Immune responses to an inactivated Johne’s disease vaccine in cattle

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ABSTRACT

The objective of this study was to develop a vaccine against Johne’s disease for calves and study its immune efficacy. A heat inactivated Johne’s disease vaccine in mineral oil adjuvant was developed using the strain predominant in Tamil Nadu and tested for its efficacy in calves for a period of 8 months by ELISA for antibodies and by Interferon-γ ELISA, MTT assay and flow cytometry for cell mediated immune responses. Vaccinated calves had high levels of seroconversion as compared to control calves from second month post vaccination (PV) and antibodies persisted throughout the study period. Lymphoproliferative response specific to MAP antigen and increase in the IFN-γ levels was observed in the vaccinated calves from 30 days PV and the response was significantly higher in vaccinated calves compared to control group up to four months PV. In flow cytometry analysis, the peak percentages of CD4+ and CD8+ T cells were noticed at three months PV among vaccinated animals. Overall, our results suggested that the inactivated Johne’s disease vaccine was effective in stimulating the immune system of the calves with significant MAP specific responses.

Keywords: Antibodies, CD4+ and CD8+ T cell response, IFN-γ, Johne’s disease, Vaccine efficacy
the final concentration such that each millilitre of vaccine contained 5 mg of bacterial culture (wet weight) which constituted one dose. The heat inactivated vaccine was tested for proper inactivation, sterility, safety and potency as per OIE (2010).

**Immunization of calves:** A total of 12 calves aged 1-3 months maintained in the University Research Farm, Tamil Nadu Veterinary and Animal Sciences University were selected for testing the vaccine during the period from 2010-2011. Calves chosen for the vaccine trial were screened using IS 900 PCR and culture of fecal samples, and found free of MAP infection. The calves were also screened for the MAP antibodies and IFN-γ titres by ELISA and are found to be free from MAP specific antibodies and IFN-γ levels on ‘0’ day.

A group of eight calves were inoculated with 1 ml of the inactivated JD vaccine, subcutaneously in the brisket region. Another group of four calves which received 1 ml of plain adjuvant subcutaneously were maintained as sham immunized controls.

**Sampling details:** Blood samples were collected on ‘0’ day and at monthly intervals post vaccination from each of the vaccinated and control calves in procoagulant vacutainers for serum separation, sodium EDTA vacutainers for lymphocyte separation and in lithium heparin vacutainers for whole blood interferon gamma assay. Whole blood samples were processed immediately for lymphocyte separation and IFN-γ assay. Serum samples were stored at -40°C until tested. At each sampling, the vaccination site of all the calves was inspected and palpated for the presence of lesions.

**Assessment of antibody response:** MAP antibody ELISA test kit (Labor Diagnostik, Leipzig, Germany), was used to measure the antibody titres against MAP in the serum, as per the manufacturer’s instructions. The serum samples were diluted 1:70 and pre-incubated with sample dilution buffer containing inactivated M. phlei extract, in order to minimize cross-reactions to atypical mycobacteria. The results were expressed in terms of S/P ratio (ratio of sample to positive control).

**IFN-γ assay:** The MAP specific IFN-γ response was estimated using an in vitro whole blood IFN-γ assay (Bovigam, Prionics Inc, USA) after stimulation of whole blood with johnin PPD. IFN-γ levels were measured in the plasma (before and after stimulation with johnin PPD), as per the manufacturer’s instructions. The results were expressed in terms of corrected OD values (OD of the sample incubated with johnin PPD − OD of the same sample incubated with PBS) as per Kumanan et al. (2009).

**Lymphocyte proliferation assay:** Isolation of lymphocytes from blood in EDTA was carried out as per Shin et al. (2005). The lymphocytes were resuspended at a concentration of 2×10^6/ml in RPMI medium. Lymphocyte proliferation in response to stimulation with mitogen Con A and MAP antigen was measured by MTT assay as per Singh et al. (2007) with some modifications. The response was reported as Stimulation Index (SI), and the average SI value for each group of animals were calculated and compared to assess the lymphocyte proliferation response induced in vaccinated and control groups at monthly intervals.

**Flow cytometry analysis of CD4+ and CD8+ lymphocyte subsets:** Peripheral Blood mononuclear cells of each animal (1×10^6 cells) were washed thrice with FACS buffer and resuspended in 50 μl of FACS buffer and 10 μl each of mouse anti bovine CD4: PE and mouse anti bovine CD8: FITC monoclonal antibodies were added and incubated on ice for 45 min. After that, the cells were washed twice with FACS buffer and finally suspended in 100 μl of 4% paraformaldehyde in FACS buffer and transferred to FACS tubes containing 400 μl of FACS buffer. Data were collected on 10,000 events using a FACS caliber flowcytometer (Becton-Dickinson, San Jose, CA) and analyzed using CellQuest software. The results were expressed as the average percent of cells stained with each monoclonal antibody, compared between vaccinated and control groups of calves at each sampling.

