SERO-PREVALENCE OF BRUCELLOSIS IN SMALL RUMINANTS

T H E S I S

Submitted

in the partial fulfillment of the requirements for the Degree of

MASTER OF VETERINARY SCIENCE

IN

VETERINARY EPIDEMIOLOGY AND PREVENTIVE MEDICINE

BY

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Enrolment No: V/12/072

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DECLARATION OF STUDENT

I hereby declare that the experimental research work and interpretation of thesis entitled **"SERO-PREVALENCE OF BRUCELLOSIS IN SMALL RUMINANTS"** or part thereof has not been submitted for any of the other degree or diploma of any university or scientific organization. The source of material used and all assistance received during the course of investigation have been duly acknowledged.

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Shri. **BAIT KAUSTUBH SURESH** has satisfactorily prosecuted his course for a period of not less than one semester and that the thesis entitled, **"SERO-PREVALENCE OF BRUCELLOSIS IN SMALL RUMINANTS"** submitted by him is the result of research work is sufficient to warrant its presentation to the examination in the subject of **VETERINARY EPIDEMIOLOGY AND PREVENTIVE MEDICINE** for the award of **MASTER OF VETERINARY SCIENCE** degree by the Maharashtra Animal and Fishery Sciences University, Nagpur.

We also certify that the thesis or part thereof has not been previously submitted by him for a degree of any other university.

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MY PARENTS, TEACHERS AND FRIENDS

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Place- Shirwal

Date - *(Bait Kaustubh Suresh)*

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LIST OF ABBREVATIONS

%	Per cent
p	pre-chosen probability
ºC	Degree Celsius
CFT	Complement fixation test
µg	Microgram
CBC	Complete Blood Count
PC	Positive control
GDP	Gross domestic product
dl	Decilitre
g	Gram
µl	Microliter
et al.	And others
Fig	Figure
Hb	Haemoglobin
i.e	That is
M	Molar
ƙ	kappa
mg	Milligram
ml	Millilitre
e.g.	Example
No.	Number
RBPT	Rose bengal plate test
pH	Power of hydrogen
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
TEC	Total Erythrocyte Count
TLC	Total Leukocyte Count
U.S.A.	United States of America
WBC	White Blood Cell

i-ELISA	Indirect linked immunosorbent assay
EDTA	Ethylene Diamine Tetra Acetic acid
SAT	Standard tube agglutination test
c-ELISA	Competitive enzyme linked immunosorbent assay
DLC	Differential Leukocyte Count
DNA	Deoxyribose Nucleic Acid
MRT	Milk ring test
MAT	Microscopic agglutination test
bp	Base pair
CI	Confidence interval
LGA	Local government areas
RNA	Ribonucleic acid
K Da	Kilo Dalton
LPS	Lipopolysaccharide
spp.	Species
HRP	Horseradish peroxidise
ZN	Ziehl-Neelsen
O.D.	Optical density
Nacl	Sodium chloride
KCL	Potassium chloride
pmol	Picomol
ATP	Adenosine triphosphate
CTP	Cytidine-5-triphosphate
GTP	Guanosine-5-triphosphate
UV	Ultraviolet
SE	Standard Error Mean
rpm	Revolutions per minute

INTRODUCTION

CHAPTER I

INTRODUCTION

Brucellosis is an infectious bacterial disease in small ruminants and having zoonotic importance but remains a neglected disease in India. The status of the brucellosis in India varies considerably from region to region. The risk of the disease is directly correlated with the changing farming practices in small ruminants. Migration of the flocks from one geographic area to other remains a major concern for the spreading of the disease in small ruminants.

India is a tropical agrarian country, with large number of the population belonging to the rural areas that primarily depends on agriculture and livestock production. In Indian economy, livestock contributed 16% to the income of small farm households as against an average of 14% for all rural households and also gives employment to about 8.8 % of the population in India (Dash, 2017). According to 19th Livestock census, 2012, livestock population of India is 512 million in which sheep population is 65.0 million which contributes around 12.68 percent and goat population is 135.17 million that contributes around 26.40 percent and of the total livestock population in India and ranks 3rd and 2nd in the world, respectively.

David Bruce in 1886 isolated a coccobacillus that he named *"Micrococcus melitensis"* from the spleen of a man who had died of "Malta Fever" (Bruce, 1887). The average annual incidence in Malta during 1901-1906 was 652 civilian and 605 military cases, with a death rate, respectively of 10.4% and 2.3% (Eyre, 1908). The human disease was primarily associated with people who consumed goat milk and had close contact with goats. The organism soon was isolated from these goats. In the year 1897 a similar microbe was isolated from the udder of cows, and in the year 1914 from swine. In about 1920 the genus was renamed *Brucella* and its species became *Brucella melitensis, Brucella abortus* and *Brucella suis,* respectively. The pathogens are not entirely species specific, e.g., Goat and sheep may be infected with

Brucella abortus. The disease has had various names, with "undulant fever" becoming predominant in the USA until the 1940s, when it began to be termed as Brucellosis. The disease has a limited geographical distribution, but remains a major crisis in the western Asia, Mediterranean region and parts of Latin America and Africa (Amato, 1995).

Brucella melitensis remains most widespread infection in sheep and goats and the incidence and prevalence of infection is high. Brucellosis is first recognized in India in the year 1942 moreover, it is endemic throughout the country. The disease occurs in all the species including sheep, goats, cattle, buffalo, camel, horses, pigs, yaks and humans. Brucellosis remains a significant public health problem in India and economic losses are considerable in an agrarian country such an India. Brucellosis in ruminants seems to be associated primarily with intensive farming practices in large organized dairy sectors. Risk behaviors such as unrestricted trade and movement of animals, use of fairs and local cattle yards for trading, sending dry animals back to rural communities for maintenance, use of semen from unscreened bulls for artificial insemination and poor farm hygiene possibly all responsible for the spread and transmission of the infection (Renukaradhya *et al.*, 2002).

Brucellosis is usually transmitted through contact with infected birthing fluids and tissues (e.g., vaginal discharges, aborted fetuses, placenta, fetal fluids) and also be spread through blood, milk, semen and urine of infected animals. Animals can get the bacteria by direct contact with mucous membranes (nose, eyes, and mouth), breaks in the skin or ingestion (oral). Brucellosis can also be transmitted by contaminated objects (fomites) such as, clothing, hay, feed, water, equipment and shoes. Some animals act as a carrier; they will have the bacteria but show no signs of illness. These animals can shed the bacteria into the environment for long periods of time, infecting other healthy animals in the herd. Abortion remains the common signs in sheep and goat during the late pregnancy period. A systemic signs like fever, depression, loss of weight may also occur in natural outbreaks in small ruminants and many times accompanied by mastitis, lameness and hygroma. As the organism is a

facultative intracellular parasite and the pathogenesis depends upon localization in lymph node, udder and uterus and an initial bacteremia.

Generally, diagnosis of brucellosis is based on clinical examination of the animals, isolation of the organism from infected individual but this is a cumbersome and time consuming task, because organisms grow slowly on primary isolation (Meyer, 1981). Moreover, it is not always practically possible to isolate Brucella every time even from infected individual, therefore, assessment of antibody response employing serological test play a key role in the routine diagnosis of brucellosis and supported where appropriate by bacteriological examination (Alton *et al.*, 1988). PCR is a quick and reliable diagnostic tool, as it is the most sensitive technique developed for the amplification of nucleic acid (Saiki *et al.*, 1988; Kramer and Coen, 2001). The high sensitivity of this technique has the advantage that it may lead to the earlier detection of the disease (Deacon and Lab, 1989; Gall and Nielsen, 2004).

Interests have been increasing to maintain brucellosis under control in endemic regions, because of the health and economic impact of brucellosis (Foulongne *et al.*, 2000). It is important to identify new endemic regions and to implement strict eradication programs beyond national boundaries. There is increasing reports on the incidence of brucellosis. Studies have revealed that in a few years to come, there is likely to be geometric increase in the incidence of brucellosis (Comerci *et al.*, 2001)

The small ruminants are considered as poor farmer's economic backbone. The study will focus on sero-prevalence of Brucellosis in and around Shirwal region of Maharashtra, which in turn will help the farmers to abide preventive measures to lower the incidence of brucellosis in their flocks. Moreover, this study will help Veterinarians to learn about the distribution pattern of the disease. The following study was carried out with the following objectives…

Objectives:

1. To study the sero-prevalence of brucellosis in and around Shirwal.
2. To confirm sero-positive cases by Polymerase chain reaction.
3. To study hematological alterations in small ruminant infected with brucellosis.

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Brucellosis disease is a major problem that is affecting small ruminants; infertility and abortions cause major losses in animal production. The early and accurate detection of brucellosis is essential so that efficacious interventions can be implemented.

2.1 Prevalence of brucellosis

Brucellosis occurs worldwide but is much controlled in developed countries due to routine screening of domestic animals and animal vaccination program. Clinical disease is still common in Middle East, Asia, Africa, South and Central America, the Mediterranean Basin and the Caribbean (Lopes *et al.*, 2010).

2.1.1 Global scenario

Hosie *et al.* (1985) collected sera from 538 Yemeni goats and 690 Yemeni sheep and screened for brucellosis by the Rose Bengal Plate Test (RBPT), reactors confirmed by the complement fixation test (CFT) and the serum agglutination test (SAT). The prevalence among goats was 0.4% and among sheep 0.6%. The prevalence among 183 imported goats and sheep was 4.4%.

Zowghi and Ebadi (1985) carried out a study on sero-prevalence of sheep and goats in Iran. Total 110817 serum samples were collected from different parts of Iran. The presence of Brucella antibodies was detected by RBPT, SAT and CFT. Prevalence was recorded as 14.7 per cent (16281) by different serological tests.

Reviriego *et al.* (2000) conducted a cross sectional study on Risk factors for ovine and caprine brucellosis in the Avila region (center of Spain) between 1996 and 1997. Sixteen (29%) flocks (3 caprine and 13 ovine) were brucellosis sero-positive. Overall, 0.7% of sheep and 0.1% of goats were sero-positive.

Al-Majali (2005) studied sero-prevalence of brucellosis and risk factors associated with this disease in Jorden. Serum samples were collected from 1100 goats randomly from 69 herds. The rose bengal plate test and the complement fixation test were used to detect antibodies against Brucella. The incidence of Brucella-specific abortion was investigated in goat herds located in the northern part of Jordan. Of the 1100 goats sampled, 305 (27.7%) had antibodies against Brucella. Thirty-seven herds (53.6%) had at least one Brucella sero-positive goat.

Samaha *et al.* (2008) collected milk and tissue samples from 813 sheep and 366 goats from production and breeding farms in various governorates in Egypt during 2007; the animals had no history of having been tested for brucellosis. Prevalence by RBPT, STAT and Rivanol test was 4.8% for sheep and 2.19% for goats.

Bertu *et al.* (2010) carried out sero-epidemiological study of brucellosis in small ruminants in Plateau state to determine the status of the disease. A total of 1347 serum samples from 851 goats and 496 sheep systematically collected from nine randomly selected Local Government Areas (LGA) of the state were tested for brucella antibodies using Rose Bengal plate test (RBPT) and serum agglutination test (SAT). The result revealed brucellosis prevalence of 14.5% in sheep and 16.1% in goats, respectively. The prevalence varied from one LGA to another and between sheep and goats in each LGA. In goats, the highest prevalence of 19.7% was recorded in Mangu LGA while the lowest (10.3%) was in Shendam LGA. In sheep, Quanpan LGA had the highest prevalence of 23.5% while Bassa LGA had the least prevalence of 6.3%.

Rahman *et al.* (2012b) conducted a cross-sectional study to determine the sero-prevalence of brucellosis in Black Bengal goats in Nilphamari Sadar and Kishoreganj upazillas of Nilphamari district of Bangladesh using Rose Bengal Test (RBT) as screening test and i-ELISA as confirmatory test. A total of 154 sera samples

from Black Bengal goats were collected from Nilphamari district. The overall sero-prevalence of brucellosis was found to be 2.59% in Black Bengal goats.

Gul *et al.* (2014) carried out study on sero-prevalence of Brucellosis in food animals in the Punjab, Pakistan. For this purpose, 1306 sheep and 282 goat serum samples were collected Samples were analyzed by using RBPT, STAT, i-ELISA and c-ELISA. Out of 1306 sheep samples 117 (8.95%), 28 (2.14), 23 (1.76), 25 (1.91) and in 282 goat samples 99 (35.10%), 27 (9.57%), 19 (6.73%), 19 (6.73%) positive by RBPT, STAT, i-ELISA and c- ELISA, respectively. Sero-prevalence was higher in mature animals as compared to the younger ones.

Kandeel *et al.* (2014) studied sero-prevalence of brucellosis in indigenous non-vaccinated sheep and goats of Saudi Arabia. Animals were screened for the sero-prevalence of brucellosis using the rose bengal test (RBT) and an indirect enzyme-linked immunosorbent assay (i-ELISA). The study was conducted from April to November 2013 and samples were collected from all the five administratively divided districts of Alkamil. The prevalence of brucellosis in sheep was more than in goats but the males and females were statistically equal in susceptibility to brucella infection. Both the RBT and i-ELISA were found to be sensitive enough for detection of antibodies of brucella while i-ELISA was more sensitive for the detection of infection.

Tsehay *et al.* (2014) conducted a cross-sectional study from November, 2013 to April, 2014 in pastoral areas of Oromia and Somali regional states to determine the prevalence of brucellosis in small ruminants and assess associated risk factors. A total of 420 serum samples were collected from 129 sheep and 291 goats in extensive management system, with no previous vaccination history. Out of 420 sera examined, 36 (8.5%) were positive to Rose Bengal plate test (RBPT). The sera screened positive by RBPT were retested using complement fixation test (CFT) and among 36 sera sample tested, 15 (3.6%) were positive for brucella antibodies. The prevalence of

brucellosis among sheep and goats was found to be 2 (0.48%) and 13 (3.09%), respectively.

Jackson *et al.* (2015) conducted a cross-sectional serological survey to find out prevalence of brucellosis in ruminants of Tajikistan. The study conducted in May 2003. Sera from 13,625 ruminants involving 3513 households in 172 kishlaks (villages) were collected and screened by the rose bengal test. Doubtful and positive results were further tested with competitive and indirect ELISAs. The overall serological prevalence were 5·8 per cent (5·2 to 6·4 per cent) for sheep, 5·5 per cent (5·0 to 6·0 per cent) for goats and 2·1 per cent (1·0 to 3·2 per cent) for cattle.

Zein *et al.* (2015) investigate the prevalence of the brucellosis in different animal species in the Northern State, Sudan. Blood samples collected over 6 Months in the period January 2011 to June 2011 form (7607) domesticated animals (2009 cattle, 2170 Sheep, 2303 goats and 1225 camels) were tested using RBPT and c-ELISA. Amongst live stocks 23.8% of cattle, 11.2% of sheep 16.3% of goats and 23.6% of camels were seropostive. The overall prevalence of brucellosis in the 7607 domesticated animals was found to be 17.9% in Northern State.

Mohamed *et al.* (2018) conducted cross-sectional study from April to July 2012 in Khartoum state, Sudan, to determine the sero-prevalence of brucellosis in goats and to investigate potential risk factors associated with this disease. A total of 307 serum samples were collected from both sexes of goats in four different localities and were subjected to testing for brucellosis using rose bengal plate test (RBPT), serum agglutination test (SAT), and competitive enzyme-linked immunosorbent assay (c-ELISA). The overall sero-prevalence was 11.4% (n=35) with a 95% confidence interval (CI) ranging from 7.80 to 15.0. Out of these 35 RBPT-positive samples, the positivity of 18 and 17 were confirmed by SAT and c-ELISA, respectively.

2.1.2 Indian scenario

Singh *et al.* (1994) studied occurrence of abortions, still births and sero-prevalence of brucellosis in native, unvaccinated and organised flocks of goats (Barbari, Jamunapari, Sirohi, Kutchi, Jakharana, and Marwari) and sheep (Muzaffarnagri) with abortion problems during the period from 1985 to 1990. In 6 years, 8.4% lambs and 13.4% kids were lost due to abortions and still births. Incidence of abortions was recorded as 12.3% and 6.8% and still births 1.0% and 1.6% in goats and sheep, respectively. Serologically, 0.6% goats and 3.6% sheep were found to be positive for brucellosis by standard tube agglutination test (SAT).

