Administering Live *Mycobacterium vaccae* to Newborn Calves Orally to Prevent Paratuberculosis – Preliminary Examinations: Safety and Immune Reaction

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**ABSTRACT**

Paratuberculosis, an enteric disease primarily of ruminants is caused by *Mycobacterium avium* subsp. *paratuberculosis*. Animals up to about six months of age are at the highest risk. Protection is based on a cellular immunity. *Mycobacterium vaccae* is an acid-fast microorganism, classified as “Generally Considered Safe” and has been shown to enhance the cellular immune reaction in humans and animals. Thus, its oral administration may be considered a suitable means to prevent paratuberculosis. Considering the early age of infection in paratuberculosis, the administration of *M. vaccae* to calves as soon as possible after parturition appears to be imperative. However, since interference with the immature enteric microflora may be detrimental to the calves, it was considered necessary to conduct a safety study. The aims of this study were to ascertain the procedure’s innocuity and assess the peripheral immune response. *M. vaccae* was administered, by gavage, within the first day after parturition and 12-13 days later. Following this, the general well-being of the calves, the shedding of *M. vaccae* and fertility and milk production were monitored. The effect of this treatment on the peripheral cellular immune system by lymphocyte proliferation, antigen specific interferon-γ production and cytometry analysis was tested. The reactions against *M. vaccae* antigen, and *M. avium* subsp. *avium* purified protein derivate (PPD) and *M. bovis* PPD were tested 2 and 7-8 weeks after administering the second dose of *M. vaccae*. *M. vaccae* administration to newborn calves was found safe. The results indicated that the administration of live *M. vaccae* has no negative short term or long-term effects. Moreover, a peripheral immune response against *M. vaccae* and *M. avium* subsp. *avium*, but not *M. bovis*, was elicited. Flow cytometry showed that stimulation with *M. vaccae* antigen resulted in the proliferation of T cells, mainly CD4 cells. These results justify further research for the potential of *M. vaccae* in preventing paratuberculosis.

**Keywords:** *Mycobacterium vaccae*; Oral; Newborn Calves; Safety; Productivity; Immunomodulation.

**INTRODUCTION**

*Mycobacterium vaccae* is a fast-growing acid-fast microorganism first isolated from a cowshed (1) and classified as Generally Considered Safe (1). It has been widely used in its inactivated form as an immunomodulatory agent to prevent or treat, in humans and animals, mycobacterial infections, such as tuberculosis and leprosy (2, 3, 4, 5), allergies (6, 7, 8), gingivitis (9) and melanomas (10). *M. vaccae* was found to seemingly be able to reduce stress reactions (11). A meta-analysis published by Yang et al. (12) indicated that in high-risk patients, *M. vaccae* elicited an efficient immune reaction against tuberculosis and its use was safe.

All these experiments used reference strains from the ATCC or NCTC, inactivated and administered *per os* or parenterally and found to be safe (13). To the best of our knowledge, the impact of this microorganism upon infection
with a major veterinary pathogen, *M. avium* subsp. *paratuberculosis* (MAP) has not been investigated.

Paratuberculosis (Johne's Disease) is a chronic infection, primarily of ruminants, caused by the acid-fast microorganism *Mycobacterium avium* subsp. *paratuberculosis* (14). The population at the highest risk is calves up to 6 months of age (15) whereas clinical signs appear only in about 10% of the infected animals, several years after the infection (16). Most commonly, exposure of the calves to the bacterium is by the fecal-oral route is the infection's source. Additional sources may be the colostrum or milk containing MAP secreted by the dam either from environmental contamination or intra-uterine transmission of the microorganism (16). Following its ingestion, MAP quickly colonizes the intestine (17), primarily the lymphatic tissue (Payer's patches) (18) and induces the creation of foci, some of which clear the microorganism while others become the source of its further dissemination and the creation of additional foci (19). These foci evolve into granulomatous lesions that, unlike those observed in tuberculosis, do not limit the spread of the microorganism (20). This reaction causes the inspissation of the intestinal wall, limiting the absorption of liquids and nutrients and leading to the severe diarrhea that characterized the disease, and emaciation.

Paratuberculosis is an incurable disease and current vaccines have a limited efficacy, being unable to prevent the infection (21). Moreover, these vaccines often cause severe reaction at the injection site (22). Thus, the most efficient current mode to reduce herd prevalence is by limiting as much as possible the risk of calves' infection by improved hygiene, restricting contact with adult cows and by removing animals shedding MAP from the herd (23).

