

T- and B-cell response analysis following calf immunisation with experimental *Mycoplasma bovis* vaccine containing saponin and lysozyme dimer

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Abstract

Introduction: *Mycoplasma bovis* is a well-known cause of various disorders in cattle, such as pneumonia, arthritis, mastitis, kerato-conjunctivitis, pharyngitis, laryngitis, otitis media, meningitis, and reproductive disorders. There are no commercial vaccines against *M. bovis* in Europe, therefore, experimental ones are still under investigation. The aim of this study was to evaluate the effect of experimental *M. bovis* vaccine, containing the Polish field *M. bovis* strain as well as saponin and lysozyme dimer adjuvants, on the T- and B-cell response in calves. **Material and Methods:** The study was carried out on 12 calves divided into two equal groups: experimental and control. The experimental group was subcutaneously injected with the vaccine composed of the field *M. bovis* strain as well as saponin and lysozyme dimer as adjuvants, whereas the control one received phosphate buffered saline (PBS). The blood samples were collected prior to the study (day 0), then in 24 h intervals up to day 7 and then each 7 days until day 84 post immunisation. The T- and B-cell response as CD2⁺ (T-cells), CD4⁺ (T-helper cells), CD8⁺ (T-cytotoxic cells), and WC4⁺ (B-cells) markers was analysed using flow cytometry. **Results:** In response to the immunisation, the general stimulation of T-cell was observed, the most seen in an increase in CD8⁺ subpopulation. Similarly, a visible rise in the percentage of WC4⁺ cells was registered in the vaccinated calves when compared to the control animals. **Conclusion:** This study demonstrated that the novel experimental *M. bovis* vaccine containing saponin and lysozyme dimer effectively stimulated the cell-mediated immunity in the calves.

Keywords: calves, *Mycoplasma bovis*, vaccine, saponin, lysozyme, T- and B-cells.

Introduction

Mycoplasma bovis is known as a causative agent of pneumonia, arthritis, mastitis, kerato-conjunctivitis, pharyngitis, laryngitis, otitis media, meningitis, and reproductive disorders in cattle (1, 9–11, 19–21). It is also the main pathogen of bovine respiratory disease (BRD) causing high morbidity and mortality in the livestock (5). Economic losses in the cattle breeding due to BRD in Europe reach approximately 600 million Euros per year (4). There is still a lack of commercial vaccines against *M. bovis* in Europe. Some commercial vaccines are available in the United States but most of them are intended to use in older calves and their efficacy is questionable, therefore experimental ones

are still under investigation (13, 18). Moreover, a decrease in antimicrobial susceptibility of *M. bovis* isolates was demonstrated, making the effective control of the infection difficult (2, 3, 22). Previously, saponin was successfully applied as an adjuvant and inactivator of mycoplasma in experimental *M. bovis* vaccines (6, 15), whereas the lysozyme dimer (Lydium-KLP™) was used to evaluate blood antioxidant status in pregnant heifers (12). In our previous and recent study Lydium-KLP™ was applied as a potential adjuvant for experimental *M. bovis* bacterin vaccine. The results of primary study indicated a visible stimulation of humoral immunity in the vaccinated calves (8). However, it is generally considered that the immune response dependent on specific antibodies to *M. bovis*

is not sufficient to eliminate the pathogen and overcome the potential infection, therefore it requires support from the cell mediated immunity (16).

The aim of this study was to evaluate the effect of experimental *M. bovis* vaccine containing saponin and lysozyme dimer adjuvants on the T- and B-cell response in calves.

Material and Methods

Animals. The study was carried out on 12 five-week-old clinically healthy female calves. After a two-week adaptive period, the animals were divided into two equal groups: experimental and control, housed in individual pens for each group and fed milk replacer twice a day and hay and water *ad libitum*.

Vaccine preparation and calf immunisation. The field Polish *M. bovis* strain (BankIt 1801634 MBovis KP795974) was cultured with a final concentration of 6.25×10^7 CFU/mL and mixed with saponin (Sigma-Aldrich, Germany) according to the procedure described by Dudek *et al.* (6). Next 2 mL of Lydium-KLP™ (Nika, Health Products, Poland) was added to 5 mL of the mycoplasma cells and the saponin mixture, and filled up with 1 mL of phosphate buffered saline (PBS) (pH 7.2) to a final volume of 8 mL. The vaccine was shown to be free of mycoplasma and bacteria contamination using a method previously described by Dudek *et al.* (6). The experimental group (S+L) was subcutaneously injected with the vaccine (8 mL per animal), whereas the control one received the same volume of PBS as the volume used for vaccine injection.

