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Ramesha CB
Dept. of Veterinary Microbiology, Veterinary College, KVAFSU, Hebbal, Bengaluru, Karnataka, India

D Rathnamma
Dept. of Veterinary Microbiology, Veterinary College, KVAFSU, Hebbal, Bengaluru, Karnataka, India

S Isloor
Dept. of Veterinary Microbiology, Veterinary College, KVAFSU, Hebbal, Bengaluru, Karnataka, India

BM Veeregowda
Dept. of Veterinary Microbiology, Veterinary College, KVAFSU, Hebbal, Bengaluru, Karnataka, India

HD Narayanaswamy
Dept. of Veterinary Microbiology, Veterinary College, KVAFSU, Hebbal, Bengaluru, Karnataka, India

M Narayana Bhat
Dept. of Veterinary Microbiology, Veterinary College, KVAFSU, Hebbal, Bengaluru, Karnataka, India

HK Siddalingamurthy
Dept. of Veterinary Microbiology, Veterinary College, KVAFSU, Hebbal, Bengaluru, Karnataka, India

SY Mukartal
Dept. of Veterinary Microbiology, Veterinary College, KVAFSU, Hebbal, Bengaluru, Karnataka, India

AG Prashanth
Dept. of Veterinary Microbiology, Veterinary College, KVAFSU, Hebbal, Bengaluru, Karnataka, India

KP Manjunatha
Dept. of Veterinary Microbiology, Veterinary College, KVAFSU, Hebbal, Bengaluru, Karnataka, India

Purushotham KM
Dept. of Veterinary Microbiology, Veterinary College, KVAFSU, Hebbal, Bengaluru, Karnataka, India

Correspondence
Ramesha CB
Dept. of Veterinary Microbiology, Veterinary College, KVAFSU, Hebbal, Bengaluru, Karnataka, India

Evaluation of immune response in sheep model following immunization with Inactivated *Mycobacterium avium* sub species *paratuberculosis* vaccine

Ramesha CB, D Rathnamma, S Isloor, BM Veeregowda, HD Narayanaswamy, M Narayana Bhat, HK Siddalingamurthy, SY Mukartal, AG Prashanth, KP Manjunatha and Purushotham KM

Abstract
Mycobacterium avium subspp. *paratuberculosis* (MAP) is an obligate pathogen causing Johne's disease (JD) in animals and responsible for significant economic loss in livestock industry. Currently no commercial vaccine is available in India for the control of disease, so the present study was conducted to evaluate efficacy of an indigenous inactivated MAP vaccine for the control of JD in experimentally challenged Mandya breed of sheep in an organized farm. A local MAP isolate S196 of sheep origin was inactivated at 72ºC for 2 hours and vaccine was prepared at a final concentration of 2.5 mg (dried) of MAP organisms per ml (approximately 12×10^8 bacilli/ml) in Aluminum hydrooxide gel and 0.01% Thiomersal. The study animals of 4-6 months of age were grouped into vaccinated and sham immunized animals and were vaccinated with inactivated MAP vaccine and challenged at 75, 77 and & 79 days post vaccination (DPV) with 3 x 10^9 colony forming units (CFU) of live MAP organisms through oral route. Two animals from each group were sacrificed at 90th, 240th and 300th DPV. Animals were evaluated for clinical symptoms, cell mediated and humoral immune responses, shedding of MAP bacilli and pathology. Vaccinated sheep gained higher bodyweights (BW 11.79 ±SE0.125221), also had high level of serum nitric oxide, significant lymphocyte stimulation by lymphocyte transformation assay and higher sero-conversion by indirect ELISA as compared to sham immunized sheep. There was significant difference between ($P < 0.05$) vaccinated and sham immunized animals in all immunological parameters. Sacrificed sham immunized animals showed pathognomonic gross lesions such as thickening and corrugation of small intestine, edematous and enlarged mesenteric lymph nodes which were not observed in vaccinated animals. Histopathologically infiltration of mononuclear cells and epitheloid cells in mucosa, submucosa of intestine and mesenteric lymph nodes were observed. In conclusion, indigenous inactivated MAP vaccine had protected the sheep against challenge infection with live MAP organisms with an effective CMI and humoral immune responses.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis,* Johne's disease, inactivated vaccine

1. Introduction
Johne's disease or Paratuberculosis, is a chronic granulomatous enteropathy of domestic and wild ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and disease was first reported by Johne & Frothingham in 1895. Paratuberculosis is found most often among domestic ruminants (cattle, sheep, goats, camelids and buffaloes) as well as wild ruminants (cervids) and has a global distribution. The disease has also been reported in horses, pigs, rabbits, stoat, fox, weasel (Greig *et al.*, 1999) [9], human beings and birds (Bennett *et al.*, 2012) [2]. Progressive weight loss and weakness are the main clinical signs in all species; in addition, there is diarrhoea in cattle (OIE, 2014) [24].

