EFFICACY OF A KILLED VACCINE (SILIRUM®) IN CALVES CHALLENGED WITH MAP

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ABSTRACT

A killed vaccine against bovine paratuberculosis (SILIRUM®, CZ Veterinaria) was evaluated in calves experimentally challenged with *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Ten calves out of 18 were injected subcutaneously when two months old with a single dose of the vaccine. The remaining 8 calves were vaccine controls, injected with PBS. Two months after vaccination 8 and 6 calves (from the vaccinated and control groups respectively) were challenged with 6 doses of 6.9×10^{10} cfu of MAP. Peripheral cellular and humoral immune responses were assessed as well as MAP fecal shedding between 0 and 330 dpv. Three vaccinated and two control calves were slaughtered at 180 dpv and the remaining 13 calves at 330 dpv. Pathologic and bacteriologic evaluation of intestine and lymph nodes samples were completed. The number of granulomas was counted in sections from both locations. No adverse reactions to the vaccine were observed in any of the calves. Humoral responses appeared in vaccinated groups at 90 dpv, whereas cellular responses were detected at 30 dpv, reaching the highest values at 120 dpv. A significant reduction in the number of granulomas present in the tissues was observed in vaccinated calves. These calves showed either no or only focal lesions confined mainly to the lymphoid tissue, except in one case of the diffuse form of infection. In non-vaccinated control calves, diffuse lesions extended to the intestinal mucosa. In this study the administration of a single dose of SILIRUM® in calves was safe and able to control the progression of disease. Vaccinated calves had fewer lesions, less severe lesions and a lower tissue burden of MAP than unvaccinated calves. Fecal shedding of MAP was not detected in any animal.

Key words

Paratuberculosis, calves, vaccination, SILURUM®, experimental study.

INTRODUCTION

Vaccination has been considered as a successful measure for controlling paratuberculosis. After its first description at the beginning of the last century (Vallée and Rinjard, 1926), different studies assessed the efficacy of vaccination in cattle (Wilesmith, 1982; Benedictus *et al.,* 1988) or small ruminants (Nisbet *et al.,* 1962; Pérez *et al.,* 1995; Corpa *et al.*, 2000b), by the evaluating the number of clinical cases or the level of fecal excretion.

The most commonly used vaccines are suspended in mineral oil to provoke a higher and more persistent immune response. The major disadvantage of this type of vaccine is the formation of a subcutaneous nodule at the site of inoculation that can be large or even ulcerated (Doyle, 1964; Chiodini *et al.,* 1984).

The subcutaneous injection of either killed or live vaccines induces both cellular and humoral peripheral immune responses (Juste *et al.,* 1994; Corpa *et al.,* 2000a) and reduces the number of animals that develop clinical disease and the level of MAP excretion (Wilesmith, 1982; Merkal, 1984; Benedictus *et al.,* 1988; Körmendy, 1994). Other studies (Kalis *et al.,* 2001) indicate that hygienic measures and the culling of shedding animals can add to vaccination efficiency.

Different experimental studies have shown that paratuberculosis vaccination does not prevent the actual infection of animals (Nisbet *et al.,* 1962; Larsen *et al.,* 1974; Juste *et al.,* 1994) but instead modifies the response to infection by limiting the progression of granulomatous lesions. Pathological methods have been previously used to assess the efficacy of vaccines mainly in small ruminants (Nisbet *et al.,* 1962; Juste *et al.,* 1994), focusing on the presence, extension and type of lesion. Recently, a classification of natural paratuberculosis lesions in sheep (Pérez *et al.,* 1996)

and cattle (González *et al.,* 2005) associates the type of lesion with the phases of MAP infection and the resultant immune response. Infected animals may show a range of pathology from focal forms (with lesions confined exclusively to the intestinal or lymph node lymphoid tissue linked to initial or latent forms of the infection and high cellular immune responses) to diffuse forms that display a severe granulomatous enteritis affecting different areas of the intestine. The latter are associated with clinical disease and high humoral (antibody) immune responses in most animals.

The main aim of this study has been the assessment of the safety and efficiency of a killed vaccine made with highly refined mineral oils as adjuvant in experimentally challenged calves.

MATERIALS AND METHODS

Animals

Eighteen two-month-old Friesian calves were used. They were selected from a tuberculosis- and paratuberculosis-free herd in which no clinical cases of paratuberculosis had been reported for 10 years. The calves were the offspring of Johne's disease test-negative dams.

