The characteristic feature concluded from the present study is that the infection of sheep with all the three genera, reaches its peak in April or May. The percentage of infection declines to its minimum in the month of September, where from it shows steady increase till April or May. The infection usually shows a gradual increase from September onwards except in some months where minor signs of outbreak of infection are indicated which is also in conformity with the percentage of infection during the winter months (December to February). In Central Provinces of Berar and in Central India, these months are climatically similar from spring to summer months in Kashmir.

Although there are minor fluctuations in the percentage of infection with these Paramphistomes, yet there is maximum infection during late spring and in early summer months, which in Kashmir are comparatively hotter, the temperature varying from 14°C to 29°C and there is enough rainfall during these months which seems to be ideal for luxurious growth of snails. Similar observations have been made by Nikitin (1996) who observed 100% infection in cattle with Gastrothylax crumnimfer in spring season in Russia.

Contrary to the present observation, Nath (1971) reported maximum infection of Paramphistomes in sheep from September to March in U.P. The present findings may be due to the fact that the climatic conditions from September to March in U.P. are similar to spring season of Kashmir. It is believed that during these periods the activity of snails is at its highest which
harbour the intermediate stage, provide there by heavy infection to the livestock during these seasons.

Rodonya (1960) has noted that the faecal egg counts fluctuate throughout the year but are highest in June and lowest in November in Georgian S.S.R., responding to the time of highest and lowest snail activity respectively. Ahmad and Ansari (1987) have also reported almost similar findings and the regulation of worm burden was correlated with the seasonal/climate conditions. The breed wise analysis revealed that the exotic sheep had higher prevalence rate than the native breed.

In genera, the results of this study are in accordance to those presented for other tropical and sub-tropical regions of the world in which the highest infection was reported in summer, autumn and at the beginning of winter (Kamburov, 1977). Nevertheless, in other latitudes some differences were observed in Australia where the prevalence of *Calicophoron calicophorum* decreased in spring and summer and increased in autumn and winter whilst in India prevalence decreased in winter and summer.

Rangal *et al.* (2002) observed that livestock infected with *Paramphistomum cervi* occurred more frequently during the rainy (July to October) and windy seasons (from Nov. to Feb.). In May during the dry seasons, similar incidence occurred to those reported in India (Varma *et al*., 1989).

Szmidt - Adjide *et al.* (2000) reported the highest prevalence of Paramphistomes in spring (0.38), followed by autumn (0.36), winter (0.30) and summer (0.11). The results of the present study were similar with the result of Talukdar (1996) who reported the highest percentage of infection in summer (35.34%) and lowest in winter (13.95%).

The infection of gastrointestinal parasites in cattle of Karanataka was found to be 67.2% (D’Souza *et al*., 1988) and 83.46% in Maharashtra (Maske
et al., 1990), while in the present observation it was 68%. The incidence of *Fasciola* and amphistome species was found to be much higher in animals in the unorganized sector as compared to animals in the organized sector. Similar observations were made on the prevalence of *Fasciola* in sheep of J&K State (Pandit et al., 1989).

El-sayed Hashem (1997) determined the fecal examination of 250 animals, where the overall infection was 56.8% as also observed in the present study.

Dube et al. (2003) reported the genus of *Cotylophoron fischeri* (1901) recovered from the inner walls of stomach of cattle slaughtered in the North and South of Nigeria with a prevalence (35%). However, the present study revealed the percentage of this parasite as 47%.

Ruiz et al. (2003) reported seasonal trends of *Paramphistomum cervi* and observed 37.10% incidence throughout the year, which is in agreement with the present study with an incidence of 42%.

Talukdar (1996) reported prevalence of seven species of trematode, in which *Paramphistomum cervi* was 80.50%, *Cotylophoron* was 32.5% and *Gastrothylax crumnifer* was 58%.

Dutt et al. (1995) showed that the incidence rate of *Cotylophoron cotylophorum* was 86%, *Gastrothylax crumnifer* 62% and also observed the rate of infection in rumen and reticulum as 58.30%.

Dubey et al. (1983) reported that out of 16,945 sheep examined in Rajasthan in 1976 and 1978, 2520 were clinically diagnosed as having parasitic gastroenteritis. Out of 1224 fecal samples examined, 528 had a low intensity, 450 moderate infection and 446 having heavy infection. The number of cases increased to peak in March. There was then an abrupt
decrease followed by a second rise in infection in July and August (i.e. during rainy months), with a lowering trend in September and October.