Ghosh and Nanda (1998) carried out study regarding the incidence of Brucellosis in goats of Tripura state. The samples were subjected to Milk Ring Test, Rose Bengal Plate Test and Standard Tube Agglutination Test. It was noticed that the percentage of positive reactors was 13.79 by MRT, 13.29 by RBPT and 6.95 by SAT.

Mrunalini and Ramasastry (1999) screened large number of serum samples from 7127 sheep and 2010 goats in a wide serosurvey in Andhra Pradesh. Prevalence of antibodies to Brucella was seen in (3.3%) in sheep and (7%) in goats by using plate and tube agglutination tests.

Hussain *et al.* (2017) conducted study on ovines of four administrative blocks of Ganderbal i.e., Lar, Ganderbal, Wakura and Kangan from May 2016 to July 2017. A multi-stage simple random sampling technique was used to determine sample size. The total serum sample size as calculated turned out to be 223 sheep. The Micro agglutination test (MAT) was performed in 96-well U-shaped microplates. The overall prevalence of ovine brucellosis was 35.87%. Prevalence was non-significantly higher in females (37.82%) having 1.99 times greater odds than males (23.33%). Age-wise prevalence was significantly higher in sheep >2 years of age. The sheep aged >2 years had 2.35 times greater odds (80.6/34.21) of having a higher prevalence of brucellosis than sheep aged < 2 years.

Saxena *et al.* (2017) investigated the frequency of brucellosis in goats in Ludhiana district of Punjab state of India. A total of 191 serum samples of goats from slaughter houses were analyzed with RBPT, STAT, MAT and ELISA. Out of these, 31 goats were positive for brucellosis by one or more of these tests. Among positives, 14 were detected by RBPT, 17 by STAT, 21 by MAT, and 21 by ELISA; 10 samples were positive and 160 negative by all methods. ELISA and MAT detected highest number of samples followed by STAT and RBPT. Frequency of brucellosis in goats in Ludhiana was found to be 5.23% by RBPT and ELISA.

Kanani *et al.* (2018) reported the sero-prevalence of brucellosis in small ruminants of Gujarat state, India, using Rose Bengal Plate test (RBPT) and indirect enzyme-linked immunosorbent assay (i-ELISA). A total of 2444 sera samples (675 sheep and 1769 goat) from unorganized sector and 1310 sera samples (861 sheep and 449 goat) from seven organized farms were collected for brucellosis screening. In unorganized sector, 23.70% sheep (160/675) and 15.99% goat (283/1769) were positive by RBPT and 24.44% sheep (165/675) and 17.24% goat (305/1769) by i-ELISA. The organized sector samples showed higher sero-prevalence in goat (7.79 %, 35/449) than sheep (4.06 %, 35/861) by RBPT. Similarly, in i-ELISA, goat samples showed a higher sero-prevalence (9.35%, 42/449) compared to sheep (7.50%, 65/861).

2.1.3 Area wise sero-prevalence

Negash *et al.* (2012) conducted a serological study on small ruminants to determine the prevalence of brucellosis in selected sites of Dire Dawa region, Eastern Ethiopia. Among the selected sites, sero-prevalence of small ruminant brucellosis was highest in sheep and goats sampled from Aseliso (11.11%) and lowest in that of Gedenser (6.84%).

Hussain *et al.* (2014) conducted survey to determine the prevalence of brucellosis in sheep and humans of district Kohat, Khyber Pakhtunkhwa. For this purpose, 100 blood samples from each humans and sheep (50 each from both sexes)

were randomly collected at three different tehsils (Lachi, Seni Gumbat and Kohat) of district Kohat. The serum samples were tested for presence of anti-Brucella antibodies by Rose Bengal Plate Test (RBPT) and Serum Agglutination Test (SAT). Moreover, a total of 50 milk samples were also collected from various sheep herds for subsequent conduction of milk ring test (MRT). The estimated prevalence of ovine brucellosis was 12.12%, 09% and 08.82% in Lachi, Seni Gumbat and Kohat, respectively, with an overall prevalence of 10% in the district.

Reddy *et al.* (2014) reported sero-prevalence of goats in Karnataka region. Total of 252 serum samples were collected subjected to serological tests, Indirect ELISA (i-ELISA) and Dot-ELISA to detect the Brucella antibodies. The sero-prevalence in goats was 9.52% by i-ELISA and 7.14% by Dot-ELISA. The prevalence of brucellosis was found to be highest among goats of northeast Karnataka followed by northwest Karnataka, central Karnataka and south Karnataka.

Ali *et al.* (2017) studied the sero-prevalence and risk factors of bovine brucellosis at animal and herd level using serological tests. The study was conducted in 253 randomly selected cattle herds of the Potohar plateau, Pakistan from which a total of 2709 serum (1462 cattle and 1247 buffaloes) samples were collected. 170 (6.3%) samples and 47 (18.6%) herds were sero-positive for brucellosis by Rose Bengal Plate test. Variations in sero-prevalence were observed across the different sampling sites. Highest Sero-prevalence is recorded in Islamabad Capital Territory as compare to Rawalpindi and Attock area.

2.1.4 Age wise sero-prevalence

Mittal *et al.* (2005) studied sero-prevalence of brucellosis in animals reared in Udham Singh Nagar of Uttaranchal. Total 339 blood samples of cattle (217), buffalo (67) goat and sheep (13) collected. RBPT, STAT and ELISA used to detect brucellosis. Maximum sero-prevalence observed in goats (39.21%) and minimum in buffaloes (29.85%). Female animals (38.24%) revealed more sero-positivity than male animals (22.50%). Age wise, animals of 4-6 years age (57.89) showed

maximum positive cases whereas animals of 0-2 years showed 23.44% sero-positivity.

Radostits *et al.* (2007a) states that sexually mature, pregnant animals were more susceptible to infection with the brucella organism than sexually immature animals.

Petersen *et al.* (2013) states that Erythritol is a four carbon sugar produced by the fetus preferentially consumed by *Brucella spp*. which leads to localization and subsequent accumulation of large amounts of bacteria to these sites, which ultimately leads to abortion.

Jarikre *et al.* (2015) carried out study to estimate the prevalence of brucellosis in sheep and goats in Northern, Ashanti and greater Accra region of Ghana. Tissues/swabs (319) and serum (370) samples were collected from sheep and goats comprising male and female West African Dwarf (WAD) and Sahelian breeds between 1 to 4 years of age in the regions. These were screened for brucellosis using Modified Ziehl Neelsen (MZN) staining method and the Rose Bengal Plate Test (RBPT). A sero-prevalence of 13.3% was recorded while 17.0% were positive with modified ZN staining. Adult animals of above two years had prevalence of 92.0% as compare to below two years old (8%).

Chahar *et al.* (2016) carried out study on evaluation of sero-prevalence of brucellosis in goats of Rajasthan. A total of 100 serum samples of goats were evaluated to determine the sero-prevalence of brucellosis. The brucella reactors were determined by using Brucheck Dot ELISA test. Overall sero-prevalence of brucellosis was 22%. The adult goats showed higher sero-prevalence (33.33%) as compared to goats below one year (12.72%) of age group.

Sharma *et al.* (2017) conducted study to determine the prevalence of brucellosis in goats in and around border areas of Jammu, India using different serological tests viz., RBPT, STAT and i-ELISA. A total of 350 serum samples from

goats were tested by RBPT, STAT and i-ELISA. Overall sero-prevalence of 1.14% was recorded in goats. Higher prevalence rates in 6-9 yr age group in goats (1.42%, 2.85% and 8.57%) were obtained by RBPT, STAT and i-ELISA, respectively.

2.1.5 Sex wise sero-prevalence

Rahman *et al.* (2012b) carried out a survey to investigate the sero-prevalence of brucellosis in sheep of selected areas (Gaibandha sadar and Gobindagonj upazilas) in the Gaibandha districts of Bangladesh. A total of 206 sera samples were collected from sheep and were tested for presence of Brucella specific antibody by Rose Bengal Plate Test (RBPT) as screening test and the RBPT positive samples were further confirmed using indirect Enzyme-linked immunosorbent assay (i-ELISA). The overall sero-prevalence of brucellosis in sheep was recorded as 3.39% in RBPT and 2.91% in i-ELISA. The prevalence of brucellosis in female sheep (3.41%) was higher than male (3.33%).

Pandeya *et al.* (2013) conducted cross-sectional study to find sero-prevalence of brucellosis in Kailali district of Nepal during a period from September, 2012 to January, 2013. A total of 233 animal blood samples (50 Cattle, 67 Buffalo and 116 Goat) were collected and tested for Brucella antibody by plate agglutination test (PAT). The sero-prevalence of Brucellosis was 12% (28/233). Thirty two percentage (16/50) of cattle, 13.4% (9/67) of buffaloes, and 2.6% (3/113) goats were sero-positive ($p<0.05$). Sero-prevalence was higher in females (14.6% vs. 10.6%) ($P>0.05$) and was higher in younger cattles and older buffalo and goats ($p>0.05$).

Suryawanshi *et al.* (2014) study the prevalence of brucellosis in sheep and goats, a total of 181 and 164 serum samples were collected from apparently healthy sheep and goats respectively of different age and sex from four districts of Maharashtra. All the samples were screened for *brucella* antibodies by employing RBPT test. An overall prevalence of 7.32% and 17.68% was observed in goats and sheep respectively. District wise prevalence indicated 15.00 % prevalence of brucellosis in goats in Akola district followed by Sangli (7.50%), Nagpur (5.00%)

and Nashik (2.30%) and 20.00% prevalence of brucellosis in sheep in Nashik district followed by Nagpur (19.50%), Akola (18.33%) and Sangli (12.50%). Age wise prevalence indicated 21.43% prevalence in sheep between 2-3years and 10.17% in goats above 3 years of age. Sex wise sero-prevalence indicated prevalence of 25.00% and 24.00% in male sheep and goats respectively and 17.16% and 4.32% in female sheep and goats respectively.

Nguna et al. (2019) carried out a cross-sectional study to find sero-prevalence of brucellosis in Kigulamo Parish, Iganga District. Human, cattle, and goat blood samples were collected and tested serologically using commercial indirect-ELISA kits manufactured by USDA, USA. Out of 451 human blood samples, 20 (4.4%) were positive, sex wise sero-prevalence in males was 6.4% (11/173) and in females 3.2% (9/278). Among 345 cattle blood samples, 4 (1.2%) were positive and among 351 goat blood samples, one (0.3%) was positive.

2.2 Enzyme linked immunosorbent assay (ELISA)

Teshale *et al.* (2006) collected sera from two sheep and goat rearing pastoral regions of Ethiopia, namely Afar and Somali, from November 2004 to April 2005. Total 2000 sheep and goats were tested by Indirect Enzyme Linked Immunosorbent Assay (i-ELISA). Out of the 2000 sera tested 9.7 % (n = 193) were positive to i-ELISA for brucellosis.

Naqshabandy *et al.* (2013) carried out work to detect the brucella antibodies in the serum of the aborted sheep and goats by indirect Enzyme-Linked Immunosorbent Assay (i-ELISA). The 82 blood samples (sheep 47 and goats 35) were collected from different flocks in Duhok governorate. Results showed 28 (34.14 %) serum samples were found positive for brucella antibodies by i-ELISA. Higher sero-positivity 17(36.17 %) was found in sheep than in goats 11 (31.42 %).

Sadhu *et al.* (2015) studied the sero-prevalence in small ruminants of Banaskantha district of North-Gujarat. Total 1000 serum samples comprising of 485

from sheep and 515 from goat tested for detection of antibodies against the Brucella species by Indirect Enzyme-linked immunosorbent assay (i-ELISA). The sero-prevalence of brucellosis in small ruminants was 8.80% by i- (ELISA).

Ahmed *et al.* (2016) carried out work for serodiagnosis of Brucellosis in Male Goats and Sheep in Baghdad, Iraq by using indirect Enzyme Linked Immunosorbent Assay (ELISA). Out of 41 sera samples (21 ram and 20 male goats) which were examined, antibodies against Brucella were detected in ram samples 16 (76.19%), whereas they were detected in 12 (60%) serum sample of goats males by i-ELISA respectively.

Alhamada *et. al.* (2017) studied brucella sero-positivity in Sheep and Goats in Duhok Province, Iraq. Sera from 432 small ruminants (335 sheep and 97 goats) from 72 farms collected. Serum samples were tested using indirect enzyme-linked immunosorbent assay (i-ELISA). Results showed that 31.7% of sheep and 34.0% of goats had antibodies against Brucella in the study area.

Padher *et al.* (2017) worked to ascertain the sero-prevalence of brucellosis in goats, sheep, and humans of Anand, districts of the Central Gujarat region. Indirect enzyme linked immunosorbent assay (i-ELISA) were employed for detecting the brucella and *Brucella melitensis* antibodies from animals and humans. A total 325 sera samples including 100 from goats, 100 from sheep, and 125 from human beings collected from the Anand district. Out of 325 sera samples tested, overall sero-prevalence was 93 (46.50%) while species wise incidence was found to be 55 (55.00%) and 38 (38.00%) among goats and sheep, respectively. Out of 93 (46.50%) sero-positive samples 65 (32.50%) samples did positive for *Brucella melitensis* comprise 46 goats and 19 sheep sera samples by i-ELISA. Sero-prevalence of *Brucella melitensis* among sheep was 19.00%while among goat was 46.00% by i-ELISA.

Abdalla *et al*. (2019) conducted survey in El-Gadarif state in the period from March 2016 to March 2017 to investigate the sero-prevalence of brucellosis in sheep.

A total number of 558 serum samples were collected from sheep form five localities. The collected samples were subjected to indirect enzyme linked Immunosorbent assay (i-ELISA). Among 558 sera samples of sheep 25 (4.5%) sera samples were positive by i-ELISA.

2.3 Polymerase chain reaction (PCR)

Baily *et al*. (1992) used suitable conditions and oligonucleotide primers for the detection of *Brucella melitensis* and *Brucella abortus* by the polymerase chain reaction. Primers were chosen from within the coding sequence of a gene encoding a 31 kDa *B. abortus* antigen. The test was shown to be sensitive, and specifically was demonstrated using DNA derived from a panel of Gram-negative pathogens. There was no detectable difference between *Brucella melitensis* and *Brucella abortus* in the sensitivity of the reaction or in the size of the amplification product.

Leal-klevezas *et al.* (1995) studied a versatile method for the extraction of Brucella DNA and PCR are presented as reliable tools for the detection of *Brucella spp.* from body fluids of infected animals. Two oligonucleotides homologous to regions of the gene encoding for an outer membrane protein (omp-2) were designed to detect the pathogen from milk and/or blood of infected goats, bovines, and human patients. The sensitivity of this test and its ability to detect the pathogen in samples from the field reveal a promising advance in the diagnosis of brucellosis in animals and humans.

Gupta *et al.* (2005) worked to overcome some of the limitations of conventional microbiological techniques in the diagnosis of brucellosis in goats, a simple single-step tissue and blood PCR, based on amplification of the 720-bp sequence of a gene coding for synthesis of an immunogenic outer membrane protein specific for *Brucella melitensis* (omp31), was evaluated on samples collected from 76 goats at time of slaughter. PCR results were compared with serology performed on

sera from the same animals. In controlled experiments, sensitivity and specificity of tissue PCR was found to be 86 and 100%, respectively. The sensitivity of blood PCR was slightly higher (93%), with a specificity of 100%. However, both serological tests exhibited a low level of sensitivity and specificity at 71 and 86%, respectively. Since this PCR assay can be performed in 1 day, is very reproducible, easily standardized and avoids the risk of infection in laboratory workers, it will complement serological testing and be a practical and reliable tool for diagnosis of brucellosis in goats.

