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Research Article

Dose Standardization Studies of 'Indigenous Vaccine' for the Control of *Mycobacterium Avium* Subspecies *Paratuberculosis* in Naturally Infected Goats

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ABSTRACT

Mycobacterium avium subspecies *paratuberculosis* (MAP) infection is most prevalent disease in Indian domestic livestock. Dose rates evaluation studies of 'indigenous vaccine' against Johne's disease (JD) were conducted in naturally infected goats. Adult female goats (29) of Barbari breed (>1.5 years) poor in body condition and suffering with clinical JD were randomly divided into four groups; Goats in group I (n=6) were given 1 mL of plane adjuvant (Gerbu Biotechnik, Germany) used in the vaccine preparation, Group II (n=7) and group III (n=8) goats were vaccinated with 2.5 mg/ml/goat and 5.0 mg/ml/goat dose rates of indigenous JD vaccine, respectively, Group IV (Control, n=8) goats were given 1 ml of PBS. Vaccinated goats were monitored for overall improvements on the basis of health (morbidity), mortality, production (body weights, reproductive efficiency), physical and clinical conditions (weakness, diarrhea, skin coat, deposition of fat in visceral organs), immunological parameters (ELISA titer) and status of shedding of MAP bacilli in feces. Average of body weights gained within one year of vaccination in four groups statistically passed normality test (P value >0.1) and vaccinated goats gained higher body weights as compared to adjuvant (Group I) and control (Group IV) groups. At 360 dpv, goats in group II (2.5 mg/ml) showed higher titer of antibodies as compared to group III (5.0 mg/ml), while it varied in control group (IV). After vaccination clinical condition of goats improved (diarrhea stopped, regeneration of hairs, body coat regained luster). Kids born to vaccinated goats had higher birth weights. Comparative evaluation of two dose rates on above parameters showed that group II goats gained higher body weights (3.10±0.52 kg) and their physical conditions also improved in comparision to group III. Dose rate of 2.5 mg/ml/goat exhibited higher efficiency against JD than 5.0 mg/ml/goat. Therefore, under optimum nutritional conditions, 2.5 mg/ml/goat dose of native vaccine was optimum for the control of JD in goats endemically infected with MAP.

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INTRODUCTION

Johne's disease (JD), caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is a global problem of domestic ruminants that adversely affect livestock productivity. Disease has been found endemic in goatherds of the country (Singh et al., 1996; Kumar et al., 2007; Singh et al., 2013a; 2013b). Production losses result from reduced milk production, shorter life expectancy, reduced fertility, longer kidding intervals, heavy premature culling, increased morbidity and higher expenditure on veterinary medicines, increased risk of other diseases (Ifearulundu and Kaneene, 1997). Annual economic losses due to ovine JD are around Rs 1,840 or $38.33 per sheep/farmer (VinodhKumar, 2013) in India. Presence of live MAP bacilli has been reported from raw and pasteurized milk and milk products (Grant et al., 2002; Shankar et al., 2010; Raguvanshi et al., 2013). MAP has potential to be categorized as zoonotic infection and has been associated with Inflammatory bowel disease (IBD) or Crohn's disease (CD) in human beings (Hermon–Taylor et al., 2000; Chamberlin and Naser, 2006).

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Treatment of JD is both expensive and impractical (Harris and Barletta, 2001), therefore, vaccination is the only promising way in controlling JD. Development of indigenous vaccine for commercial exploitation is underway and in earlier studies on 'indigenous vaccine' (Singh et al., 2007a; Singh et al., 2010a; Singh et al., 2013c; Singh et al., 2013d) though showed good response when used in herds/flocks of goats, sheep, cattle and buffaloes, however, the dose rate of 2.5 and 5.0 mg per animal in small and large ruminants, respectively for lifetime were accrued from the International literature (Perez et al., 1995). During trials of vaccine in cattle with poor nutrition status, cows exhibited increase in shedding of MAP at around 8 months post vaccination after showing decrease in shedding up to 8 months after vaccination (personal observations). Therefore, it was felt that by increasing the initial loading dose animals may develop longer duration of immunity since disease was highly endemic in native domestic livestock and infection and re–infection rates were high in animals. Under the continued validation of the 'indigenous vaccine an entirely new experiment was started on goats suffering with clinical JD symptoms (poor in body condition). Present study compared two dose rates; 2.5 and 5.0 mg/ml/goat of 'indigenous vaccine' for the therapeutic management of clinical Johne's disease in goat herds.

