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Comparative Evaluation of 'Indigenous' and Commercial Vaccines in Double Challenge Model for the Control of Caprine Paratuberculosis in India

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Abstract: Johne's Disease (JD), caused by *Mycobacterium avium* subspecies *paratuberculosis*, is endemic in domestic animals and adversely affects dairy industry worldwide. In the present study, efficacies of 'Indigenous' and commercial (Gudair, Spain) vaccines were evaluated for control of JD in experimentally challenged goats. Goats were grouped into Sham-immunized, Indigenous and Gudair vaccine groups. Vaccinated kids were challenged at 50 and 270 Days Post Vaccination (DPV), with 3×10^9 and 5×10^9 'Indian Bison Type strain 'S 5', respectively and sacrificed at 150 and 450 DPV after 1st and 2nd challenge, respectively. Vaccines were evaluated for improvements in physical condition (diarrhea, weakness, body coat color), clinical symptoms (shedding of bacilli, mortality, morbidity), immune responses (cell-mediated and humoral), pathology (gross and microscopic lesions) and production status (body weights, growth rates). Vaccinated goats gained higher body weights vis a vis sham-immunized. Mortality was higher in sham-immunized. Cell Mediated Immunity (CMI) response increased at 30 DPV and showed down regulation from 90 DPV onwards in vaccinated goats. Significant increase in humoral immune response was observed in vaccinated goats at 180 DPV and maintained till 450 DPV. Microscopical examination at 180 DPV showed reduced shedding in vaccinated groups, At 200 DPV, group 1 goats showed thickening of small intestine with corrugations specifically at ileocaecal junction, catarrhal enteritis with infiltration of mononuclear cells and epitheloid cells. In vaccinated groups, there were focal thickening of intestines at 450 DPV with lesions of chronic catarrhal enteritis and presence of lymphocyte, plasma cells and macrophages cells with a few epitheloid cells. Monitoring of MAP DNA in the blood of experimental goats of all the groups was done by testing of blood samples by Polymerase Chain Reaction (PCR) and the vaccinated groups of goats revealed MAP bacilli free status upto 300 DPV. Both the vaccines provided protection after challenge I, but since indigenous vaccine also protected goats after challenge II, was therefore superior. In conclusion, the indigenous vaccine must be exploited for its full potential for effective prevention and control of this economically important disease having public health concerns.

Key words: *Mycobacterium avium* subspecies *paratuberculosis*, caprine paratuberculosis, Johnes's disease, goat, vaccine, ELISA, PCR, LTT

INTRODUCTION

Johne's Disease (JD), caused by *Mycobacterium avium* subspecies *paratuberculosis*

(MAP), is endemic in domestic animals and adversely affects dairy industry globally (Inderlied *et al.*, 1993; Sweeney, 1996; Sweeney *et al.*, 2012). The bacterium that causes the disease in goats is genetically related to that

in cattle. Losses result from reduced milk yield, shorter life expectancy, reduced fertility, longer calving intervals, heavy premature culling, reduced value at slaughter, treatment costs and increase risk of other diseases (Johnson-Ifeorunlu and Kaneene, 1997). Economic losses in US dairy industry consist of staggering \$220 millions every year (NAHMS, 1997). Though prevalence of JD is high (Kumar *et al.*, 2006; Yadav *et al.*, 2008; Singh *et al.*, 2013a) but information on estimated losses due to JD is limited In India (Tripathi *et al.*, 2002; Vinodhkumar *et al.*, 2013). Paratuberculosis, especially the sub clinical form is difficult to diagnose as the most basic and important test bacteriological culture is time consuming and labor intensive. Usefulness of serological assays are not much as there is no any development of antibody response in animals until there is development of the clinical stage of the disease. Tests for identifying the Cell-Mediated Immunity (CMI) responses are critical to accurately detect paratuberculosis (Stabel, 1998; Whitlock *et al.*, 2000).

Risk of acquiring MAP infection is high in neonatal and juvenile animals (Cocito *et al.*, 1994). Due to lack of sensitive and specific ante-mortem tests (Hietala, 1992) culling policy was not successful in controlling JD (Singh *et al.*, 2007b; Geijo *et al.*, 2006). Treatment of JD is uneconomical and impractical (Harris and Barletta, 2001). Therefore, only way that has promise in controlling infection is vaccination (Perez *et al.*, 1995; Singh *et al.*, 2007c). For aiding in control of the disease vaccination has been practiced in several countries but under license (Halgaard, 1984). First paratuberculosis vaccine was introduced in France by Vallee and Rinjard (1926) using a subcutaneous injection of living non-virulent strain of MAP. Vaccines used in goats have been live (Saxegaard and Fodstad, 1985), heat-inactivated (Corpa *et al.*, 2000a), spheroblastic (Hines *et al.*, 2007) and recombinant antigens (Kathaperumal *et al.*, 2009). Vaccines were known to delay the onset of clinical symptoms, improve body weights and reduce mortality, faecal shedding of bacilli and modify inflammatory response so as to develop regressive-type granulomas but do not protect against infection (Rosseels and Huygen, 2008). In India, the first Johne's disease vaccine for goats was developed at Central Institute for Research on Goats (CIRG), Makhdoom (Singh *et al.*, 2007a), using highly pathogenic (Hajra *et al.*, 2006), 'Indian Bison Type' of MAP strain (Sohal *et al.*, 2009) of goat origin. The novelties of this vaccine is with its candidate strain as 'Indian Bison Type' of MAP strain is newly evolved (Sohal *et al.*, 2010) and it is most prevalent in India in comparisons to other types ('Cattle' and 'Sheep type') of MAP strain (Singh *et al.*, 2010a). Controlled experimental

trial of this vaccine showed that this indigenous vaccine was highly successful in controlling JD (Singh *et al.*, 2007a). Indigenous vaccine was better than imported commercial vaccine (Singh *et al.*, 2007a). Impact of candidate's strain of vaccine was also observed by Uzonna *et al.* (2003) and reported that native field strain based vaccine was more efficacious than commercial vaccines. Vaccination of ready to cull clinical cases of JD in sheep and goats showed marked improvement with indigenous vaccine (Singh *et al.*, 2010b). Protective response and efficacy of 'Indigenous vaccines' was compared with commercial vaccine (Gudair) in kids for shedding of MAP bacilli, immune response and patho-morphological changes in the target organs after challenge at 50 and 270 DPV (Dohoo *et al.*, 2003; <http://www.scahls.org.au/asdt.htm>). Therefore, the present study was conducted for comparative evaluation of 'indigenous' and commercial vaccines in double challenge model for the control of caprine paratuberculosis in India.

