Studies on Sero-prevalence and Risk Factors of *Mycobacterium avium* subsp. *paratuberculosis* in Sheep and Goats

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Thesis

Submitted to

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Master of Veterinary Sciences

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2021


DEDICATED
TO MY
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PARENTS
AND

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
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Shuhama Campus Srinagar-190006

Certificate – I

This is to certify that the thesis entitled, **"Studies on sero-prevalence and risk factors of *Mycobacterium avium* subsp. *paratuberculosis* in sheep and goats"** submitted in partial fulfillment of the requirements for the award of the degree of **Master of Veterinary Science (Clinical Veterinary Medicine, Ethics and Jurisprudence)**, to the **Faculty of Veterinary Sciences & Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir** is a record of bonafide research work carried out by **Dr. Ishfaq Hussain Nengroo (Regd. No. MSV-2019-435)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that information received during the course of investigation has duly been acknowledged.

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Certificate – III

This is to certify that the thesis entitled, **"Studies on sero-prevalence and risk factors of *Mycobacterium avium* subsp. *paratuberculosis* in sheep and goats"** submitted by **Dr. Ishfaq Hussain Nengroo (Regd. No. MSV-2019-435)** to the **Faculty of Post-graduate Studies, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir** in partial fulfilment of the requirements for the award of the degree of **Master of Veterinary Science (Clinical Veterinary Medicine, Ethics and Jurisprudence)** was examined and approved by the Advisory Committee and External Examiner on …….. .

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ABSTRACT

This study was undertaken to determine the seroprevalence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in sheep and goat population and to identify the risk factors associated with seroprevalence of MAP. The overall seroprevalence of MAP in sheep and goats by using indigenous indirect-ELISA was 47.26%. It was found to be significantly ($p<0.05$) higher *in* sheep (52.5 %) than goats (23.07 %). The seroprevalence did not differ significantly ($p>0.05$) with respect to age, sex and breed of the studied animals. The risk factors significantly ($P<0.05$) associated with MAP were flock size, presence of cattle at the farm, breeding system, education level of the owner, quarantine practice, and grazing with other animal species. Diarrhea was significantly ($p<0.05$) associated with MAP in goats only. The risk factor which were not significantly ($p>0.05$)associated with the occurrence of MAP in sheep and goats included transhumance, sign of emaciation, on farm replacement, cleaning pens, contamination of water or feed with fecal materials, separation of diseased animals, treatment in suspected animals, water sources. Milk feeding to the young ones could not be evaluated as risk factor for occurrence of MAP in the present study because this was practiced in all the sampled animals.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*, Seroprevalence, i-ELISA, Risk factors, Sheep, Goat.

Signature of student

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Signature of Major Advisor

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Dr. Ishfaq Hussain Nengroo

Place: Shuhama, Ganderbal

Dated

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LIST OF ABBREVIATIONS

^{o}C	:	Degree Celsius
AGID	:	Agar gel immuno diffusion test
BSA	:	Bovine Serum Albumin
CD	:	Crohn's disease
MAP	:	*Mycobacterium avium* subsp. *paratuberculosis*
CFT	:	complement fixation test
ELISA	:	Enzyme-linked immunosorbent assay ELISA
i- ELISA	:	Indirect Enzyme-linked Immunosorbent Assay
JD	:	Johne's disease
LRIC	:	Livestock Research and Information Centre
OD	:	Optical density
OIE	:	World Organisation for Animal Health
OR	:	Odds ratio
OPD	:	O-Phenylene diamine Di-hydrochloride
PBS	:	Phosphate Buffered Saline
PBST	:	Phosphate Buffered Saline with Tween
qPCR	:	Quantitative polymerase chain reaction
sPPA.	:	sonicated semi-purified protoplasmic antigen
S/P	:	Sample to positive
TDW	:	Triple distilled water
TMAD	:	Trás-os-Montes e Alto Douro
ZN	:	Ziehl–Neelsen

Chapter-1

INTRODUCTION

Sheep and goats are important species of livestock in India, mainly on account of their short generation intervals and higher rates of prolificacy. They are considered to be very important for their contribution to the development of rural people. The local initiatives to promote quality labels and innovative products for cheeses, meat and fibers could help goats in keeping a role for sustainable development in an eco-friendly environment all over the world. Sheep and goats are owned by smallholder farmers as an integral part of the livestock subsector and contribute to both subsistence and cash income generation (Ehuis *et al*., 2000). Sheep with its multi-facet utility for wool, meat, milk, skins and manure, form an important component of rural economy particularly in the arid, semi-arid and mountainous areas of the country. Sheep have a great potential to contribute more to the livelihoods of the people in low-input, small-scale mixed crop livestock production systems (Kosgey and Okeyo, 2007). The goat industry continues to grow in developing countries and to gain acceptance in modern times in countries with higher to moderate incomes, because sheep and goats are so adaptable to their environment, and they have multiple purposes including fiber, milk, and meat (Morand-Fehr *et al*., 2004).

Paratuberculosis (Johne's disease) is a chronic bacterial disease of global importance in mainly domestic and wild ruminants, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Windsor, 2014). It leads to a significant decrease in production, weight loss and ultimately death. The infection in small ruminants is considered to be of worldwide distribution, diagnosed in both sheep and goats (Okwumabua *et al*., 2010). The most common route of infection is faecal-oral. Infection can also be spread by intrauterine and transmammary transmission (Lambeth *et al*., 2004 and Verin *et al*., 2016). The intratonsillar route of infection may also play a role (Begg *et al*., 2005), although this is unlikely to be of significance in the natural infection process, mainly because the large

number of bacteria that are ingested and swallowed will heavily outweigh those that may become lodged in the tonsil. The profuse watery diarrhea is not a common feature of the disease in sheep and goats. In spite of weight loss, appetite is often good until the animal is near death. Diarrhea in sheep and goats with Johne's disease may be pasty, intermittent, present only terminally, or absent altogether. Thus, small ruminants seem to act as reservoir of bacteria for cattle and even for non-ruminant wildlife species like rodents, hares and foxes (Florou *et al*., 2008). The disease causes serious economic losses to the sheep farmers in the form of reduced body weight gain, culling and poor quality and quantity of wool (Singh *et al*., 2014).

The disease manifests as a granulomatous inflammation involving the intestinal mucosa and the mesenteric lymph nodes before involving other lymph nodes (Rathnaiah *et al*., 2017). It is a classic example of a protein-losing enteropathy, where the inflammation of the intestinal mucosa renders the absorptive epithelium incapable of adequate nutrient absorption while allowing the escape of fluid and nutrients through the faeces (Sweeney, 2011), leading to malabsorptive and secretory diarrhea, which manifests clinically as projectile diarrhea in cattle or loosely formed faeces in sheep and goats. The results of this are reduced weight gain, reduced milk, meat and wool production, emaciation and submandibular edema in the terminal stages (McAloon *et al*., 2016). Infected animals' faeces contaminate pastures, soil and water (Collins, 2003) followed by a prolonged persistence of the organism in the environment because of its tolerance to harsh environmental conditions (Lovell *et al*., 1944 and Dhand *et al*., 2009).

Ovine and caprine *paratuberculosis* involves chronic inflammatory lesions of the intestine and lymphoid organs, caused mostly by one or other of the 'S' (sheep), 'C' (cattle) or 'Bison type' strains of MAP (Windsor, 2014 and Kumar *et al*., 2010). Three distinct forms of *paratuberculosis* have been observed in sheep: multibacillary disease, paucibacillary disease and asymptomatic infection (Gillan *et al*., 2010). The multibacillary lesions of chronic granulomatous enteritis and

lymphadenitis (particularly involving the mesenteric lymph nodes) in *paratuberculosis* is characterized by accumulation of epithelioid macrophages containing numerous MAP in the lamina propria and submucosa of the intestine. The paucibacillary lesions are typically more lymphocytic in nature with MAP being far less numerous (Windsor, 2014). The enteric lesions typically develop within 6-12 months following initial detection of MAP infection and although some sheep may develop severe lesions within 12 months of infection, others progress from mild and paucibacillary to severe and multibacillary at variable rates, potentially fluctuating in severity or in the character of the inflammatory infiltrate over a period of years (Dennis *et al*., 2011).

The detection of infected animals is challenging because of the relatively poor performance of diagnostic tests in the early to mid-stage of infection (Windsor, 2015). The most definitive diagnostic test for ovine and caprine *paratuberculosis* is post-mortem evaluation with histopathological confirmation, seeking identification of characteristic anatomical and pathological changes of depletion of fat reserves, thickening of the bowel wall and enlargement of the gut associated lymphatics, including the presence of so-called 'lymphatic cords' on the serosal surface of the ileum and caecum (Windsor, 2014). For detection of subclinical and clinical *paratuberculosis*, various serological tests like complement fixation test (CFT), Agar gel immunodiffusion test (AGID) and Enzyme-linked immunosorbent assay (ELISA) have been widely used. For identifying sub clinically infected sheep AGID and ELISA are found to be more sensitive (Hilbink *et al*., 1994). The Sensitivity and specificity of ELISA using culture as a gold standard has been estimated to be 53.7 and 86.0%, respectively (Singh *et al*., 2008).

The zoonotic potential of MAP has been suggested based on the detection of MAP in the blood or mucosal tissues of the Crohn's disease patients and the similarities between *paratuberculosis* in ruminants with the Crohn's disease in humans (Sibartie *et al*., 2010). Public health issues have been raised about the transmission of MAP from animals to humans through animal products (dairy

foods, meat, and contaminated surface water) and the potential for subsequent infection and perhaps the disease (Collins, 2003). Milk and milk products are the main sources of the transmission of MAP to humans since MAP is not inactivated during pasteurization (Raguvanshi *et al*., 2010 and Singh *et al*., 2016).