**RESULTS AND DISCUSSION**

**Immunization of calves:** The calves immunized with the heat inactivated JD vaccine developed no adverse systemic reactions, except for nodule formation at the injection site with diameter ranging from 12-15 cm at one month post vaccination. Subsequently, these nodules turned into hard painless vaccine granuloma and persisted till the end of this study i.e. 8 months post vaccination. This granulomatous inflammatory reaction at the site of injection could be attributed to the inherent property of mycobacteria to stimulate cell mediated response and also to the adjuvant effect of mineral oil and the same was also reported in JD vaccination trials conducted by Kalis et al. (2001), Reddacliff et al. (2006) and Windsor (2006).

Using the ‘Bison type’ strain for vaccine preparation would be appropriate as it was found prevalent among the JD infected cattle and other livestock species of Tamil Nadu in a previous study (Chaitanya et al. 2015, 2019). The reports from North India by Singh et al. (2010) and Sohal et al. (2010) also confirmed the predominance of ‘Bison type’ strains in India. Each dose of vaccine is fixed to have 5 mg/ml wet weight of MAP in mineral oil adjuvant. Mineral oil was used for preparation of several animal vaccines. Several commercially available JD vaccines abroad also used the same dosage and adjuvant, but they contained MAP strains native to the respective countries, such as strain ID-Lelystad, Netherlands (Muskens et al. 2002); MAP 5889 Bergey strain, Hungary; MAP 316F strain in Gudair® and Silirum® (Copra et al. 2000, Reddacliff et al. 2006) which are ‘Cattle type’ strains. These vaccines may or may not be ideal for the control of JD in our livestock.

**Antibody response to vaccination:** MAP specific antibody response was determined in calves over a period of eight months. High levels of seroconversion (P<0.05) were noticed among vaccinated calves as compared to...
controls from second month PV (Fig. 1). Fifty percent of the vaccinated calves became positive for MAP antibodies by the first month PV and the percentage of reactors was 100% by six months (Fig. 2). Vaccination had marked and prolonged effect on antibody response. Copra et al. (2000) reported higher and persistent antibody response in goats and lambs vaccinated at 5 months age. Vaccination with Mycopar elicited significantly higher levels of IgG from second month and persisted for 12 months in a study by Phanse et al. (2020). A much higher percentage of vaccinated than control animals had positive MAP specific antibody levels in a large vaccination experiment in Australia (Reddacliff et al. 2006).

**Lymphocyte proliferation assay:** Lymphocyte proliferation specific to vaccination was detected at first month post vaccination in 60% of the vaccinated calves. There was a significant increase in MAP specific lymphoproliferative responses up to four months PV in vaccinated calves (P<0.01) and declined after that (Fig. 4). at two month PV. Kohler et al. (2001) reported peak IFN-γ responses at 16 weeks PV in calves vaccinated with single dose of live attenuated MAP vaccine (Neoparasec™) at 28 days age and the response persisted up to 96 weeks. This sustained IFN-γ release can be attributed to the inherent property of live attenuated vaccines that multiply in the host and cause constant stimulation of the immune system of the host. Phanse et al. (2020) also reported that live attenuated vaccines elicited robust cellular immune responses with marked increase in IFN-γ and IL-17, with little induction of humoral responses.

**IFN-γ assay:** An increase in the IFN-γ levels was noticed in the plasma of vaccinated calves from 30 days PV and increased further during the second month. Significant differences (P<0.01) in IFN-γ levels between vaccinated and control calves were observed during the initial four months PV (Fig. 3) and the titres disappeared at fifth month and later. The results are in concurrence with those obtained by vaccination with Gudair™ vaccine in lambs and kids by Copra et al. (2000), who reported peak IFN-γ gamma response at 30 days PV which persisted up to 90-120 days. Protective immunity against mycobacterial infections is cell mediated with the activation of CD4+ and CD8+ T lymphocytes reflecting a Th1 -type response with the release of Pro-Th1 cytokines such as IFN-γ (Begg and Griffin 2005). Reddacliff et al. (2006) also reported that positive IFN-γ responses are maximum among vaccines...
Immune responses to Johne’s disease vaccine

Table 1. CD4⁺ and CD8⁺ T lymphocyte counts in the peripheral blood of calves after immunization with JD vaccine