MATERIALS AND METHODS

Animal ethics: Work plan of the present study was approved by Ethics Committee of Pandit Deen Dayal Upadhyaya Veterinary University and Gau-Anusandhan Sansthan, Mathura (Uttar Pradesh) and University registered with Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Government of India. Stress was minimum on kids during experimentation.

Animals and management conditions: Forty female kids of Barbari breed (5-6 months old) were purchased from local market. Kids were screened by enzyme linked immunosorbant assay (ELISA) kit for anti-MAP antibodies and kids with strong positive S/P (sample to positive) ratios were not included in the study. Kids (40) were randomly distributed into; 'Sham Immunized' (group 1), 'Indigenous/Bison' (group 2), Gudair' (group 3) consisting of 10, 15 and 15, respectively. Kids were kept together under sub-optimal/ low plane of nutrition and subsisted on grazing in wastelands with little concentrate and straw during experimental period (450 days).

Vaccines: Two Inactivated vaccines; 'Indigenous (Singh *et al.*, 2007a), prepared using highly virulent native strain 'S 5' ('Indian Bison type' biotype of MAP) isolated from a farm goat of Jamunapari breed at CIRG terminally sick with JD and a commercial vaccine (Gudair™ manufactured by CZ Veterinaria, Spain) were compared. Efficacies were evaluated on the basis of improvements in

productivity (body weights, growth rate and reproductive performance), physical condition (diarrhea, weakness and body coat color), clinical symptoms (shedding of MAP bacilli, mortality and morbidity), immunological parameters (cell-mediated and humoral immune responses), bacteremia of MAP [blood polymerase chain reaction (PCR)] and pathological conditions (gross and microscopic lesions) before and after challenges [50 (days post infection) DPV and 270 DPV], followed upto 450 DPV. One milli-liter of 'Indigenous vaccine' contained 2.5 mg (dried) culture of MAP 'S 5' containing 12×10^8 bacilli per mL suspended in aluminium hydroxide gel with 0.01% Thiomersal as preservative (Singh *et al.*, 2007a). Inactivation was carried out in water bath at 72°C for 2 h.

Vaccination of goats: Kids of group II (Indigenous vaccine 'Indian Bison' S5 strain) and group III (Gudair™ manufactured by CZ Veterinaria, Spain) were inoculated with 1 mL of vaccine and group I [Sham immunized (Control)] with 1 mL sterile PBS sub-cutaneously on the left side of neck behind ear.

Challenge of goats: Kids of the 3 groups were challenged twice with 3×10^9 colony forming units (cfu) and 5×10^9 cfu live MAP 'S 5' strain of 'Indian Bison type' biotype at 50 and 270 DPV, respectively. Strain of MAP used in 'Indigenous vaccine' was tested for pathogenicity and was highly virulent (Hajra *et al.*, 2006).

Collection of samples: Serum samples were collected at 0, 30, 180, 360, 400 and 450 DPV for monitoring the Humoral Immune Response (HIR) by 'Indigenous ELISA kit'. Blood samples of three goats from each group were monitored for cellular immune (CMI) response by Lymphocyte Transformation Test (LTT) at 0, 30, 90, 180 and 360 DPV. Goats died during experimental period were subjected to detailed necropsy procedures.

Mortality: Mortality in experimental goats was recorded for all the groups. Based on percent mortality in Sham-immunized (group 1) and vaccinated (group 2-3) groups, Preventable Fraction (PF value) for each vaccine were calculated as per Tizard (2007) and their protective potential compared against deaths specifically due to JD after experimental challenge I and II. Physical condition of goats was monitored continuously. Detailed necropsy of the goats died during experiment was studied and analyzed. Goats as and when sick were treated symptomatically.

Cell mediated immune response: Cell Mediated Immune (CMI) response was estimated using Lymphocyte

Transformation Test (LTT) in response to fractionated MAP antigens in experimentally infected kids as per the method of Uma *et al.* (1999) and Singh *et al.* (2010b), with some modifications. Briefly, the Peripheral Blood Mononuclear Cells (PBMCs) were isolated from 10 mL of venous blood by centrifugation at 2,000 revolutions min^{-1} (rpm) on histopaque (Sigma) at room temperature for 30 min as per Boyum (1968). PBMCs were collected from the interphase layer and washed three times in sterilized Phosphate Buffered Saline (PBS) by centrifugation at 1,000 rpm for 10 min. After washing, PBMCs were suspended in growth medium [DMEM supplemented with 10% FBS, 2 mL glutamine, penicillin-streptomycin (100 U mL^{-1}), gentamicin ($50 \mu\text{g mL}^{-1}$) and 2-mercaptaethanol] were cultured at 2×10^5 cells well^{-1} in 96 wells round bottom microtiter plates in triplicate wells, three wells without antigen were used as controls. Isolated PBMCs from each goats were stimulated in triplicate with mitogen Concanavalin A (Sigma) at $20 \mu\text{g mL}^{-1}$ and sonicated protoplasmic antigen of MAP 'Bison type at $20 \mu\text{g mL}^{-1}$ concentration measured and a set of PBMCs of the same animal was kept as unstimulated control. PBMCs were cultured at 37°C at 5% CO_2 for 120 h (for 5 days) and then, 25 μL of MTT dye (5 mg mL^{-1}) was added to each well of the culture plate and the plate incubated at 37°C for 4 h. After incubation, 150 μL of dimethyl sulphoxide (DMSO) was added to each well, plate was again incubated at 37°C for 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 un-stimulated well. For data analysis purpose a signal to noise ratio, i.e., Stimulative Index (SI) was calculated for individual animal using the equation:

$$\text{Stimulative index} = \frac{\text{Average OD at 570 nm in simulated wells}}{\text{Average OD at 570 nm in non-stimulated control wells}}$$

