

Immunomodulatory effect of *Mycoplasma bovis* in experimentally infected calves

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Abstract

The effect of three different field isolates of *Mycoplasma bovis* on selected immunological parameters in experimentally infected calves was studied. Calves were kept separately in 4 experimental groups, and animals of 3 groups were infected intra-tracheally with one of the three selected isolates of *M. bovis*. The control group was inoculated intra-tracheally with sterile physiological saline. Nasal swabs and blood samples were collected just before the calf inoculation, then daily for seven days, and then weekly for another three weeks. The presence of *M. bovis* antigen, *M. bovis* antibodies, total protein, gamma globulins, IgA, IgM, IgG, acute phase proteins (haptoglobin and serum amyloid A), as well as interferon- γ and interleukin-4 concentrations were determined. *M. bovis* was detected intermittently during the study in the infected groups from day 1, whilst the control group remained free of the pathogen. *M. bovis* antibodies were detected in some of infected animals in the second, third, and fourth week after infection. The stimulation and/or immunological suppression varied between the *M. bovis* isolates used for the inoculation. All *M. bovis* isolates induced a rise of APP and gamma globulin concentrations in infected calves. However, in this study the mucosal immune response appeared to be down-regulated, which was expressed with a general lack of IgA stimulation.

Key words: calves, *Mycoplasma bovis*, immunological parameters.

Introduction

Mycoplasma bovis is one of the most widespread pathogens in cattle worldwide. Currently, New Zealand and Norway are the few countries that are believed to be free from infection (19). *M. bovis* is a causative agent of bronchopneumonia, arthritis, mastitis, keratoconjunctivitis, reproduction disorders, meningitis, and other clinical conditions (18). Additionally, its role in the aetiology of bovine respiratory disease (BRD) is well known. In many cases, *M. bovis* is considered as a primary agent of BRD, which may co-infect with other infectious agents of BRD, such as *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and others (8, 25). Houghton and Gourlay (11) reported that *M. bovis* is responsible for a colonising synergism with *M. haemolytica*, which may enhance morbidity and mortality.

It has been reported that *M. bovis* is responsible for the suppression of some immunological functions,

such as the production of pro-inflammatory agents (TNF- α and nitric oxide), inhibition of polymorphonuclear neutrophil degranulation, or induction of degenerative pathways such as an oxidative burst, or cell apoptosis (9, 12, 22, 28, 29). In contrast, a stimulating effect by *M. bovis* on gamma globulin production and acute phase response was observed in experimental and natural infections (4, 5).

The control of *M. bovis* infection is becoming increasingly difficult, as the organism appears to be less susceptible to the antibiotics routinely applied in treatment of BRD, and currently there is no commercially available vaccine against this infection in Europe (16). To improve the control methods and vaccine development, more information about the immunological mechanisms associated with *M. bovis* infection is required. Therefore, in this study the immunomodulatory effect of three different field isolates of *M. bovis* was examined in experimentally infected calves.

Material and Methods

The study was carried out on 24 calves of Black and White breed, aged approximately five weeks, with average body weight of 62 kg. No clinical signs of pneumonia were observed in the maternal herds. The calves were kept in the Institute's vivarium (room temperature of 18-20°C, air relative humidity of 60%) with no contact with other animals outside. They were housed in individual isolated pens. There was no evidence of *M. bovis* infection (the data based on the initial mycoplasma laboratory examination using microbiological and serological methods before the proper experiment). Moreover, other respiratory bacteria like *Pasteurella* sp. were not isolated from the nasal swabs of the calves. The adaptation period of the calves to new environmental conditions lasted two weeks. The calves were fed milk replacer and had an access to fodder and water *ad libitum*.

The calves were divided into four groups of six animals housed in individual pens: experimental E1, E2, E3, and the control group. Experimental procedures and animal management protocols were undertaken according to the detailed requirements of the Polish Local Ethic Committee for Animal Experimentation, which were in agreement with the EU regulations.