Vaccination

When two months old, ten of the calves were subcutaneously injected with one dose of 1 ml of SILIRUM® (CZV, Porriño, Spain in the brisket) (V); the non-vaccinated (NV) control group (8 calves) received 1 ml of sterile saline solution. SILIRUM® is a heat-inactivated vaccine containing 2.5 mg of the culture of strain 316F of MAP combined with an immunological adjuvant consisting of highly refined mineral oil.

MAP Inoculation

Two months post-vaccination, 8 vaccinated animals and 6 not vaccinated animals were inoculated with 6 oral doses of 6.9×10^{10} cfu of a virulent wild-type strain of MAP at 2 day intervals (VI vs. NVI). This challenge strain was directly isolated from the intestinal mucosa of a bovine clinical case. Two animals from the vaccinated group were kept as vaccination controls (VNI), and the remaining two calves from the control group (NVNI) served as negative controls.

Humoral immune responses

Post-vaccination and post-infection antibody response was measured by indirect ELISA in sera samples taken before vaccination (day 0) and at 15, 30, 45, 60, 90, 105, 120, 135, 150, 180, 210, 240, 270, and 330 days post-vaccination (dpv). This ELISA was performed following the methodology described by Pérez et al. (1997) and González et al. (2005), using the MAP protoplasmatic antigen PPA-3 (Allied Lab, Fayette, Missouri, USA) and protein G as a secondary antibody. The optical density (OD) result was transformed to an index value by division of the mean OD for each serum by the mean OD for the positive control for each plate. The result was considered positive when the index value was equal to or greater than 800.

An agar-gel immunodiffusion test (AGID) using the PPA-3 antigen was performed as described by Pérez et al. (1997). Samples were considered positive when a clearly definable precipitation line of identity appeared with the reference serum.

Cellular immune response

The comparative intradermal tuberculin test (ITT) was carried out at 0, 45, 150, and 330 dpv by the injection of 0.1 ml of bovine PPD tuberculin (CZV, Spain) and 0,1 ml of *M. avium* PPD tuberculin (CZV, Spain) on the left and right sides of the neck, respectively. After 72 h, increases in skin thickness equal or greater than 2 mm were considered as a positive reaction. For the evaluation of the interference of vaccination with tuberculosis, the Annex B of Council Directive 64/432/ECC was applied.

The interferon-γ production test (IFN-γ) was performed with samples of whole blood obtained at the same dates as that for the humoral response, as described by Pérez et al. (1999), using both *M. avium* and *M. bovis* tuberculins and the BOVIGAM™ commercial kit (CSL Veterinary, Australia). The OD results were transformed to an index value by dividing the mean OD of the plasma from the *M. avium* and *M. bovis* PPD-stimulated blood by the mean OD of the same plasma incubated with PBSS. A result was considered positive when the index value was equal or greater than 2.

Pathology studies

At 180 dpv (120 dpi) three calves from the vaccinated-infected group (VI) and 2 calves from the non vaccinated-infected group (NVI) were killed by intravenous injection of barbiturate. The remaining calves were sacrificed at 330 dpv (270 dpv). Complete necropsies were performed and gross lesions were recorded with special attention to the gut and related lymph nodes. Samples for histopathologic examination consisted of duodenum, jejunum (a 5-cm sample from each of the cranial, intermediate and distal zones, each sample including areas with and without Peyer´s patches), ileum (three 5-cm samples, taken at 20, 40 and 60 cm from the ileocaecal valve), ileocaecal valve, caecum, colon, rectum, jejunal lymph nodes (two sections from the most caudal part), isolated ileal lymph

nodes (two samples), and ileocaecal lymph nodes (two samples). Tissues were fixed in 10% neutral buffered formalin and dehydrated through a graded alcohol series before being embedded in paraffin wax. Sections 4-μm thick were cut from each sample and stained with hematoxylin-eosin (H-E) and the Ziehl-Neelsen (ZN) method for acid-fast bacteria (AFB).

Lesion intensity was assessed by counting the number of granulomas present in three different sections of each sample from the intestine. The number of granulomas in the lymphoid tissue and in the lamina propria of the associated mucosa was counted separately.

Bacteriology studies

Faecal samples, collected at the same dates that blood samples, and tissue samples (ileocaecal valve, distal ileum, jejunal Peyer´s patches and ileal and jejunal lymph nodes), were cultured in Herrold medium, as indicated by González *et al.,* (2005). Isolates were identified as MAP on the basis of mycobactin dependency, acid-fast staining and the appearance of typical growth appearing at least after 6 weeks of incubation.