Humoral immune response: Humoral Immune Response (HIR) at pre- and post-vaccination intervals was measured by 'Indigenous ELISA' and antibody titers were monitored from zero to 450 DPV in all the kids after 1st and 2nd challenge at 50 and 270 DPV, respectively, as per Singh *et al.* (2007b). Antigen used in 'Indigenous ELISA' was semi-purified protoplasmic antigen of native MAP isolate. Anti-MAP sero-titer for all the goats was calculated by sample to positive ratio (S/P ratio) as per method of Collins (2002).

Faecal shedding of MAP: Shedding of MAP was monitored in faeces of serially sampled goats by

microscopic examination at 0, 30, 90, 180, 270, 360, 400 and 450 DPV to check the efficacy of two vaccines in reducing number of MAP shedders. Shedding of MAP by culture was observed at 0 DPV and 200 DPV with mycobactin J on HEY medium as per Merkal (1984).

MAP bacterimia: Blood samples were collected aseptically from jugular vein of each goat for isolation of DNA and the DNA was subjected for IS900 PCR at 0, 30, 90, 180 and 360 DPV as per Singh *et al.* (2010c). Samples were kept at -20°C till further use.

Physical condition: Gain in body weight of goats (Immunized and Sham-immunized) was recorded at zero day, 30, 90, 180, 270, 360, 400 and 450 DPV and analyzed statistically using one-way analysis (ANOVA) a 5% level of significance.

Sacrifice of experimentally challenged kids: Kids of the three groups were sacrificed humanely to study the gross lesions and histopathology. Five kids from each vaccinated group and 4 from sham-immunized group were sacrificed at 200 DPV (after 150 days post challenge 1). Remaining kids were sacrificed at 450 DPV (400 and 180 days post 1 and 2 challenge, respectively) to assess the gross picture of target and non target organs in case of JD. Kids were sacrificed by injecting the super saturated solution of magnesium sulphate (about 10 mL) in jugular vein.

Carcass study: Body condition scores (live b.wt. and conformations) and carcass evaluation parameters (carcass yield, chest circumference, leg circumference, girth circumference, loin width, loin eye area measurements) and gross picture of different target and non-target organs were recorded to understand the effect of vaccine and experimental infection in three groups. For body fat scores and fat depositions (back fat thickness, cod fat, omental fat, mesenteric fat and renal fat) were measured.

Gross pathology: All segments of intestines were opened for examination of mucosal surface and length of intestines. Mucosa, especially of terminal ileum near ileocaecal valve was carefully inspected for thickening and corrugations. MLN were examined for shape, size and consistency. Smears made from the affected mucosa of the intestine and cut surfaces of MLN were stained by Ziehl Neelsen (ZN) stain for identification of MAP.

Histopathology: Tissue pieces of intestines and mesenteric lymph nodes from sacrificed animals were

collected and preserved in 10% neutral buffered formalin. Tissue after fixation were cut in pieces of 2-3 mm thickness and washed for several hours in tap water and dehydrated in ascending grade of alcohol and acetone. Clearing of tissue was performed in benzene and ultimately the tissue was embedded in paraffin. Tissue section of 4-5 µm thickness were cut and stained with Haematoxylin and Eosin (Luna, 1968). Sister sections of intestine and mesenteric lymph nodes were stained with Ziehl-Neelson's method for demonstration of acid-fast bacilli (Luna, 1968). Histological grading of the lesions were carried out on the basis of infiltrating cells, pattern of macrophage distribution, proportion macrophage containing AFB in MLN and in different layers of the small intestine as per Clarke and Little (1996) and Corpa *et al.* (2000b).

Statistical analysis: One way ANOVA analysis was adopted for comparing the live body weights, CMI response and carcasses traits among Immunised and Sham-Immunised (control) groups of goats. Mortality, shedding of MAP bacilli in faeces and presence of MAP in IS900 blood PCR was compared in percent value. Humoral immune response (sero-conversion) was interpreted with respect to Sample to positive (S/P) ratio as described by Collins (2002).

RESULTS

No adverse clinical reaction was observed in any of the goat kid after vaccination with Indigenous (group 2) and Gudair™ (group 3) vaccines.

Clinical findings: None of the vaccinated goats showed clinical symptoms of JD after challenge. In the sham-immunized goats there were distinct symptoms of JD at variable days of post infection.

Physical conditions: Despite the twice experimental challenge (at 50 and 270 DPV) physical health and overall body conditions of vaccinated goats of both groups (Indigenous group 2 and Gudair group 3) was superior as compared to sham-immunized (group 1) goats throughout monitoring period. Vaccinated animals were alert and active whereas, physical condition of sham immunized group 1 goats progressively deteriorated. Clinical symptoms of JD appeared very early in sham-immunized goats after 180 DPV which turned to advance clinical after 350 DPV. In contrast to Sham immunized group, effect of experimental infection in terms of physical condition was good in most of the vaccinated animals (group 2-3) against JD throughout monitoring period (450 DPV). Both

vaccines (Indigenous and Gudair) induced granuloma formation at the site of injection, indicating ‘take’ (positive response to vaccination). About 3-4 inches of swelling appeared within 24 h at the site of inoculation of vaccine in all the vaccinated goats. Size of ‘take’ was reduced over period of time and disappeared in most of the vaccinated goats after 400 DPV.