The three main approaches to eradicate or reduce impacts of *paratuberculosis* in sheep or goat farms are to: introduce management changes to decrease transmission of MAP, apply test and cull practices to eliminate the sources of infection, and vaccinate replacement stock to increase their resistance to infection. Importantly, vaccination does not prevent infection, although it significantly reduces the occurrence of clinical cases plus organism excretion from infected animals.

There is plenty of literature on the epidemiology of MAP in tropical region of India (Singh *et al.,* 2014 and Singh *et al.,* 2008). However, knowledge on the prevalence of this disease in sheep and goats in Kashmir is limited (Bhat *et al*., 2020) both due to lack of testing and reporting. The agroclimatic conditions of Kashmir Valley are different from the tropical mainland of India and hence there are high chances of different epidemiology of the disease in this region. The estimation of sero-prevalence and risk factor analysis of MAP in Kashmir valley will set up a basis for establishing control measures against this disease. The culture of MAP is considered difficult as MAP has a very long incubation period. To overcome this limitation, ELISA kit (i-ELISA) was used to determine the sero-prevalence of MAP in the sheep and goats.

Keeping in view the above points, the study was undertaken with following objectives:

1. To study the sero-prevalence of *Mycobacterium avium* subsp. *paratuberculosis* in sheep and goats.
2. To study the risk factors of *Mycobacterium avium* subsp. *paratuberculosis* in sheep and goats.

Chapter-2

REVIEW OF LITERATURE

Paratuberculosis or Johne's disease is a disease with a long history. "Wasting or consumptive" disease was first reported in 1807 in cattle by Edward Skellet. H. A. Johne and L. Frothingham initially reported the disease in Germany in 1894. However, it was not until 1910 that F. W. Trowt successfully fulfilled Koch's postulates by growing *M. paratuberculosis* in the laboratory and reproducing the disease in experimentally infected cattle (Chiodini *et al.,* 1984 and Harding *et al.,* 1959). Bang (1906) confirmed the non tuberculous nature of the bacteria and named this disease as Jhone's disease, later designated as pseudotuberculosis.

2.1 Micro-organism

All mycobacteria species belong to the genus Mycobacterium within the family Mycobacteriace and the order Actinomycetales. The species *Mycobacterium avium* and *Mycobacterium intracellulare* are closely related together, they belong to a large cluster of genotypically and phenotypically related organisms called the *Mycobacterium avium*-intracellulare complex (Hashizume *et al*., 2012). MAP is an aerobic, non-motile acid fast bacterium. Members of the genus Mycobacterium have a lipid-rich, hydrophobic cell wall, which is substantially thicker than most other bacteria (Ray and Ryan, 2003).The thickness and fatty composition of the cell wall render mycobacteria impermeable to hydrophilic nutrients and resist heavy metals, disinfectants and antibiotics interaction(Jarlier and Nikaido, 1994). MAP is dependent on externally provided sources of mycobactin, an iron chelating molecule, for its growth; a characteristic that distinguishes most of its strains from other mycobacteria (Tortoli, 2003). It prefers an intracellular environment that is rich in iron, (Janagama *et al*., 2010) calcium and pyruvate (De Juan *et al*., 2006). It is believed to subsist in the environment in microfilms like other mycobacteria (Chern *et al*., 2015).

Microscopically, the organism is recognized in stained smears as small (0.5 × 1.5 μm), thin, and strongly acid fast bacilli which is usually found in clumps resulting from shedding parts of intestinal mucosa where the organism has multiplied. MAP grows in rough circular colonies reaching about 1-2 mm in diameter which are usually found to be off-white or yellow in color, depending on the culture medium (Rowe and Grant, 2006).

2.2 Host diversity

*Paratuberculosis*occurs worldwide and ruminants appear to be the preferred or natural host for MAP, primarily domestic ruminants such as cattle, sheep and goat, but the disease in wild ruminants is also well documented, including red deer (*Cervus elaphus*), roe-deer (*Capreolus capreolus*), fallow deer (*Dama dama*), white-tailed deer (*Odocoileus virginianus*), alpine ibex (*Capra ibex*) and riverine buffalo (*Bubalus bubalis*) (Chiodini *et al*., 1983; Ferroglio *et al*., 2000; Pavlik *et al*., 2000; Yadav *et al*., 2008; Sleeman *et al*., 2009 and Stevenson *et al*., 2019). In non-ruminant animals, MAP was first detected in wild rabbits (*Oryctolagus cuniculus*) in Scotland (Greig *et al*., 1997 and Greig *et al*., 1999). After this finding, the research for the presence of this pathogen was extended to other wildlife species, firstly in areas with previous history of *paratuberculosis* in livestock, which allowed the detection of MAP in a very broad host range, including brown bear (*Ursus arctos*), raccoon (*Procyon lotor*), opossum (*Didelphis virginiana*), coyote (*Canis latrans*), red fox (*Vulpes vulpes*), stoat (*Mustela erminea*), weasel (*Mustela nivalis*), wood mouse (*Apodemus sylvaticus*), European badger (*Meles meles*), hare (*Lepus europaeus*) and jackdaw (*Corvus monedula*) (Beard *et al*., 1999; Beard *et al*., 2001; Corn *et al*., 2005; Kopecna *et al*., 2006; Anderson *et al*., 2007 and Stevenson *et al*., 2009).

2.3 Transmission

The introduction of MAP into a population occurs mainly when an infected animal contaminates the pasture with faeces containing viable bacteria.

Normally, animals are most susceptible to infection before birth (prenatal infection) or soon after birth (postnatal infection). Prenatal infection descends from mothers to offspring through uterine and placental barriers (Lambeth *et al*., 2004), whereas the most common route is postnatal infection. The primary mode of infection of post-weaned animals is by ingestion of feed or water contaminated with faeces from infected shedders (Lombard, 2011). Animals exposed at an older age or exposed to a very small dose of bacteria at a young age are normally not likely to develop clinical disease until they are older than 2 years (Fecteau *et al*., 2010). Although the gastrointestinal tract is the primary site of infection, some studies demonstrated the organism outside the gastrointestinal tract. MAP has been successfully isolated from milk, colostrums, semen, and placenta of infected animals (Antognoli *et al*., 2008; Carvalho *et al*., 2009 and Munster *et al*., 2012). However, the commonness of disease transmission via these alternative routes is still unclear. Embryo transfer could be another possible means of transmission.

MAP escapes pasteurization temperature(Ellingson *et al*., 2005), milk of the infected animals is the most common source of transmission of MAP from animals to human beings (Ellingson *et al*., 2005 and Grant *et al*., 2003). MAP has been incriminated as the cause of Crohn's disease (CD) in human beings (Greenstein *et al*., 2003; Behr *et al*., 2008; Pierce *et al*., 2010 and Singh *et al*., 2012). Role of MAP in causation of CD has been supported by the frequent isolations of MAP from the CD patients as compared to other suspected and ulcerative colitis patients (Sech *et al*., 2005; Schwartz *et al*., 2000; Naser *et al*., 2004; Hermon *et al*., 2000 and Singh *et al*., 2008).

2.4 Prevalence

2.4.1 Workdone abroad

Lee *et al.* (2006) conducted a study on sera from 116 black goat herds by using ELISA kit to monitor the sero-prevalence of MAP and reported the apparent sero-prevalence for herds was 18.2 to 38.2 percent.

Coelho *et al*. (2007) screened 3900 samples of sheep older than 2 years belonging to 150 sheep flocks from 12 different territorial Livestock Farmers Organizations for the presence of antibodies against MAP using a commercial ELISA test and reported true prevalence of 6.4%.

Ahmed (2010) conducted study on 92 serum samples of local Awasi sheeps by using indirect Enzyme-Linked Immunosorbent Assay (i-ELISA) to detect antibodies against MAP. The reported results showed that 7.6% samples were positive for antibodies against MAP.

Anna *et al*. (2011) investigated 2086 adult female sheep from 38 herds by using commercial ELISA. They reported mean seroprevalence of 6.29% with a greater percentage of infected sheep among female at early/late than in peak lactation stage.

Liapi *et al*. (2011) screened the sera from 8011 non vaccinated animals (3429 sheep and 4582 goats) from 83 flocks, for MAP antibodies with a commercially available enzyme linked immunosorbent assay. They reported that the within-flock mean seroprevalence in sheep and goats was 9.9% and 7.9%, respectively and mean true prevalence of infected sheep and goats was 15.0 and 11.1%, respectively.

Pithua *et al*. (2012) screened sera from goats ≥24 months of age in 25 Missouri Boer goat herds for presence of MAP antibodies using a commercial ELISA kit.They reported true animal, within-herd, and between-herd prevalences were calculated using the Rogan-Gladen estimator and were 1.4% 3% and 54.7% respectively.

Buyuk *et al.* (2014) studied 450 sheep from twenty six sheep herds, non-vaccinated against MAP. The animal, within-herd, and between-herd apparent prevalences were calculated as 6.2%, 10.2% and 57.7%, respectively. The true animal, within-herd, and between-herd prevalences were 8.3%, 14.6% and 90%, respectively.

Penda *et al*. (2014) screened 383 samples comprising of 192 goat and 191 sheep for antibodies specific to MAP using the commercially indirect ELISA kit. Twenty one of 192 goat sera were positive; however none of 191 sheep sera screened were sero-positive for *paratuberculosis*. They reported the apparent and true prevalence of MAP in goats were 10.9% and 15.5% respectively and 0% and 0% respectively in sheep.