The disease is distributed worldwide and causes significant economic losses to the livestock industry because of premature culling and production losses. In the United States, MAP positive herds experience economic losses of almost US $100 per cow and a disease cost of US $200 to 250 million annually (Park and Yoo 2016) [25]. Vinodh Kumar *et al.*, (2013) [39] reported that in India annual economic losses due to ovine JD are around Rs 1,840 per sheep per farmer.

Rawath *et al.,* (2014) [27] reported that economic losses due to Bovine JD in dairy farm was estimated from reproductive disorders (Rs. 23400/cow/year), forced removal (Rs. 41,750 /cow/year), reduced milk yield (Rs. 5,712/cow/ year) and increased mortality (Rs. 11,666 /cow/year).

The MAP is highly resistant to environmental stresses like temperature and drying and is able to persist for many years. Clinically diseased animals may shed billions of MAP organisms per day in feces and contaminate the area which results in greater risk for animal as well as human exposure.

Under natural conditions MAP infections are usually acquired during young age via the ingestion of contaminated colostrum, milk, feed and water and fecal shedding of organisms by infected animals (Chiodini *et al*., 1984; Sweeny, 1996) [3, 35]. Animals under one year are more susceptible for the disease and ingestion of feed contaminated with infected materials is the primary cause of infection (Corpa *et al.,* 2000) [5]. Infection can be spread vertically to the fetus (Larson & Kopecky, 1970) [19] and semen can be infected with the organism **(**Sweeney *et al.,* 1996) [35].Seroprevalence of 18.33% was reported in cattle in south-west Bengaluru, Karnataka by indirect ELISA (Gupta *et al.,* 2012) [10]. Mukartal *et al.,* (2016) [22] reported Prevalence of 54.7% and 16.0% in sheep in LRIC, Nagamangala and Danagur farms of Karnataka respectively.

The effective disease control programs depend on a clear understanding of the sources of infection and the routes of transmission and early detection of infected animals, thereby allowing the removal of carriers from the herd. There are three major approaches to reduce or eradicate the Paratuberculosis, it includes efficient management to reduce decrease transmission, 'testing and culling' and vaccination. Currently the JD is diagnosed by multiple tests like Direct microscopy, ELISA and IS900 PCR (Mukartal *et al*., 2016) [22] but available diagnostic tests for Johne's disease does not detect all infected animals. For these reason, 'testing and culling' strategies using the present diagnostic methods are ineffective for eradication of the disease. Under these circumstances, vaccination can be the best control method (Singh *et al.,* 2013) [31, 32].

Vaccination has been used since 1926 (Vallee and Rinjard, 1926) [38] to control Johne's disease. The types of vaccines used have included both live (attenuated and non–attenuated) and killed whole cell vaccines against Paratuberculosis (Knust *et al*., 2013) [15]. In a few cases, subunit vaccines consisting of sonicated bacteria, bacterial cell fractions or recombinant MAP antigens have been used but they have shown a much lower degree of protection (Kathaperumal *et al.,* 2009) [14]. The vaccine can also be used for therapeutic purpose in infected animal because vaccination can reduce manifestation of clinical signs, which is more cost effective than 'testing and culling' (Park and Yoo, 2016) [25].

Currently no commercial vaccine is available for the control of JD in India, so the present study was conducted to evaluate the indigenous inactivated MAP vaccines of sheep origin in experimentally challenged Mandya breed of sheep in Livestock research and Information Centre (LRIC), Nagamangala, Karnataka.

2. Materials and Methods

2.1 Animal Ethics

Approval from Institutional Animal Ethics Committee (IAEC), Veterinary College, Bengalore registered with Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Government of India, was obtained to conduct vaccination trials in sheep (No/VCH/IAEC/2016/11 Dated 29-09-2016).

2.2 Animals and management conditions

A flock of apparently healthy 20 sheep of age group between 4-6 months from Livestock Research and Information Centre (LRIC), Nagamangala, Mandya district were selected for the study. Animals are screened by indirect ELISA for the anti-MAP antibodies before start of trial. Animals with positive or strong positive reaction in indirect ELISA were not included in the experiment and rests were randomly distributed into two groups with ten animals in each group.

2.3 Vaccine

Inactivated MAP vaccine was prepared using S196 MAP isolate recovered from clinically JD suffering sheep from the LRIC Nagamangala, Mandya. MAP organisms were inactivated in water bath at 72°C for 2 hours, vaccine was prepared at a final concentration of 2.5 mg (dried) of MAP organisms per ml (Concentration of bacilli was determined by McFarland method and was about approximately 12×10^8 bacilli/ml) in Aluminum hydrooxide gel as adjuvant and 0.01% Thiomersal as preservative.