A common characteristic of the host's reaction to infections caused by acid-fast microorganisms, such as *M. tuberculosis* complex, *M. leprae* or MAP, is mounting a cellular immune response that limits the spread of infectious foci in the intestine. In the later stages of the infection, this response may be replaced by a humoral one, generally indicating the inability of the host to further contain the infection (24).

Rough colonies of *M. vaccae* have been found to potentiate the cellular immune response whereas smooth ones induced the production of antibodies (25, 26). Consequently, rough *M. vaccae* might have the potential of reducing the capacity of MAP to colonize and infect the intestine by stimulating the enteric immune system and possibly surviving in the enteric lymphatic tissue to provide a long-lasting protection. Unlike other vaccines that are administered parenterally (22), we presumed that paratuberculosis, being an enteric infection, may be prevented more efficiently by the oral administration of the antigen. Moreover, we decided to use live *M. vaccae* since the "gold standard" of anti-tuberculosis vaccines, and the only widely used one, is the attenuated BCG strain (27).

*Ulex europaeus* extract (UEE), a ligand that improves the attachment of the antigen to microfold (M) cells, and thus enhance the immune reaction (29), was added to one group of calves in the immunology experiments aim boosting the response. Since, as mentioned previously, young calves are at the highest risk, *M. vaccae* must be administered as early as possible.

The early establishment of a commensal microflora is extremely important for the calves' health and immune system (28). Thus, exposing newborn calves orally to live bacteria, even if classified as Generally Considered Safe, may be hazardous.

The experiments described in this manuscript were performed to ascertain whether the vaccination procedure had any negative effects on the calves during administration and later on in life. Moreover, various aspects of the peripheral cellular immune reactions against *M. vaccae*, *M. avium* subsp. *avium* or *M. bovis* antigens were assessed.

**MATERIALS AND METHODS**

*Mycobacterium vaccae and Ulex europaeus*

*M. vaccae* strain 10916 was purchased from the NCTC and propagated on 5% sheep blood agar (Oxoid, USA). Rough colonies were sub-cultured, suspended in inositol serum nutrient broth to 0.5 McFarland turbidity (equivalent to about $10^{10}$ colony forming units (CFU)*ml$^{-1}$) and lyophilized in aliquots of one ml. For doses prepared from cultures, bacterial concentration was determined by a turbidity meter (HI 93703, Hanna Instruments, US) according to a previously prepared curve.

Before administration, the bacteria were either reconstituted (experiment 1 and 2) or propagated (cultured of 3 days on 5% sheep blood agar plate) (experiment 3). The CFU count for this dose was determined by turbidity.

UEE, (Sigma-Aldrich, US) was added to one group of calves in the immunology experiments (see below).
Animals, inoculation and sampling
Ethical authorization (IL 491/14) was received from the Ethical committee of the Volcani Agricultural Research Center, Israel.

The study was carried out in a dairy herd of 220 lactating Israeli-Holstein cows, at the Agricultural Research Organization, the Volcani Center, Israel. The farm had a prevalence of about 1% paratuberculosis as determined by milk ELISA at the National Service for Udder Health and Milk Quality of the Israeli Dairy Board. Calves were kept in individual sheds, fed colostrum for the first 3 days and thereafter milk replacer (Denkavit, Netherlands) and hay. At about 2 months of age, the calves were transferred to groups of about 10 animals. The dairy parlor was equipped with an on-line computerized Afifarm Herd Management data acquisition system that included the Afilab milk analyzer (Afimilk, Afikim, Israel). Cows were milked thrice daily and the average milk yield in this farm ~11,500 L normalized for 305 lactation days.

In a preliminary pilot study, two calves were administered, per gavage, 3 doses of $10^{10}$ CFU M. vaccae within the first day of life, at 8 hours intervals. No general negative clinical signs (lethargy, fever, inappetence, and diarrhea) were observed.

Following the preliminary assessment, two experiments, focusing on safety and the peripheral immune reaction, were conducted. In the first experiment, twenty heifers were given a dose of $10^{10}$ CFU M. vaccae within the first day after parturition and a second similar dose 12-13 days later. Twenty additional heifers served as untreated controls. Group assignment was by order of birth. The second experiment was performed on three groups of six heifers each. Group 1 was inoculated with one dose of $10^{10}$ CFU M. vaccae reconstituted within the first day after parturition and a second similar dose 12-13 days later with M. vaccae that was propagated from the lyophilized vial on 5% sheep blood agar and incubated for 72 hours at 37°C. Group 2 received the same treatment with the addition of 100 µg UEE. Group 3 served as untreated control and received only 2 doses of UEE.