Bauman *et al*. (2016) conducted a study on blood and feces samples from 580 goats and 397 sheep that were randomly selected from 29 dairy goat herds and 21 convenience selected dairy sheep farms. They reported the Farm level prevalence of 83.0% percent for dairy goats and 66.8% for dairy sheep.

Celik and Thrutoglu (2017) studied 450 blood serum samples from 150 cattle, 150 sheep and 150 goats by using a commercial ELISA kit to investigate the presence of antibodies to MAP. The apparent and true seroprevalences based on the individual-animal, within-herd and between-herd were as 8 to 8.9%, 17.1 to 20.4%, 46.7 to 57.8% in cattle; 48 to 100%, 48 to 100%, 100 to 100% in sheep; and 24 to 35.9%, 25.7 to 38.6%, 93.3 to 100% in goats, respectively.

Mathevon *et al*. (2017) performed cross-sectional study on 1197 individual blood and fecal samples from 2 to 3 year-old sub-clinically infected ewes in 14 closed meat sheep flocks. Fecal excretion was determined using qPCR based on IS900 sequence detection, and serology was performed on serum samples using two commercial ELISA kits. They reported ELISA sensitivities were 17.4% and 17.9% with estimated specificities of 94.8% and 94.0%. Fecal qPCR demonstrated significantly higher sensitivity (47.5%) and specificity (99.0%) than the two ELISA tests.

Barrero *et al*. (2019) studied 3312 serum samples from 48 flocks of goats and observed the overall true seroprevalence was 22.54 per cent. They analysed significant association of MAP seropositivity with intensive production system,

lack of management by batches, inappropriate ventilation and seropositivity to caprine arthritis encephalitis virus.

Bauman *et al*. (2019) conducted studied 29 dairy goat herds and 21 dairy sheep flocks. The sensitivity of the BTM PCR was poor in both the dairy goat herds (0.0%) and dairy sheep flocks (25.0%), but exhibited 100% specificity in both the species. In goats, sensitivity ranged from 33.3 to 34.8% when fecal culture and PCR were the reference tests, respectively (specificities were both 100%), and 71.4 to 87.5% when the milk and serum ELISA, respectively, were the reference tests (specificities were 86.4 and 95.2%). The BTM modified ELISA in dairy sheep demonstrated comparable sensitivities, but lower specificities. When fecal culture and PCR were the reference tests, sensitivities were 50.0 and 46.7%, respectively (specificities were 77.8 and 83.3%). The sensitivities when the milk and serum ELISA were the reference tests were 87.5 and 72.7%, respectively (specificities were 92.3 and 100%).

Iarussi *et al*. (2019) screened 419 farms (16,903 sheep and 9369 goats) and reported true seroprevalence of 66.2% at flock level and 9.7% at the animal level. They also reported that the spread of infection occurs via several concomitant biological, managerial, and farmer-related factors.

Borujeni *et al*. (2020) studied blood samples from 530 cattle,568 sheep and 368 goats by using a commercial ELISA kit for detection of antibodies to MAP. They reported Apparent and true seroprevalence of MAP were 6.87% and 13.34% respectively in sheep, and 7.07% and 13.68% respectively in goats, 4.34% and 7.59%, respectively in cattle.

Shabana *et al*. (2020) screened 823 sera samples and 364 milk samples to determine the incidence of MAP using the indirect Enzyme-Linked Immunosorbent Assay and reported the seroprevalence of MAP was 11.1% in sheep and 13.8% in goats.

Hernández *et al*. (2021) screened 456 blood serum samples and at least one seropositive animal was found in 17 out of the 24 study flocks and, in total, 37 animals showed positive ELISA results (8%) also reported regarding MAP direct detection, 90 faecal pools from the 24 flocks were cultured and subjected to qPCR diagnosis. Both direct qPCR and culture detected 25 (27.7%) and 64 (71.1%) faecal pools as MAP positive, respectively.

2.4.2 Workdone in India

Singh *et al.* (2007) conducted study on 47 serum samples for comparing two MAP antigens (native S 5, 'Bison type' and commercial antigens 'Bovine'), for screening of kids against *paratuberculosis* infection by using plate ELISA test. They reported Breed-wise sero-prevalence was 10.5%, 7.6%, and nil in the Jakhrana, Sirohi, and Marwari male kids, respectively and none of the serum sample was found positive using commercial MAP 'Bovine' antigen.

Singh *et al.* (2008) reported the prevalence of MAP on the basis of tissue culture, tissue PCR and ELISA kit as 51.7, 37.9 and 46.5%, respectively. The Sensitivity and specificity of ELISA kit was 66.6 and 75.0% and 68.1 and 66.6% with tissue culture and PCR, respectively in goat herds.

Singh *et al*. (2010a) conducted a study on 829 serum samples belonging to domestic livestock (Cattle, buffaloes, goat and sheep) to estimate the seroprevalence of MAP using 'indigenous ELISA kit. They showed the seroprevalence of MAP in the domestic livestock was 23.1%. Prevalence was higher in large ruminants (24.1%) as compared to small ruminants (22.5%).

Singh *et al*. (2010b) studied serum, fecal, and blood samples of kids, young, and adult goats from farm and farmer's herds screened by ELISA, microscopy and culture. Of 111 goats (kids: 40, young: 14, adults: 57) screened, 77.5% were positive by blood PCR. Of 76 goats, 90.8% (kids: 87.5% and adults: 94.4%) were positive by PCR. From 21 kids and 14 young goats, 42.8 and 57.1% were positive. Of 21 fecal samples of kids examined by microscopy, 66.7% were

positive. In ELISA, 9.5 and 57.1% kids were positives as “type I”(S strain) and “type II” (C strain) reactors, respectively. Screening 14 young goats by culture of blood clots, 28.6% were positive.

Shah *et al*. (2012) conducted study on efficacy of three diagnostic tests viz., faecal smear examination (concentration method), rectal pinch examination and faecal polymerase chain reaction for IS-900 in 100 goats. The workers reported the prevalence of 34, 5 and 8% by faecal smear examination, rectal pinch examination and faecal PCR, respectively. Feacal smear examination appeared to be rapid, cheap and reliable test for screening both clinical and sub-clinical cases of *paratuberculosis* under field conditions.

Singh *et al*. (2013) screened 1750 faecal and 2057 serum samples, from 20 livestock (cattle, goat, sheep) farms for the bio-presence of MAP using faecal microscopy and indigenous ELISA technique and reported that 25.0% and 29.0% of the faecal and serum samples, respectively were positive for MAP infection.

Mukartal *et al*. (2016) conducted study on clinical samples (faeces-135, blood-45 and serum-100) from sheep located at Livestock Research and Information Centre (LRIC) farm and Dangur sheep, Indian breeding farm by using microscopy, ELISA and IS900 PCR. The overall prevalence of MAP was 54.7 and 16.0% in LRIC and Dangur farms, respectively.

Bhat *et al.* (2018) screened 288 faecal samples (260 sheep and 28 goats) from 23 small ruminant farms by using a multistage simple radom sampling technique. They reported 40 faecal samples positive on microscopy were subjected to DNA isolation and IS900 PCR to confirm the presence of MAP. Bio-prevalence of animals shedding acid fast bacilli (AFB), indistinguishable to MAP was 32.9%.

Biswal *et al*. (2018) conducted study on 22 serum goat samples by indirect ELISA and reported that the apparent prevalence of MAP as 68.19%.

Maity *et al*. (2018) conducted study on thirty nine serum samples from slaughtered sheep of the local slaughter places to evaluate the sero-prevalence of *paratuberculosis* in migratory Gaddi sheep of Himachal Pradesh using indigenous ELISA kit. They reported the seroprevalence of MAP to be 51.28%. It was also concluded that the indigenous ELISA was specific and sensitive as compared to AGID or acid-fast staining in detecting positive cases.

2.5 Risk factors

Kostoulas *et al.* (2006) conducted cross-sectional study in dairy-sheep and/or goat flocks to investigate the association of sub-clinical MAP infection with failing to produce a live offspring and the season of lambing/kidding. A commercial ELISA test kit was used to detect MAP antibodies in the collected serum. Random-effects logistic models were used to show the correlation between sheep and goat fertility and sub-clinical MAP. The relationship between subclinical MAP infections and live lambing/kidding is altered by parity. They concluded that when evaluating correlations between subclinical MAP infection and reproductive indices in sheep or goat flocks, parity distribution should be taken into consideration.

Dhand *et al*. (2009) reported a significant association between seroprevalence of ovine *paratuberculosis* with clay soils dedicated to agriculture (highest percentage of organic carbon, iron, and organic matter) compared with soils with a high proportion of sand and nitrogen.

Coelho *et al*. (2010) conducted a cross-sectional study on 3900 sheep from 150 flocks to evaluate the risk factors for MAP seroprevalence in sheep by using commercial ELISA. They identified a number of variables as risk factors for seropositivity like sheep pure local and/or a cross of a local breed, herd size with 31-60 head, culling during the Spring-Summer season, and the use of an anti-parasitic treatment such as Ivermectin as the only anti-parasitic medication multivariable logistic regression model.

Stau *et al*. (2012) studied serum samples from 1609 small ruminants for the presence of MAP antibodies using the commercial ELISA test kit. The intra-flock prevalence increased with the flock size, except for very large sheep flocks with more than 1000 ewes, but this correlation was not statistically significant. A low BCS correlated only in goats with a higher seroprevalence, not in sheep. No distinct correlation could be demonstrated between MAP sero status and age.