The identity and purity of three isolates of *M. bovis* used in the study were evaluated using the PCR/DGGE method (14). They were: E1 from Polish cow with mastitis; E2 from England, isolated from the lung of a five-month-old calf with pneumonia; and E3 from England, isolated from bovine foetal stomach contents. The Polish isolate (E1) was successfully used in experimental challenge in calves, which was confirmed during the preliminary study. Before the challenge, each isolate was grown in Eaton's medium at 37°C with 5% carbon dioxide for five days (17). The mycoplasma cultures were centrifuged at $10\,000 \times g$ for 40 min and then resuspended in sterile phosphate buffered saline (pH 7.4 ± 0.2). The experimental calves were inoculated intra-tracheally with 23 mL of 1.98×10^7 colony forming units (cfu)/mL of E1; 1.65×10^7 cfu/mL of E2; and 1.58×10^7 cfu/mL of E3. The calf inoculation was done using the catheter adopted for that purpose, which was inserted into the right bronchus by the nasopharyngeal cavity and the trachea, and then with lobar bronchus to the end of the diaphragmatic lobe of the right lung. Before the calf inoculation, the mycoplasma suspension was brought to the ambient temperature and then inserted by the catheter into the lung. The control group received 23 mL of sterile phosphate buffered saline (pH 7.4 ± 0.2) administered by the same route.

Before and during the experiment rectal temperature and selected respiratory parameters were recorded (cough, value, kind of nasal discharge, dyspnoea etc.). Nasal swabs and blood samples were collected just before the calf inoculation, then daily for seven days, and then weekly for the next three weeks.

Deep nasal swabs were collected and cultured for mycoplasmas in Eaton's medium as described previously (17). In addition, the antigenic ELISA kit (Pulmotest; Bio-X Diagnostics, Belgium) was used for the detection of *M. bovis*. The blood samples were collected from the *vena jugularis externa*. The indirect ELISA (Bio-X Diagnostics, Belgium) was used for the detection of antibodies specific to *M. bovis*. Further analyses were performed using commercial kits as follows: total protein (BioSystems S.A. Spain); total gamma globulin (Bio-X Diagnostics, Belgium); gamma globulin with class differentiation (for IgA, IgM and IgG) (Bethyl Laboratories, Inc. USA); acute phase protein - haptoglobin (Hp), serum amyloid A (SAA) concentrations (Tridelta Development Ltd, Ireland); interferon- γ (Bio-X Diagnostics, Belgium); and interleukin-4 (IL-4) activity (Wuhan EIIAAB Science CO., Ltd. China). The results were calculated according to the manufacturer's instructions.

The results are presented as arithmetic means. The differences between the mean values recorded in the experimental and control groups were analysed using the *t*-test with a statistically significant level of $P < 0.05$.

Results

No increase in rectal temperature ($\leq 39.5^\circ\text{C}$), neither respiratory disorders were observed in all calves before the inoculation, and in the control animals throughout the study. The significant increase in rectal temperature ($> 40.5^\circ\text{C}$) of all inoculated groups of calves was recorded from the first day after inoculation and continued for further few days (the first week of the study).

The respiratory problems, such as serous-mucosal and mucosal nasal discharge and coughing were observed in the calves of inoculated groups few days after challenge, similarly to the duration of increased rectal temperature.

Isolates were detected and identified in all of the calves in the inoculated groups the day after challenge, and at different time points of the experiment, as detailed in Table 1. All calves lacked specific antibodies to *M. bovis* at the beginning of the experiment, but antibodies developed in some of the animals of the inoculated groups from day 14 to day 28, as shown in Table 2. The control group remained negative.

For the first two days following inoculation, the total protein concentration decreased in all of the challenged groups of calves when compared to the control. The decrease was statistically significant in the E3 group, from the first day following inoculation in different time points, until the end of the study ($P < 0.05$). Between the fifth and 21st day of observation, the decrease in total protein concentration in the animals of group E2 was noticeable, but it was not statistically significant when compared with the control animals (Fig. 1).

