



Immunogenicity of chitosan coupled heat killed and fusion protein vaccine of *Mycobacterium avium* subsp. *paratuberculosis*

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Received: 17 May 2020; Accepted: 21 July 2021

Keywords: Heat killed vaccine, Johne's disease, *Mycobacterium avium* subsp. *Paratuberculosis*

Johne's disease (JD), also called as paratuberculosis is caused by *Mycobacterium avium* subsp *paratuberculosis* (MAP). JD is a chronic and economically important disease with worldwide incidence including India, and affects both domestic and wild ruminants (Singh *et al.* 2007, Singh *et al.* 2009, Vijayarani *et al.* 2009). In India, control and eradication of JD can be best done by early diagnosis and regular vaccination in endemic areas. Though a number of whole-cell based attenuated and killed vaccines have been developed to prevent MAP infections (Bastida and Juste 2011, Gupta *et al.* 2019), there are still lots of concerns about the available vaccines like cost, availability, potency, duration of immunity and ability to stop shedding of the organism, etc. Use of local strains or isolates of pathogens in vaccine preparations is always preferred. More attention is being given for the development of marker vaccines, especially for endemic diseases. In the above said scenario, the present study has been undertaken to assess the immunogenicity of chitosan coupled heat killed whole cell MAP vaccine and a recombinant fusion protein vaccine developed from a local *Mycobacterium avium* subsp. *paratuberculosis* isolate of cattle.

A cattle isolate of MAP available at the Department of Animal Biotechnology, Madras Veterinary College, Chennai was propagated in 7H9 medium with Mycobactin J and used for whole cell antigen production and genomic DNA isolation. Fusion gene was generated by the sequential linkage of the C terminal of the 85A gene fragment to the full-length ORF of 85B gene. Different sets of primers were designed for generating the 85A and 85B fusion gene. The 5' and 3' oligonucleotides to the 85A gene were designed as follows: (5' CATATG ACG CTT GTC GAC AGA TT 3') and (3' GAATTC GGT GCC CTG GCC GTT CCC 5'). The 5' oligonucleotide contained an *NdeI* and 3' oligonucleotide contained an *EcoRI* restriction sites (underlined). These oligos were used to amplify 85A, and the resulting PCR-amplified product was ligated to a pCR2.1 Topo vector. The plasmid DNA with right insert was digested with *NdeI* and *EcoRI*, and ligated into the pET22b expression vector cut

with the same enzymes. The ligated products were transformed into *E. coli* DH5 α cells and the transformants with the correct insert was identified by restriction enzymes digestion and by DNA sequencing. The 5' and 3' oligonucleotides of the 85B were designed as mentioned below:

5'(5'-GAATTC ATG ACA GAT CTG AGC GAG 3') and 3'(5'-AAGCTT TCC GCC GCC GCC CGG GGA-3').

The 5' oligonucleotide contained an *EcoRI* and the 3' oligonucleotide contained a *HindIII* restriction sites (underlined). The resulting PCR-amplified product was cloned into Topo PCR2.1 and digested with *EcoRI* and *HindIII*. The *EcoRI* and *HindIII* released product was ligated into the recombinant 85A pET plasmid digested with *EcoRI* and *HindIII*. The ligation mixture was used to transform *E. coli* DH5 α cells and the positive clones were identified by restriction digestion. The final construct, encoded a 66-kDa fusion protein (*Map 85AB*). *Map 85AB* fusion construct was transformed into *E. coli* BL21 (DE3 pLysE) cells. *Map 85AB* fusion protein was expressed by inducing the transformed clone with 1 mM IPTG and checked by SDS-PAGE.

Heat killed (HK) whole cell vaccine was prepared with the local MAP isolate by exposing the organism at 90°C for 2 h. Chitosan nanoparticles were prepared as per the method of Zhu *et al.* (2007) with minor modifications. Heat killed sonicated whole cell MAP antigen was suspended in PBS pH5.6 and mixed with 1% chitosan nanoparticles. The mixture was incubated at 28°C for 1 h 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). Fusion protein (*MAP 85AB*) was also coupled with chitosan nanoparticles and the resultant nanoparticle coupled vaccine contained 100 μ g of antigen per millilitre (One dose).