MATERIALS AND METHODS

Animal Ethics

Study plan was approved by Ethics committee of Institute registered with Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Government of India.

Experimental Animals

In October, 2011, 29 adult female goats of Barbari breed (>1.5 years old) with clinical JD were transferred to experimental unit from production herds (CIRG, Makhdoom). Randomly these goats were divided into four groups; Group I (n=6) goats were given 1 ml of plain adjuvant (Gerbu Biotechnik, Germany). Group II (n=7) and group III (n=8) goats were vaccinated with 2.5 and 5.0 mg/ml/goat doses of the 'indigenous vaccine', respectively. Group IV goats (Control, n=8) were given 1 ml of PBS subcutaneously. Animals were monitored for the response to two different doses of the vaccine.

Management and Monitoring Parameters

Goats were kept together under semi–intensive management and were provided optimum ration; concentrate feed (100 gm/day/goat), 6 hours of grazing on pasture, cultivated fodder, tree loppings and dry fodder (straw). Immune response in goats was monitored for improvements in health (morbidity), mortality, productivity (body weights, growth rate, reproductive efficiency), physical condition (weakness and body coat color), clinical symptoms, status of shedding of MAP bacilli, humoral immune responses and MAP bacterimia and were followed up to one year.

Vaccine

In India, first vaccine against JD was developed at Central Institute for Research on Goats (CIRG), Makhdoom (Singh et al., 2007a), using highly pathogenic native 'Indian Bison Type' bio–type of MAP strain 'S 5' (Singh et al., 2013c) of goat origin. Using this strain, two batches of indigenous inactivated vaccine at the dose rate of 2.5 mg and 5.0 mg wet weight of culture per milliliter was suspended in Gerbu adjuvant (Gerbu Biotechnik, Germany) and mixed properly after inactivation at 72^0C for 2 hours in water bath.

Vaccination of goats

Efficacies of group II and III batches of goats were inoculated with 2.5 mg and 5.0 mg/ml adjuvant/goat dose rates, respectively and group I with 1 ml sterile plain adjuvant (Gerbu Biotechnik, Germany) sub–cutaneously on the left side of neck behind ear. Control group (IV) was given 1 ml of sterilized PBS subcutaneously.

Collection and Analysis of Clinical Samples

Fecal, blood and serum samples were collected before and after vaccination upto one year at monthly intervals for the monitoring of shedding of MAP bacilli, MAP bacterimia and humoral immune response by microscopy (Singh et al., 2013e), IS900 PCR (Singh et al. 2010b) and serum ELISA (Singh et al. 2007b), respectively.

Mortality

Mortality in the experimental goats was recorded from 0 to 360 days post vaccination (dpv). Goats died during experimental period were subjected to detailed necropsy examination.

