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Protective Efficacy of Chitosan Coupled Johne's Disease Vaccine

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ABSTRACT

Background: Johne's disease (JD) is a chronic, economically important disease of domestic ruminants. Continuous efforts are being made to develop a potent vaccine for JD which confer a longer immunity. The present study was aimed at developing chitosan nanoparticles coupled JD vaccine and assess its efficacy.

Methods: Potency of a heat killed, chitosan nanoparticle coupled JD vaccine developed with an Indian isolate of *Mycobacterium avium* subsp *paratuberculosis* (*MAP*) was tested in 107 goats. Pre and post immunization blood samples were collected at different time points and peripheral blood mononuclear cells were used for assessing IFN-γ, IL-2, IL-10 and IL-12 gene expressions. Immunized animals were challenged with a field isolate of MAP and the immune response was assessed.

Result: Immunized goats were safe with no untoward reactions. Cytokine gene expression studies indicated a good Th1 response during 3-14 wk post immunization. The initial Th1 response was followed by a good Th2 response with a better IL-10 response than the IFN-γ and IL-2 responses in vaccinated animals at 23 wk PI. Both Th1 and Th2 responses were significantly higher in immunized animals at 23 and 34 weeks post challenge indicating a protective immune response.

Key words: Heat killed vaccine, Johne's disease, *Mycobacterium avium* subsp. *paratuberculosis*.

INTRODUCTION

Johne's disease (JD), also called as Paratuberculosis is a chronic disease affecting domestic and wild ruminants. JD, caused by *Mycobacterium avium* subsp *paratuberculosis* (MAP) is characterized by weight loss, reduced milk yield, diarrhea and granulomatous enteritis. JD is worldwide in distribution and indeed endemic in India. Prevalence of JD has been reported in several states of India and the rate of prevalence has a wide variation. In small ruminants, the prevalence percentage ranged between 3.5 and 42.3. In large ruminants the prevalence was 1.8 to 34.1 per cent (Singh *et al*. 2009). JD is an economically important disease and the loss could be attributed to meat and milk production loss, early culling of reactors and loss due to mortality.

With no systematic vaccination programme in place, high prevalence and a long, silent sub-clinical phase have placed JD control strategies in a difficult situation. Though live vaccines are available and being used for the control of JD world over, they do not give 100% protection necessitating the need for more vaccines which can prevent infection and shedding of the bacteria with longer duration of immunity. Moreover, MAP is an intracellular pathogen and effective immunization strategies aim at vaccine candidates that stimulate a robust Th1 response (Seder and Hill, 2000). Use of local isolates of pathogens in vaccine preparations has always been a preferred approach (Uzonna *et al*. 2003). Moreover, vaccine delivery also plays a major role in inducing a good protective immune response. Recent attempts involving the use of nanoparticles as vaccine carriers have yielded good results prompting the use of nanoparticles as a vaccine delivery system (Santos *et al*. 2019). A heat killed, chitosan nanoparticle coupled JD vaccine developed with an Indian MAP isolate has proved to be effective in goats under in house conditions. To further validate the potency of the vaccine and duration of immunity, the present study has been done with limited challenge studies.

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MATERIALS AND METHODS

Propagation of *Mycobacterium avium* subsp *paratuberculosis*

The MAP isolate from local cattle available at the Department of Animal Biotechnology, Madras Veterinary College, Chennai was propagated in 7H9 medium with Mycobactin J and used in the study for the preparation of heat killed, chitosan nanoparticle coupled JD vaccine during 2016.

Preparation of heat killed, chitosan coupled whole cell vaccine

MAP isolate was bulk produced in 7H9 medium. Cultured cells were suspended in PBS and exposed to 90°C for two hrs. After heat exposure, aliquots of the culture was inoculated into two tubes Heralds egg yolk (HEY) slant with Mycobactin J (MJ) and one tube of HEY slant without MJ for checking the safety of the heat killed vaccine. Chitosan nanoparticles were prepared as per the method of Zhu *et al*. (2007)

(2007) with minor modifications. Heat killed sonicated whole cell MAP antigen was suspended in PBS pH 5.6 and mixed with 1% Chitosan nanoparticles. The mixture was incubated at 28°C for one hr with intermittent shaking followed by incubation at 28°C to couple the antigen to the Nanoparticles. The final pellet was suspended in PBS pH 7.5 with 0.5% Trehalose. The resultant nanoparticle coupled vaccine contained 24 mg wet weight antigen per milliliter (One dose).

