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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Evaluation of an 'Indigenous vaccine' based on goat adapted *Mycobacterium avium* subspecies *paratuberculosis* in Patanwadi breed of sheep naturally infected with clinical Johne's disease in North Gujarat

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Abstract

Therapeutic efficacy of 'Indigenous vaccine' developed from highly pathogenic 'Indian Bison Type native goat adapted' biotype of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) was evaluated in naturally infected Patanwadi breed of sheep flock located at Livestock Research Station (LRS), Sardarkrushinagar, Dantiwada in North Gujarat. Fifty Johne's disease positive (by microscopy, PCR and ELISA), ready to cull, weak and discarded adult sheep were randomly divided into 2 groups *viz.*, 'Vaccinated' (N = 35) and 'Control' (N = 15). After vaccination sheep were monitored for physical condition, clinical symptoms (weakness and diarrhea), morbidity, mortality, body weights, shedding of MAP in feces and humoral immune responses upto 120 days at 30 days intervals. Average body weights gained were significantly higher ($P<0.01$) in 'Vaccinated group' as compared to 'Control group'. No mortality was observed in the vaccinated group during the study period. Shedding of MAP in feces was reduced in vaccinated sheep by 17.15% whereas shedding increased in control sheep. Vaccinated sheep had significantly higher ($P<0.01$) antibody titer against MAP infection in comparison to 'Control' sheep. Sheep positive on 0 DPV in fecal PCR and blood PCR were found negative on 120 DPV. 'Indigenous vaccine' effectively restricted MAP infection and improved immunity of the sheep flock exhibiting symptoms of clinical Johne's disease.

Keywords: Johne's disease; Indian Bison Type; Vaccination; Patanwadi sheep; India

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Introduction

Johne's disease or paratuberculosis, is a chronic granulomatous enteropathy of domestic and wild ruminants worldwide, caused by *Mycobacterium avium* subspecies *paratuberculosis*. MAP has been reported from wide range of domestic and wild animals including primates and human beings (Singh et al., 2013a; Singh et al., 2012) sharing of MAP between livestock species (inter-species transmission) has been frequently reported in literature (; Ayele et al., 2001; Singh et al., 2012). Major goals for controlling MAP infection are that it cause economic losses by way of reduced productivity in domestic ruminants, all over the world and may also play a role in causation of Crohn's disease in human beings (Mendoza et al., 2009). More than 68.1% of dairy cattle herds in United States are infected with MAP, causing annual loss of about $200 million to $1.5 billion to dairy industry (Ott et al., 1999). Economic losses have never been estimated

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outside developed countries, though JD has been reported to be endemic in many of developing countries like India. Knowledge of prevalence, dominant strains and husbandry practices play vital role in designing control and eradication strategies for Johne's disease. Best policy for prevention of infection is identification of infected animals and their removal ('Test and Cull' policy). Persistence of MAP bacilli in environment (soil, water, faeces etc.) is greatest challenge to test and cull policy. Test and cull policy is expensive and has not answered to its theoretical potentials (Geijo et al., 2005). Also, since JD is transmitted through semen, milk, colostrums, in-utero, etc. (Buergelt et al., 2006), test and cull policy has not been successful to its theoretical potential in controlling JD in other countries also (Geijo et al., 2005). Major reason for the failure of this policy is lack of sensitive tools to detect infection in early stages.

Vaccination is the easy and cost effective method for controlling MAP infection in animals. After the development of a live attenuated vaccine in France, most of the developed countries turned towards vaccination strategy and most of the developed countries have turned to vaccination against JD and have been able to drastically reduce National prevalence of the disease in animals (Crowther et al., 1976; Sigurdsson and Gunnarson, 1983; Benedictus, 1984; Dijkhuizen et al., 1994; Perez et al., 1995). Vaccine has been able to reduce the fecal shedding, decrease the number of clinically positive animals and markedly improve productivity. Importantly, vaccine prevents the conversion of sub-clinically infected animals becoming clinical shedders. It also helps in reducing the environmental burden of MAP thereby restrict the spread to healthy animals. It also helps in the recovery of the clinically infected animals by improving immunity and restricting the advancement of lesions and disease. Efficiency of both live attenuated and killed vaccines have been reported to be the same in small animals (Garcia Marin et al., 1999), however, in terms of safety, marketing and storage killed vaccine has more advantages (Huitema, 1967). Vaccine should be used with other management procedures to reduce the exposure of susceptible animals (Singh et al., 2007a).