Table 1: Body weights profile of different groups of goats at zero to 360 dpv

Days post vaccination (dpv)	Body weights of different groups (Kg ± SE)			
	Adjuvant (N= 6)	Old vaccine (N=7)	New vaccine (N=8)	Control (N=8)
0	23.1 ± 3.1 (N=6)	23.5 ± 3.4 (N=7)	20.4 ± 2.3 (N=8)	24.5 ± 2.0 (N=8)
30	22.8 ± 1.5 (N=6)	24.8 ± 2.5 (N=7)	21.0 ± 1.8 (N=7)	24.5 ± 2.0 (N=8)
60	24.2 ± 0.9 (N=6)	25.0 ± 2.3 (N=7)	21.7 ± 1.7 (N=7)	24.2 ± 1.6 (N=8)
90	24.6 ± 1.8 (N=5)	23.3 ± 2.0 (N=6)	19.9 ± 1.9 (N=6)	22.6 ± 2.4 (N=5)
120	25.6 ± 2.2 (N=5)	23.3 ± 1.9 (N=6)	22.5 ± 2.5 (N=4)	22.7 ± 1.7 (N=7)
150	27.8 ± 2.1 (N=6)	29.6 ± 2.4 (N=7)	25.2 ± 1.2 (N=6)	23.9 ± 1.2 (N=6)
180	29.7 ± 1.7 (N=4)	26.8 ± 3.2 (N=4)	25.9 ± 1.4 (N=5)	24.5 ± 1.5 (N=6)
210	26.0 ± 0.91 (N=4)	23.3 ± 2.6 (N=3)	21.0 ± 0.0 (N=1)	19.0 ± 1.0 (N=2)
240	26.0 ± 0.89 (N=6)	28.6 ± 2.5 (N=6)	24.0 ± 1.4 (N=6)	25.0 ± 2.2 (N=5)
270	24.0 ± 0.54 (N=5)	24.8 ± 2.7 (N=7)	22.0 ± 2.0 (N=6)	23.7 ± 1.9 (N=7)
300	24.2 ± 0.40 (N=5)	25.7 ± 2.5 (N=7)	22.3 ± 1.5 (N=6)	23.5 ± 1.8 (N=7)
360	24.2 ± 1.9 (N=5)	26.6 ± 2.5 (N=7)	23.3 ± 1.1 (N=6)	24.6 ± 1.8 (N=7)
Weight gained (in kg)	1.10 ± 0.37	3.10 ± 0.52	2.90 ± 0.64	0.10 ± 0.82

DPV– Days post vaccination

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Live Body Weights

Body weights of goats were recorded at monthly intervals from 0 to 360 dpv. Average gain in body weights of the groups I, II, III and IV were statistically analyzed using unpaired 't test' with standard error (SE) by Graph Pad InStat 3.0 software.

RESULTS

Body Weights

Average of body weights gained in 360 days of vaccination in four groups statistically passed normality test (P value › 0.1) and vaccinated goats gained significantly higher body weights as compared to groups I and IV. Average gain in body weights in 360 days post vaccination in group II and III goats were 3.10 ± 0.52 and 2.90 ± 0.64 (Kg ± SE), respectively (Table 1).

Monitoring of Vaccine Response by Fecal Microscopy and Is900 Blood PCR

Microscopic examination of fecal smears showed increased shedding of MAP in group I (8.4%) and IV (8.3%), whereas there was decrease in shedding in both the vaccinated groups [II (2.5 mg/ml/goat – 11.9%) and III (5.0 mg/ml/goat – 16.7%) (Table 2)]. Bacterimia was also reduced by 25.0% in group III, whereas there was increase by 3.5% in group IV (Table 2, Figure 2). However, in group II, none of the goat was positive for MAP infection by IS900 blood PCR at 0 dpv and this status was maintained at the end of the trial at 360 dpv. Therefore for initial diagnosis of MAP infection and for monitoring of vaccine response, multiple tests (microscopy, indigenous ELISA and blood PCR) have been used.

Monitoring of Humoral Immune Response

Screening of goats from zero to 360 dpv at monthly intervals by serum ELISA kit showed gradual increase in the sero–titer of anti MAP antibodies in group II and III goats, while it varied in group I and IV goats (Figure 1). However, vaccine using 2.5 mg/ml/goat dose showed higher increase in sero–titer of anti MAP antibodies as compared to 5.0 mg/ml/goat dose of the vaccine. In group III (5.0 mg/ml/goat) and group II (2.5 mg/ml/goat), peak titers were achieved at 150 dpv, as 1.21 ± 0.04 and 1.37 ± 0.06 (mean OD ± SE), respectively. However, in control groups slight increase in the antibodies titer (0.87 ± 0.06) was seen at 150 dpv. In majority of goats titers declined slightly after 150 dpv. In group II, the antibodies titer was higher as compared to other groups and was also maintained for longer duration (Figure 1).

