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 mce2A/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, *MbΔmce2* 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 *MbΔmce2-phoP* mutant in C57BL/6 mice.

Female C57BL/6 mice (5–6 weeks old) were immunized subcutaneously with 1.10⁵ colony forming units (CFU) in a single dose with BCG or *MbΔmce2-phoP* mutant. A group was inoculated with saline solution as unvaccinated control. After 60 days post immunization the animals were infected with 5.10⁴ CFU of virulent *M. bovis* NCTC10772 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 *MbΔmce2-phoP* mutant was equivalent to that of BCG in both spleen (Figure 1A) and lungs (Figure 1B). The fact that *MbΔmce2* 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 *MbΔmce2-phoP* in a bTB-restrictive environment.

We next compared the virulence of the double *MbΔmce2-phoP* mutant to that of the single *MbΔmce2* 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)-*Foxn1nu*) 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 *MbΔmce2-phoP* (150 days) was statistically different (p < 0.001) to that of the *MbΔmce2*-infected animals (63 days) (Figure 2). This result demonstrates that *MbΔmce2-phoP* is safer than

![Figure 1](image1.png)

**Figure 1.** Protection assays of *MbΔmce2-phoP* in C57BL/6 mice.

![Figure 2](image2.png)

**Figure 2.** Virulence assays in nude mice.

Survival of nude mice after intratracheal inoculation *M. bovis* strains (5×10⁵). Statistical analysis was performed using Mantel-Cox test. dpi: days after infection, CFU: colony forming units. (**p < 0.001).
the highly protective \(Mh\Delta mce2\) 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 \(Mb\Delta mce2\)-phoP to protect mice against bTB challenge and we demonstrated that this new attenuated strain is safer than our previous bTB vaccine \(Mh\Delta mce2\). 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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**References**


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