Gain in live body weights: On the day of vaccination there was no significant difference in the average body weight in vaccinated (group 2-2) and sham-immunized (group 1) goats. However, at 180 DPV and onwards variable trend of average body weights among the vaccinated and sham immunized goats were recorded. Increased growth rate in terms of body weights was significantly higher in vaccinated goats as compared to sham immunized at 180 DPV and onward time intervals (Fig. 1). Goats of group 2-3 gained significantly ($p < 0.01$) higher body weights as compared to sham-immunized when group wise pooled data was analyzed. There was no significant difference in the body weight gains in between the immunized animals of group 2-3.

Mortality rate: Out of 4 total goats died in all groups during study period (450 DPV), only one goat from group 2 (Indigenous vaccine) died at 85 DPV (1/15), respectively. However, case fatality rate due to JD was 20% (2/10), 0 (0/15) and 0 (0/15) in goats of group 1-3, respectively (Table 1).

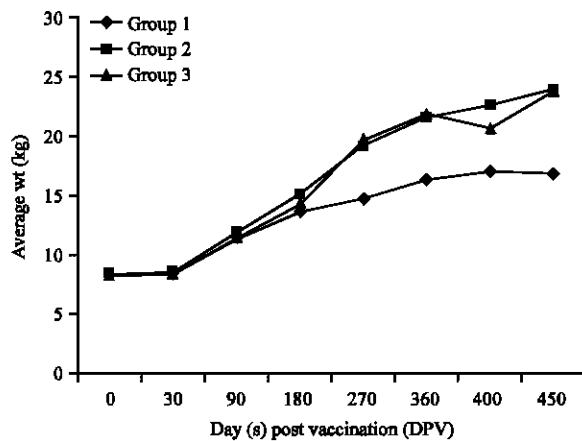


Fig. 1: Gain in average body weight in 3 groups at different time intervals

Morbidity rate: No significant illness was noticed in all three groups. However, after 180 DPV weakness was frequently observed in goats of sham-immunized group. Despite of medicaments, no significant improvement in health was observed. Goats of group 1 showed progressive weight loss and intermittent diarrhoea. Two cases from group 2-3 developed mild to moderate diarrhoea.

Cellular immune response (lymphocyte transformation test): PBMCs from both the ‘Vaccinated’ and ‘Sham immunized groups’ responded actively to non-specific stimulation (Mitogen Con A) showing that cells were viable and functional. CMI response (Stimulative index value) increased at 30 DPV and achieved highest peak at 90 DPV in goats of both vaccinated (group 2-3) and sham immunized (group 1). After 90 DPV, there was slight down regulation of SI value that was maintained till 360 DPV (Table 2). On applying the one way ANOVA tests, significant differences among the groups were recorded at 30 and 90 DPV only. At 30 DPV and 90 DPV, the CMI response in goats of group 2-3 was significantly ($p = 0.043$ and $p = 0.02$, respectively) higher than sham-immunized animals (group 1). LTT showed that vaccine induced effective CMI response during monitoring period.

Humoral immune response: Sero-conversion rate at 180 DPV and onwards increased significantly higher ($p < 0.05$) in vaccinated goats as compared to sham-immunized. Higher sero-conversion rate of vaccinated goats was maintained till 450 DPV. Highest peak in vaccinated goats (group 2-3) was recorded at 360 DPV whereas in sham-immunized group it appeared at 450 DPV with constantly increasing trend (Fig. 2).

Fecal shedding of MAP: At 180 DPV, typical acid-fast bacilli (morphologically indistinguishable to MAP) were detected in 5 ‘Sham-immunized’ goats (Fig. 5a, b). Upto 360 DPV, there was no positive goat in two vaccinated groups, however, at 400 DPV one goat of each vaccinated groups was shedding MAP. Same goat was also positive at 450 DPV along with another one goat each in vaccinated groups. In ‘Sham-immunized group’ number of MAP shedder increased with progression of time and at 450 DPV all the 4 goats were positive for the presence of MAP. Culture of faecal samples collected at 0 and

Table 1: Percent mortality in different groups during the monitoring periods

| Groups | Animals (n) | Mortality (%) | Days post vaccination (DPV) | Causes of death |
|----------------|-------------|---------------|-----------------------------|--------------------------|
| Indigenous | 15 | 1 (6.7) | 85 | Enterotoxaemia Pneumonia |
| Gudair | 15 | 1 (6.7) | 50 | Mild catarrhal enteritis |
| Sham-immunized | 10 | 2 (20.0) | 310 | Clinical cases of JD |
| | | | 360 | Clinical cases of JD |

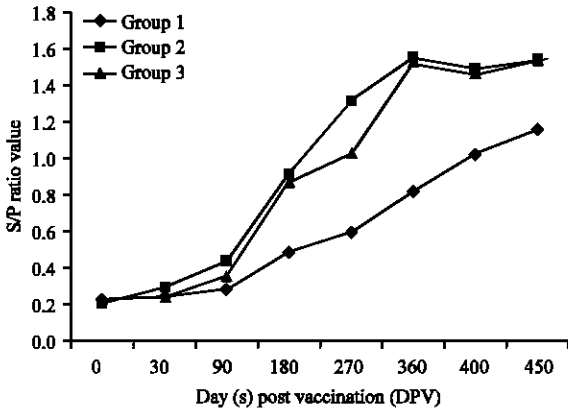


Fig. 2: Sero-conversion rate in 3 groups at different time intervals

Table 2: Estimation of stimulated index (SI) in goats of different groups at various time intervals

| Average stimulative index (SI) value±SE | | | |
|-----------------------------------------|------------------------|------------------------|------------------------|
| DPV | Group 1 (n=3) | Group 2 (n=3) | Group 3 (n=3) |
| 0 | 1.56±0.03 | 1.58±0.02 | 1.63±0.08 |
| 30 | 2.13±0.01 ^a | 2.51±0.02 ^b | 2.21±0.15 |
| 90 | 3.14±0.12 ^a | 3.77±0.14 ^b | 3.86±0.03 ^b |
| 180 | 2.73±0.06 ^a | 3.45±0.26 ^b | 3.43±0.10 ^b |
| 360 | 2.48±0.18 ^a | 3.10±0.21 ^b | 3.11±0.06 ^b |

