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INVESTIGATION ON THE OCCURRENCE OF PARATUBERCULOSIS IN APPARENTLY HEALTHY SHEEP AND GOATS SLAUGHTERED ON BISHOFTU ELFORA, MOJO MODERN AND ORGANIC EXPORT ABATTOIRS, CENTRAL ETHIOPIA

MSc Thesis

Addis Ababa University, College of Veterinary Medicine and Agriculture, Department of Veterinary Microbiology, Immunology and Veterinary Public Health

BY

Ashebr Abraha Fitwi

June, 2015

Bishoftu, Ethiopia

INVESTIGATION ON THE OCCURRENCE OF PARATUBERCULOSIS IN APPARENTLY HEALTHY SHEEP AND GOATS SLAUGHTERED ON BISHOFTU ELFORA, MOJO MODERN AND ORGANIC EXPORT ABATTOIRS, CENTRAL ETHIOPIA

A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Microbiology

BY

Ashebr Abraha Fitwi

June, 2015

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Addis Ababa University

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Department of Veterinary Microbiology, Immunology and Veterinary Public Health

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by **Ashebr Abraha Fitwi** entitled: **Investigation on the Occurrence of Paratuberculosis in Apparently Healthy Sheep and Goats Slaughtered on Bishoftu ELFORA, Mojo Modern and Organic Export Abattoirs, Central Ethiopia** and recommended that it be accepted as fulfilling the thesis requirement for the Degree of Master of Science in **Veterinary Microbiology.**

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SIGNED DECLARATION SHEET

First, I declare that this thesis is my original work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Addis Ababa University College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma or certificate.

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LIST OF ABBREVIATIONS

AAU	Addis Ababa University
AFB	Acid-fast bacilli
AGID	Agar gel immunodiffusion
ALIPB	Aklilu Lemma Institute of Pathobiology
CD	Crohn's disease
CFT	Complement fixation test
CFU	Colony forming units
CI	Confidence interval
CSA	Central Statistical Agency
CVMA	College of Veterinary Medicine and Agriculture
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
HE	Haematoxylin and eosin
HEYM	Herrold's egg yolk medium
HPC	Hexadecylpyridinium chloride
IBD	Inflammatory bowel disease
IFN	Interferon
IL	Interleukin
IS	Insertion sequence
LJ	Lowenstein Jensen medium
MAA	*Mycobacterium avium* subspecies *avium*
MAC	*Mycobacterium avium* complex
MAH	*Mycobacterium avium* subspecies *hominissuis*
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
MHC	Major histocompatability complex
MTC	*Mycobacterium tuberculosis* complex
NAHDIC	National Animal Health Diagnostic and Investigation Center

LIST OF ABBREVIATIONS (*Continued*)

OIE	Office of international des epizootics
OR	Odds ratio
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
REA	Restriction endonuclease analysis
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
Subsp.	Subspecies
Th	T helper
TNF	Tumor necrosis factor
USA	United States of America
ZN	Ziehl-Neelsen

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ABSTRACT

A cross sectional study was conducted from October 2014 to June 2015 to investigate the occurrence of paratuberculosis among apparently healthy sheep and goats slaughtered in Bishoftu ELFORA Export Abattoir, Mojo Modern Export Abattoir and Organic Export Abattoir, using gross pathology, histopathology, Ziehl-Neelsen (ZN) staining and bacteriological culture of tissue lesions from paratuberculosis suspected animals. Based on gross pathological examination, the prevalence of paratuberculosis was 5.21% (20/384) (95% CI=2.8-7.4) in sheep and 3.4% (26/768) (95% CI=2.1-4.7) in goats. Association of risk factors including species, age and origin of the animal with the occurrence of gross lesion showed statistically insignificant difference among the groups (P>0.05). However, a relatively higher prevalence was observed in sheep, age group of ≥2 years and in sheep originated from Borana. Out of 13 tissue samples from each species, histopathological examination revealed microscopic lesions consistent with paratuberculosis in 84.62% (11/13) of sheep and 76.9% (10/13) of goats, and the lesions were characterized by diffuse infiltration of many lymphocytes accompanied with few macrophages and epithelioid cells. Necrotic foci surrounded by lymphocytes, macrophages and epithelioid cells were observed in lymph nodes of 15 % (2/13) sheep and 23 % (3/13) goats. Grading of histological lesions based on the type and amount of cellular infiltrate revealed that all positive cases of sheep and goat were categorized in grade 3c lesion type and diffuse lymphocytic lesion type, respectively. On direct ZN staining of 46 tissue samples (20 sheep; 26 goats), acid fast positivity was recorded in 16 (80%) sheep and 14 (53.8%) goats. Out of 46 tissue samples (20 sheep; 26 goats) cultured on Herrold's egg yolk media (HEYM) and Lowenstein-Jensen (LJ) media with and without 1% ferric ammonium citrate, *Mycobacterium avium* subspecies *paratuberculosis* (MAP) was isolated from 2 (10%) sheep and 1 (3.8%) goat on ferric ammonium citrate (1%) supplemented media and all isolates were acid fast positive. Isolated colonies were confirmed as MAP by ferric ammonium citrate dependence, their long incubation period, colony characteristics and their morphology on ZN stained smears. A linearly weighed kappa coefficient of 0.3 was obtained between histopathology

and ZN staining, indicating fair agreement between the two tests. In conclusion the present study on sheep and goat paratuberculosis using gross pathology, histopathology, ZN staining and culture techniques revealed the occurrence of the disease in apparently healthy sheep and goats in Ethiopia. This result warrants the need for further investigation on the epidemiology, characterization of the causative agent and assessments of the economic impact of paratuberculosis on small ruminants of Ethiopia in order to design feasible control strategies.