Imaoka *et al.* (2007) developed a combinatorial polymerase chain reaction (PCR) procedure to identify four major species of the *Brucella genus* simultaneously. Four pairs of primers targeting the genes encoding a cell surface protein (bcsp31) and outer membrane proteins (omp2b, omp2a and omp31) were prepared. PCR using these primers gave rise to specific patterns of amplification for each *Brucella spp.* examined in this study. *Brucella abortus* could be identified when fragments of bcsp31 and omp2b/2a were amplified by *Brucella abortus*-specific primers. *Brucella melitensis* could be identified by the amplification of fragments of bcsp31, omp2b/2a and omp31 using pair of primers B4/B5, JRF/JPR-ab and omp31.

Mukherjee *et al.* (2007) conducted study on multiple genus-specific markers in PCR assays improve the specificity and sensitivity of diagnosis of brucellosis in field animals. Brucella-specific nucleotide sequences encoding the bcsp 31 kDa protein, Omp2 and the 16S rRNA were employed in three independent diagnostic PCR assays. The antibody-detecting ELISA results of field samples (n=87) from a serologically positive herd in India were compared separately with omp2 and bcsp PCRs of blood (n=62). While the bcsp PCR was the most sensitive, the degree of association of ELISA with omp2 blood PCR (ƙ=0.37 at $P<0.05$) was similar to that with the bcsp blood PCR (ƙ=0.34 at $P<0.05$). An improvement in the correlation between ELISA and blood PCR was noticed (ƙ=0.5 at $P<0.05$) when a consensus result of omp2 and bcsp blood PCR was considered for comparison with ELISA. The use of more than one marker-based PCR gave increased sensitivity and higher

specificity and appears to be a more reliable molecular diagnostic approach for screening of field animals.

El Kholy *et al.* (2009) carried out study to establish an accurate and sensitive polymerase chain reaction (PCR) technique for the diagnosis of active human brucellosis in Egypt. DNA extraction kit designed in-house using 2 sets of primers [B4/B5 (223 bp) and JPF/JPR (193 bp)]. The technique showed high sensitivity, specificity and accuracy. Of the 50 blood samples from the patients, 35 showed confirmation for Brucellosis.

Ling Yu and Nielsen (2010) conducted study on polymerase chain reaction (PCR) using methods like single-pair primers, multiplex primers, real-time PCRs, PCRs for marine Brucella, and PCRs for molecular biotyping. They found that these methods are becoming very important tools for the identification of Brucella, at the species level and recently also at the biovar level. These techniques require minimum biological containment and can provide results in a very short time. In addition, genetic fingerprinting of isolates, aid in the epidemiological studies of the disease and its control. PCR-based methods are more useful and practical than conventional methods used to identify *Brucella spp.*

Garshasbi *et al.* (2014) conducted study to identify *Brucella spp*. in sera of high risk individuals by a polymerase chain reaction (PCR)-based method. A total of 180 patients suspected to have Brucellosis were examined by serological tests. To establish a PCR protocol for diagnosis of active brucellosis, DNA was extracted from the serum samples by using a commercial kit. PCR amplification was done for detection of Brucella DNA using bcsp31 target gene and IS711 locus. The PCR assay showed that an amplicon of 223 bp was obtained in 73.8% (133/180) of the tested sera using primers (B4/B5) derived from a gene encoding the 31-kDa *Brucella abortus* antigen. In another PCR, an amplicon of 498 bp was obtained in 63.8% (115/180) of the samples using *Brucella abortus*-specific primers derived from a

locus adjacent to the 3'-end of IS711, and also an amplicon of 731 bp was produced in 4.4% (8/180) of the tested samples using *Brucella melitensis*-specific primers.

Ali *et al.* (2015) carried out study to determine the sero-prevalence and identify the causative agent of brucellosis in small ruminants in Pakistan. A total of 278 serum samples were collected from sheep and goats that had close contact with sero-positive bovine herds. Serum samples were initially screened using Rose Bengal plate test (RBPT). Sero-positive samples were subjected to bacterial isolation and PCR analysis using Brucella genus-specific (bcsp31) and Brucella species-specific (IS711 for Brucella abortus and Brucella melitensis) quantitative real-time polymerase chain reactions (qRT-PCR). Twenty-four (8.6%) serum samples were positive by RBPT. No Brucella isolates were obtained from the examined blood. Of the 24 sero-positive serum samples, 18 (75%) were positive in the Brucella genus-specific (bcsp31) and Brucella abortus-specific (IS711) qRT-PCR, respectively.

Saraswathy *et al.* (2015) conducted study for evaluation of clinical samples by various serological, biochemical and Polymerase Chain Reaction techniques for brucellosis. For the study 24 whole blood samples were collected from cattle. Blood DNA was isolated from these samples and tested for the detection of IS711 gene and B4/B5 gene by polymerase Chain Reaction for Brucellosis, 15 DNA samples (62.5%) were detected as positive by both gene primers.

Gujrathi (2016) studied sero-prevalence and diagnosis of brucellosis in cattle. Blood and serum samples were collected from 168 cattle from Shirwal, Pune and Nashik region of Maharashtra state (India). Out of 168 cattle studied 33 (19.64%) cattle were positive for i-ELISA and blood DNA was isolated from these sero-positive animals amongst them, 8 (4.7%) samples showed positive results at 223 bp using B4/B5 primers derived from a gene encoding the 31-kDa *Brucella abortus* antigen.

Sonekar *et al.* (2018) studied the prevalence of brucellosis among sheep flock having history of abortions. A total of 229 samples comprising of 157 blood and 72

clinical samples (vaginal swabs) were collected from 157 animals. Clinical samples were processed for the isolation of *Brucella melitensis*. Serum samples (n = 157) were tested by Rose Bengal plate test (RBPT) and i-ELISA. A total of 68 (43.31%) and 104 (66.24%) samples were positive by RBPT and ELISA, respectively. Brucella isolates (n = 2) were recovered from clinical samples. Both isolates demonstrated amplification for bcsp31 and IS711 genes. On AMOS PCR, both the isolates amplified at 731 bp, i.e., belongs to *Brucella melitensis* species.

Parthiban *et al.* (2019) conducted study on Serum based screening and molecular detection of brucellosis in ruminants. This study was undertaken in 238 samples (112 serum samples from the sheep, 82 serum samples from the goat and 44 serum samples from the cattle) suspected of brucellosis collected from the southern districts of Tamil Nadu. All the 238 samples were screened for the brucellosis by carrying out rose bengal plate agglutination test (RBPT). The sero-positive serum samples were further subjected to Brucella cell surface salt extractable protein 31 (bcsp 31) gene-based PCR for Brucella genus confirmation. The study revealed that 8.92% (n = 10) serum samples from sheep, 9.75% (n = 8) serum samples from goat and 6.81% (n = 3) serum samples from cattle were sero-positive for brucellosis by RBPT. All the twenty one sero-positive samples produced specific amplicon of 223 bp by bcsp 31-PCR confirms brucellosis.

2.4 Hematological alterations

Crosby *et al.* (1984) studied hematologic abnormalities in 38 patients with brucellosis. Anaemia was found in 74% of patients, hematocrit was <30% (range, 20-45%;mean, 31%) in 64% patients, leucopoenia in 45%, neutropenia in 21%, lymphopenia in 63%, and thrombocytopenia in 39.5%. Eight patients (21%) were pancyto-penic; seven of these individuals also had splenomegaly. Bleeding complications developed in 26% of patients and were significantly associated with

clotting abnormalities (low platelet count, low fibrinogen level, and/or prolongation of thrombin clotting time).

Galanakis *et al.* (1996) observed fifty-two cases of child-hood brucellosis which occurred in north-western Greece during the 15- year period 1979-1993. A haematological study shows that anemia, leukopenia, neutropenia, lymphocytopenia, monocytosis, eosinophilia, thrombocytopenia and pancytopenia found in brucella affected childrens.

Chahotal *et al.* (2003) investigated outbreak of brucellosis in an organized dairy farm in Himachal pradesh, India. Serological study, employing rose bengal plate test (RBPT) and serum agglutination test (SAT), revealed involvement of both B. abortus and B. melitensis in all affected cows. The haematological study revealed severe monocytosis averaging 33% (ranging from 16 to 46%) and lymphocytosis averaging 32.8% (ranging from 18 to 43%) in all animals. However, neutrophils were 26.6%, ranging from 18 to 36% in the infected animals.

Soudbakhsh *et al.* (2007) investigated 85 patients with brucellosis in Imam Khomeini Hospital during 1997-2002 to determine the hematological changes during the active course of brucella infection. Anemia was detected in 43.5% patients, leukopenia in 13.6%, and thrombocytopenia in 12.5% and pancytopenia in 2.4% of patients. Blood picture of brucella affected patient reveals anemia, leukopenia, thrombocytopenia or pancytopenia.

Sikder *et al.* (2012) carried out a haematological study to determine the considerable variations in blood parameters for brucellosis sero-positivity in commercial dairy cattle in the Chittagong region of Bangladesh from January to May 2012. The study population comprised of 250 commercial cross-breed dairy cattle. Milk Ring Test (MRT) positive 50 cows were subjected to blood collection for haematological and serological tests. After separation of sera, two serological tests specifically indirect Enzyme Linked Immuno Sorbent Assay (iELISA) and Rose Bengal Plate Test (RBPT) were done for confirmation. The results revealed that no

significant variations were found among the parameters ($p<0.05$). However, slightly increased values of TLC, monocytes, eosinophil, MCV and MCH were recorded in the positive group. In addition, a little decline in the values of TEC, and neutrophil were found in the same group. The values of Hb, PCV, ESR, lymphocytes, basophils and MCHC were remained unchanged.

Gul *et al.* (2013) conducted study to determine the sero-prevalence and hemato-biochemical manifestations of brucellosis in horses at the periphery of Faisalabad city. It was found that values of erythrocyte sedimentation rate, neutrophil, basophil and alkaline phosphatase significantly decreased in brucellosis positive animals as compared to healthy animals whereas lymphocytes and alanine aminotransferase were in opposite order.

Kushwaha *et al.* (2014) reported hematological alterations in 27 cattle which showed antibodies both in serum and milk as well as Brucella DNA in milk were selected for the study. Hb, TEC, TLC, neutrophils, eosinophils, lymphocytes, monocytes count, AST, ALT and SD values were lower whereas, PCV was higher in Brucella infected cattle.

Bozdemir *et al.* (2017) studied brucella patients with various non-specific clinical symptoms, such as fever, fatigue, sweating, joint pain, arthritis, myalgia and headache. Due to the non-specificity of clinical signs and symptoms observed, they evaluated the mean platelet volume (MPV), neutrophil to lymphocyte ratio (NLR), and platelet to lymphocyte ratio (PLR) to support diagnosis. In their study, they observed that the patients affected with brucella have increased total leukocyte count, neutrophil count and decreased lymphocyte count, which is a diagnostic marker of bacterial infection.

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

The present study was conducted to study the sero-prevalence of brucellosis in sheep and goats. Investigations comprised of evaluation of i-ELISA and PCR in diagnosis of brucellosis in Small ruminants, and hematological alterations in small ruminant infected with brucellosis.

3.2 Place of research work

Present research work was carried out in the Department of Veterinary Epidemiology and Preventive Medicine, Department of Veterinary Pathology and Central Instrumentation Facility of Krantisinh Nana Patil College of Veterinary Science, Shirwal, District-Satara.

3.3 General material

3.3.1 Glassware and plastic ware

All the glassware and plastic ware used during present study were obtained from Borosil Ltd, Mumbai (India), HiMedia Pvt Ltd, Thermofisher Scientific (India). All the glassware and plastic were properly cleaned, dried and sterilized prior to use for the research work.

3.3.2 Chemicals, Buffers and Reagents

All the chemicals, buffers used during course of study were standard and of molecular grade which were obtain from HiMedia Pvt Ltd.

3.3.3 Equipments

All required scientific instruments and equipments like ELISA Kit, ELISA Plate washer, Elisa reader, Micropipettes, Incubator, PCR machine, Gel electrophoresis, Weighing balance and Water bath, were available in Department of Veterinary Epidemiology and Preventive Medicine, Department of Veterinary Pathology and Central Instrumentation Facility of Krantisinha Nana Patil College of Veterinary Science, Shirwal and were used for carrying out the present research study.

3.4 Methods

3.4.1 Collection of blood and serum samples

For the present study, a total of 300 animals included from the flocks of small ruminants having history of abortion, retention of placenta, metritis and swelling of the testicles from Shirwal, Dahiwadi and Baramati region. Serum samples were collected in clot activator vials in morning hours and transported to the laboratory under chilling conditions. Collected serum samples were subjected to Indirect Enzyme linked Immunosorbent Assay (i-ELISA) on the same day and results were calculated. Blood samples were collected from the animals which were found sero-positive by i-ELISA in K3-EDTA vials and transported to the laboratory under chilling conditions. This blood samples were subjected for haematologial studies and DNA was isolated for Polymerase Chain Reaction (PCR) using B4/B5 primers. The serum and blood samples were stored at -20^0C till further use. Data related to age, sex, location were collected on sampling day.

Table 3.1: Area wise details of sample collected

Sr. No.	Area	No. of samples
1.	Shirwal	100
2.	Dahiwadi	100
3.	Baramati	100
	Total	300

3.4.2 Serological test

3.4.2. (a) Indirect Enzyme Linked Immunosorbent Assay (i-ELISA)

Indirect Enzyme Linked Immunosorbent Assay (i-ELISA) was performed by using ID Screen® Brucellosis Serum Indirect Multi-Species kit manufactured by ID. Vet Innovative Diagnostics. The test was performed as per protocol outline in the user manual supplied with kit.

3.4.2. (b) General Information

Indirect ELISA for detection of antibodies directed against *Brucella abortus* (bovine), *melitensis* (ovine and caprine) and *suis* (pigs). It can be used with serum or plasma from bovine, ovine, caprine and pigs.

3.4.2. (c) Principle of the assay

The qualitative immune-enzymatic determination of IgG-class antibodies against *Brucella spp.* is based on the ELISA (Enzyme-Linked Immunosorbent Assay) technique. Microwells are coated with purified *Brucella* LPS. Specimen to be tested and controls are added to the microwells diluted at 1/20. Anti-*Brucella* antibodies, if present, form an antigen-antibody complex. After elimination of the sera by washing,

a multi-species horseradish peroxidase (HRP) conjugate is added to the wells. It fixes to the anti-*Brucella* antibodies, forming an antigen-antibody-conjugate-HRP complex. Again after eliminating the excess conjugate by washing the substrate solution Tetramethylbenzidine (TMB) is added this gives a blue colour reaction product. The Intensity of this product is proportional to the amount of *Brucella*-specific IgG antibodies in the specimen. Sulphuric acid is added to stop the reaction. This produces a yellow endpoint colour. Absorbance at 450 nm is read using an ELISA microwell plate reader.

Table 3.2: i-ELISA kit Components

Sr. No.	Reagents
1.	Microplates (12 X 8 well strips) coated with purified *Brucella* LPS
2.	Concentrated Conjugate (10 X)
3.	Positive Control
4.	Negative Control
5.	Dilution Buffer 2
6.	Dilution Buffer 3
7.	Wash Concentrate (20 X)
8.	Substrate Solution (TMB)
9.	Stop Solution (H_2SO_4 0.5 M)

3.4.2. (d) Wash solution preparation

Bring the wash concentrate (20X) to room temperature and mix thoroughly to ensure that the wash concentrate (20X) is completely solubilised. Prepare the wash

solution (1X) by diluting the wash concentrate (20X) to 1/20 in distilled/ deionised water.

3.4.2. (e) Protocol for performing indirect ELISA for Brucellosis

All reagents were allowed to come at room temperature (21°C ± 5°C) before use. All reagents were homogenized by inversion.