Table 1. Isolation and identification of *Mycoplasma bovis* from nasal swabs of calves inoculated with different strains of *M. bovis*

Calf group with an individual animal No.	Days after inoculation										
	0	1	2	3	4	5	6	7	14	21	28
C											
1	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-
E1											
1	-	+	+	-	-	-	-	-	+	+	+
2	-	+	+	+	+	+	-	+	+	+	+
3	-	+	+	+	+	+	+	+	+	+	+
E2											
1	-	+	+	+	-	+	+	-	-	+	-
2	-	+	+	+	+	+	-	-	+	+	+
3	-	+	+	+	+	+	+	+	+	+	+
E3											
1	-	+	+	+	-	+	+	+	-	+	+
2	-	+	+	+	+	-	+	+	+	+	+
3	-	+	+	+	+	+	+	+	-	-	+

+ the presence of specific mycoplasmal colonies (culturing) and antigen (Pulmotest) - identical results

- the lack of specific mycoplasmal colonies (culturing) and antigen (Pulmotest) - identical results

C - control calves; E1, E2, E3 - the first/second/third subgroup of experimental calves, respectively

Table 2. The presence of *Mycoplasma bovis* antibodies in sera of calves inoculated with different strains of *M. bovis*

Calf group with an individual animal No.	Days after inoculation										
	0	1	2	3	4	5	6	7	14	21	28
C											
1	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-
E1											
1	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	1+	2+
3	-	-	-	-	-	-	-	-	-	-	-
E2											
1	-	-	-	-	-	-	-	-	-	-	1+
2	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	1+	2+
E3											
1	-	-	-	-	-	-	-	-	1+	1+	1+
2	-	-	-	-	-	-	-	-	2+	3+	3+
3	-	-	-	-	-	-	-	-	-	-	1+

+ the presence of specific *M. bovis* antibodies; 1+ - 4+ - degree of positivity from + to ++++- the lack of specific *M. bovis* antibodies

A significant difference ($P < 0.05$) was noticed in the serum gamma globulin concentration in group E2 on day 28. However, the other groups had levels similar to the control group, with a little higher values than in the controls in group E3 throughout the study, with the exception of the third day after inoculation. This difference was statistically significant ($P < 0.05$) on day two post-inoculation (Fig. 2).

The concentration of IgG in sera of E3 calves was similar to the control values throughout the study. In the E1 and E2 groups, IgG concentration was lower than in the control group until the sixth day after inoculation, with E1 being the lowest. By day seven, the IgG concentration stabilised and was similar to all other groups until the end of the study (Fig. 3).

The serum IgM concentration was higher in group E3 for the first seven days, but remained at that level, whilst groups E1 and E2 had lower concentration of this immunoglobulin than E3 and the controls during that period, but then their level increased. Calves from E1 group had the highest IgM concentration at days 14 and 28. However, these differences were not statistically significant (Fig. 4).

For all of the challenged groups, the serum IgA concentration was similar to the controls until the sixth day following inoculation. The IgA concentration then increased in control and E1 group steadily until the end of the experiment, while in E2 calves it increased to a lesser extent. The IgA concentration in E3 group increased at day 14 and then declined (Fig. 5).

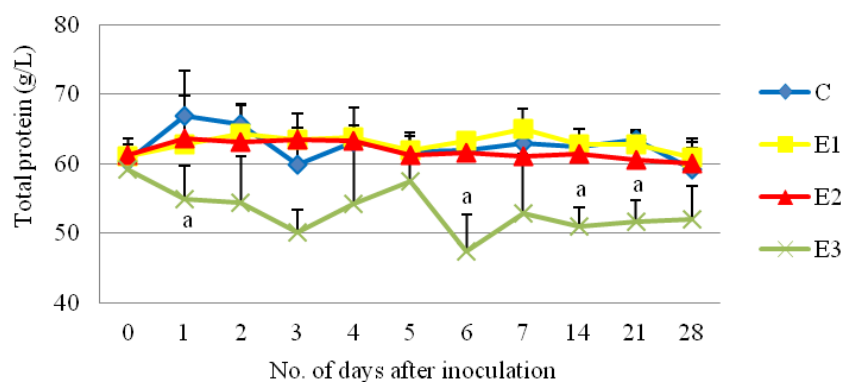


Fig. 1. Total protein concentration in sera of calves inoculated with different strains of *Mycoplasma bovis*
a - significant differences at $P < 0.05$ between E3 and control groups