Key words: *Culture, Goat, Gross pathology, Histopathology, MAP, Paratuberculosis, Sheep, Ziehl-Neelsen staining*

1. INTRODUCTION

Ethiopia is believed to have the largest livestock population in Africa with an estimated number of 56.7 million cattle, 29.33 million sheep and 29.11 million of goats. This livestock sector has been contributing considerable portion to the economy of the country, and still promising to rally round the economic development of the country (CSA, 2015). Sheep and goats contribute a quarter of the domestic meat consumption and about half of the domestic wool requirements and it is estimated that 1,078,000 sheep and 1,128,000 goats are used in Ethiopia for domestic consumption annually. The country has been earning foreign currency by exporting meat and live animals. However, different disease conditions are reducing sheep and goat production and productivity and also causing Ethiopian animals and products to be banned from export markets due to the stringent health requirements of some importing countries (Hirpa and Abebe, 2008).

Pathogens that are transmitted between environment, animals (domestic or wild) and humans present major challenges for the animal and human health. Among such pathogens, genus Mycobacterium is well represented by *M. bovis, M. tuberculosis, Mycobacterium avium* subspecies *avium* (MAA) and *Mycobacterium avium* subspecies *paratuberculosis* (MAP), which affects different species of animals. These pathogens resist adverse environmental conditions and degradation. Recently, MAP has emerged as major and successful animal pathogen with significant zoonotic and public health concerns (Verma, 2013). MAP is the causative agent of paratuberculosis (Johne's disease), a disease that infects ruminants worldwide. Although it can infect multiple species including humans, it is primarily a disease problem in ruminants (Sibartie *et al*., 2010).

Paratuberculosis is an enteric infection with a long incubation period and progressively worsening granulomatous infiltration of the small and large intestines leading to weight loss and death. It is economically significant in farmed cattle, goats, sheep and deer and is known to be capable of infecting a wide range of domesticated and free-living herbivores

and occasionally also the carnivores that prey upon them (Whittington *et al*., 2011). Paratuberculosis has become an important disease of domestic livestock placed under list B of OIE (OIE, 2014a).

The primary route of MAP transmission in all species is similar, fecal-oral. Excretion (shedding) of variable numbers of MAP in the feces of most affected animal species has been documented, as has the relative ease of experimental oral transmission in young cattle and sheep (NRC, 2003). Fecal contamination of the udder or calving environment is therefore thought to be the primary risk factor for neonatal infection (Clarke, 1997). Moreover, animals may harbor MAP in their intestine and other tissues for years without manifesting clinical signs. However sub-clinical animals develop clinical disease, if stressed (environmental, nutritional, pregnancy, parturition, lactation or any other concurrent disease). Clinically sick animals show chronic diarrhea, debility, progressive weight loss and emaciation. Decreased serum concentrations of calcium, total protein and albumin have been also reported in cattle and sheep with clinical paratuberculosis (Verma, 2013). Like many pathogenic mycobacteria, MAP is not host specific, and cross-species transmission is common. Thus, in areas where there are high contact rates it is important to understand the role of cross-species transmission in causing paratuberculosis and in maintaining infection cycles (NRC, 2003).

Because of the insidious nature of paratuberculosis, it is considered to be potentially a hidden threat to the livestock industry. No efficient treatment is known and the disease leads to economic losses due to relevant decrease in milk production, costs in the diagnosis and in disease control, the early culling of affected animals and the low carcass value at slaughter (Wiszniewska and Szteyn, 2002). Financial losses associated with paratuberculosis were mostly analyzed in dairy cattle industry as compared to sheep and goat. However, report in Australia showed that, ovine paratuberculosis affects the financial performance of individual producers primarily through increased animal mortality, reduced lambing percentages, reduced wool cut, and possibly decreased fiber quality and also through regulatory restrictions. Of these, increased mortality is considered the most serious, with annual losses of up to 15% (Eppleston and Simpson,

1999). In 1998 survey of 28 infected farms, an average loss of $23,000 in cash following diagnosis of ovine paratuberculosis was reported in Australia in that year (Webster, 2000).

A growing concern of MAP is that, it may also present a threat to individuals at risk for developing Crohn's disease (CD), a form of chronic inflammatory bowel disease in human (Sibartie *et al*., 2010). Thus, the concerns over food safety is considerable and would significantly higher economic losses associated with paratuberculosis if the steadily growing suspicion in the development of CD in humans is proven (Hruska *et al*., 2005; Hasonova and Pavlik, 2006; Skovgaard, 2007). Consequently, the isolation and cultivation of MAP are attempted in both veterinary and medical diagnostic and research laboratories worldwide (Naser *et al*., 2004; Whittington, 2010).

Detection of paratuberculosis is difficult because most cases are subclinical, most laboratory tests lack sensitivity and within-flock prevalence is often low (generally, <50%). For these reasons, regional surveillance to detect infected flocks requires the testing of many animals from each flock or herd which is expensive. Pooled faecal culture can be used as a surveillance tool to detect infected flocks or herds and appears to be both sensitive and cost effective (Whittington *et al*., 2000; Sergeant *et al*., 2002). However, in a nation where there is large flock/herd number, available resources may be insufficient for widespread testing based on pooled faecal culture. Thus, inspection of animals at slaughter in abattoirs is a relatively inexpensive surveillance method and is currently used to identify infected flocks in different areas (Allworth, 2002).

In most developed countries, as bovine tuberculosis has been brought under control, paratuberculosis represents an increasingly large part of the epizootiology of other mycobacterial infections in ruminants. According to various research reports it has been indicated that, paratuberculosis has worldwide distribution in all continents (Ayele *et al*., 2001; Harris and Barletta, 2001; Rowe and Grant, 2006). Moreover, this disease is considered as an emerging disease as its prevalence has the tendency to increase (Bakker *et al.*, 2000).