In both experiments, calves were visited daily up to two weeks after the administration of the second dose and observed for the presence general signs of sickness (fever, lethargy, inappetence and diarrhea). To assess eventual shedding of M. vaccae, Ziel-Neelsen stained slides of rectal fecal samples were examined microscopically daily for 48 hours. In addition, the samples were inoculated onto 5% sheep blood agar that were incubated at 37°C for three days and examined daily for colonies suspected to be M. vaccae. In the second experiment, blood samples (EDTA and heparinized -Vacutainer, Becton Dickinson, UK) were taken 2 and 7-8 weeks after the administration of the second dose of M. vaccae for the immunological tests. Fertility (only first experiment) was determined by the number of inseminations to pregnancy and milk yield by production adjusted for 305 days for the first pregnancy.

In vitro lymphocyte proliferation response
For M. vaccae crude antigen preparation for in vitro stimulation, the microorganism was cultured for 3 days on 5% sheep blood agar (Oxoid, USA) plates at 37°C. The lawn from 6 plates were harvested and suspended in 30ml PBS at 4°C in an ice bath. After adding 7g glass beads (Sigma, USA), the suspension was subjected to mechanical agitation for 5 minutes in a CO2 cooled cell homogenizer (Braun Melsungen AG, Germany). The glass beads and intact bacteria were removed by centrifugation for 15 minutes at 2800g and the supernatant was filtered through 0.2µm pore-size membrane (Sartorius Stedim Biotech, Germany). Protein concentration was determined by a protein assay kit (Bio-Rad, München, Germany) and the supernatant was stored at -20°C.

Peripheral blood mononuclear cells (PBMC) were isolated from EDTA whole blood by density centrifugation (650g, room temp., 30 min) over Ficoll-Paque™ Plus (d=1.077, GE Healthcare, Upsala, Sweden). PBMC were washed and resuspended to a final concentration 1×10⁶ cells/ml in RPMI-1640 with 2 mM L-glutamine supplemented with 5% fetal calf serum, and 100 U/ml antibiotics (Penicillin-Streptomycin and Amphotericin) (Biological Ind., Israel). One hundred µL of PBMC were placed in 96 well flat-bottomed plates for tissue culture (Nunc, Denmark). The reaction was tested, in triplicates, against 25 and 50 µg/ml M. vaccae antigen and 10 and 20 µg/ml M. avium subsp. avium or M. bovis purified protein derivate (PPD) (Avituber and Bovituber respectively, Symbiotics, France). The antigens, in complete media, were added to the wells at final concentrations of 5, 10, 25 and 50 µg/ml for M. vaccae and 5, 10 and 20 µg/ml for the PPDs. Wells with 10 µg/ml of Con A (Concanavalin A, Sigma, USA) were use as positive control for proliferation. Untreated cells with complete media were used as negative background. Plates were incubated for 3 days at 37°C in a 5% CO₂ enriched atmosphere. AlamarBlue® (Serotec, UK)
in a volume of 20 µL was added to each well for 4 h. Plates were read by using a fluorescent reader GENios Plus (Tecan, Austria) with a 590 nm emission filter and 535 nm excitation filters. Calculation of the Proliferative Index was as follows:

\[ \text{Index} = \frac{\text{Fluorescence test} - \text{Fluorescence mean}}{\text{Fluorescence untreated cell} - \text{Fluorescence mean}} - 1 \]

**Antigen specific interferon-γ production**

One ml heparinized blood (Vacutainer, Becton Dickinson, USA) was placed in 24 well plates for tissue culture (Nunc, Denmark) to which 50 µL of crude *M. vaccae* antigen in a final concentration of 50 µg/ml was added. Wells with final concentration of 10 µg/ml of Con A were used as positive control and wells with untreated blood were used as negative control. Plates were incubated for 24 h at 37°C in a 5% CO₂ enriched atmosphere. Subsequently, the blood was transferred from the wells to 1.5 ml Eppendorf tubes, centrifuged at 1500 g for 20 min and the clear plasma supernatant was collected and stored at -20°C. A commercial kit (Serotec Inc., UK) was used to measure bovine INF-γ according to the manufacturer’s instruction.