1. 190µl of Dilution Buffer 2 was added to all wells.
2. 10µl of Negative Control was added to wells A1 and B1.
3. 10µl of Positive Control was added to wells C1 and D1.
4. 10µl of each sample of serum was tested to the remaining wells.
5. Incubation was done for 45 min at 37°C.
6. Wells were emptied and each well was washed well 3 times with approximately 300µl of wash solution. Wash solution was prepared using 0.5 ml of Wash Solution in 9.5 ml of distilled water.
7. Conjugate was prepared by diluting the Concentrated Conjugate 10X to 1/10 (short incubation) in Dilution Buffer 3. Conjugate was made adding 100µl of Concentrated Conjugate to 900µl Dilution Buffer 3.
8. 100µl of the Conjugate 1X was added to each well.
9. Incubation was carried out for 30 min at 21°c.
10. Wells were emptied and each well was washed well 3 times with approximately 300µl of wash solution.
11. 100µl of the Substrate Solution was added to each well.
12. Incubation was done for 15 min at 21°C in the dark.
13. 100µl of the Stop Solution was added to each well in order to stop the reaction.
14. O.D was recorded at 450 nm.

3.4.2. (f) Validation

The test is validated if:

1. The mean value of the Positive Control OD (ODPC) is greater than 0.350.

$OD_{PC}>0.350$

2. The ratio of the mean values of the Positive and Negative Controls (OD_{PC} and OD_{NC}) is greater than 3.

$OD_{PC}/OD_{NC}>3$

3.4.2. (g) Interpretation

For each sample, the S/P percentage (S/P %) was calculated as follows using the sample and control values:

$$S/P\% = \frac{OD_{sample} - OD_{NC}}{OD_{PC} - OD_{NC}} \times 100$$

3.5 Molecular method

3.5.1 Polymerase Chain Reaction (PCR)

For confirmatory diagnosis of brucellosis, Polymerase Chain Reaction (PCR) technique was used on samples positive and doubtful in i-ELISA. This technique is too expensive to be used widely; it is more and appropriate for differential diagnosis. All the chemicals and reagents required for Polymerase Chain Reaction (PCR) were supplied by Himedia Pvt Ltd.

3.5.2 Principle of PCR

Polymerase Chain Reaction (PCR) uses enzyme DNA polymerase that directs the synthesis of DNA from deoxynuleotide substrate on a single-stranded DNA

template. DNA polymerase adds nucleotides at the 3' end of a custom designed oligonucleotide as it is annealed to a longer template DNA. Therefore, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can make use of the oligonucleotide as a primer and elongate its 3' end to produce an extended region of double stranded DNA.

3.5.3 DNA extraction from blood samples

Extraction of DNA is the first step in performing any PCR. DNA was isolated from the whole blood by using modified Martin's DNA isolation protocol (Martin *et al*., 2001) or commercial kits may be used.

Following method of DNA extraction was used for isolation of DNA from the blood samples of animals showing positive results in i-ELISA.

Take 500 µl of blood in a microfuge tube and 1 ml of RBC lysis buffer (0.22% NaCl, 0.015% saponin, 1Mm EDTA, ph 7.5)

The tube contents were mixed and then centrifuged at 10,000 rpm (Remi, India) for 3 minutes and the supernatant was discarded.

The pellet left at bottom of each tube was dissolved in 0.75 ml lysis buffer.

The tubes were again centrifuged at 10,000 rpm for 3 minutes and the supernatant was discarded. This process was repeated (3 times) until the pellet was clear of hemoglobin.

The pellet was then re-suspended in 100 µl of PCR buffer (50mM KCL, 10mM tris-HCL pH 8.0, 0.5% tween and 100µg proteinase-K per ml).

The tubes were incubated in water bath at 56°C for 2 hours after which they were immediately stored at -20°C until further processing.

3.5.4 Details of the primers used in PCR

The B4/B5 primers (Baily *et al.*, 1992) were used for molecular detection of *Brucella* genus that encodes a protein of *Brucella abortus* 31kDa, bcsp31 gene. For the present study; the primers were procured from Imperial Life Sciences Pvt. Ltd.

Table 3.3: Synthetic oligonucleotide design of the B4/B5 primers

Target Gene	Primer name	Sequence 5'-3'	Target Length bp	References
BCSP31	B4(F)	TGGCTCGGTTGCCAATATCAA	223 bp	Baily *et al.* (1992), Koichi *et al.* (2007)
	B5(R)	CGCGCTTGCCTTTCAGGTCTG		

3.5.5 DNA amplification

The isolated DNA samples were subjected to *Brucella* genus-specific, i.e., bcsp31 gene (Baily *et al.*1992). Details of primers are given in Table 3. The PCR reaction was carried out in 25µl volume mixture including of 12.5µl 2x PCR-Master-Mix (0.05 units/µl Taq DNA Polymerase in reaction buffer, 4mM MgCl2, 0.4mM dATP, 0.4mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP (Sigma Aldrich, USA). To formulate a final concentration of 1X, 1 µl of forward and reverse primers (10pmol/µl), 3 µl of DNA template, and nuclease free water was added to make 25 µl final volumes. The DNA amplification reaction was performed in a Master Cycler Gradient Thermocycler (Eppendorf, Germany) with a preheated lid.

Table 3.4: Steps and conditions of thermal cycling for B4/B5 primer pair used in PCR

Primers (Forward and Reverse)	Cycling conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final Extension
B4 (Forward) B5 (Reverse)	95ºC 5 minutes	95ºC 60 seconds	60ºC 30 seconds	72ºC 1 minute	72ºC 7 minutes
		Repeated for 35 cycles			

3.5.6 Electrophoresis of PCR products

After completion of PCR, the PCR products were analyzed and confirmed by Horizontal gel electrophoresis (Bio-Rad Laboratories, USA). The gel casting tray was placed on a leveled surface and gel comb was then placed across the gel casting tray, so that the teeth of the comb remains 1 mm above the base of the tray. The agarose gel was prepared and electrophoresis was performed as below:

1. Agarose gel was prepared by dissolving 1 gm Agarose (1.5%) in 0.5 x Tris Acetate- EDTA (TAE) in a glass flask and mixed it carefully in a microwave and then again repeated until clear solution was formed.
2. After cooling to about 50 °C, 3 µl of Ethidium bromide (sigma-Aldrich, India) was then added at the base of the flask and mixed thoroughly. It was allowed to cool before solidification and was poured on a plastic tray fixed on a gel caster containing a plastic comb for the wells formation.
3. The molten agarose was then poured onto the gel casting tray and was kept undisturbed for about an hour to solidify. After the solidification of gel the comb was removed.
4. The solidified gel with the gel casting tray was then submerged in the electrophoresis tank with the wells at the cathode end of the tank with sufficient quantity (about 1 mm)of electrophoresis buffer (TBE, 0.5 X) above the surface of the gel.
5. About 10 µl of PCR product was mixed with 2 µl of Bromophenol blue gel loading dye (6X) and was loaded in to the wells.
6. Simultaneously, 100 bp DNA ladder (Promega Biotech India Pvt. Ltd) was loaded in one of the wells as a molecular weight marker.
7. Electrophoresis was run at 60 volts and the progress of mobility was monitored by the forward migration dye.

3.5.7 Detection and identification of PCR product

Gel was visualized under a UV transilluminator and photographed by Bio-rad Gel Doc system. Visible band of appropriate size of 223 bp for *Brucella* genus were considered positive.

3.6 Haematological studies

Hematological estimations were carried in animals found positive for i-ELISA. The blood samples were collected from jugular vein of the sheep and goat in the K3-EDTA vials and were analyzed for hematological parameters such as

hemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC), total leukocyte count (TLC) using automated hematology analyzer Abacus Junior Vet.5 (Diatron, Hungary). Differential leukocyte count (DLC) performed as per the methods described by Benjamin (2001).

3.6.1 Differential Leucocyte Count (DLC)

3.6.1. (a) Preparation of Blood smear

1. Place a drop of blood on the end side of clean, grease free slide.
2. Using another slide let the drop to spread along its edge.
3. Hold the spreader slide at an angle of 45^0 and move it on the previous slide to the other end in a straight line with same force and pressure.
4. Allow the glass slide to dry after formation of smear, wrap it in paper and label it.

3.6.1. (b) Staining of blood smear (Leishman's staining)

1. Keep the blood smeared glass slide on a flat surface; pour drops of leishman's (5-10) drops for 1-2 minutes.
2. Add double quantity of distilled water on the slide and mix by blowing air.
3. Let the diluted stain act for 10 minutes.
4. Wash the slide with distilled water removes extra stain by keeping slide under running water and air dry.
5. Examine under oil immersion (100X) objective.

3.6.1. (c) Examination of slide

1. Set the smear on the microscope under low power.
2. Put a drop of cedar wood oil and set the smear under oil immersion lens.
3. Count up at least 100 leucocytes by battlement method i.e. count 4 fields horizontally, then 4 fields to the inside of slide, 4 fields again horizontally, 4 fields to the outside of slide and then repeat.

3.7 Statistical analysis

The data collected for various parameters were statistically analyzed by using standard methods described by Snedecor and Cochran (1994). All values in the text are expressed as percentage (%) and mean ± S.E. WASP 2.0 software was used for statistical analysis.

Plate 3.1: Study animals

Plate 3.2: Blood collection

Plate 3.3: i-ELISA plate and reagents used for i-ELISA test

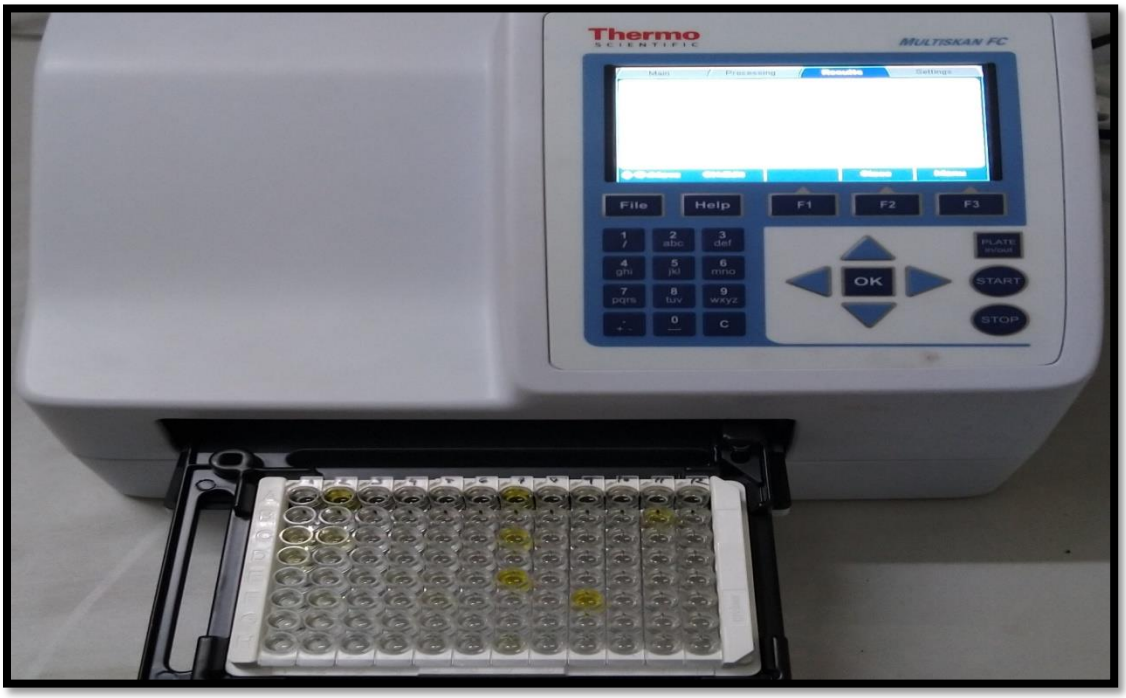

Plate 3.4: Multiscan ELISA reader

Plate 3.5: Thermal cycler

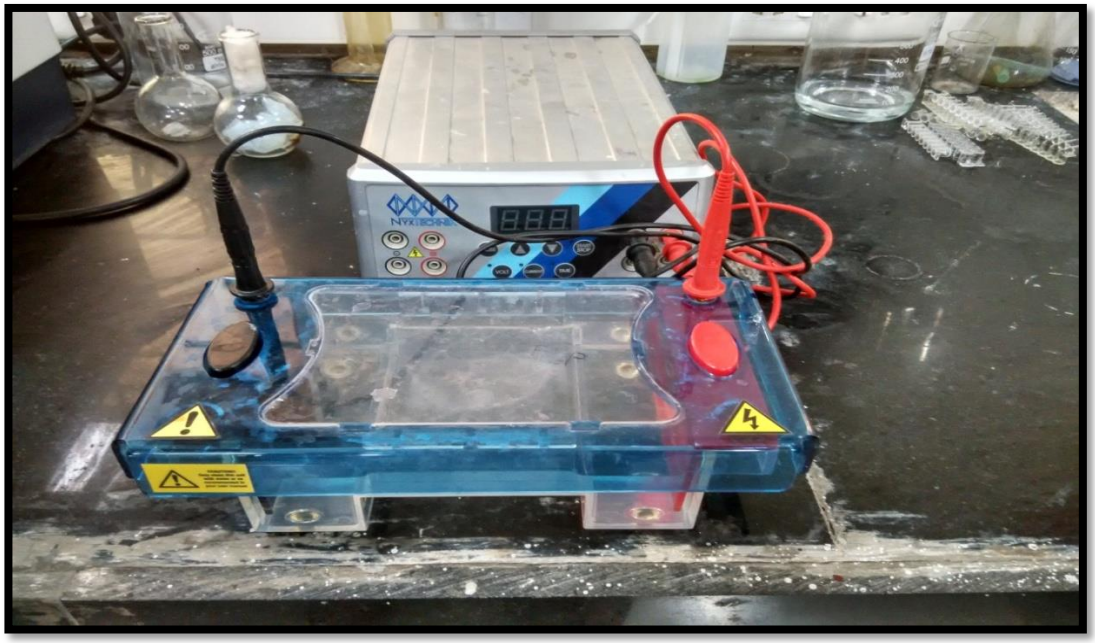

Plate 3.6: Gel electrophoresis used during the study

Plate 3.7: Gel documentation system

Plate 3.8: Automated hematology analyzer

RESULTS AND DISCUSSION

CHAPTER IV

RESULTS AND DISCUSSION

The present study was carried out, to study the sero-prevalence of brucellosis in small ruminants and confirmation of positive cases by molecular detection. In total 300 small ruminants were included this study comprising 226 goats of Sirohi, Sagmaneri, Osmanabadi, African boar, Beetal, Tellicherry breeds and 74 sheep of Madgyal, Lonand deccani, NARI suvarna breeds from Shirwal, Dahiwadi and Baramati region. The sero-prevalence study of brucellosis was done by using ID Vet Innovative Diagnostics, ID screen® Brucellosis Serum Indirect Multi-Species ELISA kit. The test was carried out as per protocol outline in the user manual supplied with kit. The test serum sample showing more than 120 percent positivity was considered as positive.

4.1 Sero-prevalence

i-ELISA technique was used for detection of the sero-prevalence of brucellosis by qualitative immunoenzymatic determination of IgG-class antibodies. Wells are coated with antigen; specimens are added to the wells. An antibody, if present forms an antigen-antibody complex then, the conjugate was added and it fixes the antibodies thus forming an antigen-antibody-conjugate complex and colour development indicates a positive result. Interpretation was done calculating the S/P percentage (S/P %) using following formula: $S/P = OD_{sample} - OD_{NC} / OD_{PC} - OD_{NC} \times 100$, then it was interpreted as % SP $\leq$ 120% (positive), 110-120% (doubtful) and $\geq$ 110% (negative).

4.1.1 Overall sero-prevalence

Total 300 serum samples (226 goats and 74 sheep) were collected from in and around Shirwal region, out of which 32 (18 goats and 14 sheep) serum samples were

positive for brucellosis with overall sero-prevalence of 10.66%, species wise as 7.96% in goats and 18.91% in sheep by i-ELISA, respectively (Table 4.1, figure 4.1).

