SEROEPIDEMIOLOGY OF CONTAGIOUS AGALACTIA AND BLUETONGUE IN SHEEP AND GOATS

Thesis

Submitted to the Guru Angad Dev Veterinary and Animal Sciences University in partial fulfillment of the requirements for the degree of

MASTER OF VETERINARY SCIENCE
in
VETERINARY MEDICINE
(Minor Subject: Veterinary Public Health and Epidemiology)

By

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CERTIFICATE – I

This is to certify that the thesis entitled, **“Seroepidemiology of Contagious Agalactia and Bluetongue in Sheep and Goats”** submitted for the degree of **M.V.Sc.** in the subject of **Veterinary Medicine** (Minor Subject: **Veterinary Public Health and Epidemiology**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Manjot Singh (L-2013-V-34-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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ABSTRACT

The present study was conducted to determine the seroprevalence of contagious agalactia and bluetongue in sheep and goat in different agro-climatic zones of Punjab state and to identify the risk factors associated with these diseases. The serum samples from animals were analyzed with ELISA for presence of antibodies to *Mycoplasma agalactiae* and bluetongue virus. Both diseases were evaluated for potential risk factors such as species (goat vs. sheep), sex (female vs. male), feeding pattern (grazing vs. stall feeding), stage of lactation (no lactating, lactation <1.5 month, 1.5-3 months, and >3 months), age (<1 year, 1-3 years, 3-5 years and >5 years), agro-climatic zone (submountainous, central and southwestern regions). For studying seroprevalence of contagious agalactia, a total of 323 animals were selected from 32 flocks. An overall apparent prevalence of 2.79% for contagious agalactia was observed. Presence of contagious agalactia revealed no significant association with species, sex, feeding pattern, stage of lactation, and age of animals. Higher prevalence was observed in submountainous zone (5.56%) as compared to central districts (3.01%) and southwestern districts (1.47%), although the difference was statistically insignificant. On the other hand, for study of seroprevalence of bluetongue, a total of 368 animals were selected from 37 flocks. The overall apparent prevalence of bluetongue disease was 52.99%. No significant association was found between bluetongue seropostivity and species of animal. The prevalence of disease was not found to (chi square value = 1.725, p>0.05) vary with respect to sex of animals (male-60.65% vs. female-51.46%). No difference was observed with respect to feeding pattern, age of animals or stage of lactation. The seroprevalence of bluetongue in (central region-64.39%) was significantly higher as compared to (submountainous region-51.85%) and (southwestern region-45.05%).

Keywords: Bluetongue, Contagious Agalactia, ELISA, Prevalence, Seroepidemiology

Signature of Major Advisor | **Signature of the Student**

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LIST OF ABBREVIATIONS

°c	:	Degree Celsius
BT	:	Bluetongue
BTV	:	Bluetongue virus
CA	:	Contagious agalactia
cELISA	:	Competitive –enzyme-linked immunosorbent assay
DIA	:	Dot immunobinding assay
DNA	:	Deoxyribonucleic acid
ELISA	:	Enzyme-linked immunosorbent assay
iELISA	:	Indirect- enzyme-linked immunosorbent assay
IgG	:	Immunoglobin G
M. agalactiae	:	*Mycoplasma agalactiae*
OD	:	Optical density
PCR	:	Polymerase chain reaction
PPLO	:	Pleuropneumonia like organism
RNA	:	Ribonucleic acid
SAT	:	Slide agglutination test

CHAPTER – I

INTRODUCTION

Small ruminants constitute significant share of the domestic animals involved in production of food for human consumption. They are able to utilize the ligno-cellulosic materials and convert them to animal products of high nutritional value viz., meat and milk. Moreover they do not compete with human and monogastric livestock species for feed resources. Sheep and goat production is an integral component of rural economy of India and serve as a major source of economic sustenance for weaker segments of the society in the hot semi arid and arid region. These species are traditionally reared by small and marginal farmers and landless laborers under extensive range management with top feed supplementation during lean season. Importance of small ruminants in Indian economy lies in their smaller size, which is easy to graze and manage.

As per Livestock census 2007, India had 71.5 sheep and 140.5 million goat population. As per literature (Karim 2008, Karim and Sankhyan 2009), the slaughter rate of sheep and goats is 32% and 36%, hence a total of 23.68 and 55.44 million heads are slaughtered annually. Small ruminants play an important role in income generation, capital storage, employment generation and improving household nutrition. In addition, goat milk is considered to be the healthiest because of its smaller fat globules and protein content which are easily digestible.

Infectious diseases play an important role in rearing and management of sheep and goats. Among the infectious diseases affecting sheep and goat, bluetongue and contagious agalactia are considered serious in nature, because of their effect on production of small ruminants and costs spent on their treatment and control.

Bluetongue is an infectious, non-contagious, arthropod borne, viral disease of domesticated and wild ruminants caused by bluetongue virus (BTV), a type specific virus of the genus *Orbivirus* in the family Reoviridae. Goats are major vertebrate hosts of the virus, but sheep usually exhibit clinical disease characterized by fever, depression, nasal discharge, drooling of saliva, oral lesion, facial edema, hyperemia of coronary bands and muscle weakness (Afshar 1994). At present, 26 serotypes have been reported throughout the world (Maan *et al* 2011). Twenty-one out of 26 serotypes (except 19, 22, 24, 25 and 26) have been reported from different states of

India (Rajkhowa *et al* 2008, Joardar *et al* 2009, Joardar *et al* 2012). BTV has now been identified on all continents except Antarctica (Gibbs and Greiner 1994). The first outbreak of bluetongue in India was recorded in 1964 among sheep and goats in Maharashtra State (Sapre 1964).

BTV is transmitted by biting of blood-feeding insect vectors of the genus *Culicoides* spp. (Diptera: Ceratopogonidae) (Mellor *et al* 2000). Though venereal transmission between ruminants has been recorded but occurs very rarely and it is not considered to be of epidemiological significance (Bishop *et al* 2000). Bluetongue typically occurs when susceptible animal species are introduced into areas with circulating virulent BTV strains, or when virulent BTV strains extend their range to previously unexposed populations of ruminants (Mahdavi *et al* 2006). Some strains of the virus can result in mortality rates as high as 70% in susceptible sheep (Breard *et al* 2004, Mozaffari *et al* 2010). Goats and cattle are considered as reservoir hosts (Maclachlan *et al* 2008).

The exact worldwide economic losses due to bluetongue has been estimated as $3 billion annually due to death, abortions, weight loss, reduced milk yield, meat efficiency, export restrictions for live animals, their semen and some products such as fetal bovine serum (Mozaffari and Khalili 2012). Various techniques have been used to detect antibodies against BTV. Agar gel immuno diffusion and competitive enzyme linked immuno sorbent assay (c-ELISA) are recommended as prescribed tests for International Trade in the OIE Manual of Standards for Diagnostic Test and Vaccines (Breard *et al* 2004).

Contagious agalactia is known to occur in small ruminants since two centuries. At present, the disease is prevalent in sheep and goats in countries where they are more densely populated such as the Mediterranean and the Balkan Peninsula in Europe, West Asia and Southwest Asia and North, Central and Eastern Africa. Contagious agalactia is a highly infectious disease of sheep and goats included in the OIE enlisted diseases. The disease spreads by ingestion of feed, water, or milk contaminated with infected milk, urine, feces, or nasal and ocular discharges. Transmission may also occur by direct entry to the teat opening at milking or by inhalation of contaminated dust. Animals with subclinical or chronic infections can carry and shed the mycoplasmas for months, and the organisms can survive in the

supramammary lymph nodes from one lactation to the next. Contaminated fomites can also transmit the organisms between premises. The morbidity rate of contagious agalactia disease is about 30-60% in a herd and the mortality rate may reach 15-20% in adult animals (Pooladgar 2014).

The disease caused by *Mycoplasma agalactiae,* is recognized by elevated temperature, inappetence and alteration in the consistency of the milk in lactating ewes with decline and subsequent failure of milk production within 2–3 days as a result of interstitial mastitis, lameness and keratoconjunctivitis affecting about 5–10% of infected animals. Fever is common in acute cases and may be accompanied by nervous signs; however, both these signs are rare in the more frequently observed subacute and chronic infections. Pregnant animals may also abort.

Bluetongue and contagious agalactia are traditionally diagnosed by culture, indirect heamagglutination, immunoblotting or phenomenon like growth inhibition; however these tests are not preferred due to delayed results. Moreover, for screening purposes ELISA is considered to be test of choice amongst all the serological assays because of its sensitivity and specificity. In addition to screen large number of samples or to determine presence/absence of disease in a farm or region, ELISA is preferred test.

These diseases are not much studied and keeping in view the considerable loss they cause, it is important to study the seroprevalence of these diseases in ovine and caprine species and to know the status in the population using active surveillance. Thus the present study was carried out with following objectives.

1. To study the seroprevalence of contagious agalactia and blue tongue in sheep and goat population of Punjab.
2. To identify risk factors associated with seroprevalence of these agents.

CHAPTER – II

REVIEW OF LITERATURE

2.1 Contagious agalactia

Contagious agalactia is a highly infectious disease of sheep and goats enlisted in OIE listed diseases. *Mycoplasma agalactiae* is the major causal agent of the disease in both sheep and goats. The infection is endemic in different regions of world, manifested by mastitis, arthritis, keratoconjunctivitis, respiratory problems and mortality of young animals. The diagnosis is based on the conventional methods, such as culture, molecular diagnostics and, routine serological and immunoenzymatic methods. In endemic regions, treatment with antibiotics or vaccination of infected animals is used. In developed countries, the disease in affected herds is controlled by elimination of infected animals or by killing them all at once.

2.1.1 Prevalence

Madanat *et al* (2002) investigated the presence of antibodies against *M. agalactiae* in herds of small ruminants on eighty serum samples collected from 60 sheep and 20 goats in the Czech Republic and 137 samples collected from 78 sheep and 59 goats in Jordan. Of the 137 Jordan sera, eight gave positive reactions, 11 reactions were dubious and 117 were negative, while all sera from the Czech animals showed negative reactions.

De la Fe *et al* (2005) conducted a microbiological survey for *Mycoplasma* spp. between 2001 and 2002 in 28 goat herds in Gran Canaria, Spain, an area where contagious agalactia is endemic. There were a total of 38.5% positive flocks from which 37 mycoplasma isolates were obtained. The results showed that the large colony variant of *M. mycoides* subsp. *mycoides* (*Mmm LC*) was the most commonly isolated agent associated with contagious agalactia. This species was isolated from 90% of the positive herds and accounted for 54.1% of all isolations. *M. agalactiae* was isolated from 40% of the positive herds (27% of isolations) and in six herds *M. arginini* was isolated (18.7% of isolations). Neither *M. capricolum* nor *M. putrefaciens* strains could be isolated.