Angelidou *et al*. (2014) screened 1599 milk samples from Does that were in the last stage of lactation in 58 randomly selected dairy goat flocks by using commercial milk ELISA. They reported that does in flocks that used common water troughs and communal grazing grounds had 4.6 times higher odds of being MAP-infected compared to does in flocks that had no contact with other flocks. Does of flocks supplied with surface water had 3.7 times higher odds of being infected compared to those in flocks watered by underground and piped water sources. When kids were spending 10 h per day with their dams they had 2.6 times higher odds of being MAP infected compared to kids that were spending less than 10 h per day with dams. Further the flocks that continuously used the same anti-parasitic compound had 2.2 times higher odds of MAP infection compared to those flocks practising alternating anti-parasitic compounds.

Mejía *et al*. (2015) screened serum samples from 368 sheep older than 2 years in 38 herds by using an indirect ELISA assay and studied variables like breed (Pelibuey, Khatadhin, Dorper, Blackbelly) sex (male or female), parity number (1, 2 3-5 and >5 lambing), body condition score (3-5), and whether or not the animal was born in the herd but none of the factors were associated with the seropositivity to MAP.

Hernández *et al*. (2017) reported that relationship between serological status to MAP and individual variables could not be established since ELISA did not reveal any positive or suspicious animals among the 59 examined animals.

Rizwan *et al*. (2017) conducted study on 100 blood samples from slaughtered and marketed sheep (irrespective of breed, age and sex)and observed that the responsible risk factors for spreading the *paratuberculosis* can be malnutrition, poor sanitation, combine housing, open grazing and tick infestation.

Rerkyusuke *et al*. (2018) evaluated risk factors for the seroprevalence of MAP from 671 serum samples. They reported the seroprevalence of MAP was 12.82% and risk factors associated with the seroprevalence of MAP antibodies included age, sex, breed, and body condition score. In addition, when using goats younger than 1 year old as baseline, older goats were at higher risk than younger goats, 3 years old and over 5 years old. Both male and female goats and goats of different breeds were at similar risk of *paratuberculosis*.

Morales *et al*. (2020) conducted a study on 1178 individual sheep to detect antibodies against MAP by immunodiffusion in agar-gel. They reported true prevalence of MAP was 7.48% and 53.5% of flocks had at least one seropositive animal. An animal was more likely to be seropositive if it was from a large flock (> 300 animals) and was born outside the farm.

Bhat *et al*. (2020) conducted a study on 450 Kashmir Merino sheep and reported the apparent and true seroprevalence of MAP as 43.3% and 73.8%, respectively. They significant association of seropositivity with age, flock size, transhumance migration, grazing with cattle, encounter with wild animals, separation of diseased animals, on farm replacement of ewes and sanitary conditions of farm.

Khamassi *et al*. (2020) screened 338 female sheep from 15 small to middle-sized, extensively managed sheep farms examined clinically before blood sampling and tested sera for the presence of MAP antibodies using a commercial ELISA kit. They reported the seroprevalence was significantly lower in 5-year-old females and animals that do not graze.

Chapter-3

MATERIALS AND METHODS

3.1 Study area

The study was conducted on the serum samples collected from different small ruminants farms of Kashmir valley including government and private farms, mainly of Budgam and Ganderbal districts. Ganderbal is located at 34.23°N latitude and 74.78°E longitude. It has an average elevation of 1,619 metres (5,312 feet) above sea level. Budgam is located at 34.015°N latitude 74.722°E longitude with average elevation 1,610 m (5,280 ft) from sea level.

3.2 Place of work

The present study was carried out at Division of Clinical Veterinary Medicine, Ethics & Jurisprudence, Faculty of Veterinary Science and Animal Husbandry, Shuhama, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir.

3.3 Study design

The study was conducted from November 2020 to June 2021 utilising a multistage simple random method, with a single animal serving as the epidemiological unit of concern, to draw a sample from the small ruminant population. Samples were collected from sheep and goats irrespective of of age, sex and breed, along with complete history of farm and individual animal. The farms were selected conveniently but the animals at a farm were selected randomly. All the animals sampled in case the herd strength is≤15.In case of farms having 16-50 animals, every third animal sampled randomly. Every 4^{th} animal was sampled at farms having more than 50 animals. Indirect Enzyme Linked Immunosorbent Assay (i-ELISA) was used to evaluate the sero-prevalence of *paratuberculosis* in sheep and goats.

3.3.1 Sample Collection

Blood samples were aseptically collected from 292 animals (240 sheep and 52 goat) by jugular vein-puncture in clot activator vials. The blood samples were kept on ice immediately and kept away from sunlight until transported to the laboratory. Care was taken to avoid the shaking of samples during transportation to prevent the destruction of red blood cells and hemolysis. Serum was separated from clotted blood by centrifugation and stored at-20ºC till further use.

3.3.2 Enzyme Linked Immunosorbent Assay

The i-ELISA was used on serum samples for detecting antibodies against MAP. ELISA kits used were procured from Department of Biotechnology GLA University, Mathura, U.P. The ELISA Kit contained following materials:

1. Antigen Coated ELISA plates
2. O-Phenylene diamine Di-hydrochloride (OPD) tablets
3. Positive and Negative controls
4. Tween 20
5. Bovine Serum Albumin (BSA)
6. PBS (10X)
7. Hydrogen peroxide(H_2O_2)
8. Substrate buffer
9. Substrate solution
10. Serum dilution buffer
11. Conjugate: Anti-species horseradish peroxides conjugate

3.3.2.1 Preparation of reagents for ELISA Test

1. ELISA-1X PBS (pH-7.4)

Sodium chloride	8.0 g
Potassium chloride	0.200 g
Di-Sodium hydrogen phosphate (Na_2HPO_4)	1.445 g
Potassium di-hydrogen phosphate	0.200 g
TDW (Triple distilled water)	1000 mL

2. Carbonated buffer (Antigen coating buffer) (pH-9.6)

Sodium bi-carbonate	2.93 g
Sodium carbonate	1.59 g
TDW	100 mL

3. Washing buffer

ELISA-1X PBS	1000 mL
Tween-20 (0.05%)	0.5 mL

4. Blocking buffer

1X PBS (pH 7.4)	100 mL
3% Skimmed milk	3.0 g

5. Substrate buffer

a) Stock solution I

2.1% Citric acid	2.100 g
TDW	100 mL

b) Stock solution II

3.56% Di-Sodium Hydrogen Phosphate (Na_2HPO_4)	2.100 g
TDW	100 mL

6. Substrate solution (pH-5.0)

Ortho-phenylene diamine dihyro chloride (OPD)	5.0 mg
Stock I	4.95 mL
Stock II	4. 95 mL
H_2O_2 (30%)	100 µl

7. Test sample

Serum	5 µl
Serum dilution buffer	245 µl

8. Serum dilution buffer

1% BSA	1.0 g
1X PBST	100 mL

9 1X PBST (Phosphate Buffered Saline with Tween)

1X PBS	1000 mL
Tween-20 (0.05%)	0.5 mL

10. Conjugate: Anti-Species HRPO conjugate

For anti-Bovine and anti-Goat (1: 5000)

Conjugate	1.0 µl
1X PBS	5.0 mL

3.3.2.2 Preparation of working antigen:

Antigen stock concentration used:	4 mg/mL
Working antigen concentration used for 1 plate:	0.1 µg/well

(add 2.5 µl of stock MAP antigen in 10 mL of antigen coating buffer)

3.3.2.3 Procedure for ELISA Test

1. Coat 96 wells of ELISA plate with 0.1µg/well concentration of sonicated semi-purified protoplasmic antigen (sPPA).
2. Keep the plate for overnight at 4^{o}C. Wash the plate once with 1X PBST.
3. Block the wells of plate with 100 µl of 3% skimmed milk (in 1X PBS) and incubate at 37^{o}C or 1 hr. Washed the plate thrice with 1X PBST.
4. To the wells of the plate add 100 µl of 1:50 diluted test serum in duplicates (diluted using buffer containing 1X PBST with 1% BSA) and incubate for 2 hr at 37 ^{o}C. Positive and Negative controls were taken in first, second and third wells of plate respectively. Wash the plate thrice with 1X PBST.
5. Then add 100 µl of optimally diluted conjugate in ratio 1:8000 (anti-human) and 1:5000 (anti-goat and anti-bovine) in 1X PBS and incubate for 1 hr at 37 ^{o}C.
6. Washed the plate 4 times with 1X PBST.
7. Finally add 100 µl of freshly prepared substrate (OPD) at concentration of 5.0 mg per plate in substrate buffer (pH 5.0) and incubate (in dark) for 3-5 min at room temperature
8. Take the absorbance at 450 nm in ELISA reader without adding stop solution (5N H_2SO_4).
9. Record the results and be sure to run blank, positive and negative controls with the test serum in each plate.

Analysis of Absorbance OD Values

OD values are converted to S/P sample to positive (S/P) ratio as per Collins (2002), using following formula:

$$\frac{\text{OD at 450 nm of test serum} - \text{OD at 450 nm of negative control}}{\text{OD at 450 nm of positive control} - \text{OD at 450 nm of negative control}}$$

Table 1: Interpretation of results as per the ELISA Kit guidelines

S. No.	S/P Ratio	Status of Animal
1	0.00-.09	Negative
2	0.10-0.24	Borderline/Suspected
3	0.25-0.39	Low Positive
4	0.4-0.99	Positive
5	1.0-10.0	Strongly Positive

For the present study, only positive and strongly positive were taken as sero-positive animals.

3.4 Risk factors associated with occurrence of *paratuberculosis*

The study population comprised of flocks from different unorganized farms and government sheep farms of Ganderbal and Budgam district. Samples were taken at random from sheep and goats, regardless of age, gender, or weight, and were accompanied with a detailed history of the farm and each individual animal. Without prior knowledge of the flock's disease condition, samples were obtained.