Immunization trial

Goats maintained at organized farms (University Research farm, Madhavaram and PGRIAS farm, Kattuppakkam) were selected and used in the study. Group I containing 107 animals was administered with one dose of heat killed, chitosan coupled JD vaccine and Group II comprising of 10 animals was kept as unvaccinated control.

Collection of samples and assessment of differential immune responses

Blood samples were collected prior to administration of the vaccine from all the animals. Post immunization blood samples were collected at 3 week, 14 week and 23 week and peripheral blood mononuclear cells (PBMCs) were used for studying the expression of IFN-γ, IL-2, IL-10 and IL-12 gene expression. Cytokine gene expression was done by Real time quantitative Polymerase chain reaction (QPCR) with Sybr green chemistry using the following designed primers at an optimum annealing temperature of 55°C.

IFN-γ: FP- 5′ AGATAACCAGGTCATTCAAAGGAG 3′
RP- 5′ GGCGACAGGTCATTCATCAC 3′
IL-2: FP- 5′ TACCAGATACCACTCTTGTC 3′
RP- 5′ TCCAGCAGCAATGACTTC 3′
IL-10: FP- 5′ TGGAGCAGGTGAAGAGAG 3′
RP- 5′ TGGGTCGGATTTCAGAGG 3′
IL-12: FP- 5′ CAGACCAGAGCAGTGAGG 3′
RP- 5′ AAGCAGGAGGAGTGAACG 3′
β-actin: 5′ FP- 5′ GCCCCTCTGAACCCCAAA 3′
RP- 5′ GCAGGAGTGTTGAAAGTCTCGAA 3′

Challenge with *Mycobacterium avium* subsp *paratuberculosis*

23 weeks after immunization, four animals from each group were challenged orally with 2×10^8 MAP cells/animal in 20 ml of milk for 5 consecutive days and assessed for the protective efficacy of the vaccine upto a period of approximately six months, by studying cytokine gene expressions until 34 wk post challenge.

RESULTS AND DISCUSSION

Johne's disease is highly endemic in India. Developed countries follow the strategy of testing, culling and vaccination for the control of JD. In India, culling of positive animals will be very challenging due to socioeconomic issues. With regular vaccination being the only option, availability of a potent and cheap vaccine inducing a good Th1 response is of paramount importance. In this context, we tested the potency and duration of immunity of a heat killed, chitosan coupled vaccine.

Pre challenge immune responses

MAP is a slow-growing intracellular pathogen and a good Th1 response than the Th2 response is required to overcome the invasion of intracellular pathogen. Th1 response for MAP vaccine was assessed by studying IFN-γ, IL-2 and IL-12 cytokine gene expressions and Th2 response by IL-10 expression. Cytokine gene expression following immunization indicated better Th1 response 3 weeks (wk) post immunization (PI). IFN-γ and IL-2 expressions were found to be the highest at 3wk PI (Fig 1) in immunized goats. IL-12 has been found to promote cell mediated immune response by stimulating IFN-γ production (Wojno *et al*. 2019). Innate ability of dendritic cells to recognize pathogens lead to IL-12 production which is responsible for activating CD4-positive T cells to differentiate into Th1 cells that produce IFN-γ. Though significant increase in IL-12 response was noticed at 3 wk PI, the response peaked at 14 wk PI, probably since it is also involved in maintenance of IFN-γ producing Th1 cells (Uzonna *et al*. 2003). As expected IL-10, the Th2 response indicator increased at 3 wk PI and peaked at 14 wk PI in immunized animals (Fig 1). Moreover, in accordance with the fact that IL-10 might have a role in immunity during later stage of exposure (Park and Scott, 2001), the response was found to be stable even at 23 wk PI (Fig 1).