Recently in India an indigenous inactivated vaccine has been developed at Central Institute of Research on Goats (CIRG), Makhdoom, using highly pathogenic 'Bison type' strain (Hajra et al., 2005; Sevilla et al., 2005). After the successful in-house trials, vaccine is now being used in goat-herds, sheep flocks and cattle herds located in different agro-climatic region of the country. Herds and flocks of large and small ruminants maintained at Livestock Research Station of veterinary college, Sardarkrushinagar, were endemic for Johne's disease (Jadhav et al., 1993; Sohal et al., 2009; Barad et al., 2013). This vaccination trial was the first randomized study in Gujarat to know the efficacy of 'Indigenous goat based vaccine' for the control of clinical Johne's disease in naturally infected Patanwadi sheep flock.

Materials and Methods

Animals and Management Conditions

Fifty weak, emaciated, diarrheic and naturally infected Patanwadi sheep with clinical JD (positive in microscopy, PCR and ELISA) from the farm herd of Livestock Research Station (LRS) were randomly divided into 2 groups: "Vaccinated" and "Control", consisting of 35 and 15 sheep, respectively. This pilot trial to study the efficacy of "Indigenous vaccine" was truly random, with respect to age, sex, stage of disease, and body weights of sheep. In each group, age of sheep varied from 6 months to more than 1 year. Sheep in both groups were maintained together under extensive system of management. Physically sheep were weak and emaciated, skin was dry, rough, and body weights were below normal.

Vaccine

'Indigenous vaccine' (Singh et al., 2007a) developed using novel, native, pathogenic, and genetically characterized "S 5" strain of MAP (Indian Bison type) of goat origin (Singh et al., 2007b) was evaluated for its therapeutic and protective attributes (immune response and improvement in the health status) of naturally infected sheep with clinical JD for a period of 120 days. "Indigenous vaccine" contained 2.5 mg (dried weight) of heat inactivated native strain of MAP (Singh et al., 2007b) with 0.01% of Thiomersal suspended in Aluminium hydroxide gel (CZ Veterinaria, Spain). Dry weight of 2.5 mg contained approximately 12×10^8 bacilli per ml (McFarland standard).

Vaccination

Sheep in "Vaccinated" group were vaccinated with 1 ml of 'indigenous vaccine' subcutaneously (in the neck region). Simultaneously both vaccinated and control groups were given 1 ml of ivermectin (on opposite side of the neck) subcutaneously.

Data recordings and collection of samples

Body weights of the sheep were recorded, zero day post vaccination (DPV) followed by recording with 30 days interval, which continued up to 120 days. Average gain in body weights by sheep of two groups was analyzed using paired t-test. Serum samples and blood samples collected during 30 days intervals of all the sheep were screened by ELISA and polymerase chain reaction, respectively. Improvements in body condition, mortality, and morbidity were also recorded.

Enzyme linked immunosorbent assay (ELISA)

Pre and post vaccination antibody response was measured by ELISA. Antibody titers were monitored in all the sheep from zero to 120 DPV at 30 days interval as per Singh et al. (2007b). OD values of serum samples were transformed to S/P ratio as described by Collins (2002) and sheep in strong positive and positive categories were considered as positive for MAP infection.

Bacteriological studies (fecal smear examination)

Microscopic examination of fecal samples from the representative animals of vaccinated and control groups was performed by Ziehl Nielson staining as per Singh et al. (2012) in all the sheep from zero to 120 DPV at 30 days interval.

Detection of MAP by IS*900* based PCR

All the 15 microscopically positive samples were subjected to PCR amplification as per Singh et al. (2012). However, MAP bacteremia was also monitored in the blood samples collected from representative animals of vaccinated and control groups using PCR from zero to 120 DPV at 30 days interval as per Vary et al. (1990) with some modification (Singh et al., 2010b).

Results

Reaction at vaccination site

No un-towards reaction or abscess formation due to vaccination was observed in sheep. Similarly no noticeable discomfort due to vaccination nodules (take) was observed in sheep from any of the vaccinated animal. A typical vaccine site reaction showed formation of palpable granuloma within 24 hrs. In few sheep the size of granuloma reduced in the mid of trial period and disappeared at the end of trial. In other sheep it was present at the end of monitoring period but in a reduced size.

Health status

Overall marked improvement was found in the body conditions of vaccinated sheep as compared to control. Vaccinated sheep were healthy, regained shining, luster of skin, regeneration of body hairs.