From the present observations it seems that sheep in Kashmir Valley can be given anthelmintics against paramphistomiasis at least twice in a year. Firstly in the month of April to May, which is highest season of infection with the hosts feeding in pasture lands and the second at the time of their return from pasture lands to the valley.

The pathology and pathogenesis of Paramphistome infections appears to be complicated by various factors including host factors (viz. Species affected, age of animal); nature of infection (viz.. Parasite species, single or mixed infection parasite load: primary or secondary infection) and managerial factors (viz. Housed or grazed) (Whitten, 1955; Horak and Clark, 1962).

In general after excystment in the duodenum to mid-jejunum, the flukes migrate towards the abomassum with the immature flukes invading the mucosa as far as muscularis mucosa while moving proximally, passing through the abomssum and omasum into the rumen (Boray, 1969; Rolfé, et al., 1994).

In present study, all the animals were adults and only a few revealed presence of low level infestation with immature Paramphistomes in duodenum along side probably reflects secondary exposures to the parasite.

Boray (1969) reported that adult sheep harbouring mature *Paramphistomum ichikawia* and exposed alongside weaners having massive infection with metacercariae, contained very few immature Paramphistomes.

Bida and Veen (1977) who observed the simultaneous presence of mature and immature *Paramphistomum microbothrium*, respectively in rumen
and duodenum of sheep dying of paramphistomiasis, ascribed the difference to the parasite species rather than age.

The occurrence of lesions in both duodenum and rumen was a consistent feature. However, the gross and histopathological lesions observed in duodenum and rumen corroborate well with the findings of earlier workers (Sharma et al., 1997; Misra et al., 1996; Dwivedi et al., 1997; Singh et al., 1984).

In present study, the nature and extent of lesions not only varied from animal to animal but also between different areas of duodenum and rumen from some animals. This point may be attributed to the fact that most of the lesions in paramphistomiasis are a result of mechanical trauma by the anchorage and feeding behaviour of the Paramphistomes (Deorani and Katiyar, 1967) and reparative process as the flukes migrate towards rumen. Presence of mature Paramphistomes in the rumen are supposed to have no clinical or sub-clinical effects. However, the severe mechanical damage in heavy infection may accentuate the intestinal effects, while severe cornification of the rumen wall has been associated with inappetence, weight loss and lethargy in lambs (Rolfe, et al., 1994).

In present study, sections of flukes were observed deep in submucosa. Other workers have also found various Paramphistomum species in the deep muscularis (Varma, 1961; Deorani and Katiyar, 1967; Deorani and Jain, 1969; Prasad et al., 1974 and Singh et al., 1984). Submucosal duodenal oedema has been observed in immature paramphistomiasis and postulated to cause biliary retention due to bile duct obstruction.

The variation in the nature and extent of inflammatory responses observed in present study may be due to difference in the parasitic load, stage of infection and involvement of local area. However in general lymphoecytic
infiltration constituted the predominant cellular response either in duodenum or rumen.

Cankovic and Batistic (1963) observed lymphocytic infiltration in the lamina propria and sometimes in the epithelium and submucosa layer of the rumen infected with *Paramphistomum cervi*.

Singh *et al.* (1984) observed a progressive increase in the severity and extent of (mononuclear and lymphocyte) infiltration in the duodenum following infection with *Paramphistomum cervi*. Appearance of eosinophils in intestinal inflammatory infiltrate have been associated with the development of resistance to parasitic infection (Moqbel, 1986). Rolfe *et al.* (1994) also observed that following *Paramphistomum ichikawia* infection, the eosinophil accumulation in small intestines coincided with the time when most parasites were rejected. The presence of globule leucocytes have also been associated with antiparasitic activity in gastrointestinal mucus and parasitic resistance (Douch *et al.*, 1986).

This might explain for the higher resistance to or expulsion of subsequent infections in the adult sheep with mature Paramphistomes in the rumen.

During present study, no metachromasia was observed in sections stained with toludine blue. Failure of globule leucocytes, which are considered to be amine-producing cells derived from subepithelial mast cells, to stain with toluidine blue is in agreement with the findings of Frigan *et al.* (2004) for the species.