Post challenge immune responses

Post challenge (PC) studies have indicated an increase in IFN-γ, IL-2 and IL-12 responses one wk after challenge (Fig 2, 3 and 4). A slight decrease in IFN-γ and IL-12 responses were noticed at 2 wk PC and 2,8 wk PC respectively. Several studies have indicated that IL-12 is the key cytokine that initiates and maintains Th1 responses (Park and Scott, 2001). All the three Th1 cytokines (IFN-γ, IL-2 and IL-12) were seen up regulated up to the observation period of 34 weeks PC indicating a strong Th1 response in the immunized animals. With increased levels in immunized goats, IFN-γ might have played a vital role in the protective immunity of the animals following challenge with MAP by IFN-γ mediated signalling of macrophages (Kumanan *et al*. 2009).

Earlier studies with MAP have suggested a dominant Th1 response initially and a predominant Th2 response at the later stage of the disease. This could be due to the ability of extracellular MAP to persist outside of macrophages and results in a changeover from cellular immunity to humoral immunity (Magombedza *et al*. 2014). In our study too, though a dip in Th2 response was noticed until 2 wk PC as evidenced by a decreased IL-10 response (Fig 5), the expression increased in immunized animals from 8 wk PC onwards. Nevertheless, both Th1 and Th2 responses were significantly higher in immunized animals at 23 and 34 wk PC indicating a protective immune response.

Initially, the protective immune response to primary vaccination was assessed for a period of up to six months.

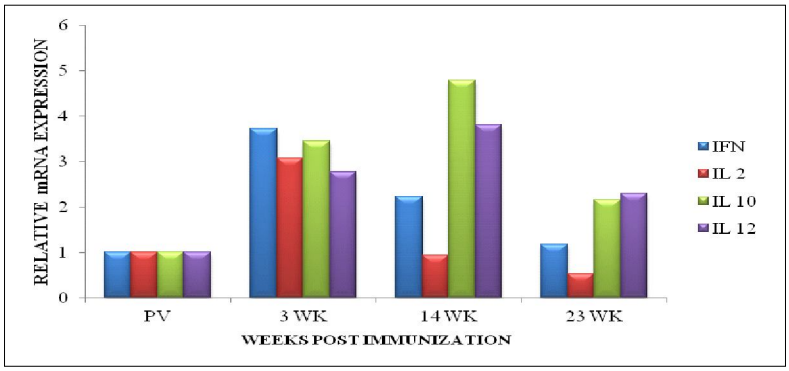

Fig 1: Cytokine gene expression following immunization with heat killed, chitosan coupled MAP vaccine.

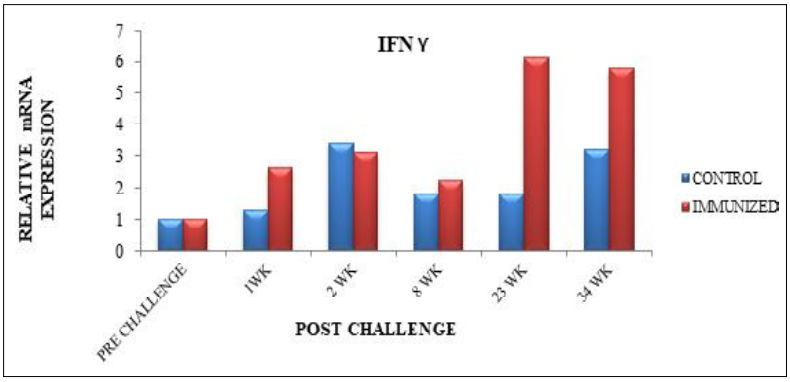

Fig 2: IFN-γ response to MAP vaccination after challenge with MAP.