**Flow cytometry analysis of proliferated cells in vitro**

PBMC, prepared from EDTA whole blood as described above was adjusted to a concentration of 2×10⁶ cells/ml. One ml of the cell suspensions in cultural medium were plated into 24 wells plates (Nunc, Denmark). The cells were stimulated with crude *M. vaccae* antigen (50 µg/ml). Con A (10 µg/ml) was used as positive control and untreated blood were used as negative control. The cells were incubated for 72 h at 5% CO₂ enriched and humidified atmosphere. Subsequently, the suspension was centrifuged at 450g for 5 minutes and washed with PBS and cells were collected and adjusted to a concentration of 1×10⁶ in 100 µL. Pre incubation (day of blood collection) and cells post incubation were labeled with double staining with the following mouse anti-bovine monoclonal antibodies (Vetmed, USA): anti-CD4 (GC50A, IgM), anti-CD8 (CACT80c, IgG1), anti-CD21 (BAQ15A, IgM) and with a proliferation marker anti CD25 (CACT116A, IgG1 or LCTB2A, IgG3).

All antibodies were used at a final dilution of 1:200. Cells were incubated for 40 min at 4°C, washed (×3) and stained with secondary antibodies (30 min at 4°C) with goat anti mouse IgG1-TRI color (1:200) conjugate (Invitrogen, USA) and goat anti-mouse IgM FITC conjugate (1:100) (Jackson, USA) or anti-mouse IgG3 FITC conjugate (1:100)(Jackson, USA) and goat anti mouse IgG1-TRI color (1:200) conjugate (Invitrogen, USA). FACS analysis was performed in a FACSCalibur flow cytometer (Becton-Dickinson, USA).

**Statistics**

The statistical significance between the groups was tested by the t test (Statistix v7.0, Analytical Software, USA). A P value below 0.05 was considered to indicate significance. Unequal variances were assumed.

**RESULTS**

**Safety**

General signs of sickness (fever, lethargy, inappetence, and diarrhea) were sporadic with no significant different among the groups. Fecal shedding of *M. vaccae* was observed only by heifers in the second experiment, following the second inoculation consisting of propagated bacteria. After having been transferred to groups, at 2 months of age, *M. vaccae* was isolated from 3 previously negative control animals, in contact with test calves. Consequently, these control calves were excluded from the second sampling cycle of the immunomodulation tests. Calves that received only one dose of cultured *M. vaccae* did not shed the microorganism.

A tuberculin test performed by the Israeli Veterinary Field Services two years after the administration of *M. vaccae* found the cows negative. At the end of first lactation, 30/40 heifers, 16 test and 14 control of the first experiment were present on the farm.

No differences between then test and control groups for number of inseminations to pregnancy and 305 days corrected milk yield were found (Table 1).

<p>| Table 1: Assessment of long-term impact of the oral administration of live <em>M. vaccae</em> to newborn heifers. |</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>Mean</th>
<th>SEM</th>
<th>95% CI</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertility (NIP)</td>
<td>Control</td>
<td>1.7647</td>
<td>0.4417</td>
<td>-1.463, 10287</td>
</tr>
<tr>
<td></td>
<td>Test</td>
<td>1.8235</td>
<td>0.2999</td>
<td></td>
</tr>
<tr>
<td>Milk Yield</td>
<td>Control</td>
<td>12968</td>
<td>353.72</td>
<td>-524.73, 1455.5</td>
</tr>
<tr>
<td></td>
<td>Test</td>
<td>12505</td>
<td>329.62</td>
<td></td>
</tr>
</tbody>
</table>

NIP: number of inseminations to pregnancy; SEM: Standard Error of the Mean, CI: Confidence Interval
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The most consistent effects were obtained when adding 50µg M. vaccae extract to the reaction. The results are presented in Figure 1. Statistically significant differences were obtained between the mean of the control group and that of the second sample of the 2 test groups: P=0.034 and P=0.031 for the group that received the UEE and the group that did not receive it, respectively. All calves responded to Con A, with no significant difference among them (data not shown).

When tested against the Mycobacterium avium subsp. avium PPD (20 µg/ml), a statistically significant difference was found between heifers that received M. vaccae without UEE and the controls (P=0.046) (Figure 2). No such difference was found for the M. bovis PPD.

Interferon γ

The antigen specific interferon-γ production was tested in vitro in whole blood activated with 50 µL of crude M. vaccae antigen in a final concentration of 50 µg/mL. Con A was used as positive control. Only 60-70% of the calves from all groups showed a response to Con A. Results shown are the average of only the responders calves. No significant difference was founded among the groups in the response to Con A. Significant difference (P=0.026) of the specific response to M. vaccae antigen was found when calves that were treated with M. vaccae but not in the calves treated with M. vaccae and Ulex and control animals (Figure 3).