According to OIE (2014a), disease information on paratuberculosis was not available in Ethiopia. Samuel and Wirtu (1995) made an attempt to report a case of paratuberculosis in cattle from the country based on history of chronic diarrhea case, clinical examination and ZN staining of rectal scraping. Recently, Mohammed (2014) reported for the first time, isolation of MAP from apparently healthy slaughtered cattle indicating the prevalence of the disease in cattle population of the country. However, so far there is no information on the occurrence of the disease in sheep and goat of Ethiopia. This indicates the need for a study on the occurrence of paratuberculosis, the isolation and molecular characterization of the causative agent in sheep and goats so as to estimate impact of the disease (economic and public health) as well as in order to implement feasible preventive and control measures. Therefore, the objectives of this study were;

General

- To investigate the occurrence of paratuberculosis and isolate the causative agent among apparently healthy sheep and goats slaughtered in Bishoftu ELFORA export abattoir, Mojo modern export abattoir and Organic export abattoir, Ethiopia.

Specific

- ✓ To determine the prevalence of paratuberculosis based on gross pathological examination in sheep and goat slaughtered in selected export abattoirs
- ✓ To characterize lesions associated with sheep and goat paratuberculosis using histopathological techniques
- ✓ To isolate and characterize *Mycobacterium avium* subspecies *paratuberculosis* from suspected paratuberculosis lesions using standard microbiological techniques

2. LITERATURE REVIEW

2.1. The Mycobacteria

Mycobacteria are obligate aerobes, non-spore forming and no motile bacilli of 0.6-1.0 ×1.0 -10 μm in size, which belongs to family *Mycobacteriaceae*, orders Actinomycetales and the phylum Actinobacteria. Their high cell wall lipid content excludes standard aniline dyes, so that once stained with special staining procedures, mycobacteria are resistant to decolorization even by acid alcohol. This property is termed acid fastness, so that mycobacteria are commonly referred to as acid fast bacilli (AFB). In contrast, these microorganisms are not readily stained with the Gram's Method and are considered weakly Gram positive (Quinn *et al*., 2011).

The genus Mycobacterium includes the *Mycobacterium tuberculosis* complex (MTC), *Mycobacterium avium* complex (MAC), other pathogenic mycobacteria and numerous species of saprophytic microorganisms present in soil and water. Growth rates for mycobacteria are slow, with generation times ranging from 2 to more than 20 hours. Based on time required to form visible colony, mycobacteria can be divided as slow and rapid growers. Slow growers require more than 7 days to form visible colonies on solid medium, whereas rapid growers form colonies within 7 days (Olsen *et al*., 2010). These correspond to the traditional fast growing mycobacteria, represented by nonpathogenic environmental isolates and the slow growing mycobacteria, containing most of the overt pathogens (Rogall *et al*., 1990). The rRNA gene copy number also reflects this division between fast and slow growing mycobacteria, in that fast-growing mycobacteria contain two sets of rRNA genes, whereas the slow growers contain only one copy (Chiodini, 1990).

2.2. The Mycobacterium avium Complex

The *Mycobacterium avium* complex comprises two species, namely *Mycobacterium intracellulare* and *Mycobacterium avium*. The *M. avium* includes MAA, *Mycobacterium*

avium subspecies *hominissuis* (MAH) and MAP. The subspecies designation is based on DNA-DNA hybridization studies and numerical taxonomy analysis (Olsen *et al*., 2010). Members of the MAC are classified as potentially pathogenic mycobacteria, characterized by their ability to multiply in favourable types of environments and to provoke mycobacterioses in susceptible hosts. The main source of potentially pathogenic mycobacteria is an environment but they can also be found in living hosts, where they colonize suitable niches on the mucous membranes. They comprise a transitional group between obligate pathogenic mycobacteria and environmental saprophytic mycobacteria. MAP is the causative agent of paratuberculosis in ruminants and is also implicated in CD in humans (Kazda, 2009). These organisms are found widely in soil and water, causing disease in animals and humans and are not readily distinguishable based on phenotype. At the subspecies level, MAP can be differentiated phenotypically from MAA and MAH by its extreme slow growth and dependency on exogenous iron chelated mycobactin for in vitro growth and supplementation and genotypically by the presence of multiple copies of an insertion element, IS*900* (Rowe and Grant, 2006).

2.3. The *Mycobacterium avium* subspecies *paratuberculosis*

Microscopically, MAP is recognized in stained smears as small, thin and strong AFB which is usually found in clumps resulting from shedding parts of intestinal mucosa where the organism has multiplied. This bacillus has a thick waxy cell wall made up of 60 % complex lipids which give it the properties of acid fastness, hydrophobicity and an increased resistance to low pH, to high temperature and to a variety of chemicals (Klanicova *et al*., 2012). Such an ability to withstand adverse environmental conditions has led to an increase in the organism's life span as well as its pathogenic potential in varied unpleasant environments (Donat *et al*., 2011). MAP is phenotypically versatile and can switch to a tough ZN negative form in which it is invisible by ordinary light microscopy in infected tissues. Furthermore, as with some other mycobacteria, MAP can shut down into latency in which state it differs both functionally and in its physical properties from activated MAP cells, especially in its resistance to lysis and the subtleties of its interaction with the immune system (Hermon-Taylor and El-Zaatari, 2004).

2.3.1. Genotypic characteristics

Sequencing and annotation of the MAP genome from strain K - 10, isolated from a cow with paratuberculosis, have been completed. The K-10 strain genome is single circular sequence of 4,829,781 base pairs. Approximately 1.5% (or 72.2 kb) of the MAP genome is comprised of repetitive DNA like insertion sequences, multigene families, and duplicated housekeeping genes. The analysis also identified 17 copies of the previously described insertion sequence IS*900*, seven copies of *IS1311*, and three copies of *ISMav2*. Three more MAP unique IS elements; ISMav2, ISMAP02 and ISMAP04 were identified in addition to previously known IS*900* (Li *et al.*, 2005). Due to this, the sequence can be used for both the identification of MAP and in its epidemiological study (Bartos *et al*., 2006).