Zendulkova *et al* (2004) carried out an epidemiological survey to find presence of contagious agalactia in herds in the Czech Republic and Jordan. A total of

99 animals were examined for the presence of *M. agalactiae* antigen (64 from the Czech Republic and 35 from Jordan). Biological materials for examination included 353 swabs (sheep 133, goats 220) collected from conjunctival, nasal and vaginal mucosae and the external ear canal. A monoclonal antibody-based sandwich ELISA could detect the antigen in 11 animals, was ambiguous in 10 animals and negative in the rest of animals.

Azevedo *et al* (2006) reported two outbreaks of contagious agalactia caused by *M. agalactiae* in Paraíba State, Northeastern Region of Brazil. Morbidity ranged from 26.1% to 100% in does, 36.5 to 100% in kids and 49% in lambs. In first farm, mortality was 14.3% in the lactating goats and 6.4% in the kids. In the other, 3.3% of the does, 36.5% of the kids and 22.9% of the lambs died and 84 affected goats were euthanized to control the disease. *M. agalactiae* was isolated from milk, joint exudates, nasal swabs and ear washings. The colonies were characteristic of Mycoplasma, did not ferment glucose and arginin and were confirmed as *M. agalactiae* by immunoperoxidase and PCR.

De la Fe *et al* (2007) reported several outbreaks of caprine contagious agalactia (CCA) in a number of flocks on the island of Lanzarote, Canary Islands, Spain. Clinical and subclinical mastitis in lactating goats and some cases of arthritis and pneumonia in kids were observed in the affected flocks. *M. capricolum* subsp. *capricolum* was isolated as the main causal agent of the outbreaks, along with *M. mycoides* subsp. *mycoides* ‘‘large colony type’’ (Mmm LC) in two flocks.

Khezri *et al* (2014) conducted a study between 2012 and 2013 on 189 small ruminants with CA signs in endemic area. Milk, synovial fluid, ear swabs, conjunctival swabs and nasal swabs were examined by PCR. *Mycoplasma* spp. was detected in 76.2% and *M. agalactiae* isolated from 16% of positive samples. *M. agalactiae* were isolated from 7 conjunctival swabs, 15 milk samples and one synovial fluid sample. Results proved that *M. agalactiae* was not the main cause of CA in small ruminants in Iran.

Lambert (1987) concluded that though contagious agalactia has long been observed in most Mediterranean countries, yet no continent is presently free of the disease. In countries like Australia, Sweden, Federal Republic of Germany, Great Britain and Canada, there have been only sporadic cases, or just a single outbreak that

was controlled successfully. Madanat *et al* (2001) revealed that a severe course of the contagious agalactia in herds can result in the death of lambs and kids (up to 40-70%). In rearing units, the losses may reach to 15-20%.Azevedo *et al* (2006) found 26.1 to 100% morbidity in goats and, approximately 50% in lambs and revealed *Mycoplasma agalactiae* by immune-peroxidase and PCR.

Kumar *et al* (2011) conducted a study on 358 samples (215 nasal discharges, 6 lung tissue, 4 pleural fluid, 5 joint fluid, 60 milk, 22 preputial swabs, 16 conjunctival swabs, 5 ear swabs and 25 vaginal discharges) for cultural isolation *Mycoplasma species* were isolated in 8.35% samples and identified as *M. mycoides, M. capri* and *M. capricolum* using PCR and PCR-RFLP.

Kashoo *et al* (2011) found the prevalence of CA to be 32.03% and 20.3% in female animals and 13.48% and 6.67% in male animals using SAT and ELISA, respectively.

Chessa *et al* (2009) developed a DNA vaccine against contagious agalactia encoding the P48 of *M. agalactiae* and evaluated specific immune responses elicited in BALB/c mice. Both total IgG and IgG1 were detected in vaccinated mice. Proliferation of mononuclear cells of the spleen, levels of gamma interferon, interleukin-12, and interleukin-2 mRNAs were enhanced in immunized animals

2.1.2 Clinical signs

M. agalactiae can affect both sheep and goats of either sex. The incubation period varies from one week to two months depending upon the virulence of organisms, and immune status of the animal (Razin *et al* 1998). *M. agalactiae* can produce acute, subacute, or chronic form of disease. In some animals atypical or asymptomatic forms have also been reported (Nicolet *et al* 1994, Bergonier *et al* 1996, Zendulkova *et al* 2007). Common clinical symptoms include fever, anorexia, lethargy, and lagging the herd, followed by the clinical symptoms depending upon the involvement of organs such as mammary glands, lungs, genitalia, joints, and conjunctiva. Rare abortions in pregnant animals have also been reported (Kizil and Ozdemir 2006, Gil *et al* 2003). Fever is common in acute cases and may be accompanied by nervous signs, but both signs are rare in the more frequently observed subacute and chronic infections.

At the beginning, the udder is usually cattarhal or parenchymatous mastitis. It is hot swollen and tender and later becomes flaccid, filled with connective and eventually become atrophic. Milk changes into a yellowish colour with a salty taste. It may have a watery consistency and separates into an upper, grayish blue and a lower yellowish green layer with clots. Milk gradually becomes purulent and, at the final stage, its production ceases.

Arthritis usually involves the carpus or tarsus joints. There are swollen and painful joints with accumulation of synovial fluid. Chronic involvement of joints and severe losses to cornea may lead to lameness along with inability to walk or stand and blindness, respectively (Mega *et al* 1983, Kwantes and Harby 1995). The conditions like pleurisy, arthritis, pneumonia, keratoconjunctivitis, and mastitis usually result from infection with *M. mycoides* too because this organism has one of the widest geographical distributions and is found wherever contagious agalactia is reported (da Massa 1983).

In sheep and goat, the signs usually become manifested shortly after parturition and lactating animals develop mastitis accompanied by yellowish green milk secretion. Ocular involvement may be found in only about 50% of cases. Lameness, which is common and may persist for a long time is more frequent in males than females.

2.1.3 Diagnosis

Kashoo *et al* (2011) compared applicability of 3 serological tests, slide agglutination test (SAT) with coloured antigen, indirect haemagglutination test (IHA) and enzyme-linked immunosorbent assay (ELISA). 493 Serum samples from goats of various parts of Uttar Pradesh and Uttrakhand were screened for CA by SAT, IHA and ELISA. ELISA was found to be a good serological test for diagnosis of CA. However, SAT can also be used in field due to its simplicity, less time consuming and easy to perform under field conditions. Further, 74% sensitivity and 96% specificity was observed in SAT and 56% and 92% of sensitivity and specificity in IHA compared with ELISA.

Imada *et al* (1987) conducted the indirect immunoperoxidase test for rapid identification of mycoplasmas. Colonies of type strains of 22 mycoplasma species, 3 acholeplasma species, and three Ureaplasma diversum serogroups were stained. All

49 isolates from bovine materials and cell cultures were easily identified by this test, and the results agreed with those obtained by growth inhibition test. Use of filter paper made it possible to add different kinds of antisera or conjugates to the same agar plate simultaneously and also to save antiserum and conjugate. The test proved to be a simple and useful technique for rapid identification of many *Mycoplasma* species grown on agar medium.

Greco *et al* (2001) optimized a multiplex polymerase chain reaction assay for the simultaneous detection of several species of small ruminant mycoplasmas. Two sets of oligonucleotide primers specific for *M. agalactiae* and *Mycoplasma 'mycoides'* cluster were used in the test. The multiplex-PCR was able to amplify a 375-bp fragment of *M. agalactiae* chromosomal DNA and 257-260-bp fragment of *M. mycoides* cluster chromosomal DNA. Four reference strains (*M. agalactiae*, *M. mycoides* subsp. *mycoides* LC, *M. capricolum* subsp. *capricolum*, *M. mycoides* subsp. *capri*) and 56 samples (44 milk samples, 2 nasal swabs, 6 ocular swabs, 3 vaginal swabs and 1 sample of fibrinous exudate from carpal joint), from sheep and goats with clinical signs of contagious agalactia, were examined. The multiplex PCR confirmed the identification of reference strains and identifed, of the 43 positive samples examined, 35 *M. agalactiae* strains, 12 *M. mycoides* cluster strains, 4 mixed *M. agalactiae* and *M. mycoides* cluster.

Abtin *et al* (2013) conducted a study for isolation and identification of *M. agalactiae* using culture from sheep of Qom province in Iran. A total of 102 samples collected from milk secretion, eye, ear and joint exudates of sheep, were cultured in supplemented PPLO broth DNAs were extracted by phenol/chloroform method and the PCR assay was applied for detecting 163bp fragment of *Mycoplasma* genus targeting 16S rRNA gene and 375bp fragment of lipoprotein gene in *M. agalactiae.* Out of the 102 samples, 19/18.63%) showed typical *Mycoplasma* colonies in PPLO agar *Mycoplasma* genus PCR was positive in 59 (57.8%) samples. Out of the 102 samples, 19 samples were positive both in the culture and PCR, 42 samples were negative both in the culture and PCR. 40 samples were negative in the culture and positive in PCR whereas only one sample was positive in culture and negative in PCR.

Kheirkhah *et al* (2011) carried out a study to detect *M. agalactiae* by culture and PCR from infected goats in Iran. A total of 57 samples were taken from

conjunctiva (n=11), joint exudates (n=35) and milk secretion (n=11). Of the 57 samples, fried egg colonies characteristic of *Mycoplasma* spp. appeared in 9 samples (16%) on the agar media. The PCR with mycoplasmal 16S rRNA was applied for detection of a variety of *Mycoplasma* species. PCR identification of genus was successful in 31 isolates (54%), of these 19 (61%) were positive for *M. agalactiae*. Of the total samples, culture and genus PCR tests were positive only in 3 samples and negative in 20 samples, 6 samples with typical colonies on the agar could not be detected by PCR and colonies were not observed in 28 PCR positive samples.

Sanna *et al* (2014) developed an immunomagnetic capture technique to detect viable *M. agalactiae* in routine milk samples using polyclonal antibodies against two *M. agalactiae* membrane surface proteins (P80 and P55) conjugated to magnetic beads. *M. agalactiae* cells were captured by a specific antigen–antibody reaction and magnetic separation.

Bidhendi *et al* (2011) conducted a study to isolate and identify *M. agalactiae* in milk samples in Kordestan province, Iran with culture and PCR. A total of 367 milk samples were collected from sheep and goat. Twenty (5.5%) out of 367 were positive in PPLO agar and 5 (25%) out of these isolates were positive in PCR. Four (75%) out of 5 isolates were from sheep and one (25%) from goat. Result of PCR on 367 milk samples showed 11 (3%) to be positive.