Table 4.1: Sero-prevalence of brucellosis in small ruminants by i-ELISA

Species	Total no. of samples	No. of positive samples	Percent positivity (%)
Goat and Sheep	300	32	10.66
Goat	226	18	7.96
Sheep	74	14	18.91

The results of present study were in agreement with the Teshale *et al.* (2006) collected serum samples from 2000 sheep and goats and these samples were evaluated by i-ELISA, results showed 193 (9.7%) serum samples were found positive for brucella antibodies and similar observations was noted by Sadhu *et al.* (2015) performed i-ELISA on total 1000 serum samples (485 sheep and 515 goats) and calculated sero-prevalence of brucellosis as 8.80%.

In contrast to present study Alhamada *et al.* (2017) collected serum samples of 432 small ruminants (335 sheep and 97 goats) from 72 farms and were tested using i-ELISA, results showed that 31.7% of sheep and 34.0% of goats had antibodies against brucella and also, Hussain *et al*. (2017) collected 223 serum samples of sheep from Kashmir Valley and observed higher sero-prevalence of brucellosis as 35.87% using Micro agglutination test (MAT).

Brucellosis is endemic in western Maharashtra region and spread of infection generally takes place through tracking of animals from one place to another place.

4.1.2 Area wise sero-prevalence of brucellosis in small ruminants

Total 300 serum samples were collected for the present study, out of which 100 samples (67 goats and 33 sheep) were collected from Dahiwadi region, 100 samples (70 goats and 30 sheep) from Shirwal region and 100 samples (89 goats and

11 sheep) from Baramati region. Highest numbers of sero-positive samples were obtained from Dahiwadi region 16 (7 goats and 9 sheep), followed by Baramati region 9 (7 goats and 2 sheep) and Shirwal region 7 (4 goats and 3 sheep) which gives overall 16%, 9% and 7% sero-prevalence in small ruminants, respectively (Table 4.2, figure 4.2). Species wise the sero-prevalence in goats of these area was 10.44%, 7.86% and 5.71%, respectively; whereas, the sero-prevalence in sheep was 27.27%, 18.18%, and 10%, respectively when subjected to i-ELISA.

Table 4.2: Area wise sero-prevalence of brucellosis in small ruminants by i-ELISA

Sr. No.	Area	Species	No. of samples	No. of positive samples	Percent positivity (%)
1.	Dahiwadi	Goat and sheep	100	16	16
		Goat	67	7	10.44
		Sheep	33	9	27.27
2.	Baramati	Goat and sheep	100	9	9
		Goat	89	7	7.86
		Sheep	11	2	18.18
3.	Shirwal	Goat and sheep	100	7	7
		Goat	70	4	5.71
		Sheep	30	3	10

The results of present study were in accordance with Hussain *et al.* (2014) who studied the sero-prevalence of brucellosis in 100 sheep in Kohat district, and found 12.12%, 09% and 08.82% sero-prevalence in Lachi, Seni Gumbat and Kohat tehsils respectively, with an overall sero- prevalence of 10%. Similarly, Negash *et al.* (2012) investigated sero-prevalence of brucellosis in small ruminants at selected sites of Dire Dawa region, Eastern Ethiopia. Highest sero-prevalence was found in Aseliso

(11.11%) and lowest in that of Gedenser (6.84%). Likewise, Ali *et al.* (2017) studied sero-prevalence of brucellosis in different areas in Potohar Plateau, Pakistan. Highest sero-prevalence is recorded in Islamabad Capital Territory as compare to Rawalpindi and Attock area in bovines.

Higher sero-prevalence was observed in Dahiwadi region because farmers use to graze their animals in free range, where transmission of infection takes place between different flocks. Whereas, in Shirwal and Baramati region farmers practiced stall feeding in which movement of animals is restricted.

4.1.3 Age wise sero-prevalence of brucellosis in small ruminants

To find out age wise sero-positivity sheep and goats were divided into 3 age groups i.e., 0.5-3 years, 3-6 years and above 6 years. A high percent of overall sero-prevalence in small ruminants was observed in the age group above 6 years as 13.72% followed by 3-6 years age group as 12.06% and 0.5-3 years age group as 5.33%. Species wise the sero-prevalence in goats of these age groups i.e. 0.5-3 years, 3-6 years and above 6 years was 3.57%, 9.16% and 10.25% respectively; whereas age wise sero-prevalence in sheep was 10.25%, 20.93% and 25% respectively when subjected to i-ELISA (Table 4.3, figure 4.3). The present study shows that adult age group of sheep and goats are more susceptible than young age group of sheep and goats for brucellosis.

Table 4.3: Age wise sero-prevalence of brucellosis in small ruminants by i-ELISA

Sr. No.	Age group	Species	No. of samples	No. of positive samples	Percent positivity (%)
1.	0.5- 3 years	Goat and sheep	75	4	5.33
		Goat	56	2	3.57
		Sheep	19	2	10.52
2.	3-6 years	Goat and sheep	174	21	12.06
		Goat	131	12	9.16
		Sheep	43	9	20.93
3.	Above 6 years	Goat and Sheep	51	7	13.72
		Goat	39	4	10.25
		Sheep	12	3	25

The results of present study were in accordance with Chahar *et al.* (2016) reported higher sero-prevalence of 33.33% in adult goats as compared to 12.72% in goats below one year of age group. Similarly, Jarikre *et al.* (2015) observed higher sero-prevalence of 92% in adult goats above two-years-old as compare to 8% in below two-years -old goats. Sharma *et al.* (2017) found higher sero-prevalence in 6-9 years age group by i-ELISA also, Mittal *et al.* (2005) reported animals of 4-6 years age (57.89%) showed maximum positive cases whereas, animals of 0-2 years showed 23.44% sero-positivity.

Higher sero-prevalence observed in adult animals because, sexually mature, pregnant animals were more susceptible to infection with the organism than sexually immature animals (Radostits *et al.,* 2007a). Erythritol is a four carbon sugar produced by the fetus preferentially consumed by *Brucella spp*. which leads to localization and subsequent accumulation of large amounts of bacteria to these sites, ultimately cause

abortion (Petersen *et al.,* 2013) and also adult animals remains a disease carrier for their entire lifespan.

4.1.4 Sex wise sero-prevalence of brucellosis in small ruminants

In present study the sex wise overall sero-prevalence in small ruminants was found higher in males as 17.77% when compare to females as 10.66% respectively. Species wise sero-prevalence in goats was observed higher in males as 16.12% when compare to female 6.66% and in sheep it was also higher in males as 21.42% when compare to female as 18.33% respectively when subjected to i-ELISA (Table 4.4, figure 4.4).

Table 4.4: Sex wise sero-prevalence of brucellosis in small ruminants by i-ELISA

Sr. No.	Sex	Species	No. of samples	No. of positive samples	Percent positivity (%)
1.	Male	Goat and sheep	45	8	17.77
		Goat	31	5	16.12
		Sheep	14	3	21.42
2.	Female	Goat and sheep	255	24	9.41
		Goat	195	13	6.66
		Sheep	60	11	18.33

The result of present study was in agreement with Suryawanshi *et al*. (2014) observed that sex wise sero-prevalence was higher in males of sheep and goats as 25.00% and 24.00% as compare to 17.16% and 4.32% in female sheep and goats. Similarly, Nguna *et al.* (2019) noticed that out of 451 human samples tested, sex wise sero-prevalence in males was 6.4% (11/173) and in females 3.2% (9/278).

In contrast to present study Rahman *et al.* (2012a) observed higher sero-prevalence in female sheep (3.41%) than male sheep (3.33%). Likewise, Pandeya *et*

al. (2013) also reported higher sero-prevalence in female 14.6% as compare to males 10.6%.

Male animals was more susceptible to brucella infection because farmers generally exchange their breeding males between farms to avoid inbreeding therefore, males had more chances to get infection than females.

4.2 Molecular detection

Polymerase Chain Reaction (PCR) is an advanced, quick and precise tool for diagnosis of brucellosis. It was first developed in the early 1990s and in recent period it has been used routinely for diagnosis of brucellosis and other infectious agents. All the sero-positive blood samples which were positive for i-ELISA technique were subjected for isolation of DNA by Modified Martin's DNA isolation protocol (Martin *et al.*, 2001) and then subjected to Polymerase Chain Reaction (PCR) using primers B4/B5 for bcsp 31 gene producing 223 bp amplicon (Baily *et al.*, 1992).

In the present study, out of 32 i-ELISA positive samples 6 (5 goats and 1 sheep) samples showed positive results at 223 bp by using B4/B5 primers for bcsp 31 gene (Table 4.5, figure 4.5). The difference in the results of i-ELISA and PCR may perhaps related to samples size, DNA isolation protocol, insufficient DNA extracted or degradation of target DNA.

Table 4.5: confirmation of sero-positive animals by PCR

Sr. No.	Total number of animals	No. of cases positive by i-ELISA	No. of cases positive by PCR
1	300	32	6

The results of present study were in concordance with Gujrathi (2016) who collected 168 blood and serum samples of cattle. Amongst the 168 cattle studied 33 (19.64%) cattle were positive for i-ELISA and 8 (4.7%) samples showed positive results at 223 bp using B4/B5 primers derived from a gene encoding the 31-kDa

Brucella abortus antigen. Similarly, Parthiban *et al.* (2019) conducted study on serum based screening and molecular detection of brucellosis in small ruminants. Out of 238 goats and sheep serum samples 21 serum samples produced specific amplicon of 223 bp by targeting bcsp 31 gene. Likewise, Saraswathy *et al.* (2015) isolated blood DNA from 24 cattle and tested for the detection of B4/B5 gene and IS711 gene by Polymerase Chain Reaction for diagnosis of Brucellosis, 15 DNA samples (62.5%) was detected as positive by both gene primers. Ali *et al.* (2015) investigated sero-prevalence of brucellosis in small ruminants. Out of 278 serum samples 24 (8.6%) samples were positive by RBPT and 18 samples were positive in the Brucella genus-specific (bcsp 31) and Brucella abortus-specific (IS711) qRT-PCR, respectively.

4.3 Hematological alterations

Hematological estimations were carried out on the samples which were sero-positive by i-ELISA and parameters like Hemoglobin (Hb), Packed Cell Volume (PCV), Total Erythrocyte count (TEC), Total Leukocyte count (TCL) and Differential leukocyte count using automated hematology cell analyzer Abacus Junior Vet.5 (Diatron, Hungary). Results of haematological analysis are given in the table 4.6, 4.7, 4.8 and 4.9, figure 4.6 and 4.7.

Table 4.6: Haematological values of goats found sero-positive for brucellosis by i-ELISA

Tag No.	Hb (g/dL)	PCV (%)	TEC ($\times 10^{6}$/µL)	TLC ($\times 10^{3}$/µL)
G1	10	25	15.65	11.2
G2	6.4	18.82	6.54	20.58
G3	9.1	24.45	13.78	15.51
G4	8.2	21.38	16.03	24.73
G5	7	19.71	6.9	18.56
G6	7.4	17.26	7.06	14.35
G7	9.3	25.96	20.3	34.13
G8	8.5	24.36	15.42	23.5
G9	7.2	20.05	15.55	20.21
G10	6.8	19.39	7.26	15.91
G11	8.8	24.17	16.15	31.48
G12	10	25.56	18.34	22.58
G13	6.3	18.48	6.72	11.12
G14	8.2	22.82	7.81	24.76
G15	5.5	17.31	6.27	35.94
G16	11.1	29.69	17.52	15.06
G17	6.8	20.19	7.22	12.42
G18	4.1	11.09	5.39	9.74
Mean ± SE	**7.81 ± 0.41**	**21.42 ± 1.01**	**11.66 ± 1.22**	**20.09 ± 1.86**
Reference Range Radostits *et al.* (2007b)	**8-12**	**22-38**	**8-18**	**4-13**

Table 4.7: Differential Leucocyte count of goats found sero-positive for brucellosis by i-ELISA

Tag No.	Lympho-cytes	Mono-cytes	Neutro-phils	Eosino-phils	Baso-phils
G1	46	3	47	4	0
G2	71	4	23	2	0
G3	55	1	43	1	0
G4	44	1	54	1	0
G5	76	5	17	2	0
G6	58	2	39	1	0
G7	17	1	79	3	0
G8	41	2	55	2	0
G9	40	1	57	2	0
G10	44	1	54	1	0
G11	22	1	74	3	0
G12	51	2	46	1	0
G13	72	3	23	2	0
G14	56	1	42	1	0
G15	29	1	69	1	0
G16	62	3	32	3	0
G17	59	3	35	3	0
G18	14	1	83	2	0
Mean ± SE	**47.61 ± 4.32**	**2 ± 0.29**	**48.44 ± 4.53**	**1.94 ± 0.22**	**0**
Reference Range Radostits *et al.* (2007b)	**50-70**	**0-4**	**30-48**	**1-8**	**0-1**

Table 4.8: Haematological values of sheep found sero-positive for brucellosis by i-ELISA

Tag No.	Hb (g/dL)	PCV (%)	TEC ($\times 10^6$/µL)	TLC ($\times 10^3$/µL)
S1	6.4	19.67	6.24	5.45
S2	8.1	23.25	9.47	9.93
S3	9.1	28.15	9.83	15.37
S4	9.7	29.26	11.82	12.03
S5	9.4	28.43	12.4	13
S6	6.7	19.32	6.79	8.63
S7	7.7	23.67	8.15	8.81
S8	7.1	20.97	8.94	3.79
S9	8.3	23.64	11.41	11
S10	9.1	22.29	7.8	13.37
S11	7.8	24.23	8.65	10.84
S12	9	30	9.64	32.3
S13	10.8	33.38	12.36	19.8
S14	9.1	24.04	9.31	14.62
Mean ± SE	**8.45 ± 0.32**	**25.02 ± 1.11**	**9.48 ± 0.52**	**12.78 ± 1.85**
Reference Range Radostits *et al.* (2007b)	**9-15**	**27-45**	**9-15**	**4-8**

Table 4.9: Differential Leucocyte count of sheep found sero-positive for brucellosis by i-ELISA

Tag No.	Lymph o-cytes	Mono-cytes	Neutro-phils	Eosino-phils	Baso-phils
S1	59	4	36	1	0
S2	46	3	48	3	0
S3	57	1	40	2	0
S4	52	2	43	3	0
S5	46	1	51	2	0
S6	16	1	82	1	0
S7	18	1	80	1	0
S8	41	2	55	2	0
S9	7	1	89	3	0
S10	35	1	62	2	0
S11	47	3	49	1	0
S12	29	1	70	0	0
S13	15	1	84	0	0
S14	32	3	63	2	0
Mean ± SE	**35.71 ± 4.47**	**1.78 ± 0.28**	**60.85 ± 4.71**	**1.64 ± 0.26**	**0**
Reference Range Radostits *et al.* (2007b)	**40-55**	**0-6**	**10-50**	**0-10**	**0-3**

4.3.1 Hemoglobin (Hb)

The mean ± S.E. value of Hemoglobin (gm/dl) in the present study in goats which found sero-positive for brucellosis were 7.81 ± 0.41. Total 9 (50%) cases of goats showed haemoglobin value below normal reference range indicating anemia, while 9 (50%) cases showed haemoglobin value within the normal reference range.

The mean ± S.E. value of Hemoglobin (gm/dl) in the present study in sheep which found sero-positive for brucellosis were 8.45 ± 0.32. Total 7 (50%) cases of sheep showed hemoglobin value below normal reference range indicating anemia, while 7 (50%) cases showed haemoglobin within the normal reference range.

All the 5 cases of goats and 1 case of sheep (G2, G5, G6, G13, G17 and S1) positive by i-ELISA and PCR had lowered Hb value indicating anemia when compared with normal reference range.

The results of present study were in agreement with Kushwaha *et al.* (2014) who observed lower values of haemoglobin in brucella affected cattle. Sikder *et al.* (2012) observed normal Hb values in brucella infected cattle. Soudbakhsh *et al.* (2007) also observed anemia in humans affected with brucellosis and similarly Crosby *et al.* (1984) reported anemia in 74% of the 38 humans affected with brucellosis. Sikder *et al.* (2012) states that the lower Hb in brucellosis could be due to intracellular position of the *Brucella* spp.

4.3.2 Packed Cell Volume (PCV)

The mean ± S.E. value of PCV (%) in the present study was 21.42 ± 1.01 in goats found sero-positive for brucellosis. Total 10 (55%) cases of goats showed PCV values below normal reference range indicating anemia and 8 (44%) cases showed PCV values within normal reference range.