For risk factor study data were collected through proper designed questionnaire (Table 2). The table questionnaire contained the following farming aspects: biological characteristics of the animals, structure of the flock, sanitary characteristics, management aspects, attitudinal and socioeconomic characteristics

Table-2: Questionnaire for risk factor analysis of *Mycobacterium avium* subsp. *paratuberculosis* in sheep and goats

	Name of owner			
	Address and phone No.			
	Species	Sheep/Goat		
1	Sex	Male/Female		
2	Age	<2yrs	2-4yrs	>4yrs
3	Breed			
4	Clinical Signs	Emaciated	Not-Emaciated	
5		Diarrhea	No Diarrhea	
6	Transhumance	Yes	No	
7	Flock size	30	30-50	>50
8	Quarantine practice	Yes	No	
9	Presence of other species at farm (cattle, equine, avian)	Yes	No	
10	Grazing with other animals species	Yes	No	
11	Breeding system	Open	Closed	
12	On farm replacement sheep/goats	Yes	No	
13	Feed supplementation	Yes	No	
14	Cleaning pens	Good	Poor	
15	contamination of water or feed with fecal materials	Yes	No	
16	Milk feeding to young ones	Yes	No	
17	Separation of diseased animals	Yes	No	
18	Treatment in suspected animals	Yes	No	
19	Encounter with wild animals (Bear/Kashmir stag etc.)	Yes	No	
20	Water source	Spring/stream/ lake	Well/water works	
21	Education level of owner	≥High school	≤High school	

of the farmer. Name and address of owner, species including, size of flock, sex, age, breed, transhumance, quarantine practice, presence of other species at farm (cattle, equine, avian), breeding system, livestock replacement, cleaning pens, contamination of water or feed with fecal materials, milk feeding to young ones, treatment in suspected animals, water source, encounter with wild animals (Bear/Kashmir stag etc),education level of owner were all filled out on the spot through face to face questionnaire based interviews.

3.5 Statistical analysis

SPSS version 20.0 software (IBM, USA) was used for statistical analysis. The association between the seroprevalence of MAP with potential risk factors was determined by chi-square test. A p-value of 0.05 or less was considered statistically significant for all the comparisons.

Chapter-4

EXPERIMENTAL FINDINGS

4.1 Seroprevalence of MAP

The present study was conducted on 292 animals (240 sheep and 52 goats) from Ganderbal and Budgam districts of Kashmir valley. The overall seroprevalence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was 47.26% (Table 3). The seroprevalence was significantly ($p<0.05$) higher in sheep (52.5%) than goats (23.07%). Further, sheep had 3.7 times higher odds of being MAP seropositive as compared to goats.

Table-3: Species-wise seroprevalence of MAP in sheep and goats

Animal	Positive	Negative	Total	Prevalence (%)	Odds	P-value	Odds ratio
Sheep	126	114	240	52.5	1.11	<0.05	1.11/0.3=3.7
Goats	12	40	52	23.07	0.3		
Total	**138**	**154**	**292**	**47.26**			

The seroprevalence of MAP did not differ significantly between male and female animals (Table 4). However, male animals had significantly higher odds (1.12 times) of developing MAP than the female animals.

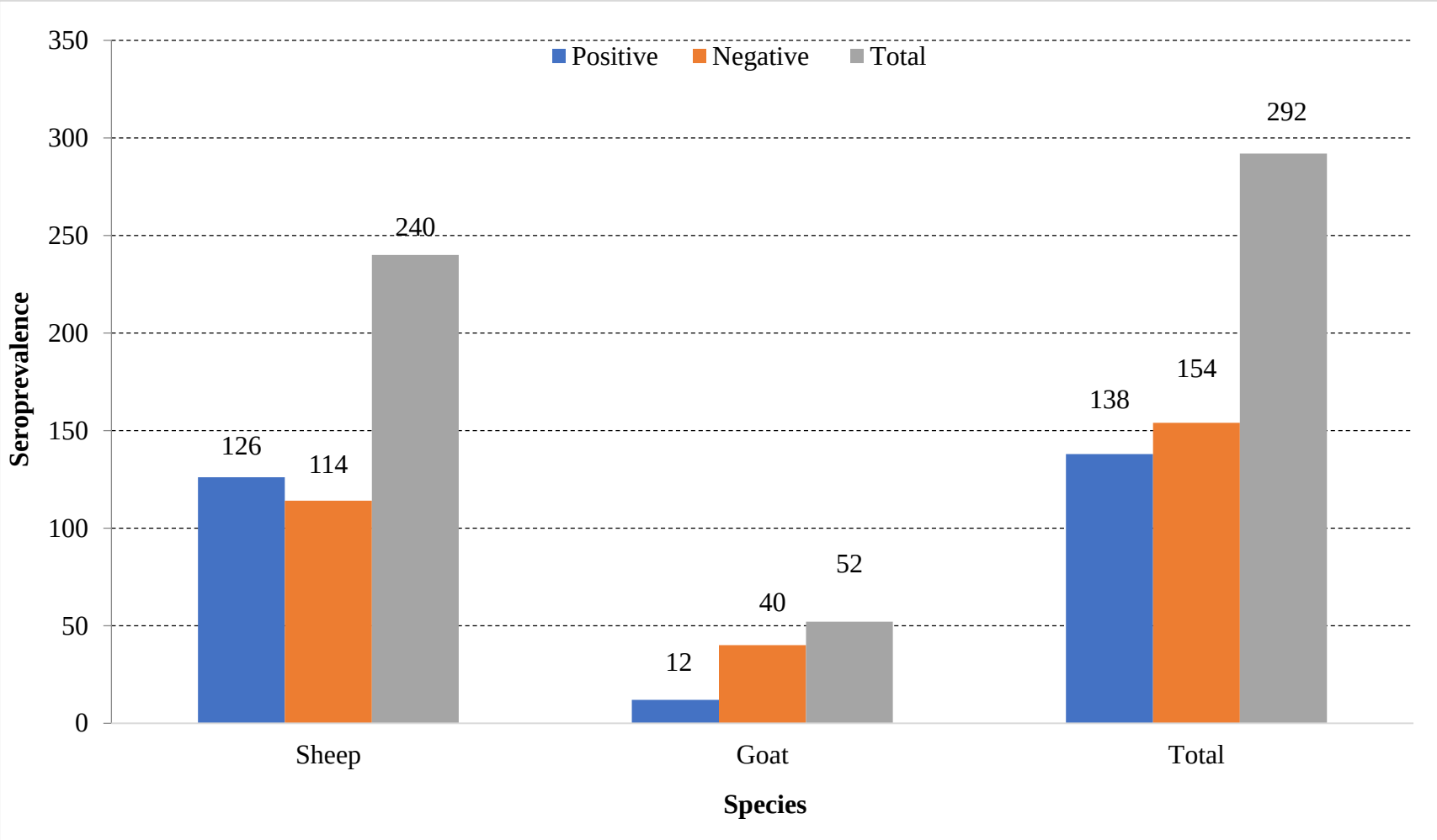

Fig. 1: Species-wise seroprevalence of MAP in sheep and goats

Table-4: Sex-wise seroprevalence of MAP in sheep and goats

Sex	Positive	Negative	Total	Prevalence (%)	Odds	Odds ratio
Male	54	56	110	49.09	0.96	0.96/0.85=1.12
Female	84	98	182	46.15	0.85	
Total	**138**	**154**	**292**	**47.26**		

On the basis of age the animals were divided into three groups as shown in Table 5. The seroprevalence of MAP did not differ significantly between the different age groups. The odds of MAP for the three age groups are present in table 5.Taking age group I as standard, the odds ratio for group I with respect to group II and group III were 1.33 and 1.25.

Table-5: Age-wise seroprevalence of MAP in sheep and goats

Age group	Positive	Negative	Total	Prevalence (%)	P value	Odds	Odds ratio
Group I (<2yrs)	35	32	67	52.2	>0.05	1.09	OR1=1.09/0.82 =1.33 OR2=1.09/0.87 =1.25
Group II (2-4yrs)	62	75	137	45.2		0.82	
Group III (>4yrs)	41	47	88	46.5		0.87	
Total	**138**	**154**	**292**				

Breed-wise seroprevalence of MAP in sheep and goats is presented in Table 6. The seroprevalence was 51.1 percent in Australian merino cross, 60.9 percent in Bakerwal, 44 percent in Corriedale, 33.3 percent in Fec-B, 60 percent in Kashmiri Merino, 50percent in Polled Cross, 25 percent in Polled Dorset and