Tola *et al* (1997) developed a simple and rapid method for DNA extraction from sheep milk for PCR diagnosis of *M. agalactiae* and tested 357 samples from 21 newly infected flocks and 87 samples from 8 flocks infected in the past. PCR results were compared with those of conventional culture. By PCR 175 positives were detected as compared to 153 by culture.

2.1.4 Risk factors

Verbisck-Bucker *et al* (2008) investigated temporal, spatial, and host-related factors in the distribution of *M. agalactiae* infection from October 1996 to November 1998 and March 2002 to May 2003. The predisposing factors were sex (females), age (young animals), and metapopulation (Sierra Nevada) among the three established metapopulations.

Al-Momani *et al* (2008) carried out serological detection of *M. agalactiae* in 104 small ruminant flocks consisting of 18 sheep, 27 goat and 59 flocks containing

both sheep and goats in northern Jordan between 2002 and 2003. At least 5 serum samples per flock were tested using an indirect ELISA for antibodies to *M. agalactiae*. To increase the chances of detecting *Mycoplasma* spp, sick or older animals were sampled. There was no significant difference in the seroprevalence of *M. agalactiae* in sheep and goats at flock level. A total of 31 variables including production and health management practices were tested as risk factors for seropositive flocks and analyzed using logistic regression analysis. Risk factors for *M. agalactiae* seropositive flocks were using outside rams, improper cleaning of the milking utensils and separating young from dam.

2.2 Bluetongue

Bluetongue (BT) is an infectious, non-contagious, vector-borne viral disease affecting wild and domestic ruminants such as sheep, goats, cattle, buffaloes, deer, most species of African antelope and various other Artiodactyla. The disease inapparent in the vast majority of animals can result in fatal form in a proportion of infected sheep, deer and wild ruminants. The manifestations of bluetongue also depend on the serotype and strain of the virus and the species, breed and age of the infected animal, older animals being more susceptible (Elbers *et al* 2008). Bluetongue typically occurs when susceptible animal species are introduced into areas with circulating virulent BTV strains, or when virulent BTV strains extend their range to previously unexposed populations of ruminants (Zientara *et al* 2010).

Culicoides (the insect vector) transmit bluetongue virus (BTV) among susceptible ruminants following feeding on viraemic vertebrate hosts (Standfast *et al* 1985). In many parts of the world, infection has a seasonal occurrence. Bluetongue virus is a double stranded RNA virus in the genus *Orbivirus,* family *Reoviridae.* The genome is made up of 10 genes encoding mRNA for seven structural and three nonstructural proteins. Two major proteins, VP2 and VP5 are contained in the outer coat. The specificity of serotypes resides in VP2 protein (Mecham *et al* 1986). Proteins VP3 and VP7 make up the inner coat, while VP7 has also been shown to be the protein involved in virus attachment (Xu *et al* 1997). Currently, there are 24 different recognized serotypes of bluetongue virus distributed differentially worldwide (Becker 2008).

The worldwide economic losses due to bluetongue have been estimated to be \$3 billion a year (Tabachnick 1996, Tatem *et al* 2003). The losses include both direct (death, abortions, weight loss or reduced milk yield and meat efficiency) and, indirect losses as a result of export restrictions on live animals, their semen and some products such as foetal bovine serum. In cases of a wider spread of bluetongue, these measures could have a serious impact on the quantity of meat and animal products available for the consumer market, therefore, bluetongue is considered a potential biological weapon (Blancou and Pearson 2003, Zendulkova and Pospisil 2007).

2.2.1 Prevalence

2.2.1.1 International

Bluetongue (BT) first identified in South African Merino sheep in 1902, was classified as malarial catarrhal fever and later named "bluetongue" in 1905. The disease expanded beyond the continent of Africa by 1943 when an epidemic occurred in Cyprus. The first case in the United States was documented as a bluetongue-like illness of sheep in 1948 but the virus could be isolated in 1953 in California from a condition known as "soremuzzle" (McKercher *et al* 1953, Price and Hardy 1954). Later on, epidemics spread throughout Portugal and Spain resulting in devastating case fatality rate ranging from 70-80% (Greiner *et al* 1990).

Avci *et al* (2014) collected a total of 168 serum samples from yaks in Kyrgyzstan between September to November 2012. Antibodies to BTV in 2.38% sera were detected using a commercially available cELISA. During the same year, Hasanpour *et al* (2014) conducted a study on 198 sheep blood samples from 20 sheep flocks in Tekab city in Iran and found 35.9% to be positive with positivity of 26.9% and 37.2% in males and females, respectively. The difference in prevalence of antibodies in serum between male and females was found to be significant.

Mahmoud and Khafagi (2014) conducted study on clinically healthy as well as suspected sheep and goats during the vector breeding season from September to November 2010 in 14 different geographical regions of Egypt. Out of 1293 animal serum samples (sheep-1028 and goats-265) tested by Agar Gel Immuno-precipitation Test (AGPT), 17.5% of sheep and 14.7% of goats serum samples were found to be positive. The overall prevalence of anti-BT antibodies in different regions was 16.9%.

Khezri and Azimi (2013) tested 996 sheep sera collected from 8 provinces in Iran for BTV using specific c-ELISA with overall seroprevalence of 34.93%, with the highest and lowest prevalence seen in West-Azerbaijan (64.86%) and Qom (12.1%) areas respectively. The results demonstrated a high prevalence of Bluetongue antibodies in Iranian sheep, giving serological evidence of extensive exposure to Bluetongue virus infection in some provinces of the country.

2.2.1.2 India

In a serological survey in Punjab, India (Sodhi *et al* 1981), 6.64% sheep were found positive for BTV antibodies by the immunodiffusion test. The prevalence of BTV antibodies was observed to be higher in exotic breeds than in indigenous animals. An outbreak of BT in sheep and goats in Bidar, Gulbarga and nine other districts of Karnataka State has been reported (Srinavas *et al* 1982), where about 50% of the population was affected. The mortality ranged between 2% and 50%. A very severe outbreak occurred in eastern Maharashtra spreading to western Marathawada and affecting sheep in all districts of the region. The morbidity was as high as 80% in village flocks (Singh *et al* 1982). (Lonkar *et al* 1983) observed an outbreak of BT in sheep at the Central Sheep and Wool Research Institute (CSWRI), Avikanagar, Rajasthan and found Rambouillet and Merino breeds to be more susceptible than indigenous breeds. The morbidity rate in Merino and Rambouillet was 33.3% and 23.5%, respectively and mortality in adult Merino to be 35.3%.

Bandyopadhyay and Mullick (1983) reported BTV antibodies in sheep sera obtained from Haryana, Uttar Pradesh, Rajasthan and Andhra Pradesh. The incidence of BTV antibodies was higher in exotic sheep than in indigenous breeds. Mehrotra and Shukla (1984) detected BTV antibodies in sheep sera obtained from Maharashtra, Andhra Pradesh, Karnataka, Rajasthan, Jammu and Kashmir, and Himachal Pradesh. The prevalence of BTV antibodies ranged between 16.4% and 61.1% in different states. Kulkarni and Kulkarni (1984), in Maharashtra, isolated BTV serotypes 8 and 18 from sheep affected with BT in developing chicken embryos. Prasad *et al* (1992) reviewed the epizootology of bluetongue in India and found that out of the 24 known serotypes of BTV, 18 have been circulating in India.

Tigga *et al* (2015) reported that out of a total of 480 serum samples (sheep-190, goats-210 and cattle-80) screened in Jharkhand, 83 (43.68%) sheep, 91 (43.33%)

goat and 46 (57.50%) cattle sera were positive. The positivity ranged between 41% and 51% in different agro-climatic zones.

Joardar *et al* (2013) conducted a study on apparently healthy as well as suspected sheep, goats and cattle from different districts of Assam. Serum samples collected from various agro-climatic zones were screened for BT antibodies using indirect enzyme linked immunosorbent assay (iELISA). Out of total 313 animal serum samples screened (sheep-68, goat-195, cattle-50), 58.82% of sheep, 31.79% of goats and 70.00% of cattle serum samples were found positive. The prevalence of anti-BT antibodies in different agro climatic zones ranged between 31 and 50%.

Thakare *et al* (2012) investigated the seroepidemiological profile of BTV using cELISA test and found that 64.9% sheep and 61.5% goats to be positive for bluetongue antibodies with an overall seroprevalence of 62.7%. The prevalence of BTV antibodies varied from 61.6% to 65.5% in different age groups with the least prevalence in 1 to 3 years age group (61.6%) and the highest prevalence (65.5%) in animals above 3 years of age. Overall seroprevalence was higher in males of sheep (75.4%) than goats (60.4%). While in females, goats were more seropositive (62.5%) than sheep (54%).

De *et al* (2009) conducted serological studies in north southern and central regions of West Bengal and the antibody prevalence to blue tongue virus was found to range from 40 to 80%. Furthermore, Ravishankar *et al* (2005) screened a total of 1010 serum samples from the 14 districts within Kerala for the presence of group specific BTV antibodies by dot enzyme-linked immune-sorbent assay (dot ELISA). Positive samples were obtained from 12 of the 14 districts. The overall BTV antibody prevalence was 5.1 ± 1.9% (at 95% confidence level).

2.2.2 Clinical signs

Bluetongue in sheep is manifested as an acute, chronic or subclinical condition, fine wool breeds being most susceptible. An incubation period of 4-8 days (Tweedle and Mellor 2002) is followed by fever and hyperaemia of the lips and nostrils with excessive salivation and serous nasal discharge that is initially clear, then becomes mucopurulent and form a dry crust around the nostrils. Oedema of the tongue, lips, submandibulum and sometimes ears appears, petechiae develop on the

conjunctiva and ulcers on the oral mucosa. Cyanotic tongues are found rarely. At the end of the fever, affected sheep may have coronitis or laminitis and as a result, they stand with an arched back and are reluctant to move. Torticollis, dermatitis and breaks in the wool may also develop (Tweedle and Mellor 2002, Darpel *et al* 2007, Kirschvink *et al* 2009). Infection in pregnant ewes may lead to abortion, foetal mummification and the birth of weak calves with potential congenital defects (Tweedle and Mellor 2002, Saegerman *et al* 2011).

Goats are less frequently infected with BTV, and rarely show typical signs of clinical disease. However, the signs are similar but less severe than in sheep. In the 2006 epidemic in the Netherlands, the diseased goats showed a fever, sudden drop in milk production, oedema of the lips and head, nasal discharge and scabs on the nose and lips, erythema of the skin of the udder and small subcutaneous haemorrhagic lesions (Dercksen *et al* 2007).