2.4 Vaccination

Recommended dose of 1ml of vaccine and 1 ml of sterile PBS was administered to vaccine group and control group animals respectively through subcutaneous route in neck region as per Kumar *et al.,* (2014) [16].

2.5 Challenge Studies

Study animals were challenged with 3×10^9 colony forming units (higher than vaccine dose 12×10^8 bacilli/ml) of S196 live MAP isolate through oral route along with milk at 75^{th}, 77^{th} and 79^{th} DPV as per Begg *et al.*,(2005) [1].

2.6 Collection of Samples

Blood samples collected from all animals at 30 days intervals were monitored for cellular immune response by lymphocyte transformation test (LTT). Similarly serum samples were collected to analyze serum total nitric oxide and MAP antibodies to monitor cell mediated immune response and humoral immune response respectively. Changes and improvements in physical traits were continuously monitored at monthly intervals. Sick animals were treated. Daily treatment records and detailed necropsy reports of the sacrificed sheep during study period were collected and analyzed.

2.7 Cellular Immune Response

Lymphocyte Transformation Test (LTT) and serum nitric oxide (NO) estimation assay were done to estimate cell mediated immune response at monthly interval in all study animals up to 300 days of study period.

2.7.1 Lymphocyte Transformation Test

LTT was performed as per Uma *et al.,* (1999) [37] with few modifications. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples of sheep using Histopaque (Sigma Aldrich) as per manufacturer protocol from both group and cells were stimulated in duplicate with mitogen Con A (Sigma) at 20mg/ml and sonicated protoplasmic antigen of MAP at 20mg/ml concentration in RPMI 1640 cell culture minimum and a set of PBMCs of the same animal was kept as unstimulated control. PBMCs were

cultured in 96-well tissue culture plates at 37°C at 5% CO_2 for 120 hours and later MTT (3,4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed. About 25 µl of MTT dye at a concentration of 5 mg/ml was added to each well of the cell culture plate. Plate was incubated at 37°C for 4 h. After incubation 150 µl of Dimethyl sulfoxide (DMSO) was added to each well, plate was again incubated at 37° C overnight and the absorbance was read at 570 nm. Proliferation of lymphocyte was indicated by Optical density (OD) value of the well with test sample that of the unpulsed well. For data analysis purpose, Stimulative Index (SI) was calculated for individual animal using the formula: Average OD at 570 nm in stimulated wells/Average OD at 570 nm in non-stimulated control wells. Then the average SI value for each group of animals were calculated and compared to assess the CMI response induced in vaccinated and sham immunized groups at monthly intervals.

2.7.2 Estimation of serum nitric oxide

Nitric oxide is a gaseous free radical which has very short half-life *in vivo* of few seconds. Therefore, levels of more stable NO metabolites, nitrite (NO2-) and nitrate (NO3-) are detected spectrophotometrically to estimate NO concentration indirectly. Serum nitric oxide in the experimental animals was estimated by using commercially available EZ assay™ Nitric Oxide Estimation kit (HiMedia).

2.8 Humoral Immune Response

Post-vaccination antibody response was measured by in-house indirect ELISA at monthly interval up to 300 days of study period as per Kumar *et al.*, (2014) [16] with little modification. Optical Density **(**OD) values of tested serum samples were transformed into sample to positive (S/P) ratio.

Method

About 125 ng of sonicated MAP antigen per well was prepared in coating buffer (pH 9.6 ± 0.05) and 100 µl of this was added to each well. The plate was incubated at 4°C for overnight. The content of the wells was discarded and the plate was washed three times with wash buffer and gently tapped over a tissue paper. The unreacted sites on the carrier surface of the wells were blocked by incubating the plate with 200µl of one per cent bovine serum albumin -PBST at 37 °C for 60 min. The content of the wells was discarded and the plate was washed three times as described earlier. One hundred µl of 1:50 diluted test sera of individual animals in serum dilution buffer was added to wells in duplicates. Incubated at 37°C for 60 min. The plate was washed as described earlier. One hundred µl of 1: 2500 dilution of Protein A HRP conjugate in conjugate dilution buffer was added to each well and incubated at 37 °C for 60 min. The plate was washed as described earlier. 100 µl of freshly prepared chromogen-substrate solution containing OPD and 3 per cent H_2O_2 as substrate (4 µl / ml of OPD) was added to each well and the plate was kept at RT for 15 min. Finally, 50 µl of 2.5 N HCl was added to each well to stop enzyme-substrate reaction. Absorbance values were read at 490 nm using ELISA reader (Biorad Labsystems). S/P ratio of samples were calculated by formula

$$S/P\,\% = \frac{\text{Sample OD} - \text{Average OD of Negative controls}}{\text{Average OD of Positive controls} - \text{Average OD of Negative controls}} \text{ X } 100$$

2.9 Live animal traits

Body weights of study animals (both vaccinated and Sham-immunized) were recorded monthly intervals and were analyzed statistically. Sheep were monitored for morbidity and mortality during study period.

