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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

Johne's disease (JD) among dairy cattle caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is gaining importance in India, as the cause of significant economic losses due to early culling of animals and decreased productivity. Herd prevalence of Johne's disease in dairy herds is estimated to be 68% in the USA (Kirkpatrick *et al*. 2011) and 20% among cattle in several countries of Europe (Nielsen and Toft 2009). Significantly high prevalence of MAP infection has been reported in sheep and goat (22.5%), and in many organized cattle and buffalo herds (29%) in India (Kumar *et al*. 2007, Singh *et al*. 2008, Narnaware and Tripathi 2017). JD could be one of the reasons for poor per animal productivity of Indian livestock, and it is also a reason for trade restrictions.

MAP infection is incurable and control by treatment of sick animals is neither practically feasible nor cost effective. Control of paratuberculosis depends on population level measures such as the culling of animals that are shedding MAP, applying hygienic measures aimed at reducing infection of neonatal /young stock, and vaccination (Whittington *et al*. 2019). Vaccination has the promise to best combat this chronic infection as it reduces the fecal shedding of MAP and prevents progression to clinical disease. Prophylactic vaccination as a control measure is in practice for many years in the USA and Australia. At present no effective vaccine is commercially available for controlling JD in India. Vaccination of cattle against JD could be a cost effective control strategy in developing countries such as India where it is not economically feasible to implement a 'test and slaughter' policy.

To be effective, the vaccines must induce a good Th 1 response characterized by rise in $CD4^+$ lymphocytes and interferon-γ levels, which are considered to be critical in resistance against JD (Phanse *et al*. 2020). Antibody response would be an indicator of the degree of activation of the immune system against MAP (Copra *et al*. 2000). Our study is aimed at developing a JD vaccine for cattle using a MAP strain which is locally prevalent and studying its efficacy in terms of stimulating antibody and cellular immune responses.

MATERIALS AND METHODS

A heat inactivated JD vaccine was developed as per the method of Singh *et al.* (2007) using a 'bison type' strain of MAP isolated from cattle in Tamil Nadu. This 'bison type' strain of MAP was isolated and maintained in the Department of Biotechnology, Madras Veterinary College and was characterized in a previous study (Chaitanya *et al*. 2015). MAP was propagated in Reid's synthetic medium, in Roux flasks incubated at 37°C for a period of 2-3 months. The culture was harvested and heat inactivated by keeping in water bath at 70°C for 2 h. The inactivated culture was adjuvanted with sterile mineral oil to obtain

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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 vaccutainers for serum separation, sodium EDTA vaccutainers for lymphocyte separation and in lithium heparin vaccutainers 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

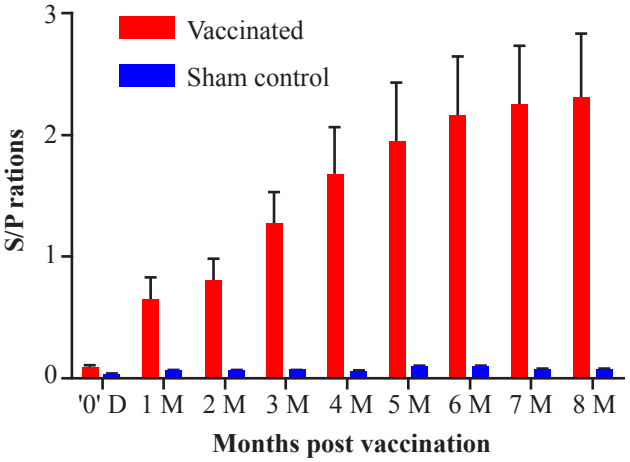

Fig. 1. Antibody response to JD vaccination in calves.

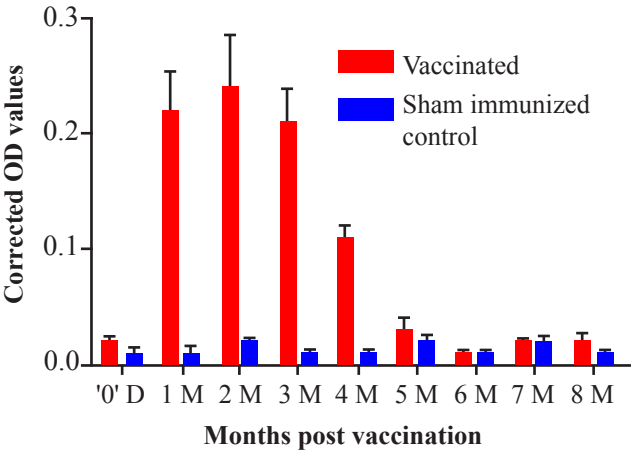

Fig. 3. Interferon gamma response to vaccination in calves.