Table 3: Detection of MAP DNA in blood of different groups of goats at various time interval

| Percent positive goats (%) | | | |
|----------------------------|-------------------------|--------------------------|--------------------------|
| DPV | Group 1 | Group 2 | Group 3 |
| 0 | 0% (0/10) | 0% (0/15) | 0% (0/15) |
| 30 | 0% (0/10) | 0% (0/14) | 0% (0/14) |
| 90 | 0% (0/10) | 0% (0/14) | 0% (0/14) |
| 180 | 30% (3/10) ^a | 0% (0/14) ^b | 0% (0/14) ^b |
| 360 | 100% (4/4) ^a | 11.9% (1/9) ^b | 11.9% (1/9) ^b |

DPV: Day (s) post vaccination. The values with different superscript in a row are different significantly in between groups. The values with no superscript/subscript in a row/column having no significant relationship

200 DPV from goats of each groups revealed that 50% goats of 'sham-immunized' were positive for MAP at 200 DPV while none was positive in both the vaccinated groups. Of the 5 positive goats, 2 were paucibacillary (<6 colonies) and 3 were multibacillary (>10 colonies). Dependency of MAP for growth on HEY medium with mycobactin J was strict, therefore confirmed goat as positive for JD.

Presence of MAP in blood: None of the goats were positive for MAP bacteremia till 90 DPV. At 180 DPV, 30% of sham-immunized goats were MAP positive and the amplicons found were of 413 bp (Table 3 and Fig. 3). Whereas goats of two vaccinated group (2-3), were negative. However, at 360 DPV blood-PCR detected 100%, 11.9 and 11.9% goats positive for presence of MAP DNA in goats of group 1-3, respectively.

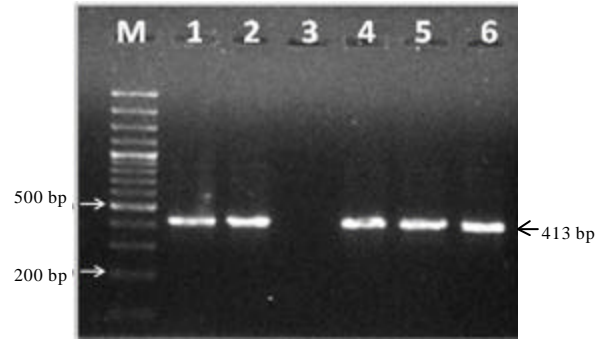


Fig. 3: MAP specific amplicons (413bp) using IS900 specific primers. Lane M: 100 bp ladder, Lane 1-2: Positive control (MAP DNA), Lane 3: Negative control and Lane 4-6: DNA samples

Carcass score: Comparative evaluation of body conformation, carcass components and fat measurements was carried out in 14 goats (4 in group 1, 5 in group 2 and 5 in group 3) and on 22 goats (4 in group 1, 9 in group 2 and 9 in group 3) on 200 and 450 DPV time intervals. In general, goats in vaccinated groups scored better than control group in most of the studied parameters. The pattern of significance remained constant at 450 DPV. The above parameters in both the vaccinated groups of goats did not differ significantly.

Pathomorphological observations

Gross examination of lesions in target tissues: MLN and intestine of goats sacrificed after challenge showed thickness of intestinal wall (Fig. 4). Mesenteric lymph nodes were enlarged and oedematous in case of sham-immunized group, whereas these lesions were more normal in vaccinated groups. Histologically, tissues revealed absence of lesions or bacilli in most of the goats vaccinated with indigenous and Gudair vaccines, whereas, Sham-immunized goats showed focal, diffused and confluent lesions (advanced) that extended into large intestine.

Histopathology: Pink colour clusters of acid fast bacilli in faecal smear of goat of group I are shown in (Fig. 5). Goats of this group slaughtered at 450 DPV revealed chronic catarrhal enteritis with atrophy, shortening and ballooning of villi with infiltration of large number of epitheloid cells forming mosaic sheet like appearance in the villi, lamina propria and glandular crypts of small intestine (Fig. 6a, b). Some of the epitheloid cells fused to form distinct granuloma with infiltration of mononuclear cells and giant



Fig. 4(a-b): (a) Ileo-caecal junction of intestine showing corrugation and thickening in goat of group 1 and (b) Enlargement of mesenteric lymph node in goat of group 1

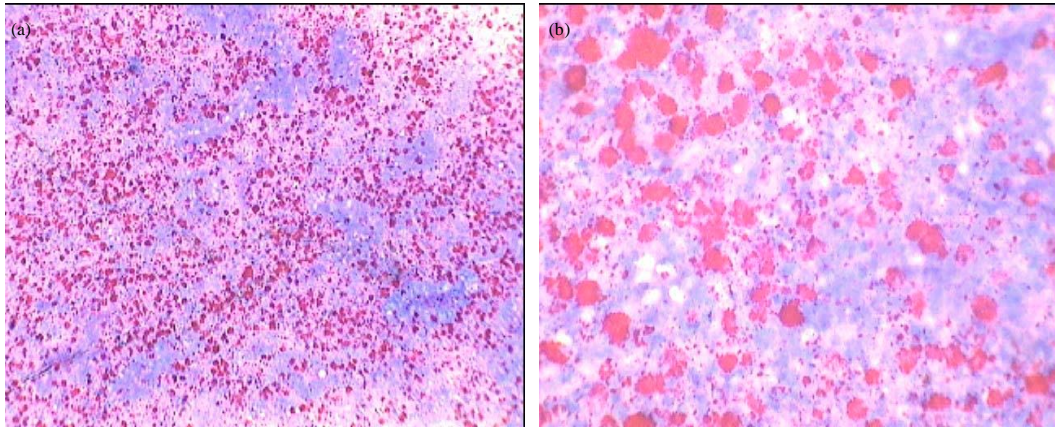


Fig. 5(a-b): (a) Pink colour clusters of acid fast *bacilli* in faecal smear of goat of group 1 (Ziehl Neelsen stain 100 X) and (b) Higher magnification of Fig. 5a (Ziehl Neelsen stain 400 X)