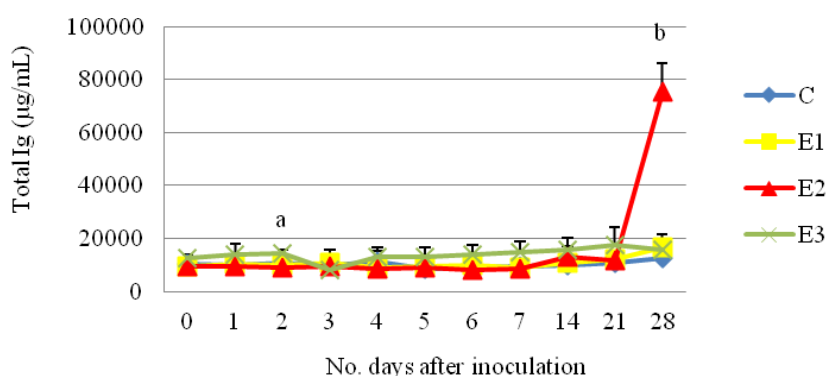


Fig. 2. Total gamma globulin concentration in sera of calves inoculated with different strains of *Mycoplasma bovis*
a - significant differences at $P < 0.05$ between E3 and control groups; b - significant differences at $P < 0.05$ between E2 and control groups

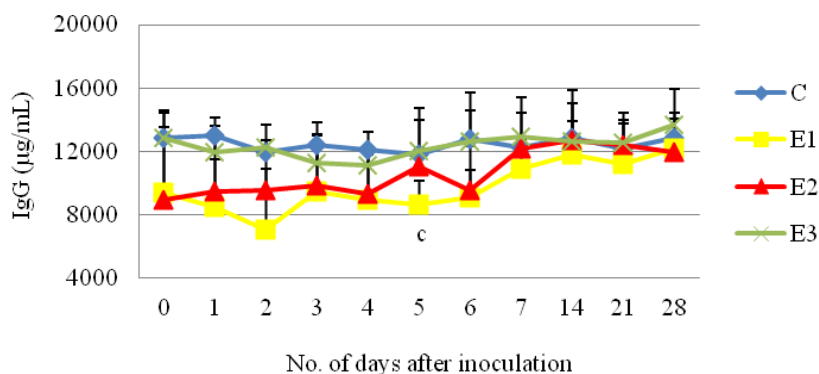


Fig. 3. IgG concentration in sera of calves inoculated with different strains of *Mycoplasma bovis*
c - significant differences at $P < 0.05$ between E1 and control groups

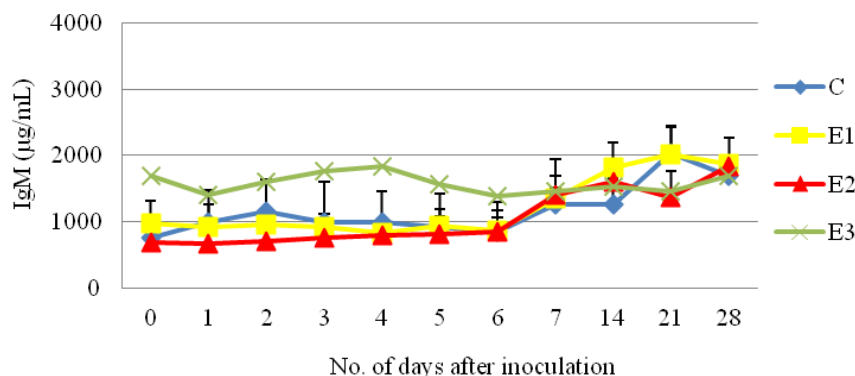


Fig. 4. IgM concentration in sera of calves inoculated with different strains of *Mycoplasma bovis*