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).

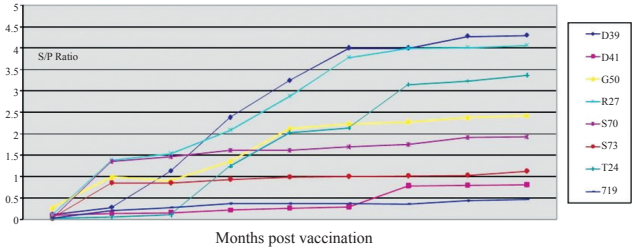

Fig. 2. Antibody response of individual calves to JD vaccination.

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 vaccinates 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.

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 lymphproliferative responses up to four months PV in vaccinated calves ($P<0.01$) and declined after that (Fig. 4).

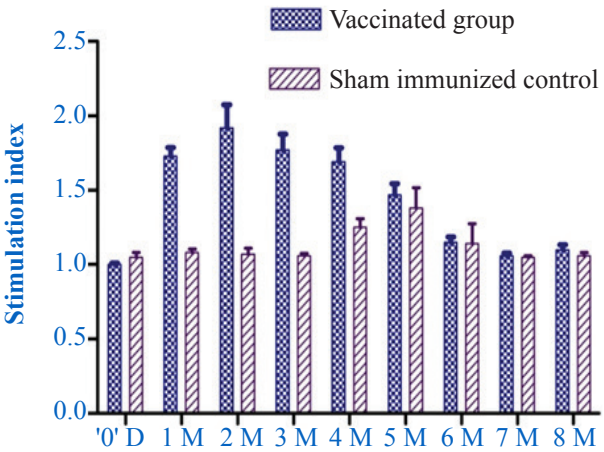

Fig. 4. Lymphoproliferative response of PBMCs of calves.

Lymphoproliferative response to antigen stimulation has been widely used as *in vitro* correlate of CMI stimulation by vaccines by several workers (Begg and Griffin 2005, Shin *et al.* 2005, Kumanan *et al.* 2009, Griffin *et al.* 2009). Singh *et al.* (2007) reported very high degree of proliferative responses to MAP 'Bison type' vaccine from first to ninth month

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

Months post vaccination	$CD4^+$ %					$CD8^+$ %				
	Vaccinated group (N=8)		Sham immunized controls (N=4)		P Value	Vaccinated group (N=8)		Sham immunized controls (N=4)		P value
	Mean	±SE	Mean	±SE		Mean	±SE	Mean	±SE	
'0' day	19.45	1.6	18.03	1.89	0.58	6.98	0.9	6.30	1.15	0.65
1 month	26.83	1.05	20.64	2.16	0.04*	9.32	1.08	5.62	1.21	0.06*
2 month	29.22	1.95	20.86	1.82	0.02*	10.72	0.74	6.18	1.41	0.03*
3 month	32.57	1.15	21.42	2.56	0.007**	12.36	1.32	6.66	1.7	0.04*
4 month	26.03	1.65	19.87	1.23	0.024*	10.28	1.03	8.74	0.77	0.28
5 month	22.52	1.01	21.36	1.64	0.56	9.12	1.57	7.53	0.80	0.82
6 month	21.70	0.95	20.32	1.17	0.39	8.98	1.28	9.04	0.64	0.96
7 month	22.34	0.65	21.04	1.33	041	8.84	1.77	8.43	0.83	0.77
8 month	21.42	1.3	20.82	1.24	074	9.22	0.74	7.73	0.7	0.97

*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.

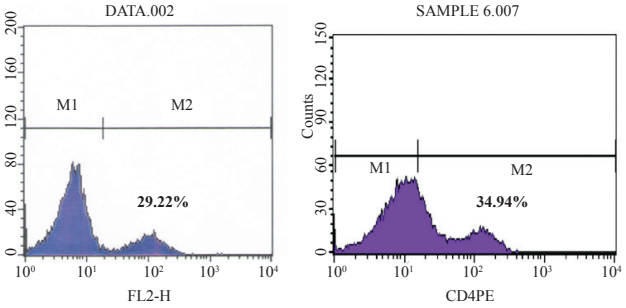

Fig. 5. A representative single parameter histogram showing $CD4^+$ lymphocyte count in PBMCs of calves 2nd and 3rd month PV.

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 fulfil DIVA is highly warranted.

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