IS900 based restriction fragment length polymorphism (RFLP) analysis confirmed two principal genotypes of MAP which correlated with epidemiological findings and in vitro cultural characteristics: (1) Cattle "C" strains, which were frequently isolated from affected cattle and grew relatively easily in artificial media; (2) sheep "S" strains, which were more often isolated from sheep and very difficult to culture. Restriction endonuclease analysis (REA) and DNA hybridization studies indicate that there may be a unique MAP strain that is isolated from Norwegian goats that falls neither in the "C" nor "S" strain groups (Collins *et al*., 1990).

2.3.2. Growth characteristics

Mycobacterium avium subspecies *paratuberculosis* is the slowest growing of the culturable mycobacteria even slower than MAA. Under identical growth conditions, MAA has a doubling time of 10-12 hours, compared to the doubling time of MAP of 22-26 hours (Bannantine *et al*., 2003).

According to *in vitro* growth capability (based on apparent colony forming unit (CFU)) at 37 ^{o}C, MAP strains may be classified as (i) growing strains; (ii) strains that do not grow in subculture; and (iii) non growing strains. Growing strains are usually detected in

domestic and wild ruminants and divided into two subgroups: (a) relatively fast growing MAP with growth after 1–3 months and (b) relatively slowly growing MAP with growth observed after 4 months of incubation (Pavlik *et al*., 1999). Strains that do not grow in subculture (primary isolations only) are usually detected in non-typical hosts such as small terrestrial mammals, carnivores, birds and others, and in the environment or foodstuffs (mostly pasteurized milk). Non-growing strains are AFB detected by histology in heavily infected tissues, especially from sheep and moufflons, and occasionally from cattle (Kazda, 2009).

In all living microorganisms, iron acquisition is critical, but this is especially true for MAP. A phenotypic hallmark that defines MAP is the requirement for mycobactin J in the culture media, which is iron-transport protein derived from a soluble component of the bacterial walls of mycobacteria. Without the presence of this siderophore, MAP cannot grow (Janagama *et al*., 2009).

Siderophores are generally secreted by the organism to compete with environmental iron binding molecules for the small amount of available iron. Siderophore synthesis is typically controlled by extracellular iron abundance, and these molecules display a high affinity for ferric iron. Four types of structurally distinct molecules are thought to be involved in iron acquisition in mycobacteria, salicylic acid and citric acid being the simplest, followed by mycobactins and exochelins. Mycobactins and exochelins are complex molecules with very high affinities for iron and are integral to the bacterial strategy for obtaining iron from the environment (DeVoss *et al*., 1999).

An alcohol extract of *Mycobacterium phlei* designated as mycobactin was used to isolate MAP. The designation of the mycobacterium of origin was later added to the name. Accordingly, mycobactin from *M. phlei* was renamed as mycobactin P, from *Mycobacterium tuberculosis* as mycobactin T and from MAP as mycobactin J. Mycobactin J, which reduces the isolation time by up to 3 weeks and increases the numbers of detected CFU by 10–20%, is used as a growth stimulator at present. MAP can

be isolated in media enriched with 1% ferric ammonium citrate and sodium pyruvate (Pavlik *et al*., 2009).

Primary colonies of MAP on solid media may be expected to appear any time from 5 weeks to 6 months after inoculation. Sheep strains, including the uncommon, bright yellow pigmented types, grow less well than cattle strains on commonly used media such as Herrold's egg yolk medium (HEYM) or Lowenstein Jensen (LJ) medium and primary cultures should not be discarded as negative without prolonged incubation (Whittington *et al*., 1999).

Primary colonies of the cattle strain of MAP on HEYM are very small, convex (hemispherical), soft, non-mucoid and initially colourless and translucent. Colony size is initially pin-point. It may remain at 0.25–1 mm, and tend to remain small when colonies are numerous on a slope. Colony margins are round and even, and their surfaces are smooth and glistening. The colonies become bigger more raised, opaque, off-white cream to buff or beige coloured as incubation continues. Older isolated colonies may reach 2 mm. The colonial morphology changes with age from smooth to rough, and from hemispherical to mammilate (Quinn *et al*., 2011).

On modified 7H10 medium, colonies of the cattle strain are less convex than those on HEYM, especially in aged cultures. They are pin-point to approximately 1 mm in diameter and, being buff coloured, are only slightly lighter than the media. Compared with colonies of cattle strains on HEYM, those on 7H10 are more difficult to detect. Colonies of the sheep strain of MAP on modified 7H10 are convex, soft, moist, glistening, off-white to buff, and very similar to the colour of the media. Colonies are typically between pinpoint and 0.5 mm, but can reach 1 mm, and rarely 1.5 mm if few colonies occur on a slope (OIE, 2014d).

2.4. Epidemiology of Paratuberculosis in Sheep and Goats

2.4.1. Occurrence and geographic distribution

Paratuberculosis is of major importance in cattle and sheep in temperate climates and some humid, tropical areas. The incidence is greatest in animals kept intensively under climatic and husbandry conditions which are conducive to the spread of infection. In Iceland the annual morbidity of sheep during the epidemic averaged 8-9 % in affected areas and was up to 40% on individual farms. Sheep are easily infected experimentally and excrete large numbers of the organism but many recover spontaneously. However, the disease can also cause significant financial losses to the sheep farmer. For example, the disease has become established in sheep flocks in Cyprus where sheep are farmed semi-intensively for cheese production, and losses can be as high as 4% per year (Radostits *et al*., 2006). In India 27.1% – 75.1% morbidity and 4.6% – 24.8% mortality was reported in sheep (Singh *et al*., 1992).