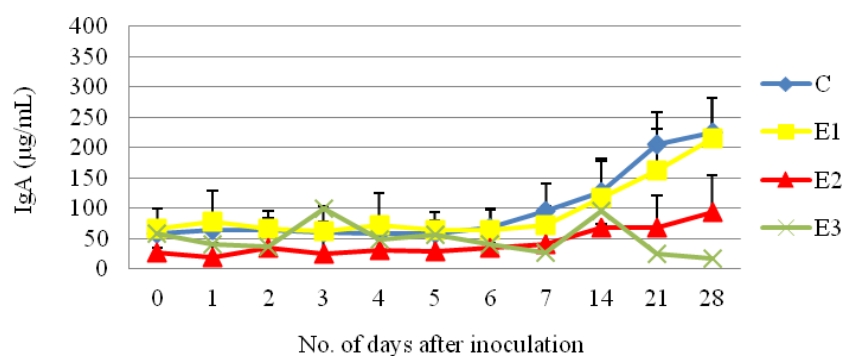


Fig. 5. IgA concentration in sera of calves inoculated with different strains of *Mycoplasma bovis*

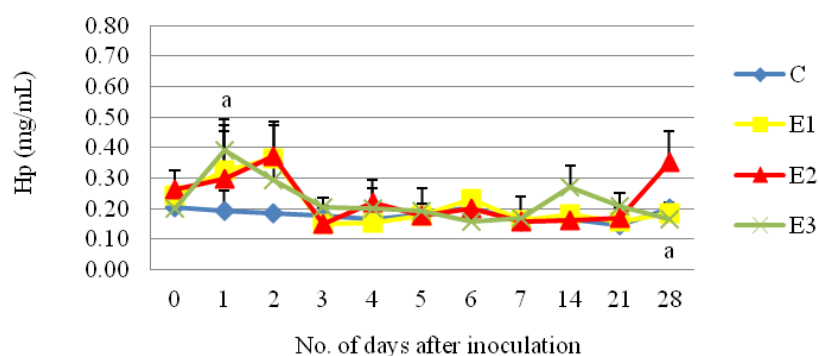


Fig. 6. Hp concentration in sera of calves inoculated with different strains of *Mycoplasma bovis*
a - significant differences at $P < 0.05$ between E3 and control groups

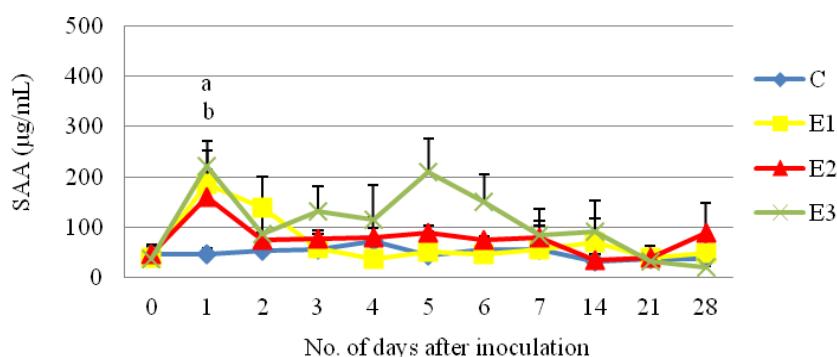


Fig. 7. SAA concentration in sera of calves inoculated with different strains of *Mycoplasma bovis*
a - significant differences at $P < 0.05$ between E3 and control groups; b - significant differences at $P < 0.05$ between E2 and control groups