Polymerase chain reaction (PCR) in tissues

A PCR technique was performed for the same sites as used for culture using both frozen and paraffin-embedded samples by the method used by González *et al.,* (2005).

Statistical analysis

The results of the OD index obtained in the ELISA and IFN-γ tests were logarithmically transformed to make them suitable for analysis of variance. The means from the OD index of each diagnostic test and the results of granuloma count were compared among the groups, through a Student-t test at each time of sampling.

RESULTS

Clinical follow-up

No adverse clinical reactions to vaccination were observed in any case. One calf from VI group died suddenly due to an abomasal ulcer at 250 dpv.

Vaccination nodules

A persistent nodule at the injection site that did not affect the animals' overall health appeared at 15 dpv, reaching the largest size around 210 dpv (Fig. 1). All nodules were hard, cool, loosely attached and with a smooth surface. These nodules were round, oval or semi-spherical and showed no ulceration or other

adverse reactions at the inoculation site at any time during the study.

Fig. 1. Nodule evolution in cm for calves vaccinated with SILIRUM®
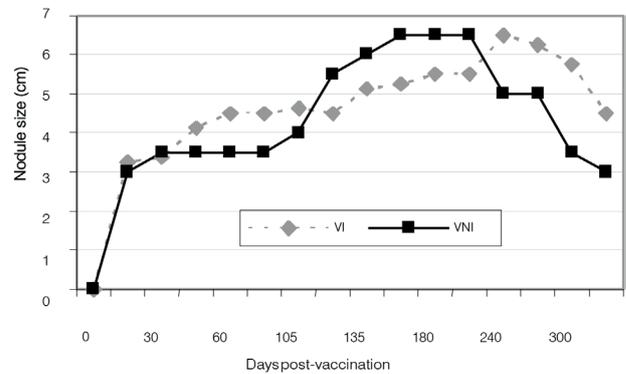
The size of the nodule is expressed as the maximum value in cm reached in any of its three dimensions. Data are the mean values for the VI (vaccinated, inoculated) and VNI (vaccinated, not inoculated) treatment groups.

The microscopic examination of the vaccination nodules at necropsy showed that they were mainly composed of various foci of caseous necrosis, with abundant neutrophils in the center. Often calcified and surrounded by a severe granulomatous reaction, they were formed by macrophages and giant cells plus abundant lymphocytes and a fibrous reaction. Only one cow from group VI had a smaller nodule and a less robust lymphocytic reaction in comparison with other calves from the group; this animal was found to have diffuse gastrointestinal lesions at necropsy.

Fig. 2. Development of antibody production in calves vaccinated with SILIRUM® and challenged with MAP at 60 dpv. by ELISA.
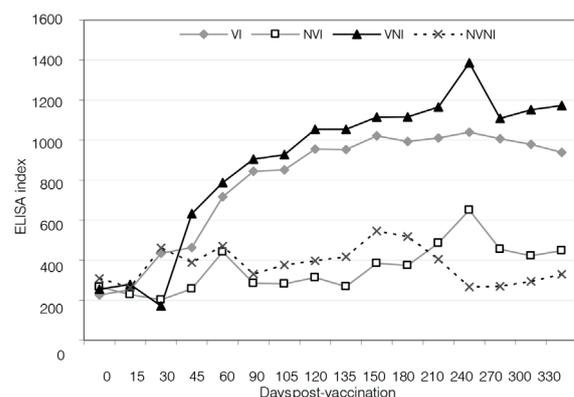
Humoral immune response

Vaccinated calves' antibody production by 45 dpv differed significantly from that of unvaccinated calves (Fig. 2). The OD level increased progressively up to 150 dpv. From then on, values remained high until the end of the experiment. In the group NVI, index values reached significant differences at 210 dpv.

Vaccinated calves were ELISA-positive (index $\geq$ 800) at 90 dpv whereas the non-vaccinated, inoculated (NVI) animals never reached the threshold interpreted as antibody ELISA positive.

Only vaccinated calves were AGID positive (data not shown). This occurred at 60 dpv and was at a lower percentage than by ELISA.

