi: days after infection, CFU: colony forming units. (***$p < 0.001$).

the highly protective *Mb*Δmce2 vaccine [10]. It also confirms the role of PhoP, the transcriptional regulator of the two-component system PhoP-PhoR [10,7], in the virulence of *M. bovis* in immunodeficient mice.

Conclusion

Here we confirm the efficacy of *Mb*Δmce2-phoP to protect mice against bTB challenge and we demonstrated that this new attenuated strain is safer than our previous bTB vaccine *Mb*Δmce2. Altogether these results support the further assessment of this candidate in cattle model of bTB.

Experiments with mice were performed in compliance with the regulations of the Institutional Animal Care and Use Committee (CICUAE) of INTA (61/2014).

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