Mortality

Only 4 goats died during the study period. One goat in group IV (Control) died of clinical JD in the 3rd month of trial. One goat died of pregnancy toxemia from group I (adjuvant). However, two goats that died from group III (5.0 mg/ml) had lesions of severe anemia, debility and pneumonia.

Table 2: Monitoring of Vaccine response by fecal microscopy, blood PCR and indigenous ELISA kit up to 360 dpv

Tests	Groups	0 dpv		360 dpv	
		animals (*n*)	Positives *n* (%)	Animals (*n*)	Positives *n* (%)
Fecal Microscopy	I – Adjuvant	6	1 (16.6)	4	1 (25.0)
	II – Old Vaccine	7	2 (28.5)	6	1 (16.6)
	III – New Vaccine	8	4 (50.0)	6	2 (33.3)
	IV – Control	8	2 (25.0)	6	2 (33.3)
	Total	29	9 (31.0)	22	6 (27.2)
IS900 blood PCR	I – Adjuvant	6	1 (16.6)	5	0 (0.0)
	II – Old Vaccine	7	0 (0.0)	7	0 (0.0)
	III – New Vaccine	8	2 (25.0)	6	0 (0.0)
	IV – Control	8	2 (25.0)	7	2 (28.5)
	Total	29	5 (17.2)	25	2 (8.0)

dpv– Days post vaccination, *Figures in parenthesis are percentage

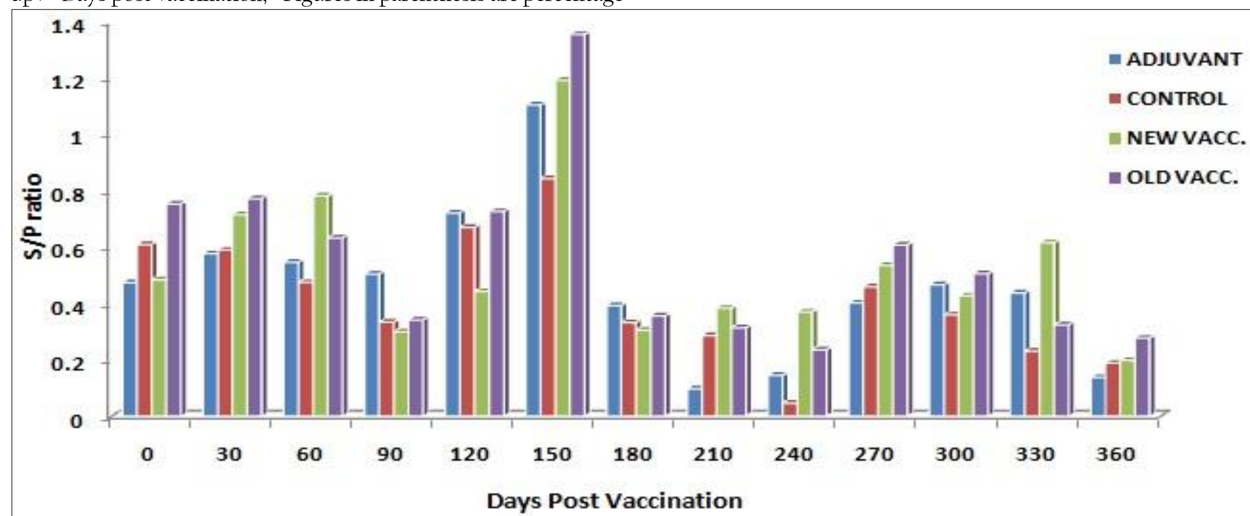

Figure 1: Sero–titer (sample to positive ratio) in different groups of animals from zero to 360 dpv