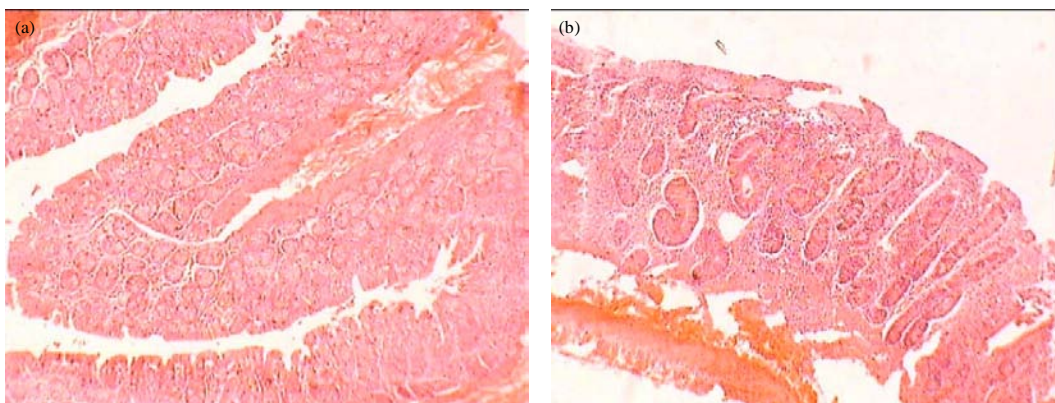


Fig. 6(a-b): (a) Intestine showing mucinous degeneration in goat of group 1 (H and E stain 100 X) and (b) Intestine showing chronic catarrhal enteritis with infiltration of mononuclear cells in goat of group 1 (H and E stain 100 X)

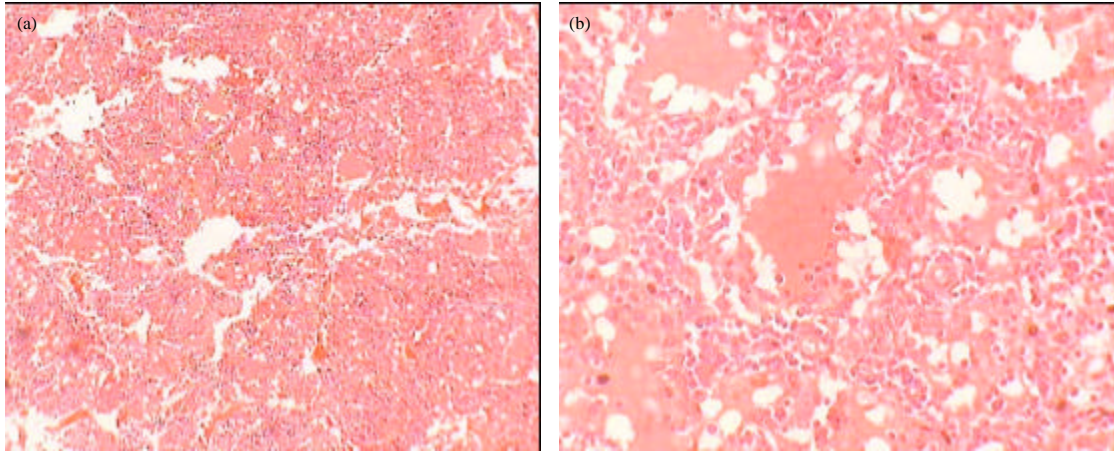


Fig. 7(a-b): (a) Mesenteric lymph node showing presence of edematous fluid in the medullary area in goat of group 1 (H and E stain 100 X) and (b) Higher magnification of Fig. 7a showing homogenous pink colour edematous fluid (H and E stain 400 X)

cells. ZN stained section revealed presence of pink colored AFB individually or in clusters in epithelioid cells. Both vaccinated groups' revealed mild chronic catarrhal enteritis with infiltration of few MNCs in intestine and mesenteric lymph nodes (Fig. 7a, b).

DISCUSSION AND CONCLUSION

An attempt has been undertaken by an International committee of Johne's Disease (JD) to standardize guidelines for prevention and control of Johne's disease and vaccination forming an integral part of it (Hines *et al.*, 2007). Corpa *et al.* (2000a) had conducted vaccination in adult goats with inactivated commercial vaccine and found the vaccine effective in causing reduction of clinical cases as well as the load of bacteria in the flock. JD is a production disease that drastically reduces the productivity of domestic animals, causing economic hardship to poor farmers and livestock owners/industries (Hasonova and Pavlik, 2006). The widely used method for the control of JD is 'test and cull'. However, the past experiences suggested that 'test and cull' policy is uneconomical and has not answered to its theoretical potentials (Geijo *et al.*, 2006; Singh *et al.*, 2007b). Therefore, for effective control of JD, development of high efficacious vaccine would be ideal. Though vaccines for JD are available commercially for many years but are not effective in preventing the disease completely (Chiodini *et al.*, 1984; Cocito *et al.*, 1994). It is considered that the 'Indian Bison Type' of MAP genotype is newly evolved and is highly virulent (Sohal *et al.*, 2009). In another study of Indigenous vaccine, Singh *et al.* (2010b)

have reported the therapeutic effect of vaccine and opened the possibility for the effective control of JD in India.