Two groups of 8 animals each were immunized with 1 ml of heat HK whole cell MAP vaccine coupled with chitosan nanoparticles (heat killed) and *MAP 85AB* (fusion protein). One group of control animals was also included in the study. Pre vaccination blood samples were collected

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for serum separation and peripheral blood mononuclear cells (PBMCs) isolation. Th1 responses to the HK vaccine and the fusion protein (FP) was assessed by lymphocyte proliferation assay, IFN- γ assay and cytokine gene expression assays employing the PBMCs collected prior to immunization and 6 and 10 wk post immunization (PI) as described earlier (Kumanan *et al.* 2008). Th2 response was assessed by serum antibody levels following immunization. A second immunogenicity trial was conducted in three more groups of goats for a longer period of six months to assess the duration of immunity. Group I received HK vaccine, group II received FP vaccine and group III was unvaccinated control animals. The animals were assessed for Th1 and Th2 response by serum antibody levels and IFN- γ response at 8 and 24 weeks PI.

Bio-incidence of JD has been reported to be on the rise and could be endemic in many herds in the India (Gupta *et al.* 2019). In India, an indigenous vaccine developed with an 'Indian Bison Type' genotype of MAP was found to improve production parameters and reduction in mortality and morbidity due to JD (Singh *et al.* 2013). Nevertheless, non-availability of a foolproof vaccine which can prevent the disease as well as prevent or stop the shedding of the bacteria from infected ruminants, the search for better vaccine candidates continues.

The chitosan nanoparticles produced were found to be uniform in size and ranged between 47 and 58 nm. Generation of 85A+85B fusion protein (MAP 85AB) resulted in a 66-kDa fusion protein. Both, the HK vaccine and the FP induced good antibody response. The response was significantly higher in the HK vaccine administered animals than those received the FP (Fig. 1). Following immunization, the lymphocyte proliferation was significantly higher in HK vaccine compared to the FP (Fig. 2). Likewise, significant differences were detected in the IFN- γ responses between the vaccinated and control animals at 6 and 10 wk post vaccination (Fig. 3). Significant increase in HK and FP specific IFN- γ gene expression was detected in the immunized animals in contrast to the control animals. The response was significantly higher with the HK vaccine than the FP (Fig. 4).

MAP being an intracellular pathogen, the hosts depend mostly on cell mediated Th1 response to control the infection. In our study, both the whole cell MAP and fusion protein MAP 85AB induced a better response than the control animals. Though the 85A and 85B proteins have been

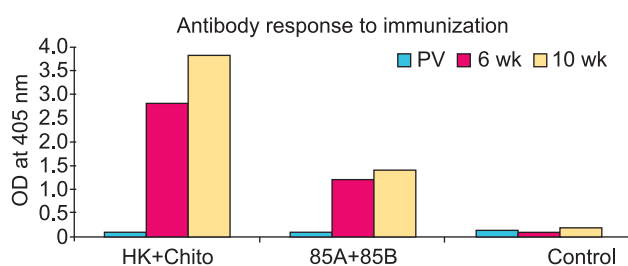


Fig.1. Antibody responses to heat killed and fusion protein vaccines in immunized and control animals.

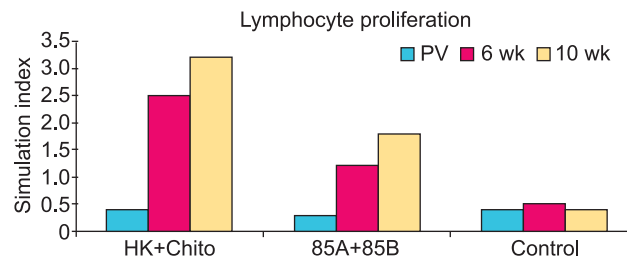


Fig. 2. Lymphoproliferative responses of PBMCs from immunized and control animals to the heat killed and fusion protein vaccines. Results are expressed as stimulation index.