Body weights

There was significant difference in the average body weights gained by the sheep of vaccinated group compared to control group at 210 DPV. 'T' test was applied to compare body weights obtained from vaccinated and control groups at zero and 120 days post vaccination (DPV). Sheep in the "Vaccinated" group gained significantly higher body weights ($P<0.01$) as compared to "control" group (Table 1).

Mortality rates and causes of deaths

No mortality was observed in the vaccinated goats as well as in unvaccinated group.

Humoral immune response (ELISA)

After vaccination high sero-conversion rates were seen in vaccinated sheep as compared to "Control" (Figure 1). However, percent of sheep sero-converted remained high in 'vaccinated group' than in "control group' at all post vaccination sampling intervals. Almost all the sheep in vaccinated groups became sero-positive (sero-converted) at 60 DPV (Table 2).

Bacteriological studies

Percentage of sheep positive for MAP was reduced from 28.57 per cent at zero DPV to 11.42 per cent at 120 DPV, in comparison to control animals where the percent increased from 33.33 per cent to 46.66 per cent

Table 1: Average body weights gained in vaccinated and control groups (0 to 120 DPV)

Trial groups	Average weight at 0 DPV (A) (in Kg ± SE)	Average weight at 120 DPV (B) (in Kg ± SE)	Average weight gained (B - A) (in Kg ± SE)
Vaccinated (35)	29.72 ± 0.61	32.64 ± 0.66	2.92 ± 0.21
Control (15)	26.79 ± 1.90	24.78 ± 1.77	- 2.01 ± 0.30[@]

DPV- Days post vaccination; @- Reduction in body weights (weight loss)

Table 2: Average S/P ratios in vaccinated and control animals from 0 to 120 DPV

Group	Animals (n)	S/P ratios (Average)				
		0 DPV	30 DPV	60 DPV	90 DPV	120 DPV
Vaccinated	35	0.471*±0.08	0.719±0.07	1.068±0.08	1.009 ±0.04	0.925*±0.04
Control	15	0.281±0.06	0.776±0.04	0.735±0.03	0.687±0.07	0.740±0.04

* S/P ratio was significant ($p \leq 0.05$) between 0 DPV and 120 DPV in vaccinated group

Table 3: Percent sheep shedding MAP before and after vaccination at monthly intervals (0 to 120 days post vaccination)

Trial groups	Sheep (*n*)	Positive sheep (%) in microscopy				
		0 DPV	30 DPV	60 DPV	90 DPV	120 DPV
Vaccinated	35	28.57	31.42	20.00	14.28	11.42
Control	15	33.33	33.33	40.00	40.00	46.66

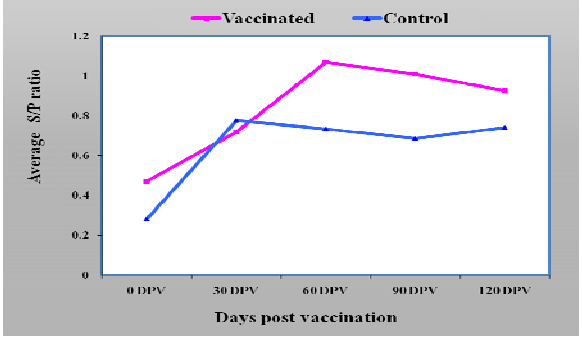

Fig. 1: Comparison of humoral response (average S/P ratios) between sheep of vaccinated group at monthly interval from 0 to 120 DPV

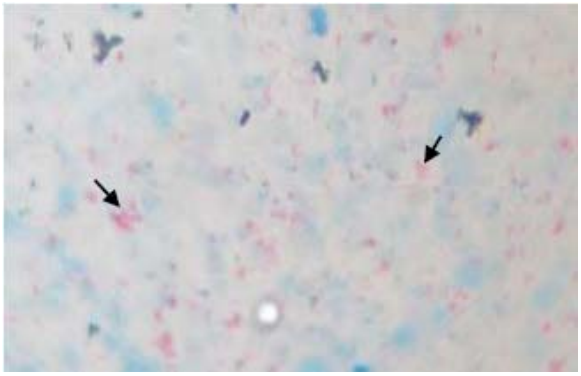

Fig. 2: Microscopic view of acid fast bacilli (indistinguishable to MAP)