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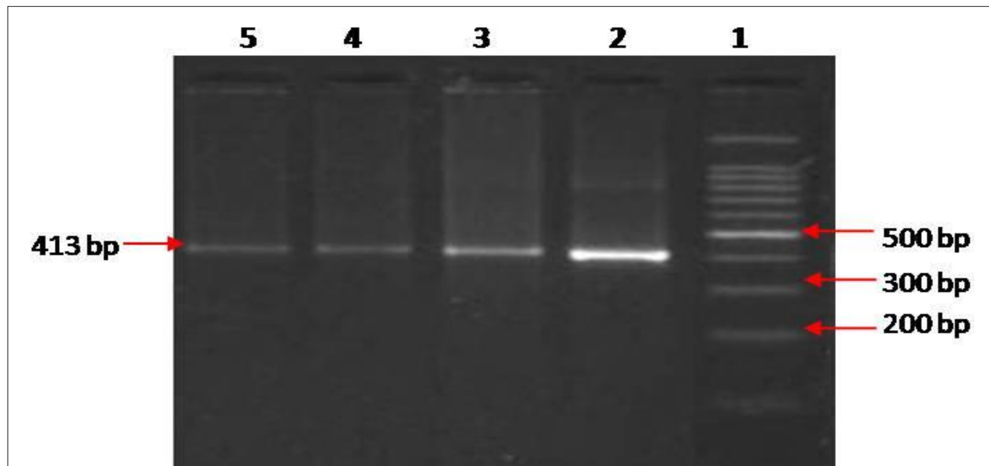

Figure 2: MAP specific amplicons (413bp) using IS900 specific primers; Lane 1: 100bp ladder, lane 2: Positive control, lane 3–5: DNA samples