Cellular immune responses

At 30 dpv, vaccinated calves IFN-γ production was noted after stimulation with *M. avium* PPD (Fig. 3). Production remained significantly higher than in the controls up to about 240 dpv. An increase in this cytokine after the stimulus with *M. bovis* PPD was also observed, although the values were not as high. The unvaccinated animals produced little to no detectable IFN-γ except for inoculated calves at one sampling period (120 dpv; 60 dpi).

For the comparative intradermal tuberculin test, all vaccinated animals were test-positive every time they were tested (Fig 4). The level of response decreased progressively however. A positive reaction against *M. bovis* PPD tuberculin was also observed, but it was always weaker than the response to *M. avium* PPD. In the unvaccinated calves (groups NVI, NVNI), a positive response was observed at 150 dpv (90 dpi) to both tuberculins, and again response to *M. avium* PPD was significantly stronger.

Fig. 3. IFN-γ production using *M. avium* PPD in calves vaccinated with SILIRUM® and challenged with MAP at 60 dpv. Results are expressed as an OD index. Positive: index $\geq$ 2.
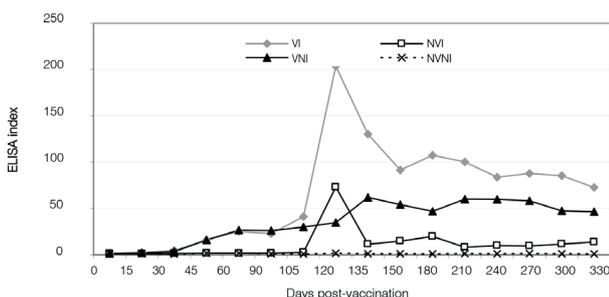
Taking into account the interpretation criteria used in the tuberculosis eradication programs, none of the vaccinated calves would have had a positive result for tuberculosis, since in each case the reactions to *M. avium* PPD were stronger than those to bovine PPD.

Fig. 4. ITT results in calves vaccinated with SILIRUM® and challenged with MAP at 60 dpv. Data are expressed as skin increase in mm recorded at 72 h after inoculation with the tuberculins, at 0, 45, 150 and 330 dpv. Av: reaction to *M. avium* PPD; Bv: reaction to *M. bovis* PPD.
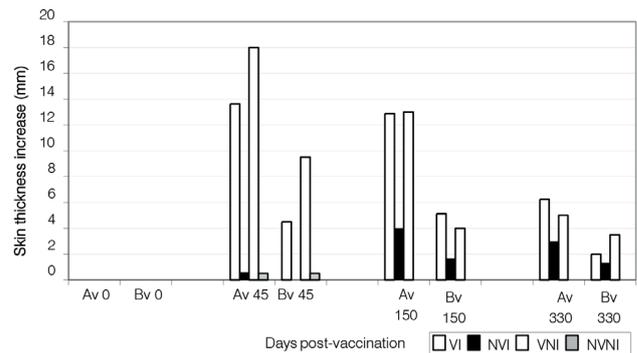
Pathology results

Lesions showed by infected calves were classified into three categories according to González et al. (2005). The categories were *focal:* characterized by the presence of small and well-defined granulomas formed by macrophages and some giant cells with scant or no AFB, mainly in the cortical areas of the mesenteric lymph nodes or in the interfollicular areas of the Peyer´s patches; *multifocal:* with granulomas with scant AFB, spreading to the intestinal lamina propria, without modifying significantly its microscopic architecture, or *diffuse:* characterized by a severe granulomatous enteritis with diffuse thickening of several parts of the ileal and jejunal mucosa, usually having large numbers of AFB. Tables 1 and 2 show the distribution of calves according to the final lesion category. Except from one calf (Nº 5931) killed at 330 dpv, all the vaccinated animals had focal or no lesions. However, the majority of unvaccinated animals had lesions that were multifocal or diffuse.

The cumulative granuloma counts corresponding to the lymph nodes and intestinal compartments examined in calves killed at 180 dpv and 330 dpv are shown in Tables 1 and 2 respectively. Significant differences (p<0.001) were noted in the number of granulomas between vaccinated and control groups, as well as the number of lesions present in the intestinal mucosa.

Table 1: Granuloma counts and lesion classification in calves killed at 180 dpv (1st sacrifice), vaccinated with SILIRUM® and infected experimentally at 60 dpv with MAP.