Therefore, in the present study, a commercial, imported and widely used Gudair™ vaccine was also tested simultaneously to check its efficacy in India where, India Bison type of genotype is more prevalent in domestic livestock species (Singh *et al.*, 2010b). Infection of MAP usually occurs soon after birth, thus traditionally vaccination has been practiced during the first weeks of animal's life, on the basis that protection gets in touch with mycobacteria would be conferred for the first (Saxegaard and Fodstad, 1985; Larsen *et al.*, 1975). However, in ovine and caprine species very good results have been achieved after vaccinating at 6 and 5 month old age, respectively (Corpa *et al.*, 2000b; Singh *et al.*, 2007a). It may be due to maturation of immune system after 5 or 6 months of age in both of the species of animals. Therefore, in the present study 5-8 months kids were used in which no untoward reaction or abscess formation due to vaccination by either of the two vaccines was observed in the experimental goats. Therefore it may be concluded that the both vaccine had potency against JD. Reddacliff *et al.* (2006) emphasized the presence of local post-vaccinal lesions as an effective guideline to positive response to vaccination.

In the earlier trial of same Indigenous vaccines in goats, Singh *et al.* (2007a) have reported significant increase ($p < 0.05$) in average body weights gain of Indigenous vaccinated goats over control after second round of challenge. Similar to the observation of Singh *et al.* (2007a), the present study also documented

even more significantly higher live body weight gain in vaccinated groups of goat as compared to goats of Sham-immunized ($p < 0.01$) goats. JD badly affects the weight of goats which in turns affect the body conformation, carcass components, non-carcass components etc. In case of JD, goats were under stress due to MAP infection and the animals lose their body weights progressively, as well as fat became consumed in order to energy demand of the body. Results were in consistent for some parameter with the earlier study of same vaccine by Singh *et al.* (2007a). Therefore, the data from sacrificed goats strengthened the superiority of vaccinated groups over Sham-immunized. This superiority of vaccinated group may be possibly due to upregulation of factors responsible for positive impact on normal growth, muscling as well as optimum productivity or in other words vaccine mediated suppression of negative factor induced by experimental infection of goats.

The two vaccines used for trial in this study were tested to determine the effect on both cellular and humoral responses as compared to the results of sham immunized (control). Statistical analysis showed significant high proliferation of PBMCs collected from the goats of Indigenous and Gudair group at 30 and 90 Days Post Vaccination (DPV). Thereafter the values started decreasing in all the groups of goat. Analyzing the trend of CMI response in both vaccinated groups, it was clear that the CMI response activated more early (at 30 DPV) in goats of vaccinated group as compared to control group. The peak titer value was achieved at 90 DPV in both vaccines groups and at 90 DPV the CMI titer value (Stimulative Index) was slightly higher in Gudair group than Indigenous group. However, subsequently at 180 DPV there was downregulation in CMI response in both the vaccinated groups. Immunity to all mycobacterial infections is dependent on cell-mediated responses and must be elicited by a vaccine to be protective (Benedictus, 1984). Among the various tools used, measurement of lymphocyte proliferation response to the specific antigen tested (Molina *et al.*, 1991; Storset *et al.*, 2001) is widely used to determine cellular immune responses (Singh *et al.*, 2007c, 2010b; Kathaperumal *et al.*, 2009). Comparably low responsiveness of PBMCs even after experimental infection in goats of sham immunized group as compared to both vaccinated groups may be endorsed to the suppressive factors secreted by monocytes and lymphocytes (Kleinhenz and Ellner, 1987). However, other possibility may be a shift from Type 1 T helper (Th1) 1 to Type 2 T helper (Th 2) type response of cytokines. Therefore, on the basis of lymphocyte transformation test it was clear that both vaccines had induced effective CMI response at least during monitoring period.

Humoral immune response in both vaccinated and Sham-immunized goats were measured by Indigenous ELISA kit having more sensitive/specific even compared to commercial kits. The sero-conversion rate increased significantly in vaccinated goats at 180 DPV and maintained till 450 DPV however, the response of sero-conversion in sham-immunized group was lower. The highest peak in vaccinated goats (group 2-3) was recorded at 360 DPV whereas in sham-immunized group it appeared at 450 DPV with constantly increasing trend. The humoral response against mycobacteria would be an indicator to degree of the activation of immune system (Corpa *et al.*, 2000b; Singh *et al.*, 2007a; Dhama *et al.*, 2011) observed comparatively late antibody titer in experimentally challenged goats using indigenous vaccine. Similar late peak antibody response (at 10 months post vaccination) was also observed by Begg and Griffin (2005) in sheep vaccinated with commercial Neoparasec™ vaccine. Early appearance of humoral immune response has been reported in the endemic areas of JD where the animals are already exposed to MAP (Singh *et al.*, 2010c). Kids are also known to respond low with respect to antibody production against infection/vaccination therefore, the ELISA test is considered low sensitive to detect the antibody in kids (Juste *et al.*, 2005; Singh *et al.*, 2007b). Significant increase in antibody level in goats of both vaccinated groups and control after 90 DPV also supported this hypothesis that kids that were possibly first exposed to MAP during vaccination and again encountered during first experimental infection thereby, producing more antibody by proliferation of plasma cells. Comparably higher serum antibody response than do naturally infected animals was also reported earlier (Gwozdz *et al.*, 2000; Juste *et al.*, 1994).

The observations indicated that the microscopical examination of faecal samples showed potential of vaccine in restriction of MAP shedding in both the vaccines had reduced detectable faecal shedding in significant number of vaccinated goats post-challenge. In non-vaccinated group (Sham-immunized) high proportion of goats were consistently positive at 180 and afterwards. After 200 DPV, out of 10 goats, 5 were detected as positive and of these 2 were heavy shedder. However, at the same time none of the vaccinated goats were positive. Faecal shedding of MAP is the most practical test for prognosis of the disease and to determine the efficacy of vaccines against paratuberculosis (Kalis *et al.*, 2001).