The mean ± S.E. value of PCV (%) in the present study was 25.02 ± 1.11 in sheep found sero-positive for brucellosis. Total 9 (64%) cases of sheep showed PCV values below normal reference range indicating anemia and 5 (35%) cases showed PCV values within normal reference range.

All the 5 cases of goats and 1 case of sheep (G2, G5, G6, G13, G17 and S1) positive by i-ELISA and PCR had lowered PCV value indicating anemia when compared with normal reference range.

The results of present study were in accordance with Sikder *et al.* (2012) who observed normal range of PCV in brucella affected cattle. Crosby *et al.* (1984) also reported hematocrit value less than 30% in 64% of the 38 humans affected with brucellosis. In contrast to present study Kushwaha *et al.* (2014) observed higher PCV in brucella infected cattle.

4.3.3 Total Erythrocyte Count (TEC)

The mean ± S.E. value of TEC $\times 10^6$ /µl in the present study was 11.66 ± 1.22 in goats found sero-positive for brucellosis. Total 9 (50%) cases of goats showed TEC value below normal reference range indicating anemia. 7 (38%) cases showed no change in TEC values however, 2 (11%) cases showed polycythemia when compare to normal reference range.

The mean ± S.E. value of TEC $\times 10^6$ /µl in the present study was 9.48 ± 0.52 in sheep found positive for brucellosis. Total 6 (42%) cases of sheep showed TEC value below normal reference range indicating anemia and 8 (57%) cases showed TEC value within normal reference range.

All the 5 cases of goats and 1 case of sheep (G2, G5, G6, G13, G17 and S1) positive by i-ELISA and PCR had lowered TEC value indicating anemia when compared with normal reference range.

The results of present study were in accordance with Kushwaha *et al.* (2014) observed low levels of TEC in brucella infected cattle. Similarly, Sikder *et al.* (2012) also reported a little decline in the values of TEC in brucella infected cattle.

4.3.4 Total Leucocyte Count (TLC)

The mean ± S.E. value of TLC $\times 10^3$/ µl in the present study was 20.09 ± 1.86 in goats found sero-positive for brucellosis. Leucocytosis was observed in 14 (77%) cases of goats and 4 (22%) cases of goats showed no change in TLC values when compared with normal reference range.

The mean ± S.E. value of TLC $\times 10^3$/ µl in the present study was 12.78 ± 1.85 in sheep found sero-positive for brucellosis. Leucocytosis was observed in 12 (85%) cases of sheep and leucopenia was observed in 1 (7%) case and in 1 (7%) case showed no change in TLC values when compared to reference range.

Total 3 cases (G2, G5 and G6) had leucocytosis and 3 cases (G13, G17 and S1) showed normal leucocyte count which were positive for both i-ELISA and PCR when compared with normal reference range.

The findings of present study was in concordance with Sikder *et al.* (2012) who observed slight increased values of TLC in brucella affected cattle. Bozdemir *et al.* (2017) reported increased total leukocyte count in humans affected with brucellosis and Kushwaha *et al.* (2014) reported lower values of TLC in brucella affected cattle.

4.3.5 Differential Leucocyte Count (DLC)

Differential leucocyte count gives relative percentage of each type of white blood cell present in blood. Leucocytes are classified into two main groups i.e. granulocytes and nongranulocytes. Granulocytes include neutrophils, eosinophils and basophils. Nongranuloctye include lymphocytes and monocytes.

4.3.5. (a) Lymphocyte

The mean ± S.E. value of Lymphocyte (%) in the present study was 47.61 ± 4.32 in goats found sero-positive for brucellosis. In the study 9 (50%) cases of goats showed lymphocytopenia. 6 (33%) cases showed no change in lymphocyte count while, 3 (16%) cases reveled lymphocytosis when compared with normal reference range.

The mean ± S.E. value of Lymphocyte (%) in the present study was 35.71 ± 4.47 in sheep found sero-positive for brucellosis. In the study 7 (50%) cases of sheep had lymphocytopenia. 5 (35%) cases showed no change in lymphocyte count while, 2 (14%) cases reveled lymphocytosis when compared with normal reference range.

Total 4 cases (G2, G5, G13 and S1) had lymphocytosis and 2 cases (G6 and G17) showed normal lymphocyte count which were positive for both i-ELISA and PCR when compared with normal reference range.

The results of present study were in accordance with Kushwaha *et al.* (2014) who observed lymphocytopenia in brucella affected cattle. Gul *et al.* (2013) noticed lymphocytosis in brucella affected horses. Chahotal *et al.* (2003) also observed lymphocytosis in brucella affected cattle and Bozdemir *et al.* (2017) reported humans affected with brucella have decreased lymphocyte count.

4.3.5. (b) Neutrophil

The Mean ± S.E. value of neutrophils (%) was 48.5 ± 4.52 in goats found sero-positive for brucellosis. Neutropenia was observed in 3 (16%) cases of goats. 7 (38%) cases had normal values of neutrophils and neutrophilia was noticed in 8 (44%) cases when compared to normal reference range.

The Mean ± S.E. value of neutrophils (%) was 60.58 ± 4.71 in sheep found sero-positive for brucellosis. In the study 5 (35%) cases of sheep had normal values of neutrophils and neutrophilia was noticed in 9 (64%) cases when compared with normal reference range.

Total 3 cases (G2, G5 and G13) had neutropenia and 3 cases (G6, G17 and S1) showed normal neutrophil count which were positive for both i-ELISA and PCR when compared with normal reference range.

The results of present study were similar with Sikder *et al.* (2012) who observed little decline in the values of neutrophils in brucella affected cattle and also Kushwaha *et al.* (2014) reported lower values of neutrophils in brucella affected cattle. However, Bozdemir *et al.* (2017) noticed humans affected with brucella have increased neutrophil count.

4.3.5. (c) Monocytes

The mean ± S.E. value of monocytes (%) was 2 ± 0.29 in goats found sero-positive for brucellosis. Monocytosis was observed in 1 (5%) case and 17 (94%)

cases of goats showed normal monocytes count when, compared with reference range.

The mean ± S.E. value of monocytes (%) was 1.78 ± 0.28 in sheep found sero-positive for brucellosis. All the cases of sheep showed normal monocytes count when, compared with reference range.

Total 5 cases (G2, G6, G13, G17 and S1) had normal monocytes count and 1 case (G5) showed monocytosis which was positive for both i-ELISA and PCR when compared with normal reference range.

The results of present study were agreement with Sikder *et al.* (2012) observed slightly increased values of monocytes in brucella affected cattle whereas, Chahotal *et al.* (2003) reported severe monocytosis averaging 33% in brucella affected cattle.

4.3.5. (d) Eosinophils

The mean ± S.E. value of Eosinophils (%) was 1.88 ± 0.24 in goats found sero-positive for brucellosis. All the cases of goats had normal count of eosinophils when, compared with reference range.

The mean ± S.E. value of Eosinophils (%) was 1.64 ± 0.26 in sheep found sero-positive for brucellosis. All the cases of sheep had normal count of eosinophils when, compared with reference range.

All the 5 cases of goats and 1 case of sheep (G2, G5, G6, G13, G17 and S1) positive by i-ELISA and PCR had normal eosinophils count value when compared with normal reference range.

The results were not in agreement with Sikder *et al.* (2012) who reported higher values of eosinophils in brucella infected cattle. However, Kushwaha *et al.* (2014) observed lower values of eosinophils in brucella affected cattle.

4.3.5. (e) Basophils

No basophils were detected in Differential Leucocyte Count of all 32 (18 goats and 14 sheep) sero-positive cases and 5 (4 goats and 1 sheep) PCR positive cases.

Hematological picture is transient and depends on the stage of disease, severity of illness, lesions, hydration status of animal etc. Hence, variation in the report can be there. In the present investigation out of total 18 goats and 14 sheep which were sero-positive by i-ELISA only, 5 goats and 1 sheep were found positive for brucellosis by PCR. Hence, only 5 goats and 1 sheep can be considered infected and others may not have active infection.

4.4 Status of animals included in research work

Small ruminants' rearing is practiced principally in dry and semi-arid district of western Maharashtra. Dhangar is the traditionally semi-nomadic pastoral society primarily located in the western Maharashtra. In Shirwal and Baramati region, samples were collected from the farmers who follow intensive type of housing system with stall feeding (Green and dry fodder + Concentrate supplement). In Dahiwadi region samples were collected from the farmers who follow semi-intensive type of housing system with stall feeding (Green and dry fodder + Concentrate supplement) and grazing (Available green and dry fodder). Deworming was performed in all of the animals included in study and vaccination was done as per standard protocol recommended by Department of animal husbandry, Maharashtra State.

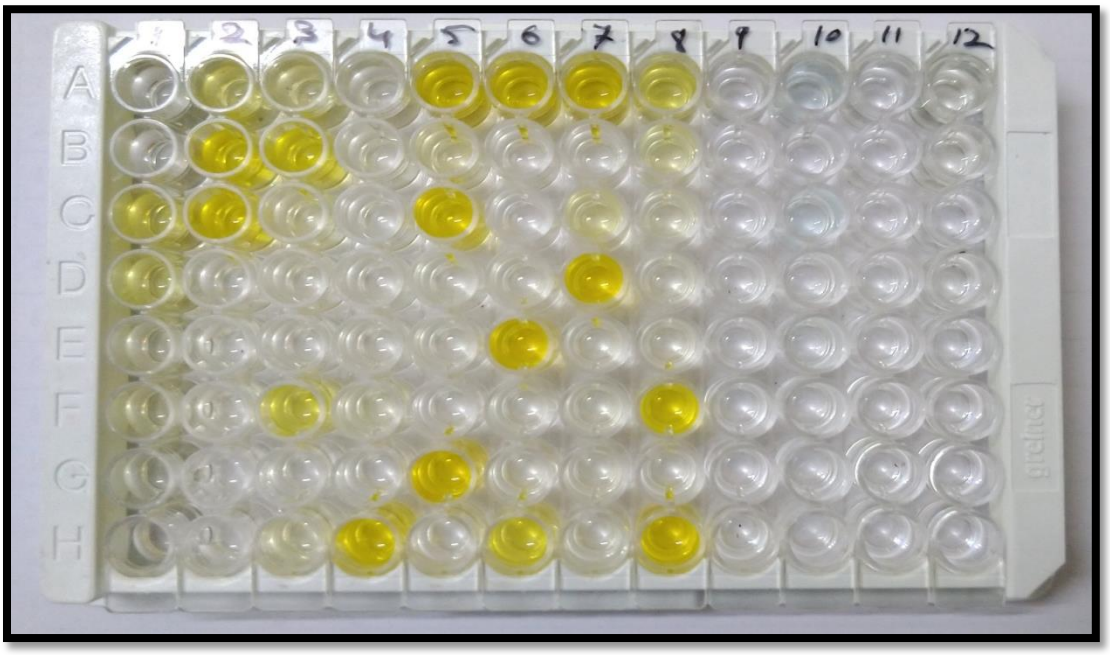

Plate 4.1: Results obtained in i-ELISA

ELISA module showing positive and negative reactions for IgG class antibody detection in small ruminants by Indirect ELISA, Wells A1 and B1: Negative Control Wells C1 and D1: Positive Control, Wells E1 to H12: Test serum samples. Yellow coloured wells denotes presence of Antibodies whereas; colourless wells denotes absence of antibodies.

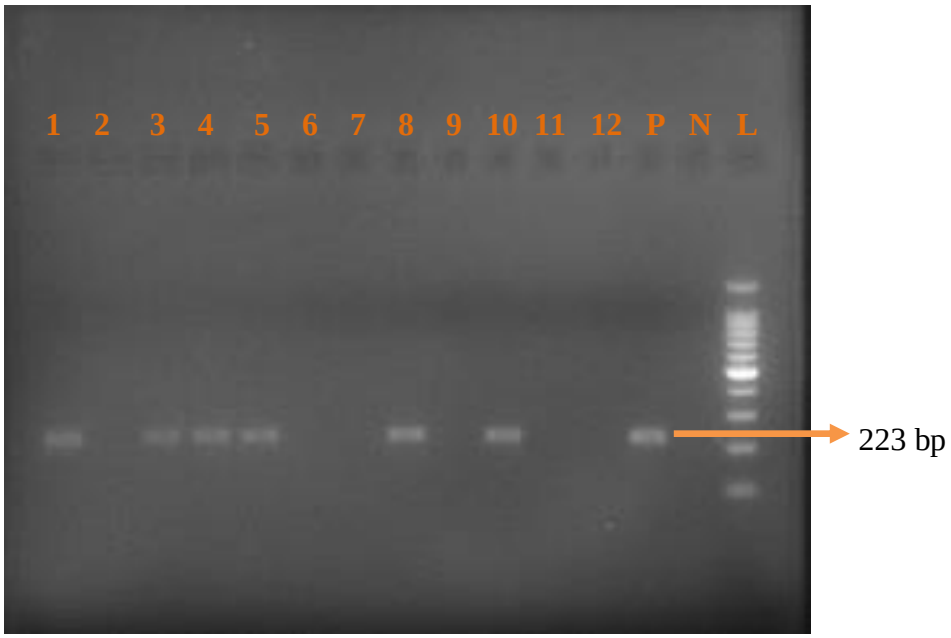

Plate 4.2: Positive results obtained at 223bp in PCR

Lane 1-12: Blood DNA isolates, Lane P: Positive control *(Brucella melitensis)*, Lane N: Negative control, Lane L: DNA ladder 100bp.