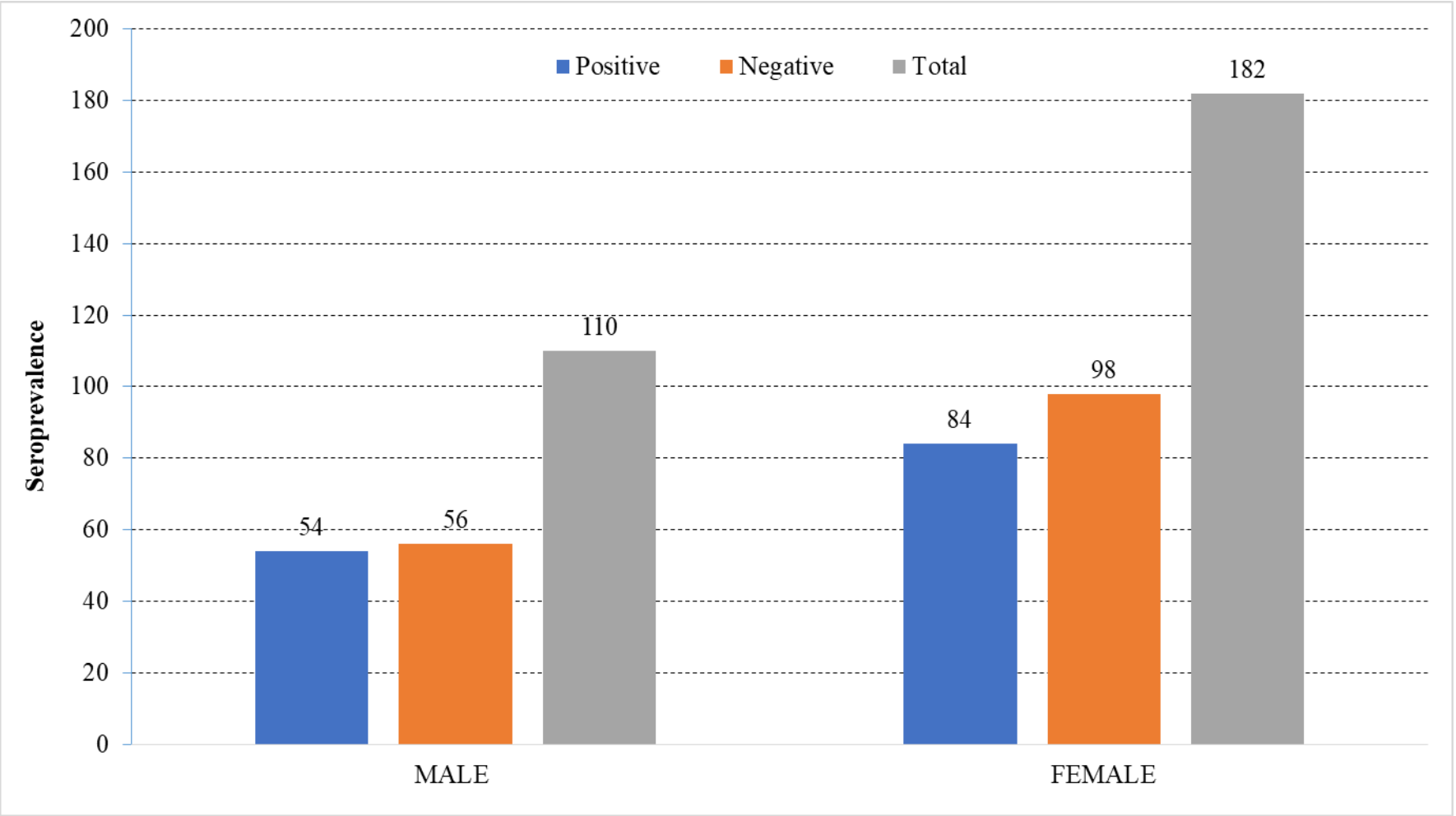

Fig. 2: Sex-wise seroprevalence of MAP in sheep and goats

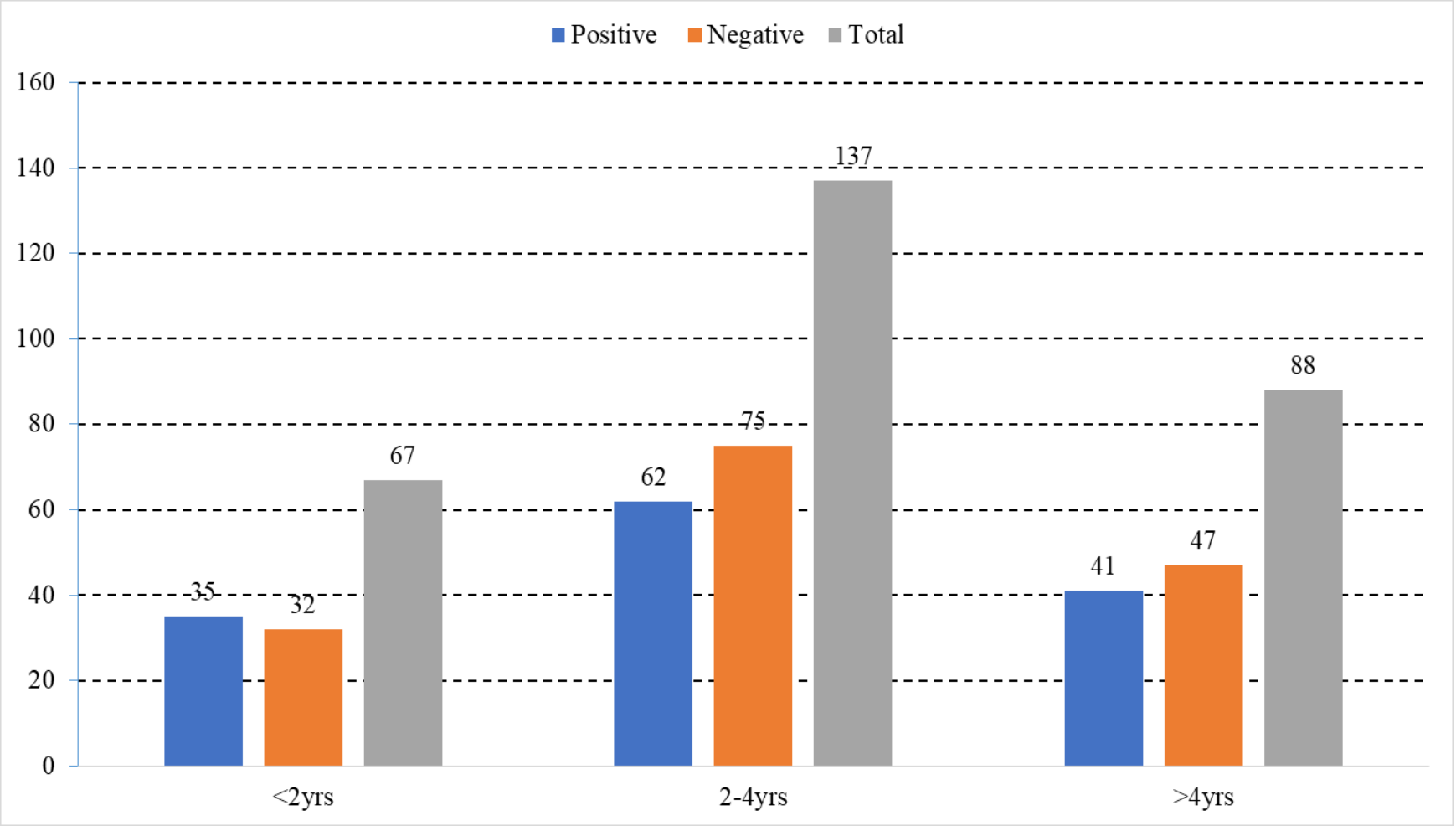

Fig. 3: Age-wise seroprevalence of MAP in sheep and goats

25 percent in South Down sheep, and in goats it was 23.8 percent in Bakerwal and 26.9 percent in Boer. None out of the five Pashmina goats tested seropositive for MAP. The seroprevalence did not differ significantly (P<0.05) between the different breeds of sheep or goats.

Table-6: Breed-wise seroprevalence of MAP in sheep and goats

Species	Breed	Positive	Negative	Total	Prevalence (%)	Odds	P Value
Sheep	Australian Merino Cross	35	33	68	51.1	1.06	>0.05
	Bakwerwal	25	16	41	60.9	1.56	
	Corriedale	11	14	25	44	0.78	
	Fec-B	3	6	9	33.3	0.5	
	Kashmiri Merino	43	28	71	60	1.53	
	Polled cross	5	5	10	50	1	
	Polled Dorset	2	6	8	25	0.33	
	South Down	2	6	8	25	0.33	
Goat	Bakerwal	5	16	21	23.8	0.31	>0.05
	Boer	7	19	26	26.9	0.36	
	Pashmina	0	5	5	0	0	

4.2 Risk factor associated with occurrence of MAP in sheep and goats

The relationship of various risk factors and seroprevalence of MAP in sheep and goats is shown in table 7-11.For risk factor analysis the data for sheep and goats is presented combinedly. The data is presented separately for sheep and goats if there existed a significant correlation between a risk factor and sheep/goats.

Flock size factor revealed significant ($p<0.05$) association between the seroprevalence of MAP with flock size (Table 7). Seroprevalence was higher in flocks with animal number less than 30.Smaller flocks (<30) had 2.17 times greater odds of developing MAP compared to flocks of >50 animals.

Transhumance factor was not significantly associated with the seroprevalence of MAP. The odds of MAP were 1.5 times higher if transhumance was absent than if it was present (Table 7). The quarantine practice was significantly ($p<0.05$) associated with seroprevalence of MAP (Table 7). Seroprevalence was higher in farms where quarantine practice was not followed. The flocks which did not adopt quarantine practice had 2.04 times higher odds of MAP than the flocks practicing the quarantine.

The presence of cattle at the farm and grazing with other animals species were significantly ($p<0.05$) associated with higher seroprevalence of MAP (Table 8). Seroprevalence was significantly higher if cattle were present at the farm. Further, the seroprevalence was significantly ($p<0.05$) higher, if sheep and goat were grazed with other animal species like cattle and horses.