In the present study, probably for the first time blood PCR was used as an additional parameter to monitor the vaccine response for Johne's disease. The test had detected 3 goats of sham-immunized as positive at 180 DPV and while none were positive in vaccinated

group at the same time interval. It is widely accepted that current serological tests lack 100% sensitivity and specificity and ability to detect infection at early stages or in young animals (McDonald *et al.*, 1999). There is presence of a haematological phase in paratuberculosis for which testing of blood by nested PCR has got the potential to detect animals suffering from subclinical infection (Gwozdz *et al.*, 1997; Buergelt and Williams, 2004). Despite inability to discriminate the live and dead MAP by blood PCR, recently it has been considered as highly sensitive test for the diagnosis of MAP infection in kids (Juste *et al.*, 2005; Singh *et al.*, 2010c). As a complementary diagnostic test, blood PCR has got a high potential compared to ELISA as it can pick up the infected animals fraction that usually the ELISA test cannot (Nielsen *et al.*, 2002). Vaccination may regulate the appearance of MAP DNA in target tissues (Collins *et al.*, 1993; Gwozdz *et al.*, 2000). Therefore, in order to measure the vaccine response, monitoring of MAP DNA in blood by PCR may be of great interest.

The significant reduction in herd mortality is well documented in earlier studies of JD vaccine (MLA, 2005; Singh *et al.*, 2007a). Vaccination of lambs against ovine Johne's disease reduced mortality up to 90% (MLA, 2005). In the present study, a total of 4 goats were died however, only 2 from Sham immunized group had typical gross lesion of JD and were showing clinical symptoms of JD before death.

The Sham immunized goats following challenge with pathogenic MAP revealed poor body conditions, emaciation and gelatinization of fat and mild gross intestinal lesions characterized by thickening and corrugation in intestine particularly at ileocaecal junction and enlargement of mesenteric lymph nodes which were oedematous and juicy at 200 DPV. At 450 DPV, grossly the body condition of the sham immunized animals was very poor, dehydrated with hide bound condition and showed moderate to marked thickening with corrugations of small intestine. Carrigan and Seaman (1990) reported diffuse thickening of intestine and in advanced cases distinct corrugations in the last few meters of the ileum and ileocaecal valves was observed. Clarke and Little (1996) described the thickening of the intestine as granular appearance of mucosa in the sheep suffering from the JD. Stamp and Watt (1954) and Clarke (1997) reported that some pigment producing MAP resulted into yellow discoloration of intestine. Clarke (1997) reported that the infection to animals usually occurs at birth/early stage of life and for clinical symptoms to appear may take a long course. Therefore, it appears that the 200 DPV (150 days of post challenge) is sufficient to develop only mild lesions in intestine and MLNs as observed in this

study. The observation of marked thickening and corrugations in the intestine in this study at 450 DPV (400 days post challenge) confirmed with the finding of MAP bacilli. Tiwari *et al.* (2006) reported the advanced lesions of JD showing severe weight loss, depletion of fat from depot and marked lesions in the intestine sufficient to cause mortality in animals in experimental cases in India. In the present study, all the mesenteric lymph nodes at 200 and 450 DPV were moderately to severely enlarge juicy and edematous in group 1. Olsen *et al.* (2002) observed pale, swollen and edematous mesenteric lymph nodes with or without nodular foci of caseation and mineralization both in the mucosa and in the lymph nodes in goats died due to JD. The mesenteric lymph nodes were also showed infiltration of mononuclear cells and scattered presence of epithelioid cells. At this stage all the goats of control group were shedding MAP in faeces at 200 DPI (150 days post challenge) and presented the lesions in the small intestine and mesenteric lymph nodes which unveiled a very mild lesions of JD in the animals. The classification made by Perez *et al.* (1996) in sheep appears comprehensive to classify the lesions observed in the present study. In vaccinated groups there were focal thickening of intestines with lesions of chronic catarrhal enteritis adorned with presence of lymphocytic, plasma cells and macrophages with a few epithelioid cells and the MLN showed infiltration of MNC and a few epithelioid cells. Earlier studies reported the reduction in progression of granulomatous lesions and in shedding of bacilli after vaccination in infected animals (Corpa *et al.*, 2000a). Similarly, Juste *et al.* (1994) also observed tuberculoid forms of lesions which were located exclusively in the intestinal organized lymphoid tissues in vaccinated animals and in the control, lesions extended to the parts other than intestine causing severe enteritis.

The advancements in the conventional, serological as well as modern molecular tools (Deb *et al.*, 2011, 2013; Dhama *et al.*, 2013a, b) have aided in the development of not only efficacious prophylactics (Dhama *et al.*, 2008, 2011, 2013c) and novel therapeutic regimens (Mahima *et al.*, 2012; Dhama *et al.*, 2013d, e; Tiwari *et al.*, 2013a) but also in the development of effective vaccines against Johne's disease. This further aids in the adaptation of effective prevention and control strategies for economic upliftment as MAP is an important pathogen of goats with significant public health concern (Grant, 2005; Tripathi *et al.*, 2002; Sweeney *et al.*, 2012). This is especially essential in the modern era of One Health, One Medicine and emerging antibiotic resistance (Momotani, 2012; Dhama *et al.*, 2013f; Tiwari *et al.*, 2013b). Therefore, vaccination may have potential to direct the inflammatory cascade into a beneficial mode and

both vaccine i.e., indigenous and commercial vaccine (Gudair) were able to prevent the paratuberculosis. Present study besides exhibiting the prophylactic efficacy of Indigenous and commercial vaccines against MAP infection, also underlined the critical role of nutrition in response to vaccination as well as in pathogenesis and management of chronic infections, as reported in goats (Singh *et al.*, 2007a, 2010b; Corpa *et al.*, 2000c), sheep (Shroff *et al.*, 2013) and cattle (Singh *et al.*, 2013b) as it hinders the precipitation of disease from sub-clinical to clinical or advanced clinical stage. It is moreover important to note for the researchers that as there is interference with eradication programmes due to vaccination for which they need to be cautious while standardizing vaccine. In conclusion, the indigenous vaccine showed applicability potential to be used as a superior vaccine for effective prevention and control of this economically important disease having zoonotic concerns.

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