<table>
<thead>
<tr>
<th>Months post vaccination</th>
<th>CD4⁺ %</th>
<th>P Value</th>
<th>CD8⁺ %</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vaccinated group (N=8)</td>
<td>Sham immunized controls (N=4)</td>
<td>Vaccinated group (N=8)</td>
<td>Sham immunized controls (N=4)</td>
</tr>
<tr>
<td></td>
<td>Mean ±SE</td>
<td>Mean ±SE</td>
<td>Mean ±SE</td>
<td>Mean ±SE</td>
</tr>
<tr>
<td>'0' day</td>
<td>19.45 ±1.6</td>
<td>18.03 ±1.89</td>
<td>0.58</td>
<td>6.98 ±0.9</td>
</tr>
<tr>
<td>1 month</td>
<td>26.83 ±1.05</td>
<td>20.64 ±2.16</td>
<td>0.04*</td>
<td>9.32 ±1.08</td>
</tr>
<tr>
<td>2 month</td>
<td>29.22 ±1.95</td>
<td>20.86 ±1.82</td>
<td>0.02*</td>
<td>10.72 ±0.74</td>
</tr>
<tr>
<td>3 month</td>
<td>32.57 ±1.15</td>
<td>21.42 ±2.56</td>
<td>0.007**</td>
<td>12.36 ±1.32</td>
</tr>
<tr>
<td>4 month</td>
<td>26.03 ±1.65</td>
<td>19.87 ±1.23</td>
<td>0.024*</td>
<td>10.28 ±1.03</td>
</tr>
<tr>
<td>5 month</td>
<td>22.52 ±1.01</td>
<td>21.36 ±1.64</td>
<td>0.56</td>
<td>9.12 ±1.57</td>
</tr>
<tr>
<td>6 month</td>
<td>21.70 ±0.95</td>
<td>20.32 ±1.17</td>
<td>0.39</td>
<td>8.98 ±1.28</td>
</tr>
<tr>
<td>7 month</td>
<td>22.34 ±0.65</td>
<td>21.04 ±1.33</td>
<td>0.041</td>
<td>8.84 ±1.77</td>
</tr>
<tr>
<td>8 month</td>
<td>21.42 ±1.3</td>
<td>20.82 ±1.24</td>
<td>0.074</td>
<td>9.22 ±0.74</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01.

Post vaccination. When antigen presenting cells process and present the killed MAP in the vaccine, they can signal antigen specific T lymphocytes in an MHC specific manner, which promotes lymphocyte proliferation (Park et al. 2008).

Flow cytometry analysis of CD4⁺ and CD8⁺ lymphocytes: The CD4⁺ and CD8⁺ T cell counts of vaccinated and control groups of calves tested at monthly intervals are expressed as mean percentages (Table 1). Representative Dot plots of two colour flow analysis showing both CD4⁺ and CD8⁺ counts and single parameter histograms showing CD4⁺ and CD8⁺ lymphocyte counts individually at different sampling intervals are provided in Fig. 5.

![Flow Cytometry Analysis](image)

Fig. 5. A representative single parameter histogram showing CD4⁺ lymphocyte count in PBMCs of calves 2nd and 3rd month PV.

Prior to vaccination, the mean percentage of CD4⁺ and CD8⁺ count in calves selected for vaccination was 19.45 and 6.98, whereas in control group calves, the percentage count was 18.03 and 6.30, respectively. At first month PV, the proportions of CD4⁺ and CD8⁺ T cells were significantly higher in the vaccinated group (P<0.05) than the control group. The peak percentages of cells were noticed at three months PV among vaccinated animals (P<0.01) with the mean values 32.57 and 12.36. At five months PV, there were no significant differences in the CD4⁺ and CD8⁺ cell percentage between vaccinated and control groups.

This increase in CD4⁺ and CD8⁺ cell populations was associated with increased IFN-γ levels and lymphoproliferative responses in vaccinated animals and could be inferred that these subsets are responsible for IFN-γ production, as reported by Kumanan et al. (2008). The results were also in correlation with the observations made by Koets et al. (2002) who reported a rise in CD4⁺ levels to 26.2% between 44-120 days PV. Animal studies indicated that IFN-γ secreting CD4⁺ T lymphocytes are critical in mediating protection (Chen et al. 2008). However, the increase in the proportion of CD4⁺ and CD8⁺ T cells in the present study may not represent vaccine specific response as the estimates were obtained by direct staining of PBMCs without being stimulated in vitro by MAP antigen.

The CD4⁺ and CD8⁺ counts before vaccination and throughout the study among sham immunized control calves were in agreement with the reference values for relative numbers of CD4⁺ (17.2 – 20.7%) and CD8⁺ cells (7.1 – 8.7%) in cattle blood as reported by Kulberg et al. (2004), who analyzed a sample size of 254 animals of different ages and suggested that data can serve as reference values for lymphocyte proportions.

Vaccination with bacterins cannot prevent infection, do not completely prevent faecal shedding (Tewari et al. 2014) and transmission of infection to calves, but limits the progression of clinical disease. Therefore hygienic management practices to prevent infection to calves remain essential (Kalis et al. 2001, Whittington et al. 2019). Furthermore, the current vaccines for MAP compromise the diagnosis of bovine tuberculosis in cattle (Serrano et al. 2017, Garvey 2020). Some countries like Sweden, completely prohibit by law the usage of vaccination for JD (Matthews et al. 2021). Development of a JD vaccine that prevent MAP infection and/or fecal shedding without interfering with bovine tuberculosis testing is essential (Barkema et al. 2017). Research for the development of live attenuated vaccines and subunit vaccines, and developing accompanying assays for differentiation of infection with M. bovis or MAP and vaccinated animals is under way (Shippy et al. 2017, Phanse et al. 2020).

Overall, the inactivated Johne’s disease vaccine was found to be effective in stimulating the immune system of the calves and eliciting MAP specific immune responses. However, development of a JD vaccine for cattle that
prevent MAP infection and/or fecal shedding, and the one that does not interfere with bovine tuberculosis testing and fulfill DIVA is highly warranted.

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