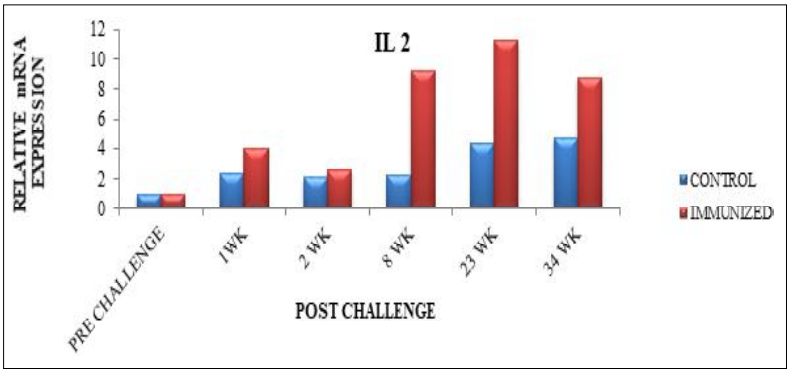

Fig 3: IL-2 response to MAP vaccination after challenge with MAP.

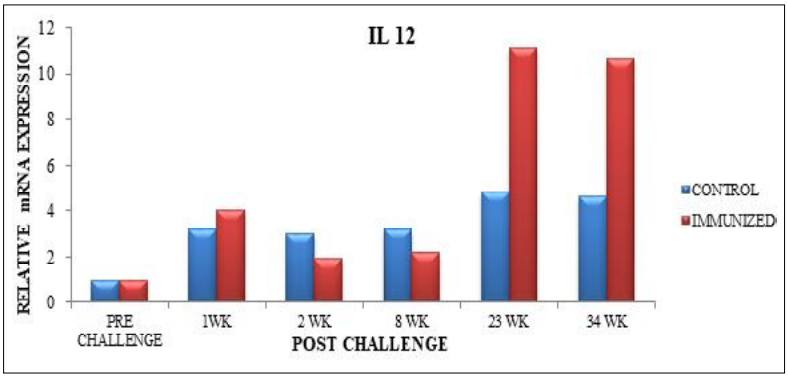

Fig 4: IL-12 response to MAP vaccination after challenge with MAP.

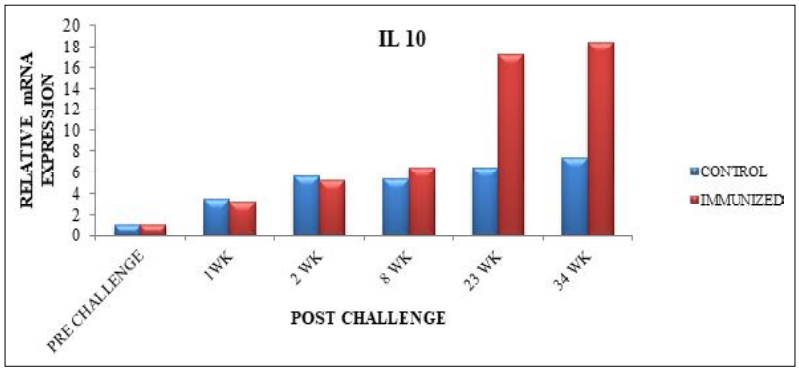

Fig 5: IL-10 response to MAP vaccination after challenge with MAP.

Following challenge, the immune response was tested up to a period of 9 months to assess the duration of immunity. It is clear from our studies that our vaccine induced a good Th1 response as required in case of intracellular pathogens like MAP as evidenced by a decent IFN-γ, IL-2 and IL-12 responses up to 14 wk PI. This initial Th1 response was followed by a good Th2 response with a better IL-10 response than the IFN-γ and IL-2 responses in the vaccinated animals at 23 wk PI. In addition to our immunological findings, we also observed that, six months post challenge, un immunized control animals developed occasional mild diarrhoea, whereas immunized animals showed no such symptoms. Post challenge, there was weight loss in un immunized control animals compared to the immunized animals.

CONCLUSION

Cytokine gene expression studies have indicated a good Th1 response until 14 wk PI. This initial Th1 response was followed by a good Th2 response with a better IL-10 response than the IFN-γ and IL-2 responses in the vaccinated animals at 23 wk PI. Following challenge, both Th1 and Th2 responses were significantly higher in immunized animals at 23 and 34 weeks post challenge indicating a protective immune response.

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