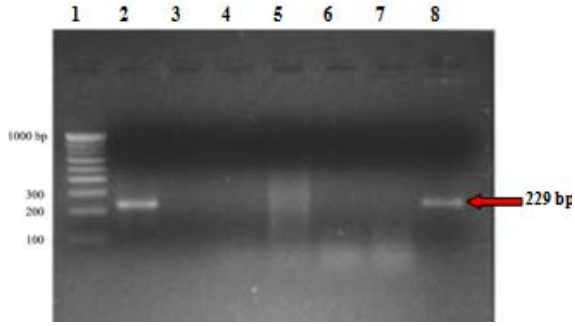

Fig. 3: Results of IS*900* fecal PCR; Lane 1: 100 bp DNA ladder; Lane 3-7: fecal sample negative; Lane 2, 8: fecal sample positive

(Figure 2). Initially, increase in the shedding of the MAP bacilli was noticed at 30 DPV in the vaccinated animals, which later declined over the period of 120 DPV, in comparison to control group (Table 3).

Detection of MAP genome using IS*900* PCR

All the 15 fecal samples, which were positive for MAP bacilli in microscopy at zero DPV were further subjected to DNA isolation and IS*900* PCR and two animals under vaccinated group were found positive at

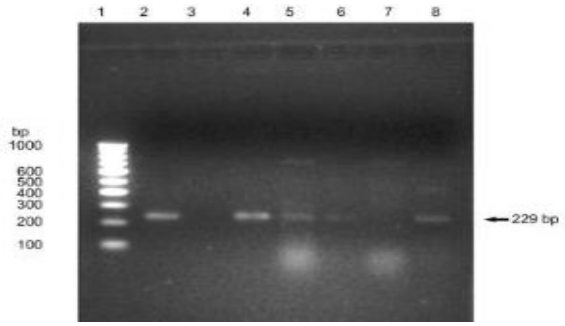

Fig. 4: Results of IS*900* blood PCR; Lane 1: 100 bp DNA ladder; Lane 2, 4, 5, 6, 8: blood sample positive; Lane 3, 7: Blood sample negative

zero DPV. Positive IS*900* PCR in fecal samples at zero day confirmed the results of fecal microscopy for the presence of MAP bacilli (Figure 3). MAP bacteremia was also monitored in the blood samples collected from representative animals of vaccinated and control groups at 0 DPV (Figure 4). Eight animals (6 vaccinated and 2 controls) were found positive out of 50 animals. Animals that were positive for MAP infection at 0 DPV were negative in IS*900* PCR on 120 DPV.

Discussion

Vaccine efficacy is defined as the reduction in the incidence of a disease among animals who have received a vaccine compared to the incidence in unvaccinated animals. Hence, the ultimate read-out of vaccine efficacy is protection against infection and disease. In another study of Indigenous vaccine developed by CIRG, Makhdoom, it has been reported that the vaccine has therapeutic potential along with prophylactic effect (Singh et al., 2010a; Singh et al., 2013b), thereby opened the possibility for the effective control of JD in India. However, before its commercial use in Indian livestock further clinical trials using well-defined parameters are essential to confirm/validate the potency of Indigenous vaccine. Therefore, in continuation of earlier Indigenous vaccine trials, present study was replicated with major aim to validate the efficacy of a 'goat based' indigenous vaccine against Johne's disease developed by CIRG, Makhdoom (Northern India) using a MAP strain recovered from a Jamunapari breed of goat suffering with terminal lesions of JD, in the flock of Patanwadi breed of sheep located in coastal region of Gujarat (Western India) and kept on extensive management system with deficient grazing area or sub-optimal nutrition. Present study also showed that vaccination could be proficient in any stage of disease (subclinical to advance clinical), age group (lambs to adults) and in any physiological stage (dry, lactating or pregnant).

Infection of MAP usually occurs soon after the birth, thus traditionally vaccination has been practiced during the first few weeks of animal's life on the basis that protection would be imparted at the time of initial contact with mycobacteria (Saxegaard and Fodstad, 1985). However, Corpa et al., in 2000 reported that in ovine and caprine species very good results were achieved after vaccination at 6 months and 5 months age, respectively. It may be due to maturation of immune system after 5-6 months of age. Therefore, sheep in the age group of 6-13 months were vaccinated in the present study. No un-towards reaction or abscess formation due to vaccination was observed in sheep. All vaccinated sheep showed the formation of palpable granuloma (at the site of injection) just after a day post vaccination. Presence of local post-vaccinal lesions may be adopted another method of assessing positive response to vaccination (Reddacliff et al., 2006). In few sheep the size of granuloma reduced in the mid of trial period and disappeared at the end of trial. In other sheep it was present at the end of monitoring period but in a reduced size. Similar observations have been made by Singh et al. (2007a) and Srivastav (2010), who used same vaccine in goats and cattle, respectively. Although in some studies, large and fistulated nodules have been reported at the vaccination site (Mckenna et al., 2006). Reddacliff et al. (2006) observed vaccination reaction site (3-45mm) in the vaccinated Merino sheep but the lesion diameter did not decline with time. This might be due to the difference in the composition of the vaccines with respect to field strain and the adjuvant used from vaccine to vaccine in previous studies. These factors apparently contributed to the reduction in size of vaccine nodule formation in the present study.