2.2.3 Diagnosis

Definitive diagnosis of BTV infection relies heavily on laboratory techniques such as isolation and demonstration of BTV antigens, viral nucleic acids and antibodies. The virus can be isolated from the erythrocyte fraction of blood collected from affected animals during the febrile phase. Semen collected from male animals at the peak of viremia and tissues from affected animals and fetuses may also be used for BTV isolation. Isolation is by inoculation of embryonated chicken eggs with a subpassage onto BHK-21 or Vero cell lines (Gard and Kirkland 1993). In particular, the inoculation of embryonated chicken eggs and passaging through cell culture is the generally accepted method for testing of animals for export and other regulatory purposes. This is, however, a laborious and time-consuming protocol that may take up to 5 weeks for completion. Consequently, alternative methods of virus detection such as antigen capture ELISA, dot immunobinding assay (DIA), immunoelectron microscopy and PCR has been developed (Mecham *et al* 1990, McColl and Gould 1991, Mecham and Nunamaker, 1994 Shad *et al* 1997, Hawkes *et al* 2000). The use of antigen capture ELISA for the detection of BTV in the blood of infected ruminants has been unsuccessful (Mecham 1993), as it could detect antigen only in animals with

high viremias (Stanislawek *et al* 1996), or was not consistent enough to allow for the reliable diagnosis of BTV (Hawkes *et al* 2000). A major problem in the diagnosis of BTV infection by immunological methods is also the cross-reactivity with proteins from other orbiviruses (Lunt *et al* 1988), although this may be circumvented by the use of cELISA using a group-specific MAb (Afshar *et al* 1989).

2.2.4 Risk factors

The clinical manifestation of BTV infection varies considerably between species, breeds and virus strains. Serotype does not determine virulence as there are both highly virulent and benign strains within the same serotype (Kirkland and Hawkes 2004). Although BTV can infect many species of ruminants, clinical signs of disease are generally found in sheep (Erasmus 1990) Indigenous breeds are rarely affected,while some European breeds, appear to be particularly susceptible (Jeggo *et al* 1987, Veronesi *et al* 2005, Darpel *et al* 2007, Worwa *et al* 2010).

A variety of factors contribute to variation of transmission by the vector including altered susceptibility to BTV among *Culicoides* spp. and variation of environmental conditions (Gerry *et al* 2001). The most important environmental factor contributing to the greatest abundance of adult Culicoides is temperature range from 28-30°C.

Pascual-Linaza *et al* (2014) reported extensive management practices, large sheep farms and Culicoides abundance on farms as risk factors in naïve animals. However, after vaccination, other factors related to BTV reservoirs, such as the proximity of cattle farms or the introduction of cattle into farms were more significant. The Talaverana sheep breed seemed to be associated with a significantly higher risk of BTV-1 occurrence.

Mohammadi *et al* (2012) reported rate of positivity in goats and sheep as 74.2% and 72.9%, respectively. In contrast, the age of the positive group was significantly more than the negative group. However, in a seroprevalence study in the southeast of Iran the results showed that seroprevalence rates decrease with the increase in age in sheep herds (Mozaffari and Khalili 2012). In another study on bluetongue epidemic in Turkey, probability of infection was much less in sheep up to 2 years old (Taylor and Mellor 1994). Australian serotypes of the virus can affect sheep 3 years old or more, obviously there is a relationship between age of infected

animals and serotype of the virus (Radostits *et al* 2007). Probably the contact with the other herds increases the chance of exposure to the viral vectors which are living with the herds, because BTV is not transmitted by direct contact with the infected animals. Further ewes with and without abortion history had 60% and 77.9% seropositivity, respectively. In goats, those with abortion history had 78.6% and those without abortion history had 69.4% seropositivity.

CHAPTER – III

MATERIALS AND METHODS

This sections deals with the research methodology which includes sample collection, test procedures and statistical analysis. These have been described in the following subsections in detail.

3.1 Study area

Punjab is north-western state of India bordering Pakistan on the west and situated between the 29.30^{o}N to 32.32^{o}N latitude and 73.55^{o}E to 76.50^{o}E longitude. Climatically, the state has 5 seasons- spring (February- March), summer (April to June, maximum temperature 43.6^{o}C), rainy season (July- August), autumn (September to November) and winter season (December- January, minimum temperature 4.4^{o}C). Punjab state is broadly divided into 3 agro-climatic zones viz. Submountainous Region (annual rainfall 800-900mm), Central Plain Region (annual rainfall 500-800mm) and SouthWestern Region (<400mm) (Table 1).

Table 1: Districts in different agro-climatic zones of Punjab

S. No.	Zones	Districts
I	Submountainous region	Dhar block of Gurdaspur, Hoshiarpur, Ropar
II	Central plain region	Amritsar, Tarantaran, Gurdaspur, Jalandhar, Nawanshahar, Ludhiana, Fatehgarh Sahib, Patiala, Sangrur, Moga
III	Southwestern region	Ferozpur, Muktsar, Mansa, Bathinda, Faridkot, Barnala

3.2 Study population

Approximately 70% of human population lives in villages and agriculture is their main occupation. India has 200 million cattle, 76 million buffaloes, 110 million goats and 46 million sheep, of which Punjab has 2.64 million cattle, 6.17 million buffaloes, 0.41 million goats and 0.44 million sheep (www.husbandrypunjab.org).

3.3 Sample size calculation

The statistical calculation of samples size has been carried out using methods described by Sergeant (2015). The state of Punjab has 12000 villages with average population of 40 sheep/goats (Livestock Census 2007). The statistical calculations revealed that significant sample size should include 31 villages, and approximately 10% blood samples from each of the selected village.

3.4 Selection of flocks and animals for sero-surveillance

A computerized list of the villages of the state was used as sampling frame and 31 villages (from 3 agro-climatic zones) were selected from the state using simple random sampling, *without replacement* and *without stratification* with 'Random Village' programme of Survey toolbox (Cameroon 1999). Out of 31 villages, 37 flocks belonging to different sheep and goat farmers were selected.

3.5 Collection of whole blood and serum separation

A total of 368 sheep and goat blood samples from the 37 flocks belonging to different agro-climatic zones of Punjab, were collected with a maximum of 14 animals from a single selected flock. Blood samples (5ml) were collected from the jugular vein in serum separation vials; the sera were separated and stored at -20°C until they were tested for antibodies to bluetongue virus and *Mycoplasma agalactiae*.

3.6 Seroprevalence studies

The serum samples were analyzed with commercial ELISA kits (Bluetongue virus antibody test kit, cELISA, VMRD, *Mycoplasma agalactiae* antibody test kit, cELISA, IDEXX) to establish the seroprevalence of bluetongue and contagious agalactia. *M. agalactiae* antibody test kit allows detection of antibodies directed against lipoprotein p48 in ovine and caprine sera. The protein is a major membrane lipoprotein of *M. agalactiae* and is recognized early during infection.

3.6.1 Contagious agalactia

3.6.1.1 ELISA procedure

M. agalactiae antigen coated microplates were brought to room temperature (25°C) before use. Briefly, 190µl dilution buffer was dispensed into individual well of microplate followed by addition of 10µl each of undiluted negative (single well), positive control (in duplicate wells) and serum samples (in remaining wells). The

plate was kept on shaker for 5 minutes then covered with aluminium foil, incubated for 1 hour at 37°C. The plate was washed thrice with 300µl of wash solution. 100µl of diluted conjugate solution was added to each well, incubated for 30 minutes at 37°C and again each well was washed thrice with 300µl wash solution. Thereafter, 100µl of TMB substrate was dispensed into each well and incubated at 25°C for 20 minutes. The reaction was stopped by adding 100µl of stop solution to each well. After addition of stop solution, optical density was read immediately at 450 nm in iMark Microplate Reader (BIO-RAD) and results were calculated.

3.6.1.2 Interpretation of results

The assay was considered valid if mean optical density of positive controls was equal to or more than 0.350 and the ratio between positive control mean and negative control was equal to or greater than 3.50. S/P% value for individual test sample was calculated.

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

Where,

OD_{sample} = optical density of sample

OD_{NC} = optical density of negative control

$OD_{PC\,\bar{x}}$ = mean optical density of positive controls

The results for contagious agalactia were interpreted as below:

- Test samples were considered positive for contagious agalactia if S/P% was >60%.
- Test samples were considered suspect for contagious agalactia if S/P% was between 50-60%.
- Test samples were considered negative for contagious agalactia if S/P% was <50%.

3.6.2 Blue tongue

3.6.2.1 ELISA Procedure

The serum samples were analyzed with commercial cELISA kits (Bluetongue virus antibody test kit, VMRD) to establish the seroprevalence of bluetongue. Briefly,

25µl test serum samples along with positive and negative controls were loaded in individual well of antigen coated microplate and incubated for 15 minutes at room temperature (25°C). This was followed by addition of 25µl of antibody-peroxidase conjugate to each well. After incubation for 15 minutes at room temperature, the plate was washed thrice with 100µl of wash solution. 50µl of substrate solution was added and the plate was again incubated for 10 minutes at room temperature. The reaction was stopped by adding 50µl stop solution in all the wells. Finally, optical density was measured at 630nm in iMark Microplate Reader (BIO-RAD).

3.6.2.2 Interpretation of results

a. Test samples were considered positive if they produce an optical density <50% of the mean of the *negative controls.*
b. Test samples were considered negative if they produce an optical density >50% of the mean of the *negative controls.*

3.7 Risk factor studies

3.7.1 Risk factors

A standardized questionnaire for animals sampled at the farms was filled at the time of blood collection. A total of 368 samples from sheep and goat were collected from 37 flocks belonging to 31 villages. The presence or absence of disease was statistically correlated using appropriate statistical tools, with various risk factors, such as:

- Species
- Age
- Sex
- Stage of lactation
- History of mastitis, arthritis and conjunctivitis
- Geographical location (zones)
- Feeding pattern i.e. stall fed/ grazing.

3.7.2 Statistical analysis

True prevalence was calculated at 95% confidence interval (CI) using the 'True prevalence' program of survey toolbox, in which sensitivity, specificity and

sample size were taken into consideration (Cameron, 1999). The data was analyzed using SPSS (Statistical Package for Social Sciences) for Window version 11.0.1©SPSS Inc. USA computer software programs. The associations were evaluated between binary outcome variable and a variety of risk factors using chi square test.

3.8. Clinical outbreak of contagious agalactia in goats

3.8.1 Clinical history

A few affected goats were presented to University Clinics with history of mortality in kids and mastitis in adult lactating goats. The farmer had purchased two sheep about one month back which later on developed clinical signs of arthritis. The farm was located in district Barnala belonging to southwestern region of Punjab.

In total, the farmer had 90 goats comprising of 55 adult goats and 35 kids, out of which 15 kids had died over a period of 10 days.