It has traditionally been considered a disease of temperate regions with sporadic occurrence in tropical environments, primarily as a result of importation of infected livestock from endemic areas. The subclinical infection rate in an infected herd is likely to be much higher than the rate of clinically apparent cases (Smith and Sherman, 2009). The disease is being recognized with increased frequency in goats than sheep and when established in goat flocks can cause large losses. In Australia, paratuberculosis occurs in dairy goat breeds with some endemic foci (Radostits *et al*., 2006).

Most information about paratuberculosis comes from dairy cattle. However, it is important to consider that the disease also is exhibited similar patterns in small ruminants (NRC, 2003). No systematic global survey has been completed for paratuberculosis or for the presence of MAP in domesticated animals, but paratuberculosis has been reported on every continent of the world except Antarctica. Some countries, principally island nations, have reported no cases of paratuberculosis, and others have reported paratuberculosis limited to specific geographic zones. Determining worldwide prevalence

with any degree of certainty is complicated by the lack of international consensus on population-testing protocols (OIE, 2014a).

Documentation of the disease in sheep is less well recorded than in cattle. The disease is endemic in many countries and its introduction into Iceland by five rams from Germany in 1933 and subsequent spread to sheep and cattle has been well documented. The introduction of paratuberculosis to New Zealand and Australia appears to be more recent and has been the subject of great efforts at control in Australia (Sharp, 2007).

Caprine paratuberculosis has been specifically described in numerous countries representing all continents but Antarctica. These countries include the Sudan, India, Nepal, Korea, Turkey, Israel, Cyprus, France, Greece, Norway, Spain, Switzerland, Canada, the United States, Mexico, Chile, Australia and New Zealand (Smith and Sherman, 2009).

OIE information database on animal health from 1980 to 2014 indicates that most of the countries in Africa have had at least one incident of the disease or the disease was suspected in cattle, sheep or goats. Algeria, Niger, South Africa are reported to have had the disease in cattle, sheep and goats at the same time. By the first half of 2012, Algeria, Egypt, Mauritius, Kenya, Sudan, Lesotho and Tunisia were reporting absence of the disease (OIE, 2014a). Based on isolation and confirmation of the etiologic agent, ovine paratuberculosis was reported in Zambia (Pandey *et al*., 1989). In addition serological and histological based study showed ovine paratuberculosis was reported as an emerging disease in provinces of South Africa (Michel and Bastianello, 2000). Paling *et al*. (1988) found antibodies to MAP in goats and camels using complement fixation test (CFT) in the coastal region of Kenya. Paratuberculosis was reported in Sudan in goats (Aradaib *et al*., 2005).

In Ethiopia, Samuel and Wirtu (1995) made an attempt to report a case of paratuberculosis in cattle from the country based on clinical history and microscopic examination of examination of the agent. Recently, Mohammed (2014) reported for the

first time the isolation of MAP from apparently healthy cattle population in the country. However, so far there is no report of the occurrence of paratuberculosis in sheep and goats of Ethiopia.

2.4.2. Predisposing factors

The causative agent of paratuberculosis is relatively resistant and survives in the environment for a long time: in river water for 270 days, in faeces and black soil for 11 months, in liquid manure at 5 ◦C for 252 days, but in urine for 7 days only. If frozen to -4 ◦C, it can survive for at least 1 year (Richards, 1981). MAP can survive inside an infected host organism for a very long time (up to several years). In the environment, the causative agent of paratuberculosis can survive in a state of metabolic quiescence for several months or years (Whittington *et al*., 2004).

Age at initial infection is an important predisposing factor. If sheep become infected as lambs, they are more likely to develop clinical signs, whereas if infected as adults, overt disease is less common. Experimentally, as few as 1000 MAP will cause infection in lambs (Sharp, 2007). In addition, some adult animals exposed to infection for the first time may develop the disease (Payne and Rankin, 1961; Larsen *et al.*, 1975). Moreover, experimental studies have demonstrated that infection is favoured by the use of young animals and high doses of the organism (Nisbet *et al.,* 1962; Larsen *et al*., 1975).

The information on breed incidence is controversial. The frequency of disease in any particular breed is proportional to the abundance of that breed, and paratuberculosis will occur with highest frequency in the most predominant breed (Radostits *et al*., 2006).

An association between high prevalence of MAP infection in ruminants and soil type has been recognized. The pH of the soil may influence the severity of the clinical signs. Cattle raised on alkaline soils, especially in limestone rich areas, may have a high incidence of infection but little clinical disease. A high prevalence of clinical paratuberculosis is recorded in the United States of America on acidic soils in contrast to alkaline soils. In addition, in Netherlands where clinical paratuberculosis was common in

areas of low calcium content and low pH, as the pH and calcium content of the soils increased, the incidence of clinical paratuberculosis decreased. In another report, lime was applied to the pastures to elevate the soil pH in excess of recommended pasture guidelines, and cases of paratuberculosis abated and had not returned for several years (NRC, 2003). Adult cattle moved from herds where the soil is alkaline to areas where the soil is acid often develop severe fatal clinical disease. Other reported risk factors include intensive farming systems, low dietary intake, stress related to transport, lactation and parturition and immunosuppression by concurrent diseases (Radostits *et al*., 2006).

This is based on the knowledge that iron is an essential trace element for most bacteria, including MAP, and iron availability is pH dependant. Thus, at neutral pH iron forms insoluble colloidal hydroxides that can only be taken up by the formation of chelate complexes with iron-binding compounds such as mycobactins. These are specifically produced and excreted by most mycobacteria for this purpose. Since MAP is a particularly poor iron chelator and cannot produce sufficient mycobactin to sustain iron transport into the cells, the availability of soluble iron at a low pH greatly enhances survival and growth in an acidic environment (Neilands, 1981).