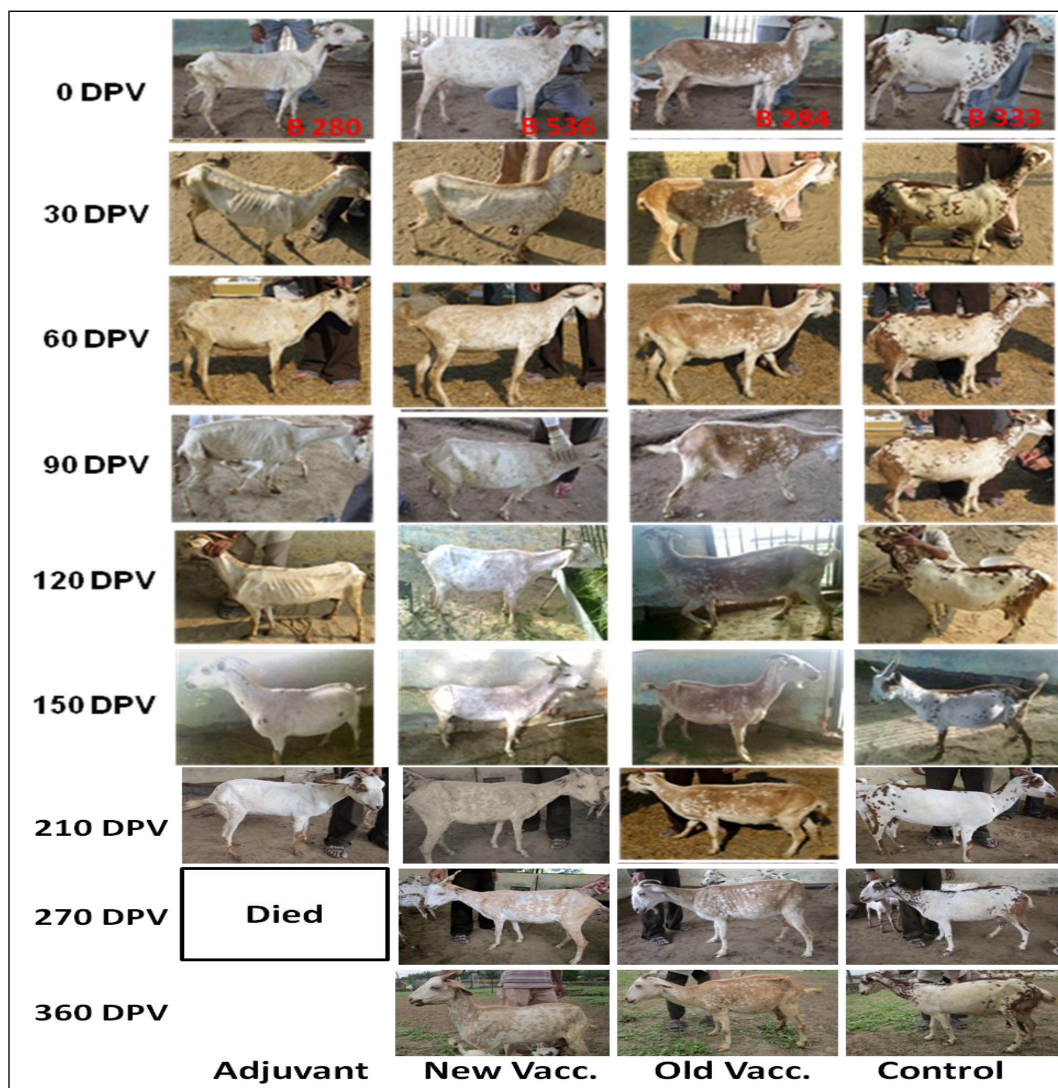

Figure3: Physical appearance of different groups of goats at zero to 360 dpv

Other Observations

There was no adverse reaction because of vaccination, except development of 'take' that was observed at site of vaccination which gradually disappeared. Physical condition of the goats improved after vaccination. There was regeneration of hairs and goats looked healthier (Figure 3). Birth weights of kids born to vaccinated goats were also superior than kids born to non–vaccinated goats.

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DISCUSSION

Johne's disease caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is chronic enteritis of ruminants and is responsible for huge economic losses in livestock productivity world–wide. Persistence of MAP bacilli in soil and water resources, resistance to environmental degradation, ban on cow slaughter and vertical transmission bacilli through semen, in–utero and by feeding of milk and colostrums (Buergelt et al., 2006), have been greater challenges for test and cull policy (Singh et al., 2012a). Therefore, there has been focus on vaccination for the control of MAP infection in domestic livestock.

Vaccine has been able to reduce the shedding of bacilli in feces, decrease the number of clinically positive animals and markedly increase productivity (Singh et al., 2007a). Vaccine prevented conversion of sub–clinically infected animals becoming clinical shedders (Singh et al., 2010a). It also helped in reducing the environmental bio–load of MAP thereby limit the spread to healthy animals (Singh et al., 2010a). Protective efficacy of both live attenuated and killed whole cell vaccines have also been reported by Garcia Marin et al. (1999). However, in terms of safety, marketing and storage killed vaccine has more advantages (Huitema, 1967).