2.10 Necropsy

A total of 6 animals, one from each group were sacrificed at 90th, 240th and 300th DPV to assess the condition of different target and non-target organs of MAP.

2.10.1 Gross Pathology

After systematic post mortem examination, gross lesions in the intestines, associated mesenteric lymph nodes and lesions in other organs were recorded. The mesenteric lymph nodes were cut longitudinally and examined for gross changes.

2.10.2 Tissue PCR

DNA extracted from the tissue samples collected from the sacrified animals and subjected for the MAP IS900 PCR as per Mukartal (2017) [21].

2.10.3 Tissue direct microscopy

Tissue sections (mesenteric lymph node and intestine near ileo-junction region) from sacrificed animals of both groups were stained with Ziehl–Neelson (ZN) staining method to demonstrate presence AFB.

3. Results

3.1 Health Status

No adverse clinical reaction was observed in any of the sheep after vaccination with inactivated MAP vaccine. No mortality was observed in the vaccinated as well as in sham immunized animals during study period however clinical signs like intermittent diarrhea, weakness, cachexia, anaemia, submandibular edema, paleness of mucus membranes, rough hair coat, wool break were observed in some of sham immunized animals after 150 days of post challenge.

3.2 Cellular immune response

Cell mediated immune response against MAP was evaluated by Lymphocyte Transformation Test (LTT) and serum nitric oxide estimation in study animals at regular interval of time.

3.2.1 Lymphocyte transformation test (LTT):

PBMCs of study animals had approximately same stimulative index to protoplasmic MAP antigen at 0 DPV whereas later during study period stimulative index of vaccinated animals was significantly higher than the sham immunized animals (Graph 1). PBMCs from both the groups responded equally to non-specific stimulation (Mitogen Con A) indicating that cells were viable and functional.

Table 1: Mean Stimulative index (SI) of PBMC of study animals from 0 – 300 DPV

Group	Vaccine	N	Mean SI
Group I	Inactivated S196 MAP Vaccine	88	3.1779 ± 0.07142 [a]
Group II	Sterile PBS	88	2.2657 ±0.05615 [b]

3.2.2 Serum Nitric oxide estimation:

Serum nitric oxide level of all study animals were approximately same at beginning of experiment after vaccination and challenge, the NO level significantly increased in vaccinated animals than sham immunized animals (Graph 2). There is significant difference between the

mean serum NO level of vaccinated and sham immunized animals.

Table 2: Mean serum nitric oxide level of study animals from 0 – 300 DPV.

Group	Vaccine	N	MeanTNO (µM)
Group I	Inactivated S196 MAP Vaccine	110	75.0435 ± 1.18475 [a]
Group II	Sterile PBS	110	48.5162 ± 0. .76774 [b]

3.3 Humoral Immune Response

The rate of seroconversion in experimental animals was compared between the groups to evaluate the humoral immune response by indirect ELISA to detect the antibodies against MAP. After vaccination high sero-conversion rates were seen in vaccinated sheep as compared to sham immunized animals. Percent of sheep sero-converted remained high in vaccinated animals than in sham immunized animals at all post vaccination sampling intervals (Graph 3).

Table 3: Mean SP ratio of indirect ELISA of study animals from 0 – 300 DPV

Group	Vaccine	N	Mean SP ratio
Group I	Inactivated S196 MAP Vaccine	110	77.9773 ± 2.20159 [a]
Group II	Sterile PBS	110	53.6323 ± 2.20323 [b]

3.4 Body Weights Gained (overall)

Before vaccination all animals were healthy and approximately having same body weight however after vaccination overall marked improvement was found in the body conditions of vaccinated sheep as compared to sham immunized animals. Initial average body weights at 0 DPV were equal (approximately) in the two groups. After challenge with live MAP, the average body weight gained by sheep in vaccinated animals is significantly higher (Plate 1) than sham immunized animals (Graph 4).

Table 4: Average body weight gained per sheep by during study period.

Group	Vaccine	Average body weight + SE(Kgs)		Average body weight gain (Kgs)
		At 0 DPV	At 300 DPV	
Group I	Inactivated S196 MAP Vaccine	12.86±0.60	24.65±0.13	11.79±0.11[a]
Group II	Sterile PBS	12.89±0.19	18.06±0.23	5.17±0.17[b]

3.5 Necropsy

Tissue samples from sacrified animals were collected aseptically and subjected to tissue MAP IS900 PCR and direct microscopy by ZN staining.