2.4.3. Means of transmission

The primary route of MAP transmission in all species is fecal-oral. Excretion (shedding) of variable numbers of MAP in the faeces/manure of most affected animal species has been documented, as has the relative ease of experimental oral transmission in young cattle and sheep. Fecal contamination of the udder or calving/lambing environment is therefore thought to be the primary risk factor for neonatal infection (Clarke, 1997).

In experimental infection of goats with clinical paratuberculosis, MAP was isolated from blood cultures and numerous tissues at necropsy, including the udder and uterus. This suggests that offspring born to clinically infected does have a very high likelihood of infection through the birth process or when suckling, if not already infected as fetuses (Smith and Sherman, 2009).

There is a claim that nematode larvae may be a suitable and efficient vehicle for indirect transmission of MAP in sheep with clinically advanced Johne's disease, as the organism was recovered from third stage larvae and ingestion of contaminated larvae would be followed by release of MAP into the lumen of the gastrointestinal tract as the larvae exsheath with subsequent release of free bacteria to attach with and penetrate specialized epithelial cells in the small intestine (Whittington *et al*., 2001).

2.4.4. Prevalence

The prevalence of infection in a region is difficult to estimate because of the uncertainty of the diagnosis and the failure to report cases unless a specific survey or eradication program is undertaken (NRC, 2003). It appears that the prevalence of infection has been increasing and there are large variations in the estimates of prevalence. The disease has a highly clustered geographic distribution in Australia and based on surveys as of the year 2000, flock prevalence were 0.04-1.5%, 8-15%, and 29-39% for low, moderate, and high prevalence regions, respectively (Radostits *et al*., 2006).

The prevalence of caprine paratuberculosis is not well documented and presumably it may vary widely between countries because of local management conditions, infectivity of MAP and other factors. The prevalence is likely to be higher in intensively managed goats than in extensively managed goats. In Norway, where goats are kept intensively for dairying, nationwide prevalence was as high as 53% before the introduction of a vaccination program (Smith and Sherman, 2009).

In Portugal, Mendes *et al*. (2004) reported 27% sero-prevalence on sheep and goat flock, with higher prevalence in goats (5.7%) than sheep (2.6%). Sirak (2010) showed culture based prevalence of 8.8% from sheep and goat slaughtered at abattoirs in Jordan. The prevalence rates reported in small ruminants in some other countries were: 73.7% in sheep in Italy (Anna-Rita *et al*., 2011); 46.7% in sheep in Portugal (Coelho *et al*., 2007); 52% in sheep and 50% in goats in Cyprus (Liapi *et al*., 2011); 48–57% in goats and

42.4% in sheep in Brazil (Medeiros *et al*., 2012) and 43.3% in sheep in India (Barad *et al*., 2014).

2.4.5. Molecular epidemiology

Although some MAP types are more prevalent in particular animal hosts, no evidence of exclusivity has been found with only weak associations between hosts and some genotypes, probably resulting from local clonal distributions (Stevenson *et al.*, 2009). Some MAP genotypes cause less severe lesions than others in the same host suggesting that subtle combinations of specific mycobacterial gene expressions may facilitate host/cell interplay (Verna *et al*., 2007) and that some MAP strains may be in the process of adapting to host environments (Wynne *et al*., 2011).

It is important to understand the role of cross-species transmission in causing paratuberculosis outbreaks and in maintaining infection cycles. Despite strain differences, cross-species transmission of MAP between sheep and cattle has been demonstrated both naturally and experimentally. Molecular epidemiology has confirmed that C strains predominate in cattle populations and S strains predominate in sheep. Nevertheless, C strains also predominate in goats, camelids, and ruminant and non-ruminant wildlife. This evidence suggests that sheep strains are less likely to be transmitted to other species than cattle strains are, but it is not clear how much of this is attributable to differences in susceptibility or to differences in exposure (NRC, 2003).

2.5. Pathogenesis of Paratuberculosis

In oral transmission, after ingestion, MAP organism is taken up by specialized M cells over Peyer's patches, lining the intestine. In some cases small numbers of villous epithelial cells adjacent to the dome regions become infected, but enterocytes in other areas are not affected (Garcia-Marin *et al.*, 1992). Moreover, experimental study showed that, caprine and bovine origin MAP showed invasion through M cells and the enterocytes, while bacterial translocation across M cells tends to be greater than the enterocytes. In addition, bacterial invasion was greater in ileal loops of lambs as

compared to jejunal loops and bacterial uptake was higher in Peyer's patch areas than that of non-Peyer's patch areas (Ponnusamy *et al.,* 2013). Uptake is through the interaction of fibronectin attachment proteins with fibronectin, followed by binding to integrins on the surface of cells. The preference of MAP for M cells is likely to be due to the abundance of β1 integrins on the cell surface (Sigurdardottir *et al*., 2004).

After uptake by the M cells, MAP bacilli are transferred to underlying lymphoid tissue and ultimately taken up by macrophages. Endocytosis of mycobacteria involves different receptors on phagocytic cells, which either bind to non-opsonized mycobacteria or recognize opsonins on the surface of mycobacteria. The macrophages in the intestinal wall and in the regional lymph nodes contain large numbers of mycobacteria. However, there might be dissemination via the bloodstream, with subsequent localization to secondary sites such as the liver, spleen and peripheral lymph nodes (Momotani *et al*., 1988). On experimental inoculation of calves with MAP strains, multiple tissue infection and colonization was reported with highest preferential colonization of the jejunum, followed by the ileum, duodenum and spiral colon along with the associated lymph nodes while, showed less preference for ileocaecal valve, spleen, transcending colon and descending colon. But, lesions consistent with paratuberculosis were most common in the ileum and ileocaecal valve, where as less commonly in jejunum and cecum (Stabel *et al.*, 2009).