Flow cytometry

No significant differences were found in the distribution of T-lymphocytes baring CD4, CD8 or γδ, and B-lymphocytes baring CD21 before in vitro incubation between vaccinated and control calves. Of the T lymphocytes, 7%-20% were double positive for CD25 and γδ T. In vitro stimulation with Con A resulted in the proliferation of mainly CD4+CD25−
(~30%) and ~10% CD8⁺CD25⁺ and 8–18% γδ⁺CD25⁺ T-lymphocytes in both control and M. vaccae vaccinated calves. In contrast, the only activated cells after the stimulation with the M. vaccae antigen were ~10% CD4⁺CD25⁺ T-lymphocytes and only of vaccinated calves. The results are presented in Figure 4.

**DISCUSSION**

The experiments described in this publication propose a novel approach to the prevention of paratuberculosis. Moreover, to the best of our knowledge, the administration of live M. vaccae, per os to cattle has never been reported before.

*Mycobacteriacea* are aerobic microorganisms and thus unlikely to survive for long on the intestinal mucosa, an anaerobic environment. Since the protection from mycobacterial infections in general and from paratuberculosis in particular depends primarily on the cellular component of the immune system, its stimulation by oral administered live M. vaccae to newborn calves was assessed.

Our results indicate that, since no shedding of M. vaccae was observed in heifers receiving the microorganisms directly after reconstitution, they might have been destroyed. However, the protocol in which the first dose was administered directly after reconstitution and the second one consisted of bacteria from culture resulted in their shedding it up to the end of the experiment, indicating that they survived and multiplied. The absence of M. vaccae in the feces is not, however, an unequivocal indication of the lack of colonization of intestine by the bacterium since, as shown by Wu *et al.*, (17), this organ may be colonized by MAP without fecal shedding.

Our results indicate that the oral administration of live M. vaccae to newborn heifers is safe, in both the short and long terms. The immunological tests were conducted on a relatively low number of animals and the statistical analysis of the results was likely to be influenced by the high variance of the observations. Consequently the results of these tests must be considered preliminary.

We found a significant difference between lymphocyte stimulation for samples taken 7–8 weeks after the 2nd administration of M. vaccae and that of control animals. Since the animals were not administered M. vaccae between the two samplings, this seems to indicate continued activity of the bacterium. No statistically significant differences were found between the first samples and the control animals and between calves that received or did not receive UEE. Results of the interferon γ reaction seem to support the assumption that the M. vaccae continued to stimulate the immune system.

![Figure 4: Flow cytometry: test (A) and control (B) heifers.](image)
in time as the difference between test and control animals became statistically significant for the 2nd sample. The lack of reaction in the UEE treated calves remains unexplained.

Since the oral administration of live \( M. \) \textit{vaccae} is expected to impact the calves' reaction to enteric exposure to MAP, it is important to assess not only the homologous cellular immune reaction but that to the paratuberculosis agent as well. Being used routinely in comparative tuberculin testing \( M. \) \textit{avium} subsp. \textit{avium} PPD was used as the nearest reagent available to MAP. Although the stimulation index was higher in the heifers the received \( M. \) \textit{vaccae} with the UEE, the high variability of the results made them statistically not significant. This may be the result of the low number of the tested animals. Our findings that live \( M. \) \textit{vaccae} induces an immune reaction to this antigen is promising as to its ability to positively influence the calves capabilities to withstand its intestine's colonization by the pathogen. Conversely, the lack of reaction of \( M. \) \textit{bovis} PPD confirms the negative tuberculin results in test animals and the previously reported lack of protective capacity of killed \( M. \) \textit{vaccae} against \( M. \) \textit{bovis} in cattle (30).

Another positive outcome is the reaction observed by the flow cytometry assay that assay showed a specific CD4+ cell proliferation in the test animals. The same type of cells has been shown (31) to be the main component in the immune reaction in the early stages of MAP infection.

In conclusion the results of this study, although based on a limited number of animals, indicate that the immune response induced by the oral administration of live \( M. \) \textit{vaccae} may have a positive impact on the calves' ability to resist MAP infection.

These findings open possibilities to investigate the impact of this practice on a variety of enteric infections, primarily paratuberculosis due to the taxonomic proximity of \( M. \) \textit{vaccae} and MAP and the latter's pathogenicity being focused in the intestine. Moreover, since the population of animals at risk is primarily that of calves of up to six months of age, it may suffice to protect them only for this period, following which, if they are raised on an infected farm, the exposure to MAP may act as a booster and provide the necessary protection.

### CONFLICT OF INTEREST STATEMENT

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

### FUNDING STATEMENT

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### REFERENCES