Table-7: Association of flock size, transhumance and quarantine practice with occurrence of MAP in sheep and goats

		Positive	Negative	Total	Prevalence (%)	P Value	Odds	Odds ratio (OR)
Flock size	<30	58	40	98	59.1	<0.05	1.45	OR =1.4/0.7=2.07
	30-50	0	0	0	0		-	
	>50	80	114	194	41.2		0.70	
	Total	138	154	292	47.2			
Transhumance	Yes	114	135	249	47.7	>0.05	0.84	OR =1.26/0.84=1.5
	No	24	19	43	55.8		1.26	
Quarantine practice	Yes	95	126	221	42.9	<0.05	0.75	OR =1.53/.75=2.04
	No	43	28	71	60.5		1.53	

Table-8: Association of cattle at farm, grazing with other animals species and breeding system with occurrence of MAP in sheep and goats

Risk factor		Positive	Negative	Total	Prevalence (%)	P-value	Odds	Odds ratio
Presence of cattle at farm	Yes	83	73	156	53.2	<0.05	1.13	1.13/0.68=1.66
	No	55	81	136	40.4		0.68	
Grazing with other animal species	Yes	24	13	37	64.86	<0.05	1.85	1.85/.0.80=2.31
	No	114	141	255	44.7		0.80	
Breeding system	Open	46	32	78	58.9	<0.05	1.43	1.43/0.75=1.91
	Closed	92	122	214	42.9		0.75	

Breeding of ewes (open or closed) was significantly ($p<0.05$) associated with the seroprevalence MAP (Table 8). The farms practicing open breeding system had significantly higher seroprevalence than the farms with closed breeding system. Also open breeding farms had 1.91 times higher odds if having MAP than farms with closed breeding.

On overall basis the sign of diarrhea was not significantly associated with seroprevalence of MAP (Table 9). However, in goats the sign of diarrhea was significantly ($p<0.05$) associated with seroprevalence of MAP. Also the animals with signs of diarrhea had higher odds of disease than animals without sign of diarrhea.

Unlike other previous studies various risks were not associated with the seropositivity of MAP in the present study. These factors included signs of emaciation, on farm replacement, cleaning of pens, water resource, encounter with wild animals, contamination of feed and water, separation of diseased animals and treatment in animals with signs of emaciation and diarrhea (Table 9-11).

The education level of the owner was significantly ($p<0.05$) associated with the seroprevalence of MAP (Table 11).The seroprevalence was significantly ($P<0.05$) higher in farms where owners had education level upto high school than the farms with owners educated above high school level.

Table-9: Association of diarrhea and emaciation with occurrence of MAP in sheep and goats

	Diarrhea	Positive	Negative	Total	Prevalence (%)	P value	Odds	Odds ratio
Sheep	Yes	34	29	63	53.9	>0.05	1.17	1.17/1.08=1.08
	No	92	85	117	78.6		1.08	
Goats	Yes	5	6	11	45.4	<0.05	0.83	0.83/0.20=4.15
	No	7	34	41	17		0.20	
Total	Yes	39	35	74	52.7	>0.05	1.11	1.11/0.83=1.33
	No	99	119	218	45.4		0.83	
Emaciation	Yes	46	45	91	50.5	>0.05	1.02	1.02/0.84=1.21
	No	92	109	201	45.7		0.84	

Table-10: Association of on farm replacement, cleaning pens, water source and encounter with wild animals with occurrence of MAP in sheep and goats

Risk factor		Positive	Negative	Total	Prevalence (%)	P value	Odds	Odds ratio
On farm replacement	Yes	62	65	127	48.8	>0.05	0.95	0.95/0.85=1.11
	No	76	89	165	46		0.85	
Cleanings pens	Good	104	125	229	45.4	>0.05	0.83	1.17/0.83=1.40
	Poor	34	29	63	53.9		1.17	
Water source	Stream	110	133	243	45.2	>0.05	0.82	1.33/0.82=1.62
	Well	28	21	49	57.1		1.33	
Encounter with wild animals	Yes	106	122	228	46.4	>0.05	0.86	1/0.86=1.16
	No	32	32	64	50		1	

Table-11: Association of contamination of water or feed with fecal materials, separation of diseased animals, treatment in animals with signs of emaciation and education level of owner with occurrence of MAP in sheep and goats

Risk factor		Positive	Negative	Total	Prevalence (%)	P value	Odds	Odds ratio
Contamination of water or feed with fecal materials	Yes	80	74	154	51.9	>0.05	1.08	1.08/0.75=1.44
	No	58	80	138	42		0.72	
Separation of diseased animals	Yes	109	129	238	45.7	>0.05	0.84	1.53/.75=2.04
	No	29	25	54	53.7		1.16	
Treatment in animals with signs of emaciation and diarrhea	Yes	134	149	283	47.3	>0.05	0.89	0.89/0.8=1.11
	No	4	5	9	44.4		0.8	
Education level of owner	≤High school	68	58	126	53.9	<0.05	1.17	1.17/0.73=1.60
	≥ High school	70	96	166	42.1		0.73	

Chapter-5

DISCUSSION

Paratuberculosis is a worldwide distributed chronic granulomatous intestinal infection that affects wild and domestic ruminant species (Singh *et al*., 2010). The purpose of this study was to determine the seroprevalence of *Mycobacterium avium* subsp. *Paratuberculosis (*MAP) in the population of small ruminants. Another objective of this study was to identify the potential risk factors for MAP in small ruminants, which could be used to develop an effective MAP control programme in the future.

The identification of the pathogen by bacterial culture is the gold standard for diagnosing *paratuberculosis* (OIE, 2013 and Gilardoni *et al*., 2012). However, due to a lack of shedding in the early stages, sporadic shedding, or limitations in culture methods, this test does not provide an adequate identification of actually infected animals (Thrusfield, 2005). Nielsen (Nielsen, 2008) proposed that the presence of antibodies was a strong measure of MAP infection progression, particularly in non-shedding and transiently shedding animals.In the current study i-ELISA was used to catagorize the animals as seropositive or seronegative.The sample to positive ratio (S/P) for each sample was calculated and compared with the interpretation values provided by the manufacturer of the kit. Only positive and strongly positive animals were considered as positive in serum i-ELISA test.

5.1 Seroprevalence of MAP in sheep and goats

The study provides data about seroprevalence and risk factors of MAP in different breeds of sheep and goats from Ganderbal and Budgam districts of Kashmir valley. The seroprevalence of MAP in present study (47.26%) was higher as compared to other parts of India. In India seroprevalence of MAP in sheep is reported to be 16-45% (Singh *et al.*, 2010 and Dixit *et al*., 2013).Variable prevalence of MAP has been reported while taking into consideration different variables e.g. in a study on sheep and goats the apparent and true seroprevalences

based on the individual-animal, within-herd and between-herd have been reported to 8%-8.9%, 17.1%-20.4%, 46.7%-57.8% in cattle; 48%-100%, 48%-100%, 100%-100% in sheep; and 24%-35.9%, 25.7%-38.6%, 93.3%-100% in goats, respectively (Celik and Thrutoglu., 2017).There have been reports of disease in sheep and goats of Kashmir valley (Bhat *et al.,* 2018; Shah *et al*., 2012 and Mir *et al*., 2009). In Kashmir, Merino sheep, the apparent and true seroprevalence of MAP is reported be 43.3% and 73.8%, respectively (Bhat *et al.,* 2020)

Barrero *et al*. (2019) observed the overall true seroprevalence of 22.54 per cent in goat flocks. Singh *et al.* (2010) reported the seroprevalence of MAP in small ruminants was 22.5% by using 'indigenous ELISA. Lee *et al.* (2006) reported the apparent sero-prevalence for herds to a range from 18.2 to 38.2 percent in black goat herds. Our observations are in agreement with these seroprevalence reports. Maity *et al*. (2018) reported the seroprevalence of MAP to be 51.28% in migratory Gaddi sheep of Himachal Pradesh. Iarussi *et al*. (2019) reported true seroprevalence of 66.2% at flock and of 9.7% at the animal level.

There have been reports of low seroprevalence by many workers. Ahmed (2010) showed that 7.6% samples were positive for antibodies against MAP. Liapi *et al*. (2011) reported the within-flock mean seroprevalence in sheep and goats was 9.9% and 7.9%.Kostoulas *et al.* (2006) reported the true prevalence of sub-clinically infected animals in sheep and goats was 14.9% and 35.9%, respectively. Khamassi *et al*. (2020) showed 3.25% were seropositive to MAP in extensively managed sheep farms. Mejía *et al*. (2015) showed the apparent and true prevalences were 5.16% and 7.6%, respectively. The farm level average bio-load of MAP has been reported to be 23.0% in sheep of India (Singh *et al*., 2014). Borujeni *et al*. (2020) reported apparent and true seroprevalence of MAP were 6.87% and 13.34% respectively in sheep, and 7.07% and 13.68%, respectively in goats.

However, there have been reports of high prevalences by many workers. Bauman *et al*. (2016) reported the farm level prevalence of 83.0 percent for dairy

goats and 66.8% for dairy sheep. Singh *et al.* (2008) reported the prevalence of MAP on the basis of tissue culture, tissue PCR and ELISA kit as 51.7, 37.9 and 46.5%, respectively. In another study, Singh *et al.* (2013) reported the prevalence of MAP infection by microscopy in sheep (32.0%) and goats (21.6%) of eight Indian states by Ziehl Neelsen staining of faecal smears.

In present study, the seroprevalence of MAP was significantly higher in sheep than goats, and sheep had 3.7 times higher odds of developing MAP than goats.The prevalence of MAP in goats was higher than reported by Shah *et al.* (2012), who reported the prevalence of 34, 5 and 8% by faecal smear examination, rectal pinch examination and faecal PCR, respectively in goats of Kashmir. Shabana *et al*. (2020) reported that the seroprevalence of MAP was 11.1% in sheep and 13.8% in goats. Similar to our study, a lower seroprevalence rate of 19.6-31.8 percent and 30.9-50.0 percent has been reported in goats of western Rajasthan and Uttar Pradesh, respectively (Singh *et al.,* 2007). Goswami *et al*. (2000) has reported a very low seroprevalence of 13.5 per cent in an organized farm of Pashmina goats.