To investigate the dose rate a trial of 'Indigenous vaccine' was started with the 'new batch' of vaccine prepared with double dose (5.0 mg/ml of adjuvant) as compared to existing (2.5mg/ml/goat) dose rates. 'Indigenous Vaccine' developed (Singh et al., 2007a) using native 'Indian Bison type' strain ('S 5') of goat origin (a new biotype; Singh et al., 2013c) was both 'therapeutic' (Singh et al., 2010a) and preventive (Singh et al., 2007a) in goats at 2.5 mg/mL of adjuvant for life. After vaccination of goats with 2.5 and 5.0 mg./ml/goat (double dose) of vaccine, no un–towards reaction or abscess formation was observed in goats except the formation of 'take' at the site of injection within 3–5 days post vaccination. Size of 'take' was reduced in the middle (4–6 months post vaccination) of trial period and disappeared at the end of trial. Presence of local post–vaccinal 'take' may be adopted as another method of assessing positive response to vaccination (Reddacliff et al., 2006). Similar observations have been made by Singh et al. (2007a) and Singh et al. (2013d), when 'indigenous vaccine' was used in goats and cattle, respectively. Other studies reported formation of large and fistulated nodules at the site of vaccination (Mckenna et al., 2006). Hence indigenous vaccine can considered safe in vaccination programs.

Vaccinated goats gained significantly higher weights and values passed the normality test (P value > 0.1) as compared to group I and IV. Comparative evaluation of body weights profiles of different groups of goats at different intervals of days post vaccination showed that group of goats vaccinated with 2.5 mg/ml/goat gained higher body weights. These observations are in agreement to the earlier findings (Singh et al., 2007a; Shroff et al., 2013).

Fecal samples profile for shedding of MAP at zero and 360 dpv showed reduction in number of shedders in vaccinated groups and increase in shedding of MAP in group I and II. The improvements in vaccinated goats may partly be due to reduced shedding by vaccinated goats, therefore lowered chances of re–infection of goats in view of reduced contamination of environment. This is why the improvement was reported in goats of group I and II (Singh et al., 2013d).

Results of IS*900* blood PCR showed decrease in MAP bacterimia in group III up to 360 dpv and increased by 3.5% in group IV. Serum ELISA kit showed gradual increase in sero–titer of anti MAP antibodies in group II and III, while it was varying in groups I and IV. The 2.5 mg/ml/goat dose rate showed higher increase in sero–titer of anti MAP antibodies as compared to group III with double dose (2.5 mg/ml/goat) of MAP bacilli. Singh et al. (2013f) reported sero–monitoring of the Bharat Merino sheep flock by 'indigenous ELISA kit' showed improved 'flock immunity' in successive generations and reduced clinical disease burden in flocks endemic for Johne's disease. High sero–conversion rates were observed in goats of vaccinated group as compared to the groups I (adjuvant) and IV (control). Singh et al. (2007a, 2010a) and Eppleston et al. (2005) have also reported peak ELISA titers at 60–150 days post–vaccination in goats using heat inactivated vaccine.

Since JD is endemic in goatherds located at CIRG, Makhdoom and goats in general suffer from weakness (both from immunological and nutritional angle), despite good feeding schedule. MAP bacilli grows within endothelium of intestines (digested food is not absorbed due to denudation of endothelium of intestines slowly) and within mesenteric lymph nodes (production of antibodies is disturbed), therefore it is usual that the carcass of goats is anemic and debilitated at necropsy. Pneumonia is actually leading cause of deaths in CIRG herds besides diarrhea and weakness (debility). Irrespective of cause of death, part of lung is always involved, in case of goats and sheep whenever necropsy is done. Higher dose (5.0 mg/ml/goat) of vaccine may have resulted in additional stress on the immune system, which is already damaged, besides the stresses caused by damage to immune system, negative energy balance and protein losing entero–pathy. Therefore, in weak animals (immunologically and physically), the secondary invaders present in respiratory system gain upper hand and become cause of death in immunologically weak animals.

Overall marked improvement was found in physical condition of the goats after vaccination. In–contact goats also showed improvements in body weights though gain in body weights was not significant. Birth weight of kids born to vaccinated goats was also better. Compiling all observations, group II goats showed higher improvements compared to group III goats, therefore, 2.5 mg/ml wet weight of 'Indian Bison Type' biotype of MAP of goat origin may be considered optimum dose for goats maintained under optimum nutrition conditions and infected with MAP.

CONFLICT OF INTEREST

No conflict of interest to declare.

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