Mycobacteria have evolved as pathogens to survive and replicate in macrophages and to overcome multifactorial hostile innate and acquired host immune mechanisms, so that they can survive and eventually multiply and transmit to other animals (Bartow and McMurray, 1998). Mycobacteria survives intracellular killing by inhibiting phagosome maturation. This is achieved by reducing the activity of a macrophage enzyme, sphingosine kinase; by secretion of a lipid phosphatase that inhibits phosphatidylinositol 3-phosphate production, thereby disallowing the acquisition of lysosomal constituents by phagosomes; and interaction of mannose-capped lipoarabinomannan (ManLAM) with mannose receptors on macrophage resulting in limited phagolysosome fusion (Deretic, 2008; Sohal *et al*., 2008). Gollnick *et al*. (2007) indicated that bacterial genotype is

important in the survival and host specificity of MAP, in that sheep strains of MAP initially showed very high number of bacterial infection in bovine monocyte-derived macrophages (MDMs), but subsequently showed a significant decline in bacteria per infected cell as compared with the cattle strain.

An important aspect of MAP infection is its ability to modulate host immune response. For instance genes coding pro-inflammatory factors such as IL-1β, IL-1α, chemokine ligand 2 (CXCL2), prostaglandin-endoperoxide synthase 2 (PTGS2/COX2), lipocalin (LCN2) and TNF-α were down-regulated in MAP infected macrophages as compared with MAA infected. These are important in the development of innate and adaptive cell mediate immune response to mycobacteria (Basler *et al*., 2008). In addition there is down-regulation of MHC antigen expression in infected macrophages. Hence, the bacteria are able to replicate inside nonactivated macrophages, eventually leading to the death of the infected cells as MHC antigen expression activates CD4+ T cells, which leads to macrophage activation, nitrogen monoxide (NO) production and MHC class II expression (Olsen *et al*., 2010).

In animals that fail to contain the infection, replication within tissues progresses and more of the intestinal lining (mucosa) and regional lymph nodes are infiltrated by macrophages filled with mycobacteria. This phase of infection corresponds with the prolonged incubation period observed clinically. Eventually, the accumulating mycobacteria laden macrophages interfere with intestinal absorption, resulting in weight loss and an initially intermittent diarrhea. Formation of mycobacterial antigen-antibody complexes in the infected intestine results in histamine release, thus exacerbating the diarrhea. Failure of the immune system to contain the infection results in a continuously increasing mycobacterial burden and progressively more severe clinical disease. In the terminal stages of the infection, immune cells become functionally nonresponsive, resulting in uncontrolled replication and spread of MAP in tissues (Chiodini *et al*., 1984).

2.6. Host Immune Response to MAP Infection

The first line of defense against invading MAP in the ruminant intestine involves M cells and phagocytic macrophages. In early stages of infection the organism is found in phagocytic macrophages in the intestine. In addition, at early stage of MAP infection, there is an elevated level of defensins, which are host antimicrobial peptides and pro-inflammatory cytokines that disrupt microbial cytoplasmic membranes (Khare *et al*., 2009).

During the early subclinical stages of infection, the organism elicits a cell mediated response by the host that can be characterized by strong delayed-type IV hypersensitivity reactions, lymphocyte proliferation responses to mitogens and production of cytokines by stimulated T lymphocytes (Waters *et al.,* 2003). Activated T cells traffic back to the site of mycobacterial infection and interact with infected phagocytes, generating the characteristic granulomatous inflammatory response. The arrival of Th1-type CD4+ cells to the site of granuloma formation is an important event in halting replication of the mycobacterium as a result of IFNγ production by these cells which enhances the mycobactericidal activity of macrophages (Cooper *et al*., 1997). Increased numbers of CD4+ cells and γδ T cells are seen in the ileal and jejunal Peyer's patches in young sheep a few weeks after exposure to MAP (Reddacliff *et al*., 2004). In calves inoculated surgically with MAP, intestinal lesions containing lymphocytes were detected at 6 months post challenge even though mycobacteria were detected in these tissues by day 30 (Wu *et al*., 2007a). In vitro γδ T cells from calves do not produce significant amounts of IFNγ in response to interactions with MAP infected macrophages (Simutis *et al*., 2007). Perhaps the role of these cells in paratuberculosis is in monitoring cellular traffic to the granuloma, allowing access to lymphocytes and macrophages but limiting the movement of inflammatory cells such as neutrophils that can cause tissue damage (D'Souza *et al*., 1997).

In paratuberculosis, lesions are seen at the site of predilection, intestinal tissues and also in lymph nodes draining the site. Granuloma formation is necessary to contain and

control infection but is also responsible for the damaging pathology of these diseases (McIlroy *et al*., 1986). The cellular micro-architecture of a granuloma comprises a mixture of macrophages and lymphocytes (Schlesinger, 1996). The macrophages are capable of differentiating into multinucleate giant cells which are generally located centrally and are surrounded by epithelioid macrophages (Pieters, 2008) and a zone of CD4+, CD8+ T cells and B cells (Serbina and Flynn, 2001; Serbina *et al*., 2001).

In paratuberculosis the granulomas do not caseate (except in deer) and lack a fibrous capsule, rather they are more diffuse and may occupy and distend most of the lamina propria. Lymphocytes are interspersed throughout MAP lesions, which can vary from small foci to larger granuloma (Perez *et al*., 1996; Clarke, 1997). Paucibacillary lesions contain few mycobacteria and macrophages but have numerous lymphocytes. Multibacillary lesions are densely packed with macrophages and intracellular mycobacteria. These enteric lesions appear to progress from paucibacillary to multibacillary, but mild paucibacillary lesions can regress (Dennis *et al*., 2010).