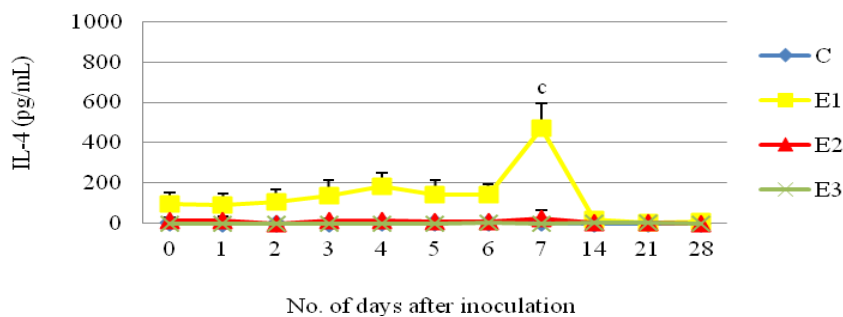


Fig. 8. IL-4 concentration in sera of calves inoculated with different strains of *Mycoplasma bovis*
c - significant differences at $P < 0.05$ between E1 and control groups

All of the experimental calves had higher Hp concentrations than the control group for the first two days following inoculation. There were significant differences in the E3 group on day one, and the levels remained higher until the fifth day, and were then higher on the seventh, 14th, and 21st day following inoculation. On day 28, the Hp concentration in E3 calves was significantly lower ($P < 0.05$) than in the controls. The E2 group had higher Hp values when compared to the control on days four, 21, and 28 following challenge. However, these differences were not statistically significant. Hp concentrations in E1 group were similar to the controls, but with a slightly lower value on day four (Fig. 6).

For the first three days, the SAA concentrations in the challenged calves were higher than in the controls. A significant difference ($P < 0.05$) was observed in the E2 and E3 groups on day one following inoculation. Increased values were recorded for the E3 group until the 14th day, whereas for the E2 group SAA concentrations were higher than in the control group up to day seven and also on day 28. In the E1 group, the concentration remained higher than in the control group on days 14 and 28. On the other days in all the groups SAA values were similar to, or were slightly lower than in the control group (Fig. 7).

In the E1 group, the serum IL-4 concentration was higher than in the controls during the whole experimental period with a significant increase on day seven ($P < 0.05$) following inoculation. In the E2 group, a slight increase in the IL-4 production was observed when compared with the controls. The E3 group values were similar to the controls throughout the study (Fig. 8).

Following the challenge with the different isolates of *M. bovis*, no changes in IFN- γ concentration were found and remained at zero level in both challenged and control calves.

Discussion

In 2011, the seroprevalence of *M. bovis* in Polish cattle affected with BRD was reported in 64.3% from the 841 serum samples tested (6). Reports from other countries confirm the role and importance of *M. bovis* infection in cattle affected with BRD (1, 3, 13, 21, 25). The clinical signs and disease caused by *M. bovis* can be various. Chronic nature of the pathogen has been associated with its ability to survive inside lesions and then cause reinfections (23).

In this challenge study, three different *M. bovis* isolates were used to assess the host response using a number of parameters, which included immunoglobulins, acute phase proteins, and some cytokines. *M. bovis* was detected in all of the challenged calves during the study, and the majority of calves had a specific anti-*M. bovis* immune response detected by day 28 of the study. This indicated that the challenge

was successful in causing infection with all three isolates. However, the immune response determined using different parameters measured in this study varied between the different isolates. In calves of the E3 group, the total protein concentration fell after challenge and remained lower than in the animals challenged with the other isolates and the control group throughout the study. In contrast, the total gamma globulin concentration in E3 group was higher throughout the study with the exception of day three; whereas in the E2 group it increased substantially comparing to the other groups at day 28, although an increase was recorded in all groups at day 28. IgG production was stimulated in all of the challenged groups around day seven. The IgG values in E1 and E2 groups were lower than in controls, which was probably due to the lower initial concentration. The data presented by Vanden Bush and Rosenbusch (28) indicated a prevailing stimulation of IgG1 production rather than IgG2 in an *M. bovis* experimental infection. Some stimulation of IgM production was observed in the E1 group of calves, whereas in other groups IgM values were generally lower than in the controls. For IgA, on the first day of the study, there was a slight increase in concentration in the E1 group, and on the third day in the case of E2 group. Otherwise, the IgA concentration in these groups was lower than in the controls, although it increased from day seven to 28, with a single peak on day 14 in E3 calves. Therefore, this data indicates that the mucosal system is down-regulated rather than stimulated during *M. bovis* infection in calves.