1st SACRIFICE (180 dpv/120 dpi)			
Animal ID	Mucosal granulomas[1]	Total granulomas[2]	Overall classification
Vaccinated group (inoculated at 60 dpv)			
3303	0	2	Focal
5953	0	0	No lesions
9108	0	0	No lesions
Mean ± SD	0	0.67 ± 1.15	----
Control group (Not vaccinated, inoculated at 60 dpv)			
5933	22.73	58.46	Multifocal
7460	0	34.66	Focal
Mean ± SD	11.37 ± 16.07	46.56 ± 16.83	----

Bacteriology and PCR results

In tissues from two animals (No 5931 and 9109) belonging to VI group, MAP could be isolated, as well as in four calves from NVI group (No 3300, 7460, 5956 and 9107). Culture isolates were made in more organ types and the number of colonies was higher in calves from the NVI group than those from VI group. No positive isolation was made from fecal samples in any of the animals.

By PCR, only one unvaccinated animal (No 5956) had test-negative results for all tissue samples, while in the vaccinated group, three animals had all negative results (No 3303, 5953 and 7461).

conditions.

No noticeable discomfort due to the presence of the vaccination nodules was observed in any of the vaccinated calves and no ulceration occurred. Other experiments have reported large and fistulated nodules (Doyle, 1964; Pérez *et al.,* 1995). The quality and amount of the mineral oil used plus the site of inoculation for this vaccine differs from vaccines in previous studies. These factors apparently contributed to the reduction of vaccination nodule formation (Hanly, 1995).

Another well-known adverse effect of vaccination is interference in the official diagnostic test for tuberculosis. (Vallé and Rinjard, 1926; Stuart, 1962;

Table 2: Granuloma counts and lesion classification in calves killed at 330 dpv (2nd sacrifice), vaccinated with SILIRUM® and inoculated experimentally at 60 dpv with MAP. Four cows were lesion-free (VNI n=2; NVNI n=2) and are not shown.

2nd SACRIFICE (330 dpv/270 dpi)			
Animal ID	Mucosal granulomas[1]	Total granulomas[2]	Overall classification
Vaccinated group (infected at 60 dpv)			
746[3]1	0	0	No lesions
3301	0	3.29	Focal
7930	0	83.26	Focal
9109	0	9.6	Focal
5931	416	1287	Diffuse
Mean ± SD	83.2 ± 186	276.63 ± 565.85	----
Control group (Not vaccinated, inoculated at 60 dpv)			
3300	0.66	8.59	Focal
3302	193.37	326.9	Multifocal
5956	7.99	83.26	Multifocal
9107	495	1741	Diffuse
Mean ± SD	174.26 ± 231.67	548.94 ± 812.16	----

DISCUSSION

The main aim of this study was to assess the efficacy of a killed vaccine (SILIRUM®) against bovine paratuberculosis in experimentally infected calves. From the results obtained, a protective effect of the compound was demonstrated even when taking into account a challenge dose that was considerably higher than usually experienced by an animal under field

Gilmour and Brothersson, 1966; Merkal, 1984) In this trial, as in the majority of the cases in other studies, the *M. bovis* tuberculin reaction was lower than the *M. avium* tuberculin for both the INF-γ production and ITT test. (Stuart, 1962; Huitema, 1967) The conclusion may be made that vaccination against paratuberculosis may not interfere with the official diagnostic test for tuberculosis when comparative tests are used. A stronger reaction to *M. bovis* PPD may be due to actual *M. bovis* infection rather than paratuberculosis

vaccination. However, further studies would be needed to evaluate the immune response to the vaccine and its effect on diagnostic testing in tuberculosis-infected herds.

The main goal of vaccination is to elicit protective immune responses in animals. As has been previously observed in other experiments (Spangler *et al.,* 1991; Juste *et al.,* 1994; Corpa *et al.,* 2000a), the vaccine used in this study stimulated both a cellular and humoral peripheral immune response in all the vaccinated calves. Immunity to all mycobacterial infections is dependent on cell-mediated immune responses and must be elicited by a vaccine for it to be effective (Gilmour, 1976). Humoral immune factors have little or no protective value (Chiodini, 1996). The strong and long-standing cellular immune responses induced by this study's vaccination protocol in all the calves as measured by INF-γ or ITT are evidence of the protective effect of the vaccine. Moreover, high levels of INF-γ have been associated with an increase in the macrophage ability to limit the intracellular growth of MAP (Zhao *et al.,* 1997) and with the presence of focal lesions, interpreted as latent or "resistant" forms (Perez *et al.,* 1999). Although the antibody response may not have any protective effect, it would be an indicator of the degree of activation of the immune system against mycobacteria, since both cellular and humoral responses are mounted after the processing of mycobacteria by macrophages and antigen presentation to CD4+T cells (Munk and Emoto, 1995). In natural paratuberculosis, cellular immune responses appear in the first stages of the infection (Stabel, 2000), as was observed in the NVI group of this study, whereas in vaccinated animals both humoral and cellular appeared simultaneously. This pattern could be related to the immune system's exposure to large amounts of antigen (Abbas *et al.,* 1996).