As the disease progresses, the cell mediated immune response wanes, while a strong humoral response becomes dominant. This is associated with a decreased ability of mononuclear cells to produce IFN-gamma, both specifically and nonspecifically at the site of infection and in the blood (Waters *et al.*, 2003). In support of this, increases in the expression of genes for Th2 type cytokines have been observed from animals with multibacillary disease while paucibacillary type lesions are associated with a Th-1 biased immune profile (Smeed *et al*., 2007; Gillan *et al*., 2010).

The loss of putatively protective CD4+ T cells leads to a lack of control of mycobacterial replications and, subsequently, to the progressive granulomatous enteritis. Antibody to MAP does not protect the host against disease; rather, Th 1-mediated immunity appears to be essential to keep the infection under control. The host immune system begins a series of attacks against MAP infected macrophages, including the rapid development of activated T cells, CD4 + T cells, and cytolytic CD8+ cells. These cells interact with the persistently infected macrophage and with each other through a complex network of

cytokines and receptors. Despite these aggressive efforts to clear the infection, MAP persists and the constant struggle of the immune system leads to pronounced injury to the intestinal epithelial cells (Stabel, 2000). During the final stages of disease, lack of antigen specific cell-mediated immune response or complete anergy may result, allowing for rapid dissemination of the infection throughout the host (Koets *et al*., 2002).

2.7. Clinical Courses of Paratuberculosis in Sheep and Goat

A distinguishing characteristic of paratuberculosis is that infection occurs in animals at an early age, usually under 30 days of age, and clinical disease does not occur until 3-5 years of age. Exact details of the effect of exposure to infection in adults are not available, but it is probable that some animals exposed for the first time as adults develop clinical disease while others may become carriers of the organism without manifesting clinical signs (Radostits *et al*., 2006).

Clinical signs usually develop months to years after the initial exposure, depending on the species and strain of MAP. In cattle, the incubation period is generally 3–10 years while in sheep and goats the period of subclinical disease tends to be shorter, although there is no defined time frame and individual animals vary enormously in disease progression and outcome. In a study of infected sheep flocks, the majority of deaths occurred at 3–4 years of age (Bush *et al*., 2006). All ruminants enter a subclinical phase of the infection that varies in length and is eventually subject to intermittent shedding of bacteria in the faeces (Fecteau and Whitlock, 2010). Intestinal and lymph node lesions develop during the sub-clinical period (Whitlock and Buergelt, 1996). A recent longitudinal study following individual sheep through biopsy revealed evidence for recovery in some cases (Dennis *et al*., 2010).

Infected animals can, according to the intensity of the shedding of MAP, be categorized as non-shedders, low shedders and high shedders. Based on this aspect and on the clinical status, the animals in an infected herd may be classified as susceptible animals, resistant animals and recovered or “super-resistant” animals. Susceptible animals are clinically

normal or ill animals, which continuously shed MAP or are clinically normal animals which shed MAP non-intensively/non-regularly. Resistant animals are clinically normal animals, and never shedding MAP. Recovered or “super-resistant” animals are formerly MAP shedding animals who have spontaneously recovered (Toman *et al*., 2003).

Clinical disease in sheep and goat is characterized by production loss, roughening of hair coat and gradual weight loss despite normal appetite. In advanced clinical stage there is lethargy, weakness, emaciation, intermandibular edema due to hypoproteinemia, cachexia and profuse diarrhea. Diarrhea is less frequent and onset occurs in younger animals. When diarrhea does occur, it typically attends end-stage disease (Sharp, 2007). In goat overt clinical disease rarely occurs before one year of age and is most common in goats two and three years of age. Clinical disease is often triggered by some episode of stress, such as parturition or recent introduction into a new herd. Soft stool, like dog feces in appearance, may occur intermittently through the course of disease, but normal pelleted feces are the rule (Smith and Sherman, 2009).

2.8. Pathology of MAP Infection

2.8.1. Gross pathology

2.8.1.1. Sheep

In sheep the principal pathological changes centre on the intestine and the related lymphatic and lymphoid tissues, although, in advanced cases, there is wasting, with gelatinous atrophy of fat depots and serous effusion into body cavities. Thickening and corrugation of the intestinal mucosa, lymphatic cording and lymphadenopathy were evident. Carcass emaciation and oedema, ascites and hydropericardium, intermandibular oedema, and atrophy and necrosis of fat were also noted. The intestinal mucosa was frequently reddened, showed crevices, and had a granular appearance. Some severe clinical cases showed only minor macroscopmal abnormalities. Lesions occurred

particularly in the terminal ileum but commonly extended to the colon and jejunum, and occasionally the duodenum (Perez *et al.*, 1996).

In ileum more usually visible change is a slight fleshy or velvety thickening, or a faint granularity of the surface, perhaps with slight congestion. These subtle changes may be overlooked in a cursory examination. Occasionally, there may be a tendency for the mucosa to form fissures when bent over the fingers. Gross changes in sheep with paratuberculosis are often difficult to detect, and do not resemble those caused by the disease in cattle (Clarke, 1997). A particular feature of many ovine cases, especially in British studies, was the orange-yellow colour of the intestinal mucosa, caused by pigmented strains of mycobacteria (Clarke and Little, 1996).

2.8.1.2. Goat

In natural case of paratuberculosis goat showed emaciation, myxomatous connective tissue replacing the fat deposits. The ileal and jejunal walls are often thickened and occasionally corrugated. In most cases jejunal thickening are clearly seen, whereas the ileum showed little if any thickening. The serosal and mesenteric lymphatic vessels appeared as enlarged white cords with small necrotic foci. Fibrosis of the contiguous mesentery is also a common feature. The mesenteric and ileocaecal lymph nodes may enlarge and showing oedema with necrotic