3.5.1 Gross Lesions

Carcasses of sham immunized animals showed emaciation and hide bound condition. Paleness of mucus membranes and internal organs with very less or no subcutaneous, mesenteric and omental fat was observed. Lesions were more prominent in carcasses of animals sacrificed at 240 and 300 DPV than in carcass of animal sacrificed at 90 DPV. Carcasses of vaccinated animals had good body conditions with good amount of omental fat was observed. No abnormal lesions were detected in internal organs of vaccinated animals.

3.5.2 Gross pathology of intestines and associated lymph nodes

Sham immunized animals sacrificed at 240 and 300 DPV showed pathognomonic gross lesions such as the thickening of small intestine with corrugation specifically at ileocaecal junction, catarrhal enteritis, cordoning of lymphatics with edematous and enlarged mesenteric lymph nodes (Plate 3 and 5). However in carcasses of vaccinated animals no pathognomonic lesions of Paratuberculosis were observed (Plate 2 and 4).

3.5.3 Tissue Direct Microscopy

The microscopic changes observed in small intestine and associated mesenteric lymph nodes varied in their severity between the animals. Pink color small bacilli were observed in bunches in submucosa of small intestine (Plate 8) of sham immunized animal however no acid fast bacilli were observed in small intestine (Plate 7) of vaccinated animal.

3.5.4 Tissue MAP IS900 PCR

Tissues collected from the scarified animals were subjected to tissue MAP IS900 PCR. A total of 12 samples were subjected to IS900 PCR, out of 6 samples from vaccinated group none showed positive for MAP genome however 66.66% of samples from sham immunized animals showed positive for MAP by IS900 PCR (Plate 6).

4. Discussion

Paratuberculosis in one of the economically important disease of livestock caused by *Mycobacterium avium* subspp. *paratuberculosis.* Economic losses occur by the way of death or premature culling, loss of body conditions, productivity and increased predispositions to diseases. Control of Paratuberculosis by 'test and cull strategy' have proved counterproductive due to lack of sensitive diagnostic tests. At this point vaccination is the most economic method for control of Paratuberculosis in animals. An attempt has been undertaken by an International Committee of Johne's Disease (ICJD) to standardize the guidelines for prevention and control of JD and vaccination forming the integral part of it (Hines *et al.,* 2007). The present study was carried out to evaluate an indigenous inactivated MAP vaccine for the control of Paratuberculosis. The results of the study are discussed hereunder.

4.1 Clinical Symptoms

In the present study, out of ten sham immunized animals four animals showed clinical form of John's disease, clinical signs observed in those animals included pasty feces, weakness, cachexia, anemia, submandibular edema, paleness of mucus membranes, rough hair coat, wool break. The clinical manifestations of the study are in accordance with the observations of earlier reports by several workers (Gilmour, 1976; Chiodini *et al.,* 1984; Clarke, 1997; Windsor, 2014, Srikanth, 2017) [7, 3, 4, 40, 34]. They also reported similar symptoms in the JD affected sheep. The development of these manifestations could be due to wasting and chronic nature of the disease which results in improper ingestion and malabsorption of nutrients (Reddy *et al.,* 1984; Srikanth, 2017) [28, 34].

4.2 Cell mediated immune response

Cell mediated immune response elicited by inactivated MAP vaccine was analyzed by Lymphocyte Transformation Test

and serum total nitric oxide estimation.

4.3 Lymphocyte transformation test

Strong and long lasting cellular immune response induced by vaccine was measured by LTT exhibited protective effect of this vaccine. Lympho-proliferative response to antigen stimulation has been widely used as *in vitro* correlates of cell-mediated immunity (Gurang *et al.*, 2014) [11]

In the present study, PBMCs from vaccinated and shamimmunized animals were used to assess the response to MAP protoplasmic antigen. Proliferative response in terms of SI was greater in PBMCs from the vaccinated groups (Mean SI 3.1779 ± 0.07142 [a]) to MAP protoplasmic antigen at all the sampling periods, ($P < 0.05$) than PBMCs of Sham-immunized (Mean SI 2.2657 ±0.05615 [b]) group.

The results of the study are in accordance with the observations of earlier reports by several researchers (Begg *et al.*, 2005; Singh *et al.*, 2007; Kumudika *et al.*, 2009**;** Singh *et al.*, 2013; Kumar *et al.*,2014) [1, 7, 31, 32, 16].