Samples. The blood samples were collected into a 1-mL vacutainer with EDTA-K2 anticoagulant prior to the study (day 0), then in 24 h intervals up to day 7 and then each 7 days until day 84 post immunisation and analysed immediately after collection.

Methods. The CD2⁺ (T-cells), CD4⁺ (T-helper

cells), CD8⁺ (T-cytotoxic cells), and WC4⁺ (B-cells) antigens were detected by specific mono- and polyclonal antibodies (Bio-Rad Laboratories Inc.) and analysed using a flow cytometer (Coulter Epics XL 4C, Beckman Coulter Company, USA) according to the procedure described by Dudek *et al.* (7).

Statistical analysis. The results are presented as arithmetic means \pm standard deviation. The differences between the mean values recorded in the S+L and control groups were analysed at the same time point using *t* test with a statistically significant level of $P < 0.05$.

Results

Following the immunisation no adverse reactions were observed in the vaccinated calves. The changes in the *M. bovis* specific antibody titres and total concentration of IgG, IgA, and IgM in the vaccinated calves were described in our previous study (8).

The percentage of CD2⁺ cells was visibly increased in the vaccinated group up to day 21 when compared to the controls. The rise was repeated between days 63 and 77, whereas on days 35 and 42 this percentage was lower than in the control group (Fig. 1). On days 6, 7, and 70 post immunisation, the CD4⁺ cell percentage was visibly higher than in the control. At the remaining days of the study this percentage was slightly lower or comparable to the controls (Fig. 2). In the vaccinated calves, the percentage of CD8⁺ cells was increased throughout the study when compared to the control, with the exception of day 3 when the values were comparable to the control group (Fig. 3). Starting from day 2 post immunisation, the WC4⁺ cell percentage was visibly higher than in the control up to the end of the study *i.e.* on day 84, with the exception of day 63 when the values were slightly lower than in the control group (Fig. 4).

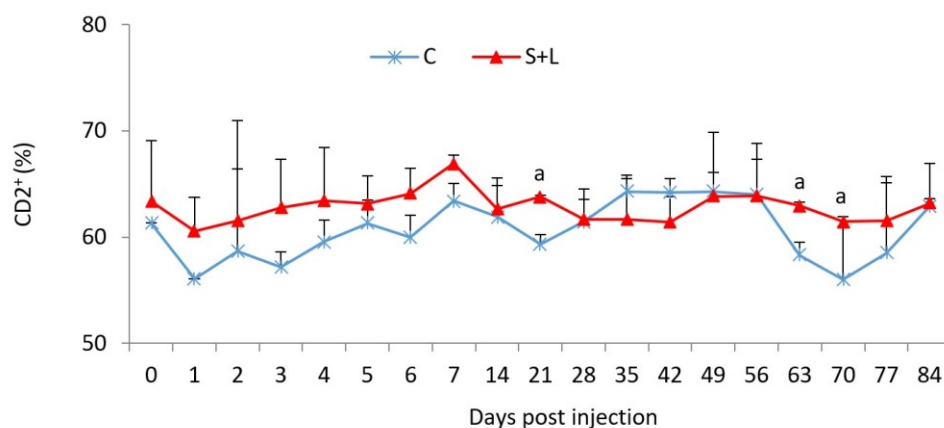


Fig. 1. Percentage of CD2⁺ cells in the blood of vaccinated (S+L) and control calves (C). a – $P < 0.05$ between groups C and S+L