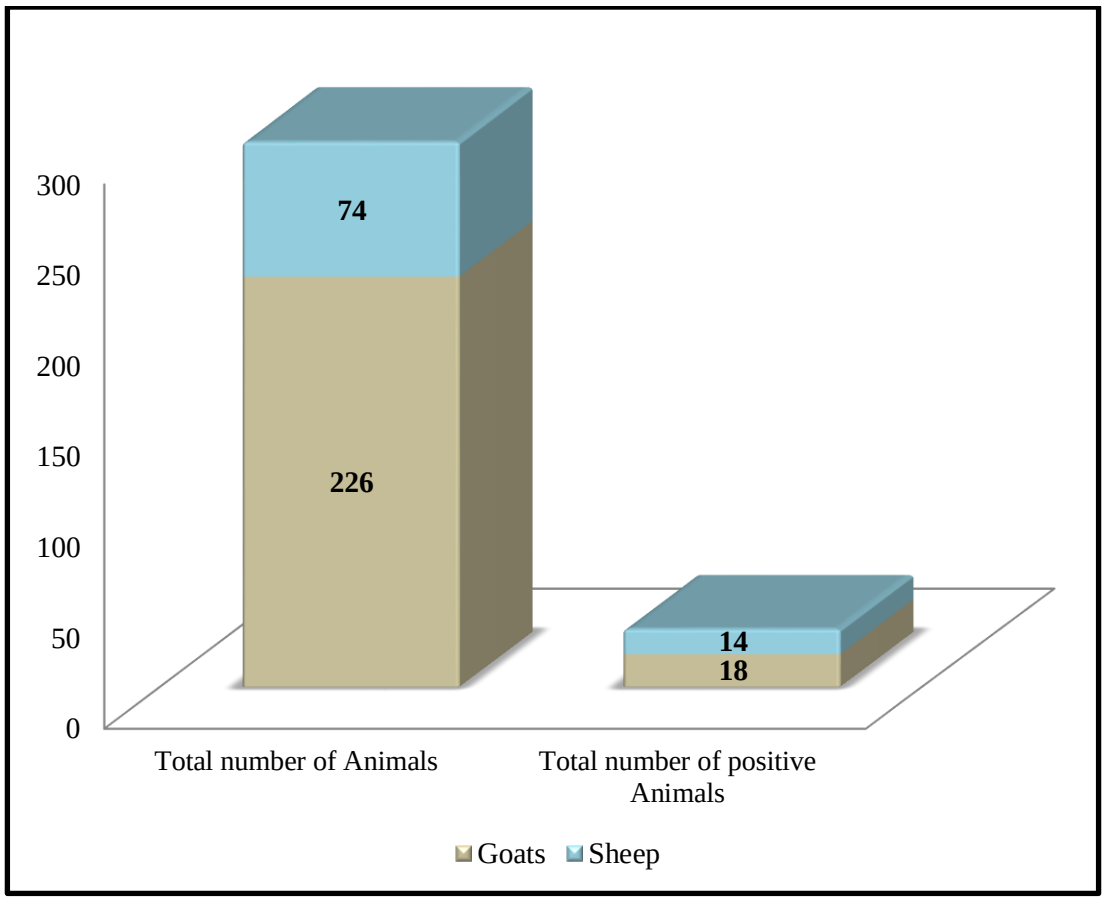

Figure 4.1: Sero-prevalence of Brucellosis in small ruminants by i-ELISA

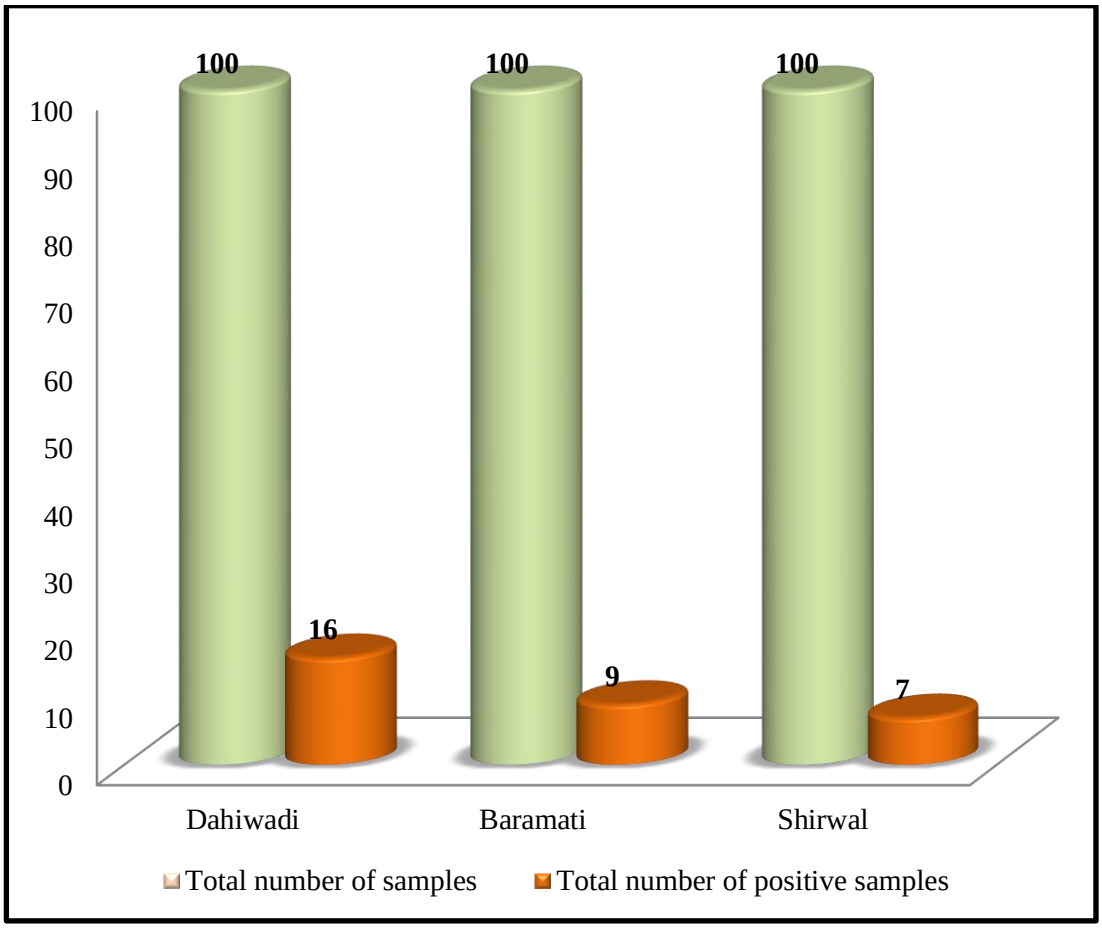

Figure 4.2: Area wise sero-prevalence in small ruminants by i-ELISA

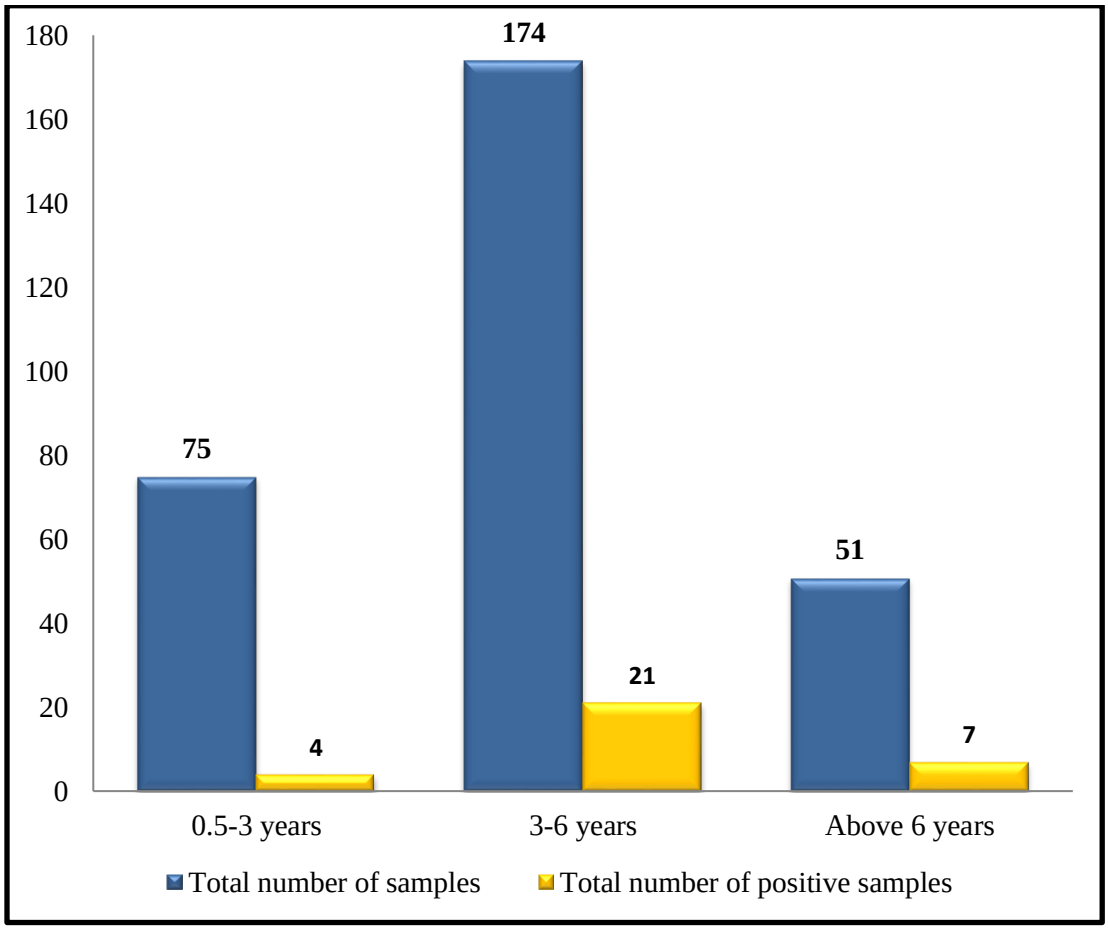

Figure 4.3: Age wise sero-prevalence of brucellosis in small ruminants by i-ELISA