4.4 Nitric Oxide Estimation

Immunity to all mycobacterial infections is dependent on cell-mediated responses and mustbe elicited by a vaccine to be protective (Corpa *et al.*, 2000) [5] and the NO assay is an indirect reflector of CMIR (Goswami *et al.*, 2014) [8]. Mullerad *et al.*, (2003) [23] reported that the reactive nitrogen such as nitric oxide is most effective in direct killing of Mycobacteria. In the present study, mean serum NO level of vaccinated animals is significantly higher (75.0435 ± 1.18475 [a] µM) than sham immunized animals (48.5162 ± 0. .76774 [b] µM) during study period. The results of the study are in accordance with the observations of several workers Goswami *et al.*, (2014) [8]; Singh *et al.*, (2013) [31, 32]; Jubeda *et al.*, (2014) [13].

4.5 Humoral Immune Response

Humoral immune response in study animals measured by i-ELISA. SP ratio of study animals are approximately same before vaccination but after vaccination SP ratio of vaccinated animals (mean SP ratio 77.9773 ± 2.20159 [a]) higher than sham immunized animals (mean SP ratio 53.6323 ± 2.20323 [b]) however SP ratio of sham immunized animal showed with a constantly increasing trend than vaccinated animals. The results of the study are in accordance with the observations of earlier reports by several workers Begg *et al.*, (2005) [1]; Singh *et al.*, (2013) [31, 32]; Faisal *et al.*, (2013) [6]; Gurung *et al.*, (2014) [11].

4.6 Body Weight

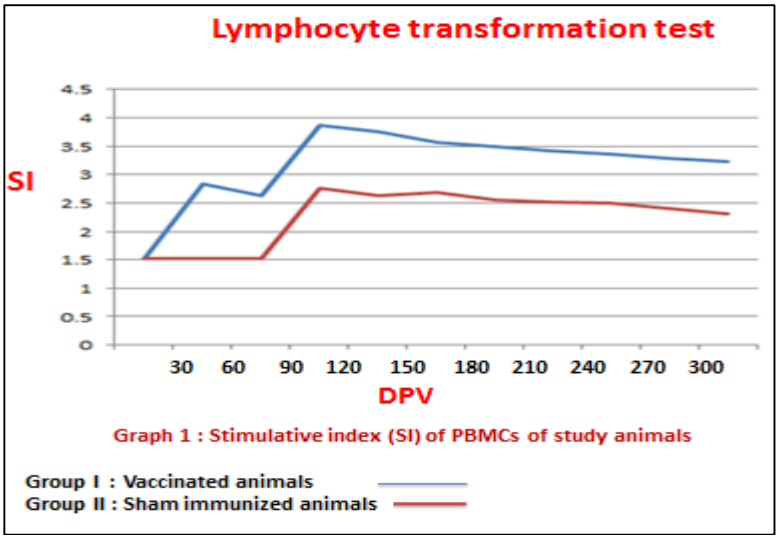
In the present study, average body weight of vaccinated animals (12.86 ±0.60 Kgs) and sham immunized animals (12.89±0.19 Kgs) approximately same at 0 DPV but later vaccinated animals (11.79±0.11[a] Kgs) gained higher body than the sham immunized (5.17±0.17[b] Kgs) animals after 300 DPV. There is significant difference between the vaccininated and sham immunized animal in gaining body weight after vaccination. The results of the study are in accordance with the observations of earlier reports by several workers Shroff *et al.*,(2013); Singh *et al.*, (2013) [31, 32]; Kumar *et al.*, (2014) [16]. They also reported inactivated MAP vaccinated animals gained higher body weight than sham immunized animals.