3.8.2 Collection of blood samples

About 5-10ml of blood samples was collected aseptically in sterile vials through jugular vein-puncture from 47 animals of flock. The samples in the clot activating vials were allowed to clot and transported to laboratory immediately at 4°C and sera were separated by centrifuging the samples at 2500 rpm for 5 min. The separated sera were stored at -20°C till further use.

3.8.3 Collection of milk samples

Milk samples were obtained from the lactating animals from which the blood samples have been collected without any preservatives in sterile vials. For collection of milk samples, udder and teats of animals were cleaned well by brushing off any dirt, loose straw and mud etc. In case of need, the udder and teats were washed with water and dried completely using individual paper towel. The teat orifice was swabbed with 70% alcohol (spirit), and first few streaks of milk were discarded. Following, the 8-10 ml of individual quarter foremilk samples were collected in sterilized glass test tubes taking all the possible aseptic precautions. The collected milk samples were put into mobile refrigerator and transferred to the Mastitis Research Laboratory of the department for microbial analysis.

3.8.4 ELISA

M. agalactiae antibody test kit (IDEXX) was used for detection of antibodies directed against lipoprotein p48 as detailed in section 3.6.1.1

3.8.5 Isolation of *M. agalactiae*

Milk samples were inoculated directly on PPLO agar plates (HiMedia). The plates were allowed to dry and stored in the dark in an airtight canister at 37°C and examined for colony development from the fifth day of incubation. The colonies were observed under microscope for fried egg appearance. Individual colonies that had formed were picked using a Pasteur pipette and cultured in 500 µl PPLO broth (HiMedia).

3.8.6 Molecular Diagnosis

3.8.6.1 DNA extraction from milk

The genomic DNA was extracted from 10 milk samples according to the QIAamp DNA blood Mini kit protocol (QIAGEN). In brief, 200µl of milk was taken into 1.5 ml micro centrifuge tube containing 20µl of proteinase K (20mg/ml). To this, 200µl lysis buffer was added and vortexed immediately for 1 minute. The mixture was incubated at 56°C for 20 minutes, and the sample was vortexed in between for better lysis. Then, 200µl of absolute ethanol was added and vortexed vigoursly to yield homogenous suspension. All the contents were transferred into the spin column with 2 ml collection tube and centrifuged at 8000 rpm for 2 minutes and the filtrate was discarded. The spin column was placed into new collection tube and 500µl of washing buffer (AW_1) was added and centrifuged at 8000 rpm for 2 minutes and filtrate was discarded again. The above step was repeated with drying buffer (AW_2) and centrifuged at 14000 rpm for 3 min and filtrate was discarded. The spin column was finally placed into 1.5 ml micro centrifuge tube and 30µl of elution buffer was added and incubated for 1 min at room temperature and centrifuged at 14000 rpm for 1 minute. The filtrate containing DNA was stored at -20°C till further use.

3.8.6.2 PCR amplification of DNA

DNA amplification was carried out using specific forward and reverse primers as described by van Kuppeveld *et al* (1994).

GPO-3 (5'-GGGAGCAAACAGGATTAGATACCCT-3') and

MGSO (5'-TGCACCATCTG TCACTCTGTTAACCTC-3').

PCR reactions were performed in an iCycler^{TM} thermocycler (Bio-Rad) with 100 pg of genomic DNA as template in a 25µl reaction containing 13µl of master mix (Qiagen) and 250nM of each primer (Genaxy).

After an initial denaturation step at 94°C for 5 min, 40 amplification cycles were performed, each consisting of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C and followed by a final extension step at 72°C for 5 min.

3.8.6.3 Agarose gel electrophoresis

PCR products were analyzed using conventional agarose gel electrophoresis in 1% w/v agarose (Genaxy). The amplified products were run in agarose gel in 1x TBE buffer (Genaxy) containing ethidium bromide at 0.1 μg/ml. Quantitative DNA Markers (Genaxy) were used as molecular size markers. The DNA bands were visualized and imaged using the Molecular Imager® ChemiDoc™ XRS+ imaging system (Bio-Rad).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Contagious agalactia

4.1.1 Seroprevalence

A total of 323 animals from 32 flocks in 3 agro-climatic zones of Punjab were selected and their serum samples were collected. The serum samples were analyzed with ELISA for presence of antibodies to p48 gene of *Mycoplasma agalactiae*. Out of 32 flocks, 9 animals from 6 flocks were found to be positive for *M. agalactiae* revealing farm wise prevalence to be 16.2%. The prevalence in goats and sheep was found to be 3.30% and 1.80%, respectively; the overall apparent prevalence being 2.79%. The prevalence in the present study is quite low as compared to recent studies from Iran which reported the prevalence of *M. agalactiae* as 16% (Khezri *et al* 2012, Moslemi *et al* 2013).

The presence of asymptomatic carriers in a herd appears to be a serious health risk. These animals carry the infectious agent in their genital tracts; the carrier state is less obvious in males than females (Bergonier *et al* 1997). *M. agalactiae* is a highly persistent pathogen, remaining in the animals for years (Bergonier *et al* 1997,Corrales *et al* 2007), and it is very difficult to eliminate from infected herds. It has been shown that other animal species, such as cattle, camels or small wild ruminants, may act as reservoirs for mycoplasmas (Perrin *et al* 1994). Gil *et al* (1999) reported *M. agalactiae* and *M. mycoides* subspecies *mycoides* LC type were the species most frequently isolated from ear canals (70% and 25.5%, respectively). *Mycoplasma spp* were isolated from 26 (96.3%) of the 27 nasal swab, 20 (77%) of the 26 ear swabs and 10 (55.5%) of the 18 synovial fluid samples. *M. agalactiae* was isolated from only one (10%) of the 10 synovial fluid samples.

4.1.2 Risk factors

Various host factors associated with the disease were studied so as to help in formulation of control programs.

4.1.2.1 Species

Species-wise serological prevalence was found to be statistically similar (chi square 0.605, $p > 0.05$) in goats (3.30%) and sheep (1.80%) (Table 2).

Table 2: **Species wise seroprevalence of *M. agalactiae***

	Positive	**Total**	**% Prevalence**
Goat	7	212	3.30%
Sheep	2	111	1.80%
Overall	9	323	2.79%

Chi square uncorrected = 0.605 (p>0.05)

Chi square Mantel – Haenszel = 0.603 (p>0.05)

Chi square - Yates corrected = 0.178 (p>0.05)

Odds ratio = 1.86 (95% CI = 0.347–13.201)

Relative Risk = 1.83 (95% CI = 0.358–12.703)

Risk of disease was found to be almost twice higher in goats in comparison to sheep populations as revealed by risk ratio and odds ratio. Similar to present results, most of the studies conducted earlier have also reported similar prevalence of disease in goats and sheep (Al-Momani *et al* 2008). The results indicate that both sheep and goats are equally susceptible to *M. agalactiae*. This may be due to the fact that they are equally exposed to the infection in the mixed sheep and goat practice adopted by the majority of flock owners. Infected mixed flocks put equal exposure pressure on both sheep and goats and subsequent transmission of *M. agalactiae*. Thus, for any control program to be successful, both the species will have to be included in the program.

4.1.2.2 Sex

Other host factor studied was sex of animals. In present study, none of male sampled was found to be positive while a prevalence rate of 3.43% was found in the female animals. The prevalence of disease was not found to vary with respect to sex (chi square 2.155, p>0.05) (Table 3).

Table 3: **Sex wise seroprevalence of *M. agalactiae***

	Positive	**Total**	**% Prevalence**
Female	9	262	3.43%
Male	0	61	0%

Chi square uncorrected = 2.155(p>0.05)

Chi square Mantel – Haenszel = 2.149(p>0.05)

Chi square - Yates corrected = 1.074(p>0.05)

Odds ratio = inf (95% CI =0.3952–inf)

Relative Risk = inf (95% CI =0.409–inf)

There is a little published information on the potential effects of gender as a risk factor for *M. agalactiae*. Domestic sheep and goats of both sexes can be infected at the same frequency (Madanat *et al* 2001), but morbidity is most often associated with pregnant and lactating females rather than males (Ruffin 2001). It probably is related to changes in immunologic competence caused by physiologic and hormonal changes associated with reproduction (Real *et al* 1994).

Kashoo *et al* (2012) conducted a serological survey of contagious agalactia in different parts of Jammu and Kashmir and recorded a prevalence of 32.0% by slide agglutination test (SAT) and 20.3% by ELISA in female animals and 13.5% by SAT and 6.7% by ELISA in male goats. Verbisck-Bucker *et al* (2008) investigated host-related factors in the distribution of *M. agalactiae* infection and found sex of animal to be significantly associated with presence of *M. agalactiae*. Prevalence rates of infection in males (8.4%) and females (16.9%) were significantly different. In addition, females were more at risk of infection as compared to males. Inspite of low frequency of infection observed in males, it is important to recognize that males may be infected and may play an important role in the epidemiology and the maintenance of *M. agalactiae*.

4.1.2.3 Feeding pattern

No difference (chi square 0.759, p>0.05) in prevalence rate was observed with respect to feeding pattern viz., grazing (2.48%) and stall feeding (4.88%) (Table 4).

Table 4: **Association of feeding pattern with seroprevalence of *M. agalactiae***

	Positive	**Total**	**% Prevalence**
Grazing	7	282	2.48%
Stall fed	2	41	4.88%

Chi square uncorrected = 0.759 (p>0.05)

Chi square Mantel – Haenszel = 0.756 (p>0.05)

Chi square - Yates corrected = 0.132 (p>0.05)

Odds ratio = 0.496 (95% CI =0.090 – 3.596)

Relative Risk = 0.509 (95% CI =0.102–3.516)

4.1.2.4 Stage of lactation

In present study, female animals were grouped into different lactation groups viz. not lactating, lactating from <1.5 month, lactating between 1.5 and 3 months, and lactating for >3 months. The prevalence of disease did not vary with respect to lactation status (chi square 0.706, p>0.05) (Table 5). Verbisck-Bucker *et al* (2008) suggested that *M. agalactiae* infection is strongly associated with reproductive cycle and a greater frequency of infection was observed during the lactation. In addition, lactating females often have morbidity rates between 10% and 90% (Bergonier *et al* 1997).

Table 5: **Association of stage of lactation with seroprevalence of *M. agalactiae***

	Positive	**Total**	**% Prevalence**
No	3	89	3.37%
<1.5 month	1	55	1.82%
1.5-3 months	2	52	3.85%
≥3 months	3	66	4.54%
	9	262	3.43%

Chi square uncorrected = 0.706 (p>0.05)

4.1.2.5 Age

The comparison was made with respect to age viz., <1 year, 1-3 years, 3-5 years and >5 years. The prevalence rate was found to increase with respect to age until 3-5 years age and decrease thereafter, however, the difference was statistically insignificant (chi square 1.803, $p>0.05$) (Table 6). Verbisck-Bucker *et al* (2008) recorded significant association between age and *M. agalactiae* infection. Most (70%) of the infected animals were between one and four year age; the highest being in 3-year age (27%).