The similar findings was reported by Bida and Veen (1977), the attachment of the immature conical flukes to the small intestinal mucosa and the haemorrhages and erosions which then developed probably lead to plasma albumin loss and the subsequent generalized oedema observed in many cases.
Chhabra and Bali (1972) observed during post-mortem examination, the subcutaneous tissue and bristles was markedly oedematous and gelatinized. The abnormal part was much thickened, oedematus and gelatinized but the parasite were not present in the abomassum. almost the results are same with the present study. At the site of attachment of flukes the stratum corneum and granulosum of the duodenum was found to be roughened and slightly thickened but there were no changes seen in the abomassum. The serosa of the small intestine was redened and the blood vessels were enlarged and prominent.

Rothwell and Dineen (1973) found no differences in the number of mucosal mast cells in uninfected, infected or immune animals, although there was some difficulty in the identification of these cells.

Mast cells were not recruited by the presence of *Trichostrongylus colubriformis* until after antigenic stimulation and subsequent re-challenge (Douch *et al.*, 1986). The present study reveals the diffused infiltration of lymphocytes and eosinophils associated with globule leucocytes, but no mast cells could be demonstrated by Toluidine blue method. Noble (1956) found similar results in *Paramphistomum cervi* infection in cattle and sheep causing cellular infiltration mainly of eosinophils.

The flukes caused damage to the duodenal wall, not only by their embedding habit, but also exerts an additional pulling action and consequently increased tissue damage with the help of their powerful acetabulum (Prasad *et al.*, 1974; Cankovic and Batistic, 1963 and Graubmann *et al.*, 1978).

In the present study, the rumen showed increased cornification of the stratum corneum of the papillae and increase in size and number of cells in the stratum granulosum. Atrophy, severe infiltration, cornification and thickening of mucosa occurred in the rumen papillae (Mukherjee and
Deorani, 1962; Maqsood, 1944; Mudhaliar, 1945 and Guelhorn and Priouzean, 1945) in sheeps and goats with immature amphistomes (Katiyar and Varshney, 1963; Deorani and Katiyar, 1969; Deorani and Jain, 1969 and Prasad et al., 1974).

Cornification was found in rumen which lead to weight loss and lethargy in sheep (Horak, 1971). The number of circulating neutrophils and eosinophils was increased and that of lymphocytes decreased in heavy infested lambs (Misra et al., 1996). The results are similar with the present study that the infection with Paramphistomum cervi causes destruction and desquamation of the mucosa leading to the loss of cells into the lumen. Further accumulation of eosinophils was observed in the small intestine similar to that observed in Trichostrongylus colubriformis infection in guinea pigs (Rothwell and Dineen, 1973).

Thus, it is concluded that the immature forms of Paramphistomum cervi caused more severe damage in the duodenal tissue, where as adult forms inflicted mild tissue damage in the rumen of sheep.

It is a well known fact that helminth parasites during their development undergo antigenic polymorphism which induces drastic alteration in immune response. so use of these different developmental stage antigens in the immunodioagnosis is very important. Development of simple and specific immunological tests for the diagnosis of helminth infections has been a major goal for recent immunological research. Several immunodiagnostic methods have been used recently for the diagnosis of trematode infections in ruminants and also to assess the immune response elicited by these parasites in the experimental animals. Immunodiagnostic methods for the detection of these parasitic infections, however, usually suffers from problems of low sensitivity. Immunodiagnosis of parainphistomiasis at early stage i.e., the diagnosis of immature parainphistomiasis is very limited.
Agar gel immunodiffusion test of complete somatic antigen of *Paramphistomum* species and *Fasciola hepatica*, using the respective homologic and heterologic hyperimmune rabbit sera have been studied and the number of precipitation lines produced were six to *Paramphistomum* and four to *Fasciola* antigen (Bratnov *et al.*, 1981). The immunodiffusion tests (DID and CIEP) and ELISA have been found suitable and test of choice for serodiagnosis of fascioliasis (Hillyer, 1975; Cechini and Kasalin, 1989; Gorman *et al.*, 1993 and Mousa, 1994) due to their high specificity and satisfactory sensitivity. The results revealed that the routine serological technique such as AGPT, IEP, IHA and ELISA can detect antibodies to *Paramphistomum cervi* in sheep infected under natural conditions and hyperimmunized rabbits.

The agar gel immunodiffusion test (AGPT) was a simple and economical procedure and required no special equipment, but was found to be least sensitive in the present observations. In the present study Ouchterlony test revealed the appearance of one precipitin arc in sheep infected sera and 2-3 precipitin arcs in immunized rabbit sera against Paramphistome antigen, which indicated the presence of multiple antigenic determinants. In rabbits the number of precipitin bands increased from the day 1st to day 20 upto 3 bands, but the number of precipitin bands decreased to one from day 40 to 50 post immunization. The decrease in precipitin bands may be due to decrease in antibody titre in the immunized rabbit sera.