There was no significant association of MAP seroprevalence with sex of the animals sampled. This was in agreement to findings of Bhat *et al*. (2020).In our study there was no association between age of animals and seroprevalence of MAP.Different studies have reported varied seroprevalence in different age groups of sheep and goats. Singh *et al*. (2013) reported that the age-wise seroprevalence of Johne's disease in Mehsana goat flock was 0.0 percent in 0-3 months, 0.0 percent in 3-6 months, 22.0 percent in 6-12 months, 90.4 percent in >12 months.Bhat *et al*. (2020) reported significantly higher seroprevalence in sheep older than 3 years than sheep of <3years of age. Similar to our results,Singh *et al.* (2007) observed no association of seroprevalence of MAP with age in sheep. Further, the higher odds of disease in young animals was in agreement to earlier published findings (Khamassi *et al*., 2020 and Iarussi *et al*., 2019).

5.2 Risk factor associated with occurrence of MAP in sheep and goats

The epidemiology of MAP is complicated, with a long incubation period, the ability to infect and survive in multiple mammalian hosts, the ability to evade the host immune response, a latent period ranging from months to years, and longer survival in the environment. These characteristics, combined with the lack of good diagnostic tests, have impeded the eradication efforts, with only minimal results. In subclinically infected animals, diagnosis and control of disease is exceedingly difficult as the disease is spread before clinical indications appear (Sohal *et al*., 2007). Even now, some epidemiological elements of *paratuberculosis* have not been identified, and no country or region has succeeded in eradicating the disease worldwide (OIE, 2013).

To determine the disease's prevalence, many researchers evaluate and quantified management elements that may be associated with MAP. The majority of current Johne's disease control suggestions rely on management strategies aimed at limiting the introduction and spread of MAP. There is very inadequate data available on the risk factors for MAP in sheep and goats of Kashmir valley. A questionnaire was designed in the present study to evaluate individual risk factors associated with seroprevalence of MAP. The questionnaire included many risk factors which have been presented in table 2.

The significant association of smaller flock size with the seroprevalence of MAP was in contrast to findings of Bhat *et al.* (2020). However, similar to our study seroprevalence of MAP has been reported to be higher in small sized flocks as compared to large sized flocks in southern Italy (Iarussi *et al*., 2019). Higher stocking rate has been associated with increased prevalence of MAP infection in sheep (Dhand *et al*., 2007 and Attili *et al*., 2011). It is important to mention that in Kashmir valley the stocking density rate is usually higher in sheep/goats farms with less number of animals than the farms with higher number of animals. So we

suggest that rather than the flock size, the stocking rate is the actual risk factor for MAP in this study. Similar to our suggestion, Attili *et al*. (2011) reported that higher prevalence in large flocks was related to density dependent effects where housing provided inadequate space for large numbers of animals thus favoring the spread of contagious infections. Stock density, as a function of flock size, increases the exposure chance to infection in these situations.

With respect to transhumance, our results are in contrast to Bhat *et al*. (2020) who reported that transhumance of the small ruminants was significantly associated with the seroprevalence of MAP.A single animal (clinically or sub-clinically infected) is enough to contaminate a flock and spread MAP to other flocks in the absence of biosecurity precautions. As a result, animals entering a flock without being quarantined was significant risk factors for MAP seropositivity in the present study. Bauman *et al*. (2016) recommended implementation of strategic management practices like quarantine practices that should be applied for prevention and control of *paratuberculosis*. The results of present study are in agreement to previous published data in Kashmir valley (Bhat *et al.*, 2020).

Presence of cattle at farm was significantly associated with prevalence of MAP. Khamassi *et al*. (2020) also reported higher MAP infection in ovine mixed with cattle. Other workers have also observed a significant positive association between presences of other species with seroprevalence of MAP (Iarussi *et al*., 2019).In the present study, grazing of sheep and goat with other animals species was found to be significantly associated with seroprevalence of MAP. Bhat *et al*. (2020) reported that grazing with cattle was a risk factor significantly ($P<0.05$) association with seroprevalence of MAP ($\chi 2=4.04$; $P<0.05$; OR=7.2). However, Iarussi *et al*. (2019) reported that grazing with cattle was not significantly associated with the seroprevalence of MAP.

The implementation of biosecurity strategies like closed breeding on goat and dairy sheep farms are recommended to reduce plausible risk factors for MAP (Bauman *et al*., 2016).The present study also advocates the use of closed breeding system for prevention of MAP because the seroprevalence was significantly higher in farms practicing open breeding. The use of infected rams can poses a danger of MAP dissemination by horizontal sexual transmission (Buergelt *et al.*, 2004).Similar to our results, exchange or share of rams to the flock has been reported to be a critical risk factor associated with MAP seropositivity (Morales *et al*., 2020).

In the present study, milk feeding to young ones was practiced in all sampled animals, so association of this factor with the occurrence of MAP could not be established. However, this factor has been observed to be significantly associated with MAP in other study.

Diarrhea was significantly associated with the occurrence of MAP at the time of sampling in goats but not in sheep and overall basis. MAP infected sheep are reported to be silent, subclinical or clinical disease and same was observed in our study. The non-association of emaciation with seroprevalence of MAP indicated that most of the seropositive animals had not the clinical form of the disease, at the time of sampling.

Education level of owner was significantly associated with seroprevalence of MAP. In agreement to present findings, likelihood of seropositivity has been reported to increase by 2.26 if farmers were poorly educated (Iarussi *et al*., 2019).This risk factor has not been previously studied in sheep and goat of Kashmir valley. We suggest that, suitable strategies for effective technical training of farmers for control and spread of MAP are needed to address the biological, structural, and management shortcomings of semi-extended sheep and goat farms.

Non-significant association was observed between seroprevalence of MAP and on farm replacement, cleaning pens, contamination of water or feed with fecal materials, treatment in animals with signs of emaciation and diarrhea, water source, encounter with wild animals (Bear/Kashmir stag etc.). Among these factors, on farm replacement, sanitary condition of farm have been reported to be associated with seroprevalence of MAP (Bhat *et al*., 2020 and Attili *et al*., 2011). Similar to our results, wildlife has not been found to be associated with MAP infection in sheep of Australia (Dhand *et al*., 2007).

Chapter 6

SUMMARY AND CONCLUSIONS

The infectious, chronic and occasionally fatal infection of *paratuberculosis*, known also as John's disease, mostly affects ruminants' small intestine. Slowly progressive wasting and diarrhoea that are intermittent at initially but get more severe until consistently present are clinical indicators of *Paratuberculosis*. The affected animals usually die as a result from dehydration and acute cachexia.

In this study 292 animals (240 sheep and 52 goats) were screened for seroprevalence of MAP. The seroprevalence was estimated on the basis of antibody titer against MAP in serum. The blood samples were aseptically collected by jugular vein-puncture in clot activator vials. Serum i-ELISA identified serum samples as positive out of a total of 292 serum samples. The seroprevalence was significantly higher in sheep (52.5%) as compared to goats. The seroprevalence did not differ significantly between different age groups of sheep and goats. Further, the seroprevalence did not differ significantly ($P<0.05$) between male and female animals and various breeds of sheep and goats.

There is very inadequate data available on the risk factors for MAP in sheep and goats of Kashmir valley. A questionnaire was designed in the present study to evaluate individual risk factors associated with MAP. It was based on species, sex, age, breed, size of flock, transhumance, quarantine practice, presence of other species at farm (cattle, equine, avian), grazing with other animals species, breeding system, livestock replacement, cleaning pens, contamination of water or feed with fecal materials, milk feeding to young ones, treatment in animals with signs of emaciation and diarrhea, water source, encounter with wild animals (Bear/Kashmir stag etc.) and education level of the owner. Flock size, quarantine practice, presence of cattle at farm, grazing with other animals species, breeding system, education level of owner were significantly ($p<0.05$) associated with

occurrence of MAP in goats and sheep. The sign of diarrhea was associated with occurrence of MAP in goats only. Various risk factors were not significantly associated with the occurrence of MAP in sheep and goats, like transhumance, sign of emaciation, on farm replacement, cleaning pens, contamination of water or feed with fecal materials, separation of diseased animals, treatment in suspected animals, water sources. However, out of these non-significantly associated risk factors, various factors had odds of greater than 1.5. Milk feeding to the young ones could not be evaluated as risk factor for occurrence of MAP in the present study because this was practiced in all the sampled animals.

Conclusions

(1) The overall seroprevalence of MAP was significantly ($p < 0.05$) higher in sheep than goats.

(2) Sheep had 3.68 times higher odds of developing MAP than goats.

(3) The risk factors significantly ($p<0.05$) associated with seropositivity of MAP in sheep and goats were flock size, presence of cattle at the farm, breeding system, education level of the owner, quarantine practice and grazing with other animals species.

(4) The clinical sign of diarrhea was significantly ($p<0.05$) associated with occurrence of MAP in goats but not in sheep.

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CERTIFICATE

Certified that all the corrections/amendments as suggested by External Examiner **Dr D.K. Gupta, Associate Professor, Veterinary Medicine, COVS, GADVASU, Ludhiana, Punjab** during Viva-Voce examination held on 11-11-2021, have been incorporated in the manuscript entitled "**Studies on sero-prevalence and risk factors of *Mycobacterium avium* subsp. *paratuberculosis*** submitted by **Ishfaq Hussain Nengroo (MSV-2019-435).**

(Dr. Syed Ashaq Hussain)
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