reported to be good vaccine candidates (Shin *et al.* 2005, Kumanan *et al.* 2008), as a fusion protein, performance has not been as good as the whole cell MAP. Quite understandingly, the fusion protein, MAP 85AB was not inducing as good a response as the HK vaccine. However, by improving its efficacy with other adjuvants or including other immunogenic proteins in the construct, FP vaccine could be useful as a 'Marker vaccine' in DIVA strategies. Nevertheless, both Th1 and Th2 responses of the fusion protein were significantly higher than the control animals. It is observed that, the immune response has been well maintained during the entire observation period of 10 weeks for both the HK and the FP vaccine. Vaccines prepared from local field isolate strain has been found to enhance the immune responses better than the commercial vaccine if the vaccination with the field isolate strain is done prior to exposure of the animals to the field isolate (Uzonna *et al.* 2003). This timing is important since prior exposure to field MAP might alter the cytokine environment and thus, interfere with the efficacy of the vaccine. IFN- γ , the Th1 type cytokine is produced by antigen specific T cells and plays a vital role in the activation of host macrophages and thereby killing intracellular parasites like MAP. IFN- γ response is upregulated in both vaccinated groups than the control group, the response being significantly higher in HK MAP vaccinated animals (Figs 3 and 4).

In the second trial, interferon gamma response assessed after 8 weeks and 24 weeks post immunization indicated better response in HK vaccine. Similarly, the sero conversion was also found to be better in the HK vaccine than the FP vaccine. Conjugating both vaccine candidates with chitosan nanoparticles have given the adjuvant effect, with the HK vaccine performing better than the FP. The adjuvant effect of chitosan nanoparticles is clearly evident

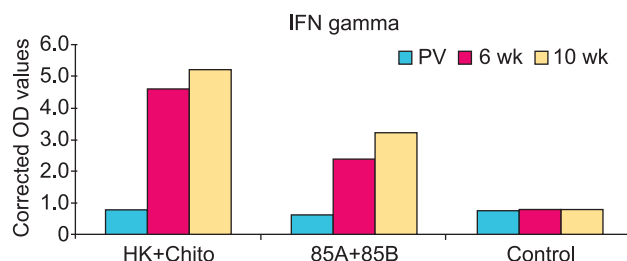


Fig. 3. Antigen specific IFN- γ response of PBMCs from immunized and control animals. Results are expressed as corrected OD values.

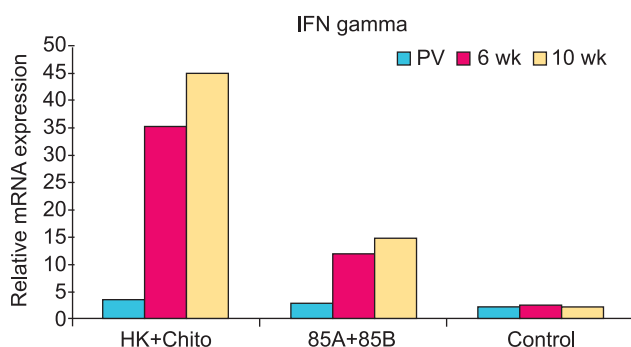


Fig. 4. IFN- γ mRNA expression in the immunized groups and control group. Results are expressed as the mean fold increase over unstimulated PBMCs served as calibrators.

in the second trial wherein the animals were observed for a longer period of 24 weeks. With increased IFN- γ and antibody levels, vaccinated animals responded well as compared to the control ones.

It is concluded that the HK chitosan coupled whole cell MAP vaccine was found to be safe with good immune response for up to 24 weeks. With some more on farm and field trials, this vaccine could be further processed for commercialization.