Baqui (1983) also reported a maximum of 3 precipitin lines at day 20 by Ouchterlony test in experimentally infected rabbits to whole worm antigen of *Setaria cervi*.

Stewart (1953) studied the production of antibodies against the infective larval challenge of *Trichostrongylus* and concluded that the
continued presence of higher titre of antibodies was due to persistent infection of *Trichostrongylus*.

Varma *et al.* (1991) tested rabbit hyperimmune sera with crude extract of adult *Paramphistomum cervi* by DID and found only faint precipitin lines from 8th week of post infection. However, there was no reports of absence of precipitation reaction in DID with experimental hyperimmune sera.

These results are in agreement with those of (Hafeez *et al.*, 1984; Verma *et al.*, 1990) who observed a progressive increase in antibody titre from 2-6 weeks post infection by *Paramphistomum cervi* and *Paramphistomum epiclitum*.

Yadav and Gupta (1993) reported faint band of precipitation’s observed in the serum samples of single rabbit each at 4th and 6th weeks post infection.

Rao *et al.* (1993) also reported presence of 3 bands with hyperimmune sera raised against saline extract of *Gastrothylax exaplanatum* by immunodiffusion and immunoelectrophoresis and concluded that after 8th week of post immunization, precipitin bands decline. The absence of the precipitin lines in naturally infected buffaloes and hyperimmunized rabbit might be due to low level of precipitating antibodies. Similar opinion has been expressed by Gaur and Deo (1972) in *Ascaris suum* infection in pigs.

In the present study, immunoelectrophoretic technique and Indirect haemagglutination tests were employed for detection of antibody levels in infected, uninfected sheep and immunized rabbit sera. In immunoelectrophoresis, the appearance of 2 precipitin bands were observed in hyperimmunized rabbit sera at day 20 where as only one precipitin band was observed against the infected sheep serum and no precipitin band with normal uninfected serum. Simple electrophoresis of serum samples revealed increased gamma globulins in sheep serum due to paramphistomiasis.
infection. Our results are also similar with the findings of Wrights and Gonzalez (1943), Leland et al. (1995), Baqui and Ansari (1975) and Ahmad et al. (1990) who studied serum protein changes of lambs experimentally infected with *Haemonchus contortus* or *Trichinella spiralis* and *Microfilaria* in rats, experimentally infected with bovine setariid. They have reported a marked decrease in albumin, whereas α-globulin, β-globulin and γ-globulin increased at peak of the infection as direct result of antibody production. Zharikov et al. (1976) observed 5-6 precipitin arc at anodic and one arc at cathodic fraction. Swarup et al. (1987) reported that sharing was not noticed between *Gastrothylax explanatum* and *Fasciola gigantica* (Song and Kim, 1982 and Larramendy and Pedrosa, 1984).

Our results are in accordance with (Yoshihara et al., 1981; Hillyer et al., 1980; Yamaguchi et al., 1989 and Santiago Weil, 1984) who observed one main identical band in sheep serum by immunoelectrophoresis and two bands in immunodiffusion with *Fasciola* antigen.

In the present study, indirect haemagglutination test of naturally infected sheep sera and hyperimmunized sera in rabbits was done. The antibody titre of hyperimmunized sera was 1:64 titre and in naturally infected sera was 1:32 titre. It showed a higher sensitivity than agar gel precipitation test but was less sensitive than IEP. The results are in accordance with the results of Swarup et al. (1987) who reported antibody titre in IIA as 1:128 against *Fasciola gigantica*.

Muralidhara and Sastry (1997) showed 1:160 titre of antibody with a protein concentration of 7.2 gm% against hydatidosis. IIA test was carried out in sheep, indicated its specificity and sensitivity (De-Rosa and Puccini, 1979; Werner and Keurim, 1971). Sharma et al. (1987) reported counter immunoelectrophoresis as most sensitive than IIA and AGPI. Ogunrinade (1983) and Arriaga et al. (1983) have reported 100% specificity of some of
the immunological tests including immunodiffusion in the diagnosis of *Fasciola gigantica* and *Fasciola hepatica* of cattle.