Previously, a stimulatory effect of *M. bovis* experimental infection on an acute phase response and arachidonic acid cascade changes in the affected calves was confirmed. The challenge caused a significant increase of Hp concentration until the seventh day after the infection, whereas a stimulation of SAA synthesis and proinflammatory arachidonic mediators (eicosanoids: PGE₂, PGF_{2 α} , TXB₂, LTB₄) was noted throughout the study, up to the ninth day of observation (5). In this study, a significantly higher Hp concentration in all of the challenged groups of calves than in the controls was recorded earlier, on days one and two, with an increase at different time points, between the fourth and 28th day for each of experimental groups of calves. It is known that Hp is a slow reactive acute phase protein and its rise is not generally observed until 24 h after the action of the pro-inflammatory factor, whereas the peak values occur between 72 and 96 h. Stefaniak (26) reported that increased concentrations usually return to the reference values between the eighth and 14th day after initiation of the inflammatory process. In this study in spite of higher Hp concentrations than the reference values in all calves also before the challenge, a distinct stimulation of Hp production was found after inoculation with *M. bovis* when compared to the controls, even on day 28. SAA is the second acute

phase protein, which has a great diagnostic significance in cattle (20). It is a fast reactive protein, and its concentration usually increases within 6–8 h, and peaks at between 24 and 48 h, after the initiation of the pro-inflammatory process. The enhanced response returns to the reference values within a few days (26). Here we observed a significant increase in SAA concentration in all challenged groups of calves on day one following challenge with *M. bovis*. Considerably higher concentrations of SAA than the control values were still observed in the E3 and E2 groups on the 14th and 28th day after the challenge, respectively.

Changes in Hp and SAA concentrations were previously reported in response to various bovine infections, including *Mannheimia haemolytica*, BRSV, and BVD/MD (20). The diagnostic significance of Hp in cattle was also reported for enteritis, peritonitis, endocarditis, endometritis, and abscesses (15, 20). In most clinical cases, Hp has inhibitory effect for granulocyte chemotaxis, phagocytosis, bactericidal activity (24), and T-cell or mast cell proliferation (2, 7). It may, therefore, be anticipated that a stimulation of Hp production during the *M. bovis* infection would impair those immune functions, and allow the progress of infection. The immunomodulatory activity of SAA during inflammation is less known, but some studies indicate its inhibitory effect on lymphocyte and endothelial cell proliferation, platelet aggregation, and T lymphocyte adhesion (27). SAA is also responsible for chemotaxis of T cells and neutrophils (10, 30) and modulation of some neutrophil functions, such as myeloperoxidase release and O₂ production (10).

In this study, a significant stimulation of IL-4 was only observed in calves of E1 group. The stimulation could result from a higher IL-4 concentration than the control values released following the challenge. However, the major differences were observed on the seventh day and it coincided with the increased synthesis of the cytokine as a result of the *M. bovis* infection. In contrast, the IFN- γ production was not demonstrated in calves after challenge with *M. bovis*. The data presented by Vanden Bush *et al.* (28) showed a significant increase in peripheral blood mononuclear cells (PBMCs) producing both IL-4 and IFN- γ isolated from *M. bovis* infected calves. However, that was probably an antigen specific immune response as those cells were incubated with heat inactivated *M. bovis* antigen.

The inoculation with different isolates of *M. bovis* in calves caused both stimulation and suppression of immune response, depending on the *M. bovis* isolate used. However, all *M. bovis* isolates induced a rise of APP and gamma globulin concentrations in infected calves.

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