Overall marked improvement was found in the body conditions of vaccinated sheep as compared to animals under control group. Vaccinated sheep were healthy, regained shining, luster of skin and regeneration of body hairs. Retrospective data analysis revealed that there were frequent abortions in pregnant sheep in the previous years. However, when this study was undertaken 90 per cent of vaccinated sheep were pregnant and no incidence of abortion was noticed after vaccination and all the pregnant sheep lambed normally, indicating that vaccine was safe in any stage of pregnancy. Though, the MAP is not considered as abortificient agent, clinical condition caused by JD and low body weight might have precipitated the condition for miscarriage of fetus. Hence, this study proved to be very much useful for regaining production as well as reproductive efficiency of pregnant sheep.

Paratuberculosis is a chronic disease and it directly affects the body growth and other production and reproduction performance of animals (McKenna et al., 2006). Therefore, monitoring of live body weight is used as important parameters for assessing the vaccine mediated protective value (Singh et al., 2007a) as well to assess the therapeutic value of vaccine in naturally infected goats (Singh et al., 2010a). A significant ($P < 0.05$) increase was observed in the monthly live weight gained in the Patanwadi sheep under vaccinated group, as compared to control group, between 0 to 120 DPV. Sheep were maintained on grazing and under ordinary management conditions. There was decline in the body weights in maximum number of sheep under both groups, at the 30 DPV. This might be due to the unfavorable weather *viz.,* delay in monsoon, there was scarcity of vegetation for grazing and as the animals were maintained only on grazing they were under stress. This observation was similar to the findings of Singh et al. (2007a) and Singh et al. (2013b), when they used the same vaccine in goats. Our findings are also in agreement with the findings of Eppleston et al. (2005), when he used a commercial killed vaccine in sheep. These observations showed that vaccinated group had clear advantage in terms of body weights and body condition over the control group.

Being a chronic infection, mortality rate is comparatively low in JD. However, progressive weakness leads to emaciation and death. Significant reduction in herd mortality is well documented in earlier studies of JD vaccine (Singh et al., 2007a; Singh et al., 2013b). Before vaccination, retrospective analysis of the previous records suggested that there was 40 per cent mortality at the farm. Present study and Barad et al. (2013) showed the evidence that JD was prevalent / endemic in the LRS farms. During course of this study, no mortality was observed in the vaccinated sheep. Singh et al. (2010a) and Srivastav (2010) also noticed lower mortality rate under vaccinated groups following vaccination by the same vaccine as compared to control group, in goats and cattle herds, respectively. Singh et al. (2011) recently reported significant reduction of mortality in Jamunapari goat herds in post vaccination year as compared to preceding year (non-vaccinated). Reddacliff et al. (2006) also observed that there had been only seven OJD mortalities in vaccinates compared to 80 from the controls (a reduction of about 90%).

The vaccine used in this study elicited humoral immune response. Significant difference ($P<0.05$) in number of ELISA reactors was observed after 30 DPV. All the sheep in vaccinated group became ELISA positive after 60 DPV of vaccination. Significantly high ($P<0.05$) number of sheep in vaccinated group retained high titer of anti-MAP antibodies throughout experiment (Figure 6). High sero-conversion rates were observed in sheep of vaccinated group as compared to the control group. Similar observations has also been made by Singh et al. (2007a) using the killed vaccine in goats maintained at CIRG, Makhdoom. Eppleston et al. (2005) using a killed vaccine also observed maximum ELISA reactors around 2 months post-vaccination.