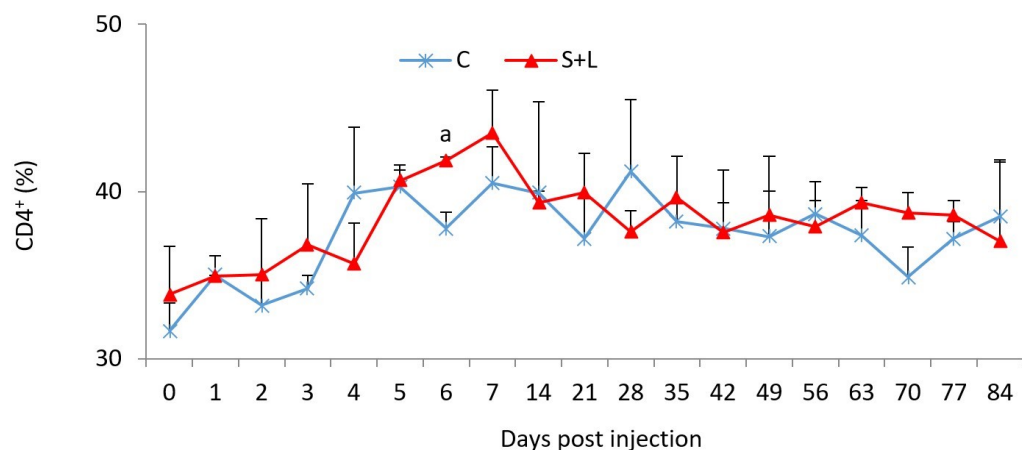


Fig. 2. Percentage of CD4⁺ cells in the blood of vaccinated (S+L) and control calves (C). a – $P < 0.05$ between groups C and S+L

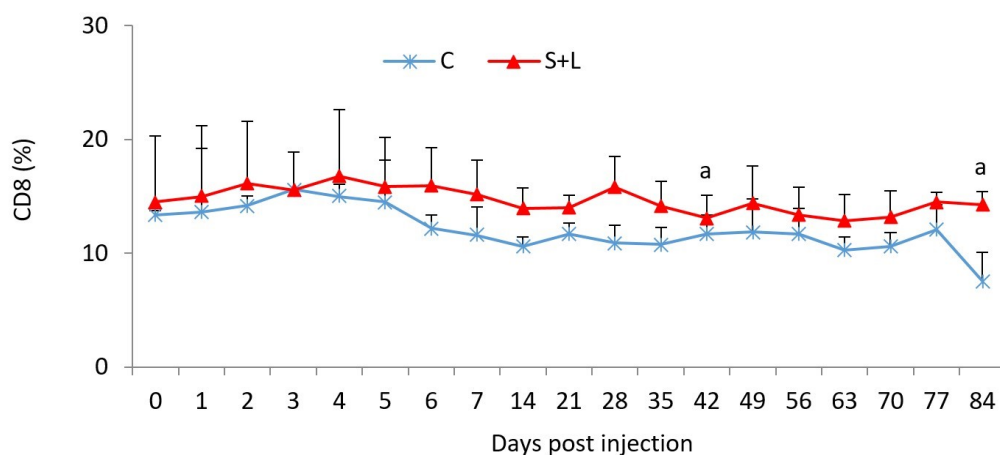


Fig. 3. Percentage of CD8⁺ cells in the blood of vaccinated (S+L) and control calves (C). a – $P < 0.05$ between groups C and S+L

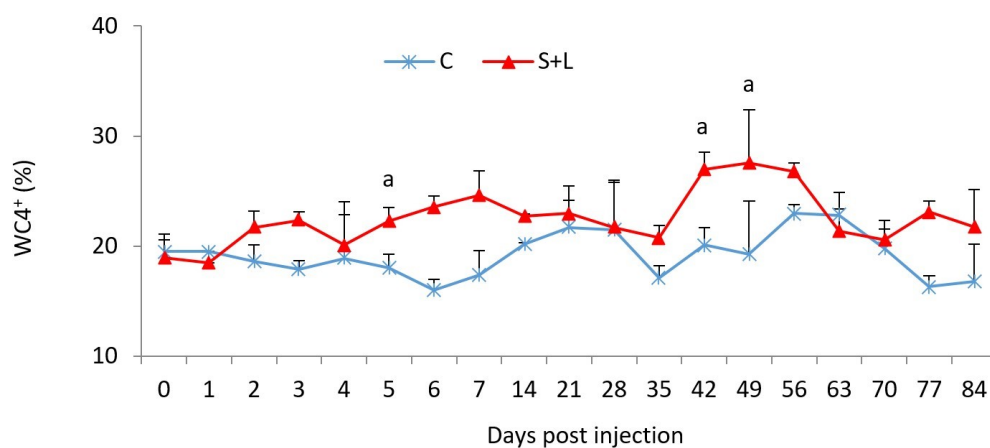


Fig. 4. Percentage of WC4⁺ cells in the blood of vaccinated (S+L) and control calves (C). a – $P < 0.05$ between groups C and S+L