ELISA test has been reported to be good from the point of sensitivity, specificity and repeatability (Maisonnave, 1999). In the present study in comparison to gel diffusion, IEP, IHA and the ELISA test has been found to be highly effective and may be employed for the wide use in seroepidemiological survey (Ibarra *et al.*, 1998) of paramphistomiasis. In *Haemonchus contortus* infection also a high titre of 1:40,000 was observed with rabbit hyperimmune sera raised by somatic antigen of *Haemonchus contortus* (Kaur *et al.*, 2002). The present study revealed antibody titre of sheep sera diluted from 1:100 to 1:16400 dilution, whereas the immunised rabbit sera ranged from 1:100 to 12800 dilution. At 6th – 8th week of infection, antibody was detected by ELISA, then due to increasing dilution, the antibody absorbance values were found to be decreasing. The highest titre observed in the present study might be of good significance in diagnosis of paramphistomiasis in field. The serodiagnosis of the disease is the only alternate to coproscopic detection of the fluke eggs (Hammond, 1973; El-Hareth, 1980 and Gupta and Yadav, 1992).

The immunodiffusion test and ELISA have been found suitable and test of choice for serodiagnosis of fasciolasis (Hillyer, 1975; Gomez *et al.*, 1984; Cechini and Kasalin, 1989; Gormen *et al.*, 1993 and Mousa, 1994) due to their high specificity and satisfactory sensitivity.

The ELISA is clearly more sensitive than the faecal count method, partly because antibodies are present approximately by the 8th week before the infection matures and eggs are shed in the faeces (Castro *et al.*, 2000 and Reichel, 2002) and partly many animals with mature infection do not have detectable numbers of eggs in faeces. The ability to diagnose the infections by ELISA and to treat early is a big advantage because it minimizes tissue
damage due to flukes as they migrate through the small intestine. More importantly, early treatment prevents shedding of eggs in faeces, thus contributing to effective management by reducing the rate of infection.

The present study aimed to identify and partially purify antigenic polypeptides of *Paramphistomum cervi* paramphistomiasis. There is no information on the antigenic polypeptides and cross reactivity among three species of Paramphistomes.

The preliminary investigation indicated that IgG antibodies can be detected even up to a dilution of 1:2800 by the ELISA. It is thus concluded that the soluble extracts of *Paramphistomum cervi* are highly antigenic.

Sanchez *et al.* (2000) compared Sandwich ELISA with indirect ELISA for the diagnosis of natural *Fasciola hepatica* infection. They reported the Sandwich ELISA to be more specific but less sensitive than indirect ELISA and concluded that antigen detection combined with results of antibody detection to be a reliable diagnostic technique for epidemiological studies.

Grelick (1977) compared IFAT with indirect ELISA technique for routine diagnosis of *Fasciola hepatica* in cattle and found IFAT more sensitive than the ELISA but gave more false positive results.

Hillyer *et al.* (1996) used *Fasciola hepatica* excretory/secretory antigen-antibody for evaluation by ELISA and reported 100% sensitivity in sheep and 82% in cattle.

An evaluation was made by Intapan *et al.* (1988) on somatic antigen, excretory/secretory antigen and egg antigen in serodiagnosis of the disease in sheep using DID, CIEP and ELISA. They found that in ELISA excretory/secretory antigens were the most reactive with no significant differences. A similar result was obtained in goats (Mandal *et al.*, 1999).
Hira Ram (2000) used ELISA for assessing immune response in experimental infected buffalo calf. There was an increase in the titre of antibody from 1st week of post infection and peak titre was found at 3rd week of post infection. However, during the present study the peak titre was found at 6th weeks post infection in experimental animal rabbits, which was not much significant indicating that immune response was weakly developed in experimental animals.

Paramphistomiasis in ruminants caused by *Paramphistomum* species is widely prevalent in Indian sub-continent resulting in immense economic loss due to high morbidity and mortality. For the effective control of the disease, a sensitive and specific method to diagnose a symptomatic or latent parasitic infections in endemic population is mandatory. Coprological examination fails to detect infection during prepatency. When maximum damage is caused by migration and developmental stages of *Paramphistomum* species. However, serodiagnosis of the disease is possible by detecting circulating antifluke antibodies during early prepatency period (Yadav and Gupta, 1992).

Thus, it could be concluded that ELISA technique was better than other methods employed during the study for detection of antibody levels and could be used for mass screening of sheep for *Paramphistomum cervi* infection and accordingly better management strategies could be adopted to minimize economic losses due to this infection.