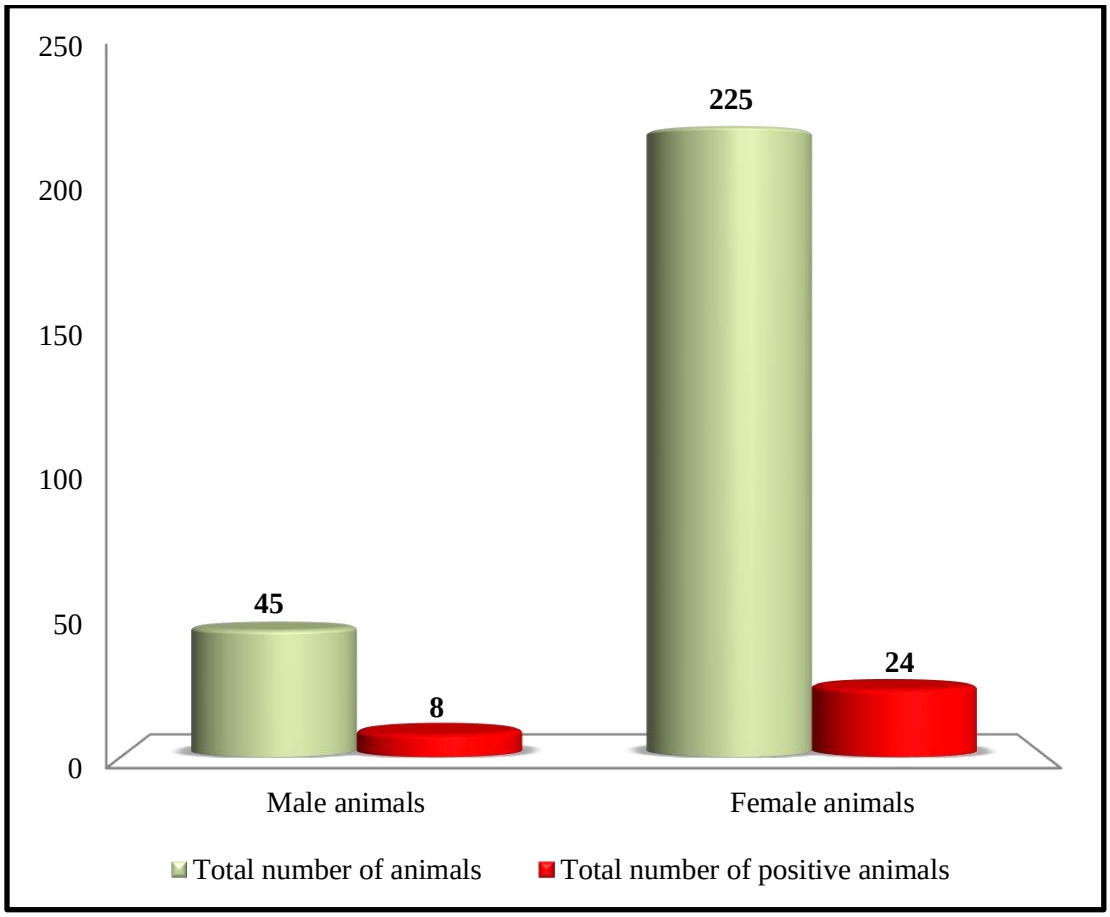

Figure 4.4: Sex wise sero-prevalence in small ruminants by i-ELISA

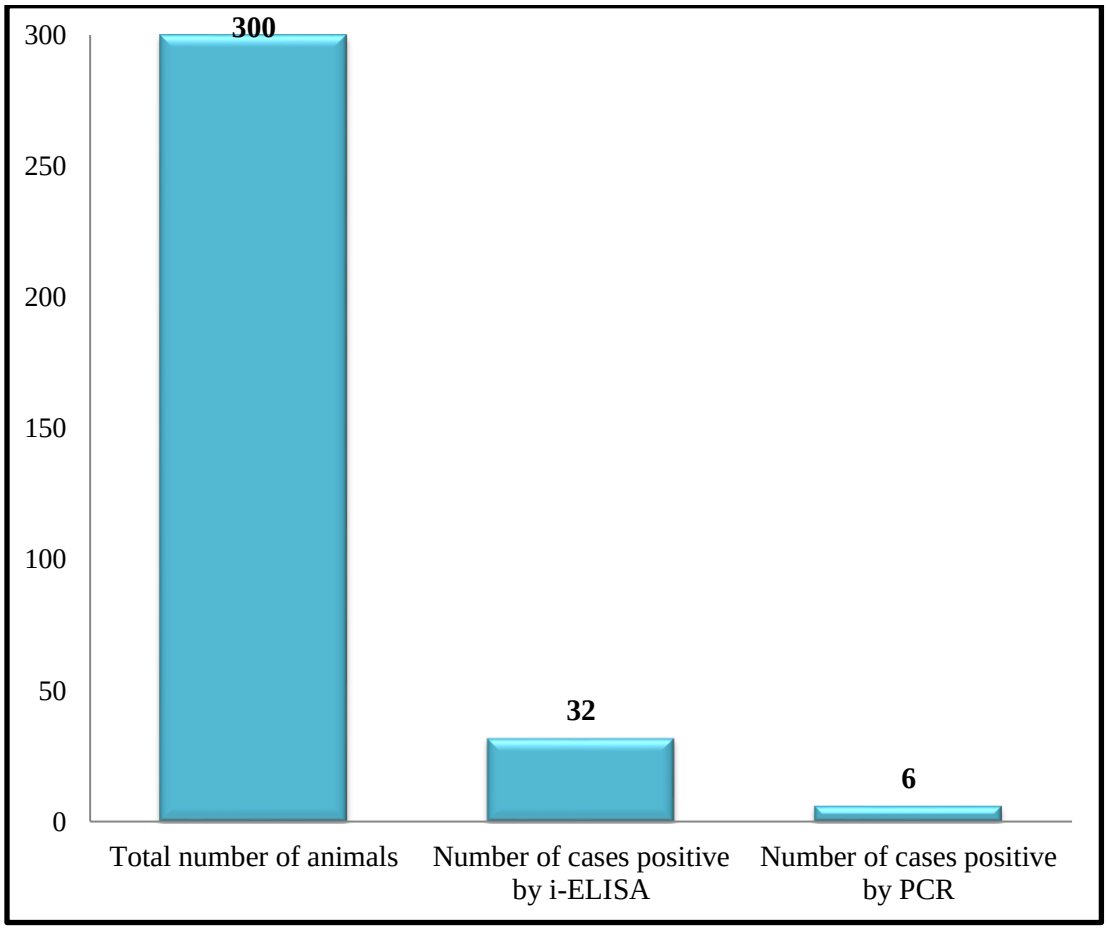

Figure 4.5: Conformation of sero-positive animals by PCR

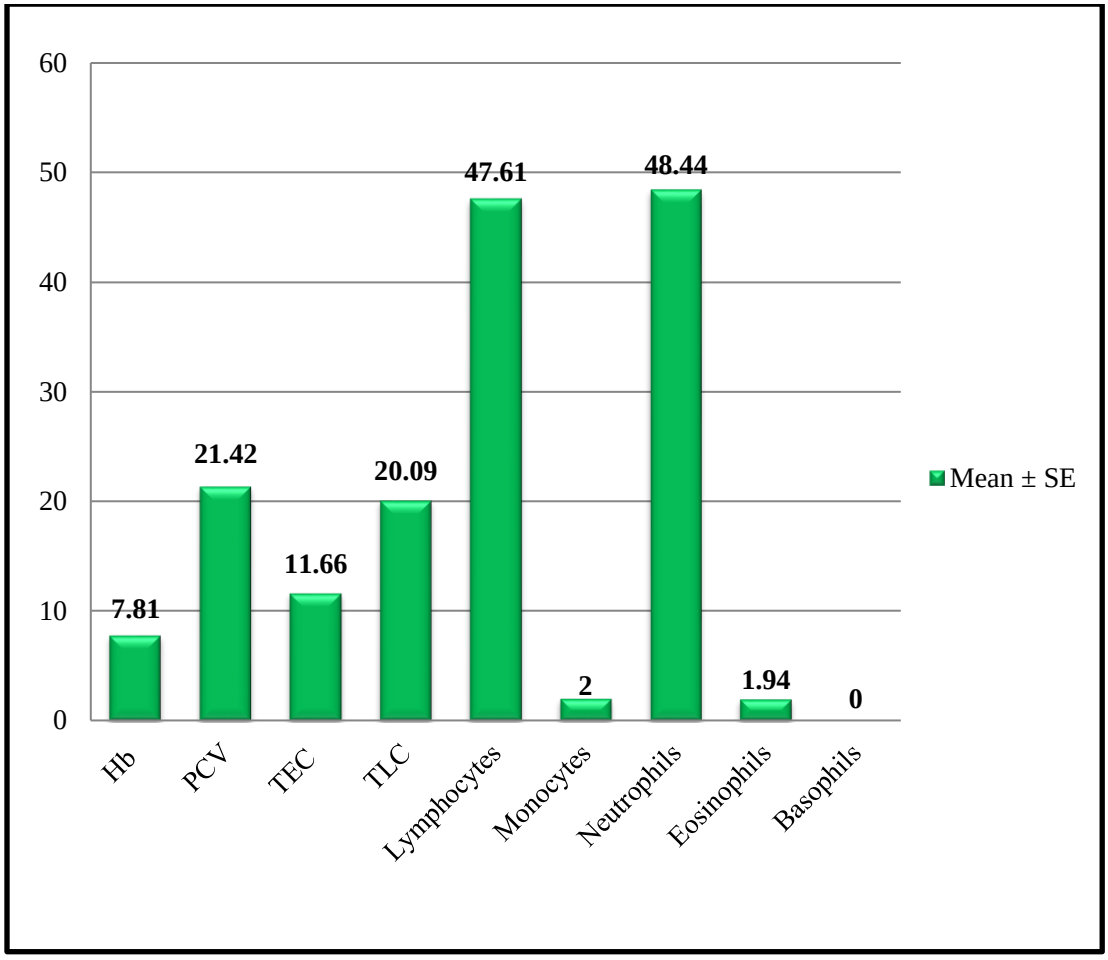

Figure 4.6: Results of haematological analysis in brucella sero-positive goats

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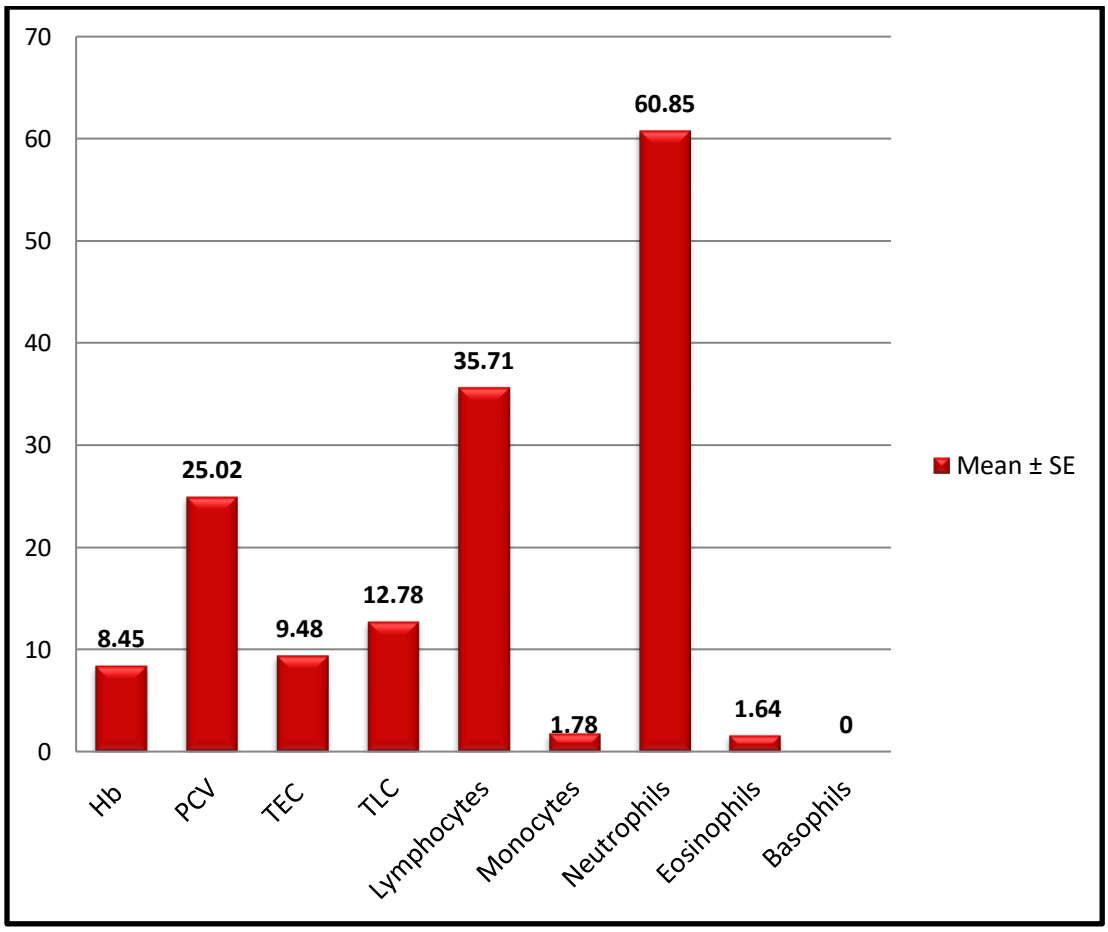

Figure 4.7: Results of haematological analysis in brucella sero-positive sheep

SUMMARY AND CONCLUSIONS

CHAPTER V

SUMMARY AND CONCLUSIONS

Brucellosis is a major zoonotic disease affecting humans as well as in livestock. The prevalence of disease in the present study was found variable from region to region. The aim of the study was to focus diagnosis of brucellosis in small ruminants thus helping to inculcate preventive measures to control the spread of disease.

In the present study, a total 300 animals (226 goats and 74 sheep) included from the flocks of small ruminants from Shirwal, Dahiwadi and Baramati. Serum samples collected were screened by using Indirect-Enzyme Linked Immunosorbent Assay (i-ELISA). The overall sero-prevalence was recorded at 10.66%; species wise overall sero-prevalence was 7.96% in goats and 18.91% in sheep, respectively.

Highest sero-prevalence was observed from Dahiwadi region 16% followed by Baramati region 9% and 7% in Shirwal region, respectively. Species wise the sero-prevalence in goats of these regions were 10.44%, 7.86% and 5.71%, respectively; whereas, the sero-prevalence in sheep of these regions were 27.27%, 18.18%, and 10%, respectively when subjected to i-ELISA.

Age wise sero-prevalence in these animals was calculated. A high percentage of overall sero-prevalence in small ruminants was observed in the age group above 6 years as 13.72% followed by 3-6 years age group as 12.06% and 0.5-3 years age group as 5.33%. Age wise the sero-prevalence in goats of these groups were 10.25%, 9.16% and 3.57%, respectively; whereas, the age wise sero-prevalence in sheep of these groups were 25%, 20.93% and 10.25%, respectively in above mentioned age groups, when subjected to i-ELISA. The present study shows that adult age group of goats and sheep were more susceptible than young age group of goats and sheep.

In present study, the overall sex wise sero-prevalence in small ruminants was found higher in males as 17.77% when compared to females as 10.66%. Species wise sero-prevalence in goats was observed higher in males as 16.12% when compared to females 6.66% and in case of sheep it was also higher in males as 21.42% when compared to females as 18.33%.

A total 32 sero-positive animals were screened for molecular detection of *brucella genus* by Polymerase Chain Reaction (PCR). Blood DNA was isolated from these 32 sero-positive animals for the detection of bcsp 31 gene using primers B4/B5. Six samples were found positive producing 223 bp amplicon.

The hematological alterations in infected animal are useful for the therapeutic management of disease. The mean Hb, PCV, TEC, TLC values found in the cases which were sero-positive for brucellosis by i- ELISA were 7.81 ± 0.41 g/dL, 21.42 ± 1.01%, 11.66 ± 1.22×10^6 /µl, 20.09 ± 1.86×10^3/ µl, respectively in case of goats and 8.45 ± 0.32 g/dL, 25.02 ± 1.11%, 9.48 ± 0.52×10^6 /µl, 12.78 ± 1.85×10^3/ µl, respectively in case of sheep.

The average values of Lymphocyte, Monocytes, Neutrophil, Eosinophils and Basophils found in the cases which were sero-positive for brucellosis by i-ELISA were 47.61 ± 4.32%, 2 ± 0.29%, 48.44 ± 4.53%, 1.94 ± 0.22% and 0%, respectively in case of goats and 35.71 ± 4.47%, 1.78 ± 0.28%, 60.85 ± 4.71%, 1.64 ± 0.26% and 0%, respectively in case of sheep.

The haematological values in the cases positive by both i-ELISA and PCR revealed anemia in all 6 (100%) cases, leucocytosis was found in 3 (50%) cases and 3 cases 50% shows normal leucocyte count, 4 (66%) cases showed lymphocytosis while, in 2 (33%) cases showed normal lymphocyte count, 3 cases (50%) had neutropenia and 3 cases (50%) showed normal neutrophil count, 5 (83%) cases showed normal monocytes count and 1 (16%) case had monocytosis when compared with normal reference values.

Thus, it may be concluded from the above study that,

- The overall sero-prevalence observed was 10.66% with highest sero-prevalence was observed from Dahiwadi region 16%.
- The prevalence of brucellosis was recorded highest in the age group above 6 years as 13.72% and high sero-prevalence was noticed in males as 17.77% when compared to females.
- PCR method was found useful in molecular detection of Brucellosis by isolating blood DNA.
- The haematological indices in small ruminants were valuable to find assessment of disease condition.
- To prevent economic losses due to brucellosis the surveillance and monitoring of the diseases is important. Effective vaccination should be done in endemic areas to prevent and control incidence rate.

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THESIS ABSTRACT

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a)	Title of the thesis	:	SERO PREVALENCE OF BRUCELLOSIS IN SMALL RUMINANTS
b)	Full name of student	:	Bait Kaustubh Suresh
c)	Name and address of Major Advisor	:	Dr. Milind D. Meshram Professor, Department of Veterinary Epidemiology and Preventive Medicine, Krantisinh Nana Patil College of Veterinary Science Shirwal, Dist. Satara
d)	Degree to be awarded	:	M. V. Sc.
e)	Year of award of degree	:	2019
f)	Major subject	:	Veterinary Epidemiology and Preventive Medicine
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ABSTRACT

Brucellosis is a highly contagious disease in small ruminants and having zoonotic importance but remains a neglected disease in India. The present study entitled, "Sero-prevalence of brucellosis in small ruminants" was undertaken to detect the prevalence of brucellosis. Total 300 animals were included from the flocks of small ruminants having history of abortion, retention of placenta, metritis and swelling of the testicles from Shirwal, Dahiwadi and Baramati region. Blood and serum samples were collected. Sero-prevalence was calculated by using i-ELISA and confirmation of sero-positive cases was done by PCR.

Out of 300 animals 32 were positive by i-ELISA and the overall sero-prevalence was found to be 10.66% with highest sero-prevalence was observed from Dahiwadi region as 16%. Maximum number of sero-positive cases found in the age group above 6 years as 13.72% and high sero-prevalence was noticed in males as 17.77% when compared to females. Sero-positive animals were screened for molecular detection of *brucella genus* by Polymerase Chain Reaction (PCR). Blood DNA was isolated from these 32 sero-positive animals for the detection of bcsp 31 gene using primers B4/B5. Six samples were found positive producing 223 bp amplicon. Haematological study in animals which were positive by i-ELISA and PCR revealed anemia, leucocytosis, lymphocytosis and neutropenia.

The small ruminants are considered as poor farmer's economic backbone. This present study will help the farmers to abide preventive measures to lower the incidence of brucellosis in their flocks and also act as a guideline to field Veterinarians to know the distribution pattern of disease.

प्रबंध सारांश

अ)	प्रबंधाचे शीर्षक	:	ब्रुसेलोसीस या आजाराचे शेळ्या मेंढ्यामधील प्रमाण
ब)	विद्यार्थ्यांचे पूर्ण नाव	:	बाईत कौस्तुभ सुरेश
क)	मार्गदर्शकाचे नाव व पत्ता	:	डॉ. एम. डी. मेश्राम प्राध्यापक पशुवैद्यकीय साथरोग व प्रतिबंधात्मक औषध शास्त्र विभाग क्रां. ना. पा. पशुवैद्यकीय महाविद्यालय शिरवळ जि. सातारा
ड)	पदवीचे नाव	:	एम.व्ही. एस्सी
इ)	पदवीचे वर्ष	:	२०१९
फ)	विषय	:	पशुवैद्यकीय साथरोग व प्रतिबंधात्मक औषध शास्त्र
ग)	प्रबंधातील एकूण पाने	:	५५
ह)	प्रबंध सारांशामधील एकूण शब्द	:	२५०
म)	विद्यार्थ्यांची स्वाक्षरी	:	
भ)	सादरकर्ता अधिकाऱ्याची स्वाक्षरी नाव व पत्ता	:	डॉ. एम. डी. मेश्राम प्राध्यापक पशुवैद्यकीय साथरोग व प्रतिबंधात्मक औषध शास्त्र विभाग क्रां. ना. पा. पशुवैद्यकीय महाविद्यालय शिरवळ जि. सातारा

सारांश

ब्रुसेलोसीस हा शेळ्यामेंढ्यांमधील एक संसर्गजन्य आजार आहे जो प्राण्यांपासून माणसांना संक्रमित होतो. पण भारतात हा एक दुर्लक्षित आजार

आहे. सदर अभ्यास हा "ब्रुसेलोसीस या आजाराचे शेळ्यामेंढ्यांमधील प्रमाण" या विषयावर करण्यात आला आहे आणि त्या आजाराची व्याप्ती शोधण्यात आली आहे. शिरवळ, दहिवडी आणि बारामती या ठिकाणांवरील गर्भपात, नाळेची धारणा, गर्भाशय दाह आणि अंडकोश सूज असलेल्या ३०० शेळ्या,मेंढ्याची अभ्यासासाठी निवड करण्यात आली. रक्त आणि रक्तजल नमुने गोळा करण्यात आले. आजाराची व्याप्ती आय इलायसा चाचणीने काढण्यात आली व पीसीआरद्वारे पुष्टी केली गेली.

३०० जनावरांपैकी ३२ जनावरे आय इलायसाद्वारे आजाराला बाधित आढळली. एकूणच आजाराची व्याप्ती १०.६६ टक्के होती. व त्यामध्ये दहिवडीमध्ये सर्वाधीक १६ टक्के आजाराची व्याप्ती आढळली. सहावर्षांवरील शेळ्यामेंढ्यामध्ये सर्वात जास्त १३.७२ टक्के आजाराची व्याप्ती आढळली. नरांमध्ये माद्यांच्या तुलनेत जास्त १७.७७ टक्के इतकी आजाराची व्याप्ती आढळली. पीसीआरद्वारे ब्रुसेला वंशाचे आण्विक शोधण्यासाठी आय इलायसाद्वारे आजाराला बाधित असणाऱ्या ३२ जनावरांचे परीक्षण केले गेले. त्यांच्या रक्तामधून डीएनए वेगळा करण्यात आला व बी४/बी५ प्रायमरने बीसीएसपी ३१ जनुकांची तपासणी केली असता ६ नमुने २२३ बिपी ऑप्लिकॉन उत्पादन करणारे सकारात्मक आढळले. आय इलायसा आणि पीसीआरद्वारे ब्रुसेला आजाराला बाधित असणा-या जनावरांची रक्ततपासणी केली असता त्यात अशक्तपणा, ल्युकोसाइटोसीस, लिम्फोसाइटोसीस आणि न्यूट्रोपेनिया आढळला.

शेळी-मेंढीपालन हा शेतकऱ्याचा आर्थिक कणा मानला जातो. वरील अभ्यास हा शेतकऱ्यांना त्यांच्या कळपातील ब्रुसेलोसीसचा प्रादुर्भाव कमी होण्यास

व प्रतिबंधात्मक उपाय करण्यास मदत करेल. शिवाय हा अभ्यास पशुवैद्यकांना या आजाराच्या वितरणाची पद्धत समजण्यास मार्गदर्शन करेल.