In control group of some of the sheep showed high titer of ELISA upto 30 days post vaccination and then they showed decline in ELISA titer. The possible reason could be that the animals might have been suffering from parasitic infection; hence the animals under stress with low protein levels were unable to generate the antibodies against the MAP. When ivermectin was administered to the animals, due to removal of parasitic infection the host immune system started generating the antibodies against MAP and showed the peak at 60 DPV, but were unable to maintain this titer in the animals belonging to control group as compared to vaccinated group where it continued to increase and showed the peak at 60 DPV. This proved that the vaccine was able to maintain high titer of antibodies against the MAP in vaccinated animals. In the vaccinated group, as the infection might have been controlled due to high titer of antibodies generated after vaccination there is decrease in the MAP shedding and environmental contamination, leading to decrease in exposure of the animals to MAP and this might have resulted in controlling the natural infection in the non vaccinated sheep of control group and ultimately resulted into decreased ELISA titer in this group. Comparative higher serum antibody response in vaccinated animals than in naturally infected animals was well established earlier (Gwozdz et al., 2000; Singh et al., 2007a, 2010a, 2011, 2013b).

Fecal shedding is the most practical test for ante-mortem evaluation of the efficacy of paratuberculosis vaccines (Kalis et al., 2001). The most valuable information comes not only from determining whether an animal is fecal culture positive but also by obtaining an estimate of the level of shedding (Eppleston et al., 2005; Reddacliff et al., 2006). On the microscopic examination of 50 fecal samples from representative animals (35 vaccinated and 15 controls), significant reduction in shedding of MAP in the feces of vaccinated group animals was observed in comparison to control group animals. Percent of sheep positive for MAP was reduced from 28.57 per cent at zero DPV to 11.42 per cent at 120 DPV, in comparison to control animals where the percent increased from 33.33 to 46.66 per cent. Initially, increase in the shedding of the MAP bacilli was noticed at 30 DPV in the vaccinated animals, which later declined over the period of 120 DPV, in comparison to control group. Hence, vaccination reduced fecal shedding in significant number of vaccinated sheep, leading to reduction in total amount of MAP organisms shed on the pasture. Thus thereby, decreased the infective dose rates to the successive generations of sheep and possibly increasing the proportions of exposed animals that resist infection or at least do not shed MAP bacilli during their time on farm, leading to reduction in JD prevalence. Increase in the MAP shedding in the feces at 30 DPV might be due to the un-favorable (summer) weather, the animals were under stress. Subsequently, after monsoon, as the animals got relief from environmental stress, the vaccine efficiently worked and reduction in the MAP shedding have been observed. Our findings are in agreement with Singh et al. (2007a), who also reported that vaccination reduced fecal shedding in significant number of vaccinated goats at Makhdoom. Reddacliff et al. (2006) also found the reduction in prevalence of shedders by about 90 per cent on using the killed vaccine for the control of JD in sheep.

All the 15 fecal samples positive (10 vaccinated and 5 control animals) in microscopy at zero DPV were further subjected to IS*900* based PCR amplification. Three animals under vaccinated group showed an amplicon of 229 bp at 0 DPV. MAP bacteremia was also monitored in the blood samples collected from representative animals of vaccinated and control groups at 0 DPV. Eight animals (6 vaccinated and 2 controls) were found positive out of 50 animals. The animals that were positive for MAP at 0 DPV, all were found negative for both blood and fecal PCR on 120 DPV. Our findings are in agreement with Srivastav (2010), who reported reduction in the blood and fecal PCR positives in the vaccinated cattle while using the same vaccine in Uttar Pradesh. On comparing the results of microscopy, fecal PCR and blood PCR at different time intervals the sensitivity was slightly higher for microscopy. The high rate of detection by microscopy may sometimes be due to low specificity, lack of standardization of test and proper training of the person behind the test, where chances of false positive diagnosis by microscopy or detection of other fast growing acid fast bacilli exist (Singh et al., 2007a).

Reduction in new clinical cases of JD was achieved by following immunization of infected sheep flock endemic for Johne's disease. Similar results have been observed by Uzonna et al. (2003), where vaccine prepared from native isolate reduced more MAP shedders than commercially available vaccine.

Conclusions

Hence, single dose of 'Indigenous vaccine' developed from highly pathogenic locally isolated 'Indian Bison Type' biotype of MAP of goat origin significantly reduced morbidity, mortality and shedding of MAP, reduced clinical signs (diarrhoea) and enhanced flock immunity in the Patanwadi sheep flock naturally infected and endemic for JD. Therefore 'Indigenous vaccine' developed against Johne's disease was both 'therapeutic' and 'preventive' and routine use might reduce the overall disease burden and transmission to susceptible sheep population. Further, a long term monitoring of the vaccinated animals is suggested to analyze the proper effects of 'Indigenous vaccine'.

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