SUMMARY

In search for better vaccine candidates for Johne's disease, chitosan nanoparticle coupled heat killed whole cell and fusion protein (Antigen 85A+85B) vaccines were developed using a local isolate of *Mycobacterium avium* subsp. *paratuberculosis*. Immunization studies in goats indicated that the heat killed whole cell vaccine and the fusion protein vaccine were found to be safe and resulted in Th1 and Th2 responses. Antibody response was significantly higher in the heat killed whole cell immunized animals than those received the fusion protein. Significant increase in heat killed whole cell MAP and fusion protein specific IFN- γ gene expression was detected in the immunized animals in contrast to the controls. The response was significantly higher with the heat killed whole cell MAP than the fusion protein. The adjuvant effect of chitosan nanoparticles was clearly evident in the second trial wherein the animals were observed for a longer period of 24 weeks. With increased IFN- γ and antibody levels, vaccinated animals responded well as compared to the control ones and the response was better in heat killed whole cell vaccine.

ACKNOWLEDGEMENTS

The authors thank the Department of Biotechnology,

Government of India, New Delhi for funding this work. The authors also thank the Dean, FBS, TANUVAS; Professor and Head, PGRIAS, Kattupakkam and the Professor and Head, Department of Animal Biotechnology, MVC for necessary facilities.

REFERENCES

- Bastida F and Juste R A. 2011. Paratuberculosis control: A review with a focus on vaccination. *Journal of Immune Based Therapies and Vaccines* **9**: 8.
- Gupta S, Singh S V, Singh M, Chaubey K K, Karthik K, Bhatia A K, Naveen Kumar and Dhama K. 2019. Vaccine approaches for the 'therapeutic management' of *Mycobacterium avium* subspecies *paratuberculosis* infection in domestic livestock. *Veterinary Quarterly* **39**: 143–52.
- Kumanan K, Park S U, McDonough S, Stehman S, Akey B, Huntley J, Wong S, Chang C F and Chang Y F. 2008. Vaccination with recombinant *Mycobacterium avium* subsp. *paratuberculosis* proteins induces differential immune responses and protects calves against infection by oral challenge. *Vaccine* **26**: 1652–63.
- Shin S J, Chang C F, Chang C D, McDonough S P, Thompson B, Yoo H S and Chang Y F. 2005. *In vitro* cellular immune responses to recombinant antigens of *Mycobacterium avium* subsp. *paratuberculosis*. *Infection and Immunity* **73**: 5074–85.
- Singh S V, Singh A V, Singh P K, Gupta V K, Kumar S and Vohra J. 2007. Sero prevalence of paratuberculosis in young kids using 'Bison type' *Mycobacterium avium* subsp. *paratuberculosis* antigen in plate ELISA. *Small Ruminant Research* **70**: 89–92.
- Singh S V, Sharma M C, Sohail J S, Singh A V and Singh P K. 2009. Johne's disease status, diagnosis and control: Indian Scenario. Central Institute for Research on Goats, Makhdoom, India. pp 10–16.
- Singh K, Chandel B S, Chauhan H C, Dadawala A, Singh S V and Singh P K. 2013. Efficacy of 'indigenous vaccine' using native 'Indian bison type' genotype of *Mycobacterium avium* subspecies *paratuberculosis* for the control of clinical Johne's disease in an organized goat herd. *Veterinary Research Communications* **37**: 109–14.
- Uzonna J E, Chilton P, Whitlock R H, Habecker P L, Scott P and Sweeney R W. 2003. Efficacy of commercial and field strain *Mycobacterium paratuberculosis* vaccinations with recombinant IL-12 in a bovine experimental infection model. *Vaccine* **21**: 3101–09.
- Vijayarani K, Shrine Nagalakshmi K, Balachandran C and Kumanan K. 2009. Simultaneous infection of Johne's and tuberculosis in a cow. *Indian Veterinary Journal* **86**: 960–61.
- Zhu B D, Qie Y Q, Wang J L, Zhang Y, Wang Q Z, Xu Y and Wang H H. 2007. Chitosan microspheres enhance the immunogenicity of an ag85b-based fusion protein containing multiple t-cell epitopes of *Mycobacterium tuberculosis*. *European Journal of Pharmaceutics Biopharmaceutics* **66**: 318–26.