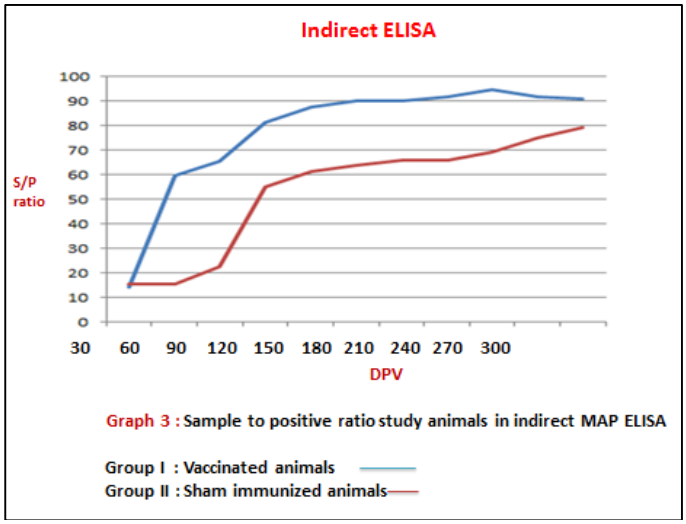
4.7 Gross Pathology

Necropsy of the sham immunized animals sacrificed at 240 and 300 DPV exhibited the marked gross lesions of JD. Lymph nodes were highly enlarged and swollen, intestinal mucosa was thickened withprominent ridges (corrugation), and degeneration of visceral fat was marked in these sheep. Sheep from vaccinated groups did not showed severe lesion of JD grossly due to inhibition of localization of MAP organisms in intestine and associated lymph nodes. The imperceptible thickening of the mucosa noticed in the present study was in accordance with the observations of Lal (1958) and Jubb *et al.*, (2007) [12]. The observation of dilated, prominent, tortuous and firm mesenteric lymphatics was in accordance with the findings of Lenghus *et al.* (1977) [20], Tripathi *et al.*, 2006 [36], Jubb *et al.*, (2007) [12], Rajendrakumar T (2008) [26], Srikant, (2017) [34].

4.8 Tissue MAP IS900 PCR and direct microscopy

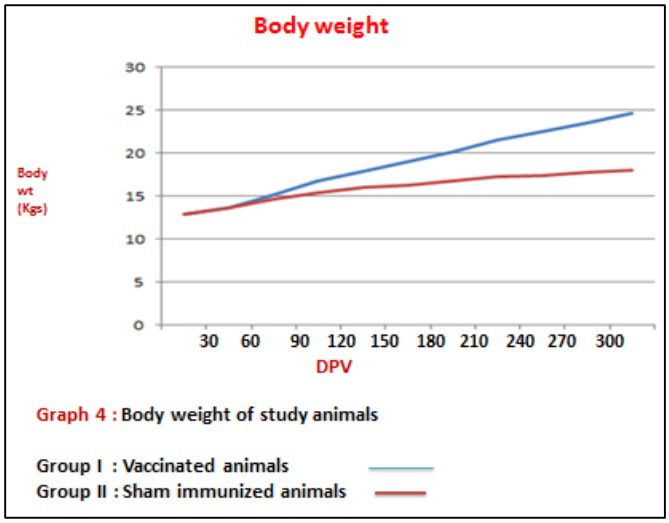
Tissue samples from sacrified animals were subjected to MAP IS 900 PCR and direct microscopy by ZN staining to detect the presence of MAP. 66.66% of samples from sham immunized animals showed positive for MAP by IS900 PCR however none sample from vaccinated animal positive for the MAP DNA. Pink colored acid fast small bacilli were observed sub mucosa of small intestine of sham immunized animals but organisms were not observed in vaccinated animals indicates inactivated MAP vaccine effectively inhibited the localization of organisms by effective immune response. The results of the study are in accordance with the observations of earlier reports by Singh *et al.*, (2010) [33].