Table 6: **Association of age with seroprevalence of *M. agalactiae***

	Positive	Total	% Prevalence
< 1 year	0	22	0%
1-3 years	2	100	2%
3-5 years	6	150	4%
>5 years	1	51	1.96%

Chi square uncorrected = 1.803 ($p>0.05$)

4.1.2.6 Agro-climatic zone

Higher prevalence was observed in submountainous region (5.56%) as compared to central region (3.01%) and southwestern region (1.47%), although the difference was statistically insignificant (chi square 2.422, $p>0.05$) (Table 7).

Table 7: **Association of agro-climatic zones with seroprevalence of *M. agalactiae***

Agro-climatic zones	Positive	Total	% Prevalence
Submountainous zone	3	54	5.56%
Central plain region	4	133	3.01%
Southwestern region	2	136	1.47%

Chi square uncorrected = 2.422 ($p>0.05$)

4.1.2.7 Association with clinical signs

In the present study, clinical signs suspected of contagious agalactia were found in 5 animals, out of which only 3 were found positive in ELISA. Whereas, out

of 318 healthy animals without any clinical signs of contagious agalactia, 6 animals were found to be seropositive. Of the five animals with clinical signs, 4 showed signs of mastitis, 2 showed signs of arthritis and 1 had conjunctivitis.

Contagious agalactia is a very contagious disease, characterized by fever, malaise, mastitis, arthritis, and keratoconjunctivitis. *M. agalactiae* can produce acute, subacute, or chronic form of disease. In some animals atypical or asymptomatic forms have also been reported (Nicolet *et al* 1994, Bergonier *et al* 1996, Zendulkova *et al* 2007). Common clinical symptoms include fever, anorexia, lethargy, and lagging the herd, followed by the clinical symptoms depending upon the involvement of organs such as mammary glands, lungs, genitalia, joints, and conjunctiva. Rare abortions in pregnant animals have also been reported (Kizil and Ozdemir 2006, Gil *et al* 2003). Fever is common in acute cases and may be accompanied by nervous signs, but both signs are rare in the more frequently observed subacute and chronic infections. Arthritis usually involves the carpus or tarsus; swollen and painful joints with accumulation of synovial fluid is common finding. Chronic involvement of joints and severe losses to cornea lead to lameness along with inability to walk or stand and blindness, respectively (Mega *et al* 1983, Kwantes and Harby 1995).

4.1.2.8 Other risk factors

In a recent study, 31 health management and production variables were tested and only three, namely, using outsider rams, improper cleaning the milking utensils and keeping the young separate from dam were found to increase *M. agalactiae* seropositivity (Al-Momani *et al* 2008). The disease can be introduced by carrier animals from other flocks used for breeding (Nicholas 1999), as using rams from other flocks for breeding is accompanied with increasing flock-level seroprevalence of *M. agalactiae*.

4.2. Clinical outbreak of contagious agalactia in goats

4.2.1 Epidemiological and clinical observations

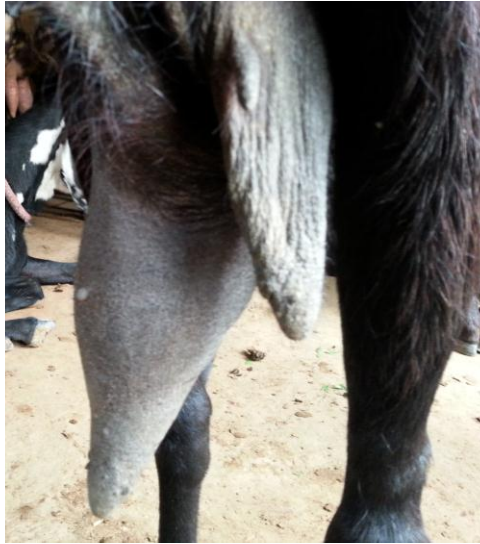
There were a total of 90 goats comprising of 55 adult goats and 35 kids, out of which 42 adults and 25 kids were affected with clinical symptoms and15 kids had died over a period of 10 days(Table 8). On clinical examination, fever was recorded in

some of the affected animals. There were clinical signs of mastitis in most of the lactating goats, arthritis in young as well as adult goats and keratoconjunctivitis (Fig. 1). On the basis of characteristic clinical signs, the cases were suspected of contagious agalactia.

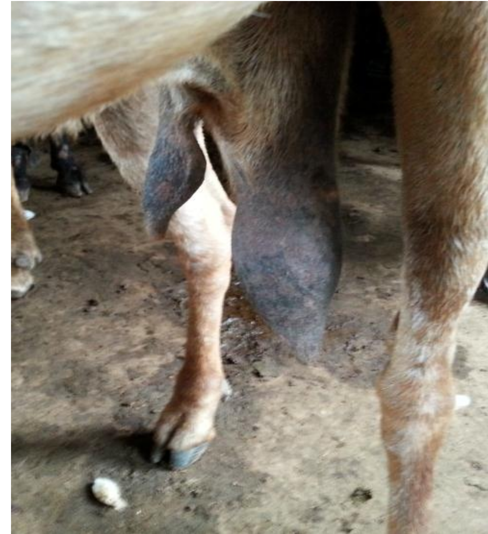
Table 8: Epidemiological data of outbreak

Outbreak	**Adult**	**Kids**	**Total**
Animals at risk	55	35	90
Affected animals	42	25	67
Died animals	1	15	16
Cumulative incidence	76.4	71.4	74.4
Cumulative mortality	1.8	42.9	17.8
Case fatality	2.4	60.0	23.9

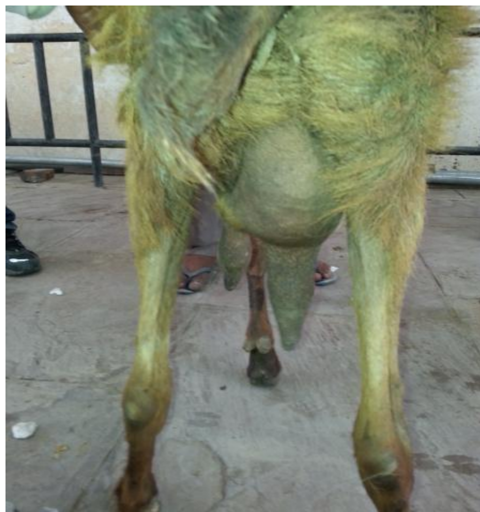
The main clinical signs observed in the outbreak in this study were mastitis and agalactia in the does, and polyarthritis in kids, but numerous does also had polyarthritis. The high frequency of mastitis and agtalactia in goats with some of them having arthritis is highly suggestive of infection by *M. agalactiae.* In non milking goats other common causes of arthritis can be confused with infection by *M. agalactiae*, including other Mycoplasma and bacterial infections, virus infections and traumatic injuries, which are common in young animals, but the high frequency of polyarthritis is suggestive of Mycoplasma infection. In cases of arthritis by *M. agalactiae*, the thickening of the joint capsule, the liquid aspect of the exudates, the absence of umbilical lesions or abscesses in other organs, associated with the high prevalence of polyarthritis suggest the infection by *M. agalactiae* (Bajmocy *et al* 2000), but other Mycoplasma, including *M. mycoides subsp. capri* can also cause high frequency of arthritis (Nascimento *et al* 1986, Real *et al* 1994). The treatment of mycoplasmosis is currently based on antibiotics, such as the tetracycline and fluoroquinolones (Bergonier *et al* 1997, Madanat *et al* 2001). Antibiotics can result in symptomatic improvement, but they may not be effective in chronic joint infections or keratoconjunctivitis. If the therapeutic dose is not exactly defined and the relevant antibiotic is not administered for a sufficiently long period, the efficacy may be very poor. The causal agents may be shed into the environment and there is a possibility of development of resistant (Madanat *et al* 2001).



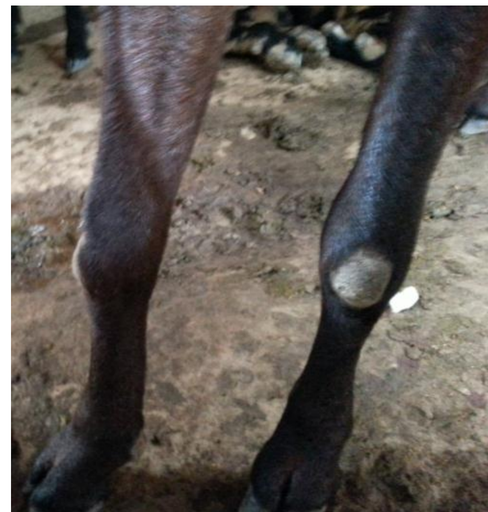
(a)



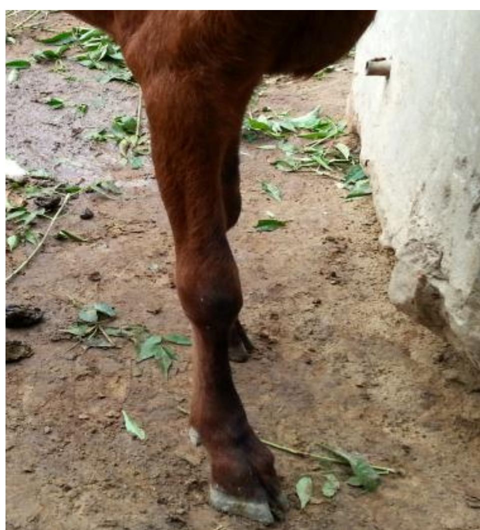
(b)



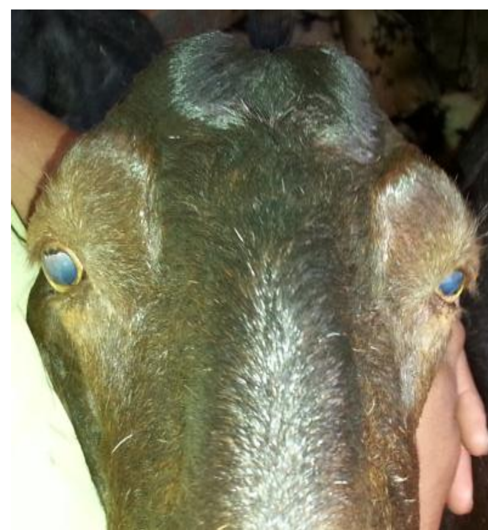
(c)



(d)



(e)