Discussion

Our novel experimental *M. bovis* vaccine containing saponin and Lydium-KLP™ adjuvants was previously examined for selected parameters of humoral immunity in calves (8). The cited study demonstrated a general activation of humoral immune response in the vaccinated animals, which was the most evidenced in markedly increased titres of specific *M. bovis* antibodies and stimulation of IgG production. Similarly, a strong stimulation of specific *M. bovis* antibody titres and increased IgG and IgA levels following calf immunisation was shown in our previous study, in which the vaccine comprised of the same *M. bovis* strain but formulated with saponin and Emulsigen as adjuvants (6). In the same study a strong humoral immunity was correlated with the protection of the vaccinated calves against *M. bovis* nasal colonisation and the pathogen dissemination in the host and also with visible reduction of clinical response to the experimental challenge. A positive correlation between the increased ELISA titres of *M. bovis* specific antibodies and the protective properties of saponin-based *M. bovis* vaccine was also demonstrated by Nicholas *et al.* (15). In contrast, results of other study showed that even strong humoral immune response following immunisation with total extracts and/or membrane fractions of *M. bovis* formulated with CpG ODN 2007/Emulsigen adjuvants was not sufficient to protect the vaccinated calves against the challenge with the pathogen (14). This state was confirmed by other study where a *M. bovis* sub-unit vaccine containing Emulsigen™ was used (17). Therefore, the cell-mediated immunity seems to play an important role in an elimination of *M. bovis* from the host.

The current study showed a stimulation of cell-mediated immunity in the vaccinated calves, depending on T- and B-cell response. A general activation of T-cells was manifested by a significant increase in the percentage of CD2⁺ cells following the immunisation. Detailed immunophenotyping of peripheral blood lymphocytes demonstrated some stimulation of the T-helper cells, especially during first days post vaccination and last days of observation between days 63 and 77. In contrast, the T-cytotoxic and B-cells were generally stimulated in the vaccinated calves throughout the study. In our previous study the same Polish *M. bovis* strain was used for calf challenge (7). The results of this study showed general T-cell suppression in the affected calves, especially concerning the CD8⁺ cell subpopulation. In contrast, some stimulation was demonstrated in the T-helper cells, whereas for the B-cell response it was evident. The two English *M. bovis* isolates examined in this study gave the strongest T-helper response and some T-cytotoxic cell stimulation, but this difference resulted from their affinity to the other common clonal complex which was obtained by MLST analysis (7). Both CD4⁺ and CD8⁺ cell activation was also observed following

incubation of peripheral blood mononuclear cells (PBMCs) derived from the calves inoculated with *M. bovis* with heat-inactivated *M. bovis* antigen (24). Another study demonstrated that *M. bovis* was able to adhere to the surfaces of T-cells and their subsets (T-helper and cytotoxic T cells) as well as B-cells and penetrate them to be found intracellularly (23). On the other hand, Mulongo *et al.* (14) showed changes in the antigen-specific lympho-proliferation of PBMCs derived from the calves vaccinated with both total extracts and membrane fractions of *M. bovis* or *M. bovis* membrane fractions alone, both formulated with CpG ODN 2007/Emulsigen adjuvants, depending on the kind of *M. bovis* antigen used for the cells stimulation. In response to both soluble extracts and membrane fractions of *M. bovis*, the lymphocyte suppression was observed in both vaccinated groups of calves. In contrast, the visible blastogenesis was induced when the PBMCs were stimulated with the *M. bovis* whole cells but only in the calves vaccinated with both total extracts and membrane fractions of *M. bovis* and only in the post-challenge samples. It is worth noting that in this study the lymphocyte proliferation was evaluated following the recall response to *M. bovis* antigen and the vaccinated calves were challenged with BHV-1 prior to *M. bovis* challenge (14). Based on these results it is possible to speculate that *M. bovis* whole cells are able to induce bovine lymphocyte proliferation as was demonstrated in our recent study (7).

In conclusion, the novel experimental *M. bovis* vaccine containing the field Polish *M. bovis* strain as well as saponin and Lydium-KLP™ adjuvants effectively stimulated the cell-mediated immunity in the vaccinated calves.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: The authors declare that the experiments on animals were conducted in accordance with laws and regulations of the 2nd Local Ethical Committee for Animal Experiments in Lublin as regards care and use of laboratory animals.

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