Histopathologic methods have been commonly used in the evaluation of the vaccination efficacy (Nisbet *et al.,* 1962; Juste *et al.,* 1994; Wentink *et al.,* 1994; García Marín *et al.,* 1995; Pérez *et al.,* 1995; Corpa *et al.,* 2000b). In this work, a quantification method for granulomatous lesions showed significant differences between study groups, with the vaccinated animals showing less gastrointestinal tract and lymphatic tissue pathology.

In experimental studies, the first lesions usually associated with MAP infection appear in lymphoid tissue (intestinal Peyer´s patches or lymph nodes) (Payne and Rankin, 1961; Nisbet *et al.,* 1962; Larsen *et al.,* 1975; Juste *et al.,* 1994; Clarke, 1997; Kurade *et al.,* 2004). Animals with these lesions are usually subclinical and the lesions are of the tuberculoid type. They may remain even until the death of the animal from other causes (Pérez *et al.,* 1996). This lesion type is associated with strong cellular immune responses (Pérez *et al.,*

1999; 2002) capable of controlling multiplication of the bacillus and progression of the lesions toward more severe forms. When this effective immune response is overcome, the lesions progress first toward the lamina propria of the mucosa associated with the lymphoid tissue, and then to different section of the intestinal mucosa causing severe diffuse granulomatous enteritis.

In our study, except for one case, vaccinated animals were either free of lesions or developed only the focal form. In contrast, multifocal or diffuse lesions were described in NVI calves. This finding supports the hypothesis that the vaccine limits granulomatous lesions to a tuberculoid form in which regressive granulomas with no or scarce mycobacteria are confined to the organized lymphoid tissue, as previously suggested in sheep (Juste *et al.,* 1994; García Marín *et al.,* 1995).

The above findings suggest a considerable protective effect of vaccination for bovine paratuberculosis. The protection linked with limiting the progression of pathology may be even greater than the observed in this study because the challenge dose was so large. Nevertheless, the protection provided by the vaccine product was not absolute since one vaccinated calf (No 5931) had diffuse lesions. This absence of absolute protection seems to be a common finding in paratuberculosis vaccines (Doyle, 1964; Larsen *et al.,* 1978; Körmendy, 1994; Pérez *et al.,* 1995; Corpa *et al.,* 2000b) and is believed to be a function of factors that predispose an individual animal to a weaker or incomplete immune response (Doyle, 1964). One vaccinated calf in this study may be such a case, given its small nodule and meagre inflammatory response to vaccination. Coupling this calf's muted immune response with the high challenge dose resulted in a clinically severe infection.

Fewer MAP isolates were obtained from VI calves' tissues than what was seen with NVI calves. This finding was expected since diffuse forms of pathology, mostly seen in calves from the NVI group, usually bear high numbers of the bacilli. These results are in agreement with previous studies (Brotherston *et al.,* 1961; Stuart, 1962; Juste *et al.,* 1994; Pérez *et al.,* 1995) and suggest that MAP multiplication is being suppressed. However, there are field studies (Kalis *et al.,* 2001) that have not found a clear reduction in MAP shedding after a number of years in vaccinated flocks. In this study, no fecal shedding was detected in any animal.

CONCLUSION

SILIRUM® , a killed vaccine against paratuberculosis, was shown to be safe in calves experimentally challenged with MAP. It induced strong cellular and humoral immune responses. Although cellular

immune response cross reactions to *M. bovis* PPD appeared, reactions were always higher to *M. avium* PPD suggesting that if comparative TB assays are used the paratuberculosis vaccine need not interfere in surveillance for tuberculosis.

The vaccine had a marked, although not absolute, protective effect against paratuberculosis. Vaccinated and challenged calves had fewer lesions, less severe lesions and a lower tissue burden of MAP than unvaccinated calves. The histopathological method employed has been shown to be useful in the assessment of the efficacy of a vaccine against paratuberculosis.

ACKNOWLEDGMENTS

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