(f)

Fig. 1: Clinical symptoms of contagious agalactia in goats.
a-c: Mastitis, d-e: Arthritis, f: keratoconjuctivitis

Fig. 2: Detection of antibodies to p48 lipoprotein of *Mycoplasma agalactiae*

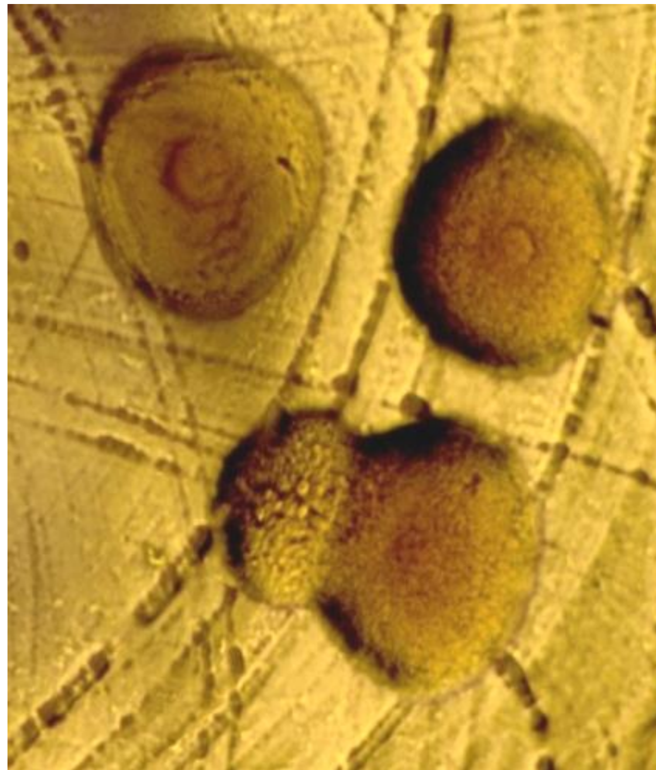


Fig. 3: Typical fried egg colonies on PPLO agar characteristic of Mycoplasma infection

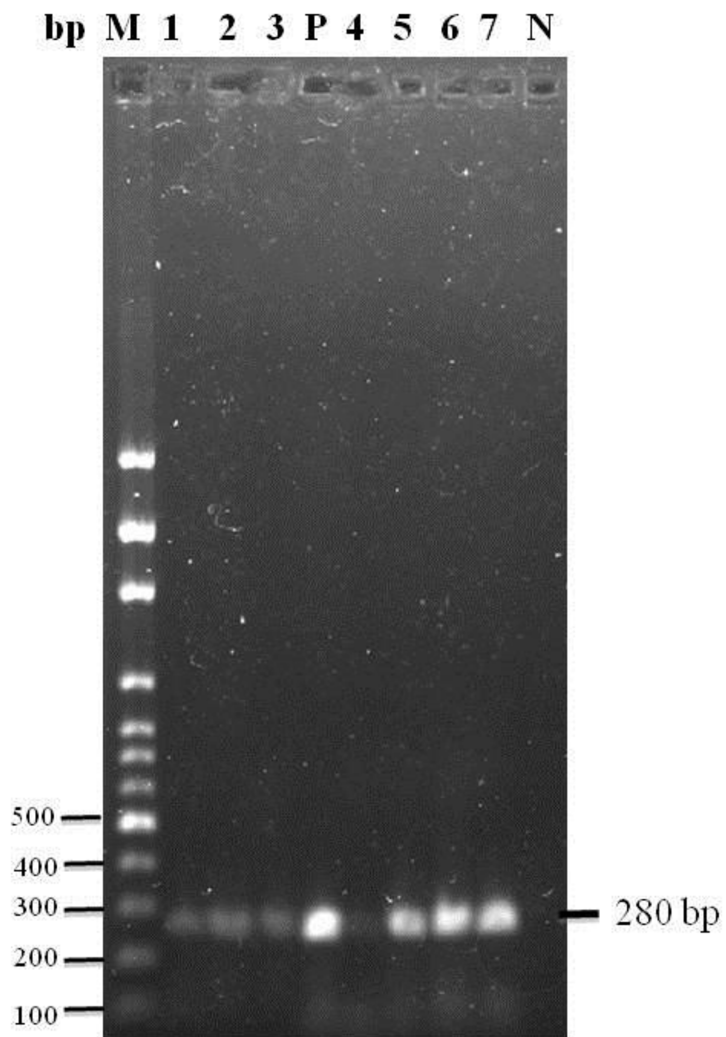


Fig. 4: PCR products run on 1.5% agarose gel revealing 280bp bands indicative of Mycoplasma infection

4.2.2 ELISA

A total of 47 serum samples comprising of 46 females (suspected=40, healthy=6) and 1 male were collected from the farm. ELISA employed on these samples revealed 36 to be positive (females=35, male=1), 4 were suspected and 7 were negative for *M. agalactiae* antibodies (Fig. 2).

4.2.3 Isolation of *Mycoplasma species*

Milk samples from affected animals were inoculated directly on PPLO agar plates and observed daily for growth from 5th day onwards. The fried egg colonies characteristic of mycoplasma were recognized microscopically (Fig. 3).

4.2.4 Molecular Diagnosis

PCR amplification was carried out to detect *Mycoplasma species* following extraction of DNA from milk samples. The genus specific forward and reverse primers, GPO-3 and MGSO as suggested by van Kuppeveld *et al* (1994), were employed. The amplified products were visualized and imaged revealing a product of 280 bp in positive samples (Fig. 4).

4.3 Bluetongue

4.3.1 Seroprevalence

A total of 368 animals were selected from 37 flocks in 3 agro-climatic zones of Punjab. The serum samples from these animals were analyzed with cELISA for presence of antibodies to bluetongue virus, which revealed the overall apparent prevalence of disease to be 52.99%.

De *et al* (2009) conducted serological studies in different regions of West Bengal and the antibody prevalence to bluetongue virus was found to range from 40 to 80%. In contrast, Ravishankar *et al* (2005) reported a lesser rate of prevalence (5.1 ± 1.9%) in Kerela state.

4.3.2 Risk factors

4.3.2.1 Species

In this study, out of 368 serum samples (goat-257, sheep-111), 130 (50.58%) goats and 65 (58.56%) sheep were found positive. Presence of anti-bluetongue antibody was found to be higher in sheep population as compared to goats, although the difference was not statistically significant (Table 9). Recent study in Jammu

region of Jammu and Kashmir revealed an overall seroprevalence of 29.5%, which was significantly higher (p<0.05) in goats (37.4%) as compared to sheep (11.5%) (Singh *et al* 2009). In more recent study in Jharkhand, 43.68% of sheep and 43.33% of goat sera were found positive by indirect enzyme linked immunosorbent assay (iELISA). Tigga *et al* (2015) reported prevalence of 43.68% in sheep and 43.33% in goat in Jharkhand. Joardar *et al* (2013) conducted a study on apparently healthy as well as suspected sheep, goat and cattle from different districts of Assam. Out of total 313 animal serum samples screened (sheep-68, goat-195, cattle-50), 58.82% of sheep, 31.79% of goat and 70.00% of cattle serum samples were found positive.

Table 9: **Species wise seroprevalence of bluetongue**

	Positive	Total	% Prevalence
Goat	130	257	50.58%
Sheep	65	111	58.56%
	195	368	52.99%

Chi square uncorrected = 1.979 (p>0.05))

Chi square Mantel – Haenszel = 1.974 (p>0.05))

Chi square - Yates corrected = 1.672 (p>0.05))

Odds ratio = 0.724(95% CI =0.450 – 1.161)

Relative Risk = 0.864 (95% CI =0.710– 1.075)

In sheep, the present values are in accordance with the result of Panda *et al* (2011), who reported prevalence of 57.66% in sheep by performing iELISA in West Bengal. However, Naresh and Prasad (1995) reported a much lower (23.5%) seroprevalence from Haryana, Himachal Pradesh and Punjab. In goat, the prevalence was almost similar to findings from De *et al* (2008), where 47% goats were found seropositive in Sunderban area of West Bengal using iELISA. Singh *et al* (2009), described 45.0% goats as seropositive in Udhampur district, Jammu province. Relatively higher (66.95%) seroprevalence was reported by Panda *et al* (2011) in West Bengal, while lower (31.79%) seroprevalence was reported by Joardar *et al* (2013) in goats of Assam after performing iELISA. Bluetongue disease is endemic and frequent outbreaks in small ruminants have been reported from many parts of the country (Jain *et al* 1986, Mehrotra *et al* 1996, Aruni *et al* 2006). Serological

surveillance for BTV antibodies in small ruminants has been conducted in the endemic states in the northern, central and southern areas of the country and a high degree of seropositivity (40%-80%) has been reported (Naresh and Prasad 1995, Sreenivasulu *et al* 2004, Ravishankar *et al* 2005).

4.3.2.2 Sex

In present study, 60.65% of male sampled were found to be positive while a prevalence rate of 51.46% was found in the female animals. The prevalence of disease was not found to vary with respect to sex of animals (chi square value = 1.725, p>0.05) (Table 10). Risk of disease was found to be almost similar in goat and sheep populations as revealed by risk ratio and odds ratio. Singh *et al* (2009), reported an overall sero-prevalence of 31.8% in females as compared to 26.4% in males and no significant difference was observed with respect to sex of animals. Similarly, Shringi and Shringi (2005) indicated that the prevalence of BTV was not markedly affected by the sex. Morbidity, mortality and case fatality rates were 8.83%, 4.03% and 45.65% in male and 5.83%, 3.32% and 55.29% in female sheep, respectively (Selvaraju 2014). Tigga *et al* (2015) observed that the overall seroprevalence was higher in males of sheep (75.4%) than goats (60.4%). While in females, goats were more seropositive (62.5%) than sheep (54%).

Table 10: **Sex wise seroprevalence of bluetongue**

	Positive	Total	% Prevalence
Female	158	307	51.46%
Male	37	61	60.65%

Chi square uncorrected = 1.725 (p>0.05)

Chi square Mantel – Haenszel = 1.721 (p>0.05)

Chi square - Yates corrected = 1.376 (p>0.05)

Odds ratio = 0.688 (95% CI =0.378 – 1.247)

Relative Risk = 0.848 (95% CI =0.684 – 1.114)

4.3.2.3 Feeding pattern

No difference (chi square 0.073, p>0.05) in prevalence was observed with respect to feeding pattern viz., grazing (52.74%) and stall feeding (55.00%) (Table 11).