Graph 3 : Sample to positive ratio study animals in indirect MAP ELISA

Group I : Vaccinated animals
Group II : Sham immunized animals



Graph 4 : Body weight of study animals

Group I : Vaccinated animals
Group II : Sham immunized animals

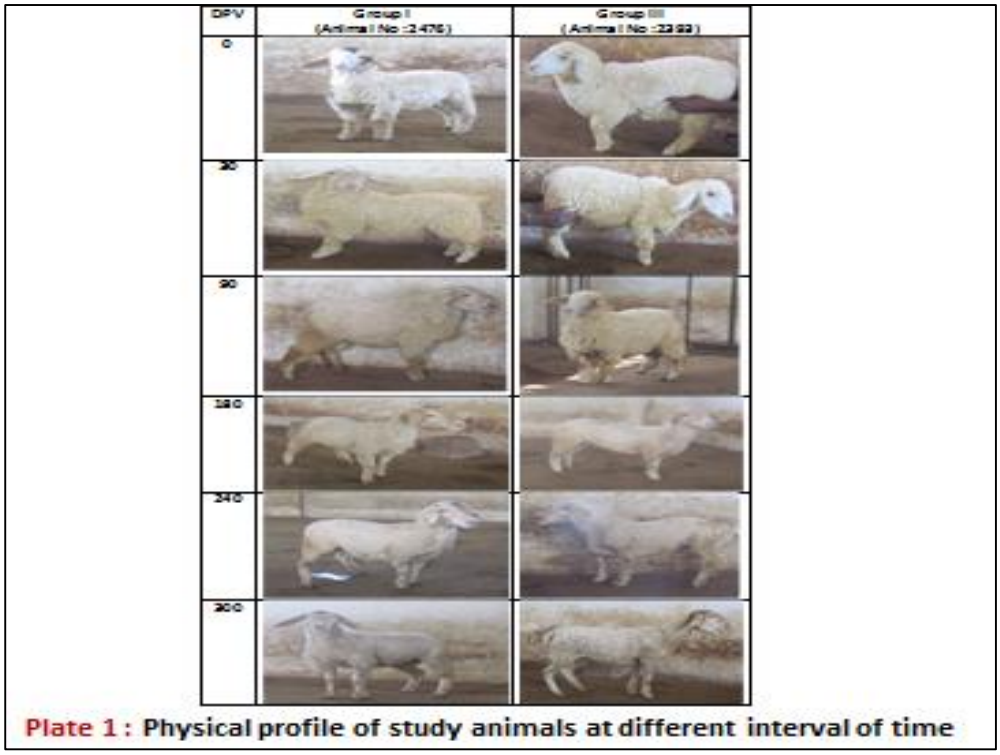


Plate 1 : Physical profile of study animals at different interval of time

Necropsy

Plate 2 : Carcass of Vaccinated animal (no : 2317) showing omental fat and no oabnormalities detected in viscera.

Plate 3 : Carcass of Sham immunized animal(no :2453) showing congested blood vessels and Cordening of mesenteric lymph node

Necropsy

Plate 4 : small intestine of Vaccinated animal (no : 2317) showing no corrugation

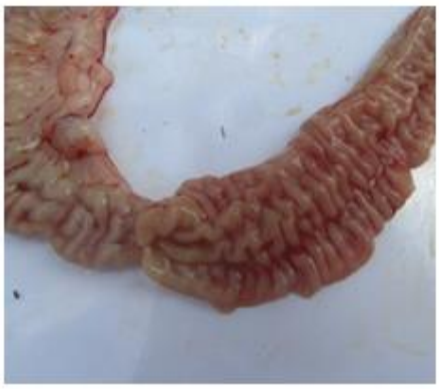


Plate 5 : small intestine of Sham immunized (no: 2453) showing severe corrugation

Tissue PCR..... MAP IS900 PCR

Time : 300 DPV
Organs : Intestine ,Mesentric lymph node

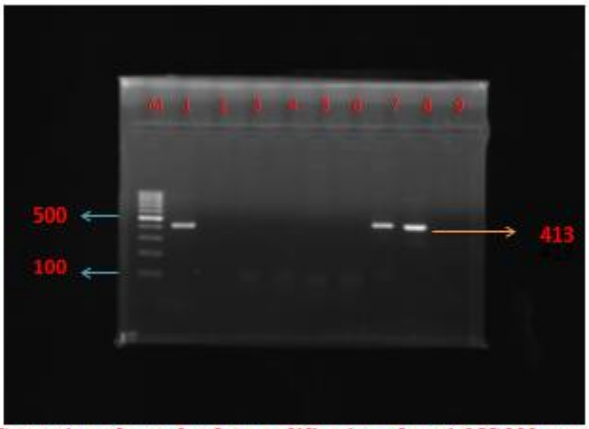


Plate 6 : Screening of samples for amplification of partial IS*900* gene of MAP (413bp)
Lane M: 100bp ladder,
Lane 1 : Positive control
Lane 2 and 9: Negative control and NTC
Lane 3-6: DNA samples from vaccinated animals
Lane 7-8 : DNA samples from sham immunized animals

Tissue direct microscopy ..... ZN staining

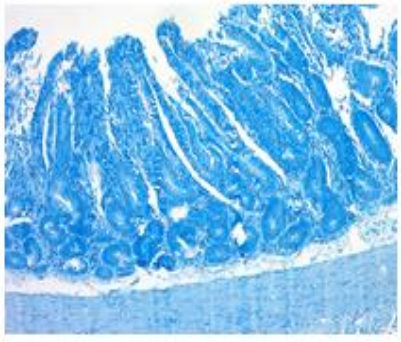


Plate 7 : No AFB in sub mucosa of small intestine of Vaccinated animal (x50)

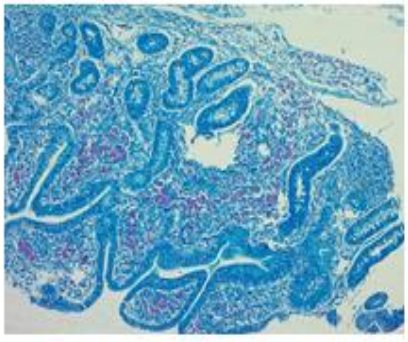


Plate 8 :Pink color AFB in sub mucosa of small intestine of Sham immunized animal (x100)

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6. Conclusion
"Indigenous vaccine" made from S196 *Mycobacterium avium* subspp *paratuberculosis* isolate of sheep origin was effective in inhibiting the localization of MAP organisms in vaccinated animals and made animals resistant to MAP infection. However sham immunized animals were susceptible to MAP infection and showed increased morbidity, typical clinical signs of Paratuberculosis. Therefore, indigenous inactivated MAP vaccine against Johne's disease had good prophylactic effect on MAP infection by eliciting effective immune responses.

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