Table 11: **Association of feeding pattern with seroprevalence of bluetongue**

	Positive	Total	% Prevalence
Grazing	173	328	52.74%
Stall fed	22	40	55.00%

Chi square uncorrected = 0.073 (p>0.05)

Chi square Mantel – Haenszel = 0.073 (p>0.05)

Chi square - Yates corrected = 0.010(P>0.05)

Odds ratio = 0.913 (95% CI =0.449 – 1.851)

Relative Risk = 0.959 (95% CI =0.730 –1.386)

4.3.2.4 Stage of lactation

In present study, no significant (Chi square uncorrected = 5.793, p>0.05) difference in prevalence rates was observed with respect to stage of lactation in female animals. The prevalence rates were 51.68%, 63.64%, 44.61% and 47.13% in different lactation-groups viz. not lactating, lactating since <1.5 month, lactating since 1.5-3 months, and lactating since >3 months, respectively (Table 12).

Table 12: **Association of stage of lactation with seroprevalence of bluetongue**

	Positive	Total	% Prevalence
Not lactating	46	89	51.68%
<1.5 month	42	66	63.64%
1.5-3 months	29	65	44.61%
≥3 months	41	87	47.13%
	158	307	51.46%

Chi square uncorrected = 5.793 (p>0.05)

4.3.2.5 Age

In the present study, the prevalence rate of bluetongue did not vary with respect to age of animals (Chi square = 6.95, p>0.05). The prevalence rate of 68.18%, 59.80%, 46.81% and 55.36% in was observed <1 year, 1-3 years, 3-5 years and ≥5 years age groups, respectively (Table 13). The prevalence of BTV antibodies varied from 61.6% to 65.5% in different age groups with the least prevalence in 1 to 3 years age group (61.6%) and the highest prevalence (65.5%) in animals of above 3 years of age (Tigga *et al* 2015). However, Singh *et al*. (2009) reported that adult animals showed greater sero-positivity, the difference between >2 years (33.3%) and 1–2 years (3.6%) age group in sheep being significant. Morbidity, mortality and case fatality rates were 5.72%, 1.94% and 33.87% in young sheep and 6.27%, 3.79% and 58.99% in adult sheep respectively (Selvaraju 2014). Infection rate and severity of the disease were more in adults than young animals. Shringi and Shringi (2005) also indicated that BTV infection was common among adult animals. But, Sreenivasulu *et al* (2004) observed that sheep aged (6 -12) months were more susceptible than adults. Higher frequency of disease in adults might be due to increased nomadic activity for search of fodder and movement of adult stock for marketing.

Table 13: **Age wise seroprevalence of bluetongue**

	Positive	Total	% Prevalence
< 1 year	15	22	68.18%
1-3 years	61	102	59.80%
3-5 years	88	188	46.81%
≥5 years	31	56	55.36%

Chi square uncorrected = 6.95 (p>0.05)

The results of an earlier study also indicated that the seroprevalence rates increase with the increasing of age in the studied herds, which is another reason for the disease to be endemic, because newborn lambs can be protected by maternal immunity (Mohammadi *et al* 2012). However, in a seroprevalence study in the southeast of Iran, the results showed that seroprevalence rates decrease with the increase in age in sheep herds (Mozaffari and Khalili 2012). Also, Taylor and Mellor reported that after BT epidemic in Turkey, the disease became endemic and

probability of infection was much less in sheep up to 2 years old (Taylor and Mellor 1994). In addition, according to the fact that Australian serotypes of the virus can affect sheep 3 years old or older, obviously there is a relationship between age of infected animals and serotype of the virus (Radostits *et al* 2007)

4.3.2.6 Agro-climatic zone

In the present study, the seroprevalence of bluetongue in (central plain region) was significantly (Chi square = 11.52, $p<0.01$) higher as compared to (submountainous region) and (southwestern region) (Table 14).

Table 14: **Association of agro-climatic zones with seroprevalence of bluetongue**

Agro-climatic zones	Positive	Total	% Prevalence
Submountainous region	28	54	51.85%
Central plain region	85	132	64.39%
Southwestern region	82	182	45.05%

Chi square uncorrected = 11.52 ($p<0.01$)

The distribution and intensity of infection in different regions are determined by the climate, geography and altitude, as they affect the occurrence and activity of the Culicoides vectors and by the presence of susceptible mammalian hosts (Mellor and Boorman 1995, Erasmus and Potgieter 2009). The climate is a major risk factor as *Culicoides* require warmth and moisture for breeding and calm, warm humid weather for feeding (Purse *et al* 2005). A cold winter or a dry summer can markedly reduce vector numbers and risk for the disease. Moisture may be in the form of rivers and streams or irrigation, but rainfall is the predominant influence and rainfall in the preceding months is a major determinant of infection. Optimal temperature is also essential and in endemic areas the temperature for survival of the adults and larvae requires temperatures sustained above a mean of 12.5ºC for the cooler months and temperatures in the range of 18 to 30ºC in the summer and autumn for optimum recruitment to adults and for optimal activity (Erasmus and Potgieter 2009). Tigga *et al* (2015), on the other hand, did not found any difference in seroprevalence of bluetongue in three agro-climatic zones.

CHAPTER – V

SUMMARY AND CONCLUSION

Small ruminants constitute significant share of the domestic animals involved in production of food for human consumption. Sheep and goat production is an integral component of rural economy of India and serves as a major source of economic sustenance for weaker segments of the society. These species are traditionally reared by small and marginal farmers and landless laborers under extensive range management. Small ruminants play an important role in income generation, capital storage, employment generation and improving household nutrition. Bluetongue and contagious agalactia are serious infectious diseases due to their effect on production of small ruminants and costs spent on their treatment and control.

Bluetongue is an infectious, non-contagious, arthropod borne, viral disease of domesticated and wild ruminants caused by buetongue virus (BTV). The disease is characterized by fever, nasal discharge, drooling of saliva, oral lesion, facial edema, hyperemia of coronary bands and muscle weakness. Some strains of the virus can result in mortality rates as high as 70% in susceptible sheep.

Contagious agalactia, on the other hand, is a highly infectious disease of sheep and goats that spreads by ingestion of feed, water, or milk contaminated with infected milk, urine, feces, or nasal and ocular discharges. Animals with subclinical or chronic infections can carry and shed the mycoplasmas for months, and the organisms can survive in the supramammary lymph nodes. The morbidity rate of contagious agalactia disease is about 30-60% in a herd and the mortality rate may reach 15-20% in adult animals. The disease caused by *Mycoplasma agalactiae* is recognized by elevated temperature, mastitis, lameness and keratoconjunctivitis.

Bluetongue and contagious agalactia are traditionally diagnosed by culture, or immunoblotting however these tests are not preferred due to delayed results. Moreover, for screening purposes ELISA is considered to be test of choice amongst all the serological assays because of its sensitivity and specificity. In addition to screen large number of samples or to determine presence/absence of disease in a farm or region, ELISA is preferred test.

The present study was conducted with study the seroprevalence of contagious agalactia and bluetongue in sheep and goat in 3 different agro-climatic zones of Punjab state and to identify the risk factors associated with these diseases.

To study seroprevalence of contagious agalactia, a total of 323 animals were selected in 3 agro-climatic zones of Punjab. The serum samples from these animals were analyzed with ELISA for presence of antibodies to *Mycoplasma agalactiae*, which revealed an overall apparent prevalence of disease being 2.79%. Presence of contagious agalactia revealed no significant association with species (goats-3.30% vs. sheep-1.80%, chi square 0.605, p> 0.05), sex (female-3.43% vs. male-0%, chi square 2.155, p>0.05) or feeding pattern (grazing-2.48% vs. stall feeding-4.88%, chi square 0.759, p>0.05). Similarly, presence of contagious agalactia had no association with other risk factors studied viz., stage of lactation (not lactating-3.37%, lactating since <1.5 month-1.82%, lactating since 1.5-3 months-3.85%, and lactating since >3 months-4.54%, chi square 0.706, p>0.05), age (<1 year-0%, 1-3 years-2%, 3-5 years-4% and >5 years-1.96%, chi square 1.803, p>0.05) in both species. Higher prevalence was observed in submountainous region (5.56%) as compared to central (3.01%) and southwestern regions (1.47%), although the difference was statistically insignificant (chi square 2.422, p>0.05).

To study seroprevalence of bluetongue, a total of 368 animals were selected in 3 agro-climatic zones of Punjab. The serum samples from these animals were analyzed with cELISA for presence of antibodies to bluetongue virus, which revealed the overall apparent prevalence of disease being 52.99%. In this study, out of total 368 serum samples (goat-257, sheep-111), 130 (50.58%) of goat and 65 (58.56%) of sheep were found positive. No significant association was found between occurrence of bluetongue disease and species of animal (Chi square = 1.979, p>0.05). In present study, 60.65% of male sampled were found to be positive while a prevalence rate of 51.46% was found in the female animals. The prevalence of disease was found not to vary with respect to sex of animals (chi square value = 1.725, p>0.05). Risk of disease was found to be almost similar in goat and sheep populations as revealed by risk ratio and odds ratio. No difference (chi square 0.073, p>0.05) in prevalence rates of flocks

was observed with respect to feeding pattern viz., grazing (52.74%) and stall feeding (55.00%). Similarly, the prevalence rate of bluetongue did not vary with respect to age of animals (Chi square = 6.95, p>0.05). The prevalence rate of 68.18%, 59.80%, 46.81% and 55.36% in <1 year, 1-3 years, 3-5 years and ≥5 years age groups, respectively. In addition, no significant (Chi square uncorrected = 5.793, p>0.05) difference in prevalence rates was observed with respect to stage of lactation in female animals of both species. The prevalence rates of were 51.68%, 63.64%, 41.54% and 47.13% in different lactation-groups viz. not lactating, lactating since <1.5 month, lactating since 1.5-3 months, and lactating since >3 months, respectively. The seroprevalence of bluetongue in central plain region (64.39%) was significantly (Chi square = 11.52, p<0.01) higher as compared to sub mountainous region (51.85%) and southwestern region (45.05%).

CONCLUSIONS

- The seroprevalence of contagious agalactia and blue tongue was found to be 2.79% and 52.99%, respectively in small ruminants.
- Though there was a low prevalence of contagious agalactia in Punjab, one clinical outbreak was recorded in goats characterized by mastitis, arthritis and keratoconjunctivitis. Cumulative incidence, mortality and case fatality during the outbreak was 74.4%, 17.8% and 23.9%, respectively.
- Clinical cases of contagious agalactia could be confirmed based on ELISA, Isolation of mycoplasma on PPLO agar and PCR based DNA amplification.
- Bluetongue and contagious agalactia can affect both sheep and goat population irrespective of sex, age, lactation status, feeding pattern and geographical location. Thus for any control program, measures will have to be applied on both the species.
- The search for risk factors should be broadened to include other potential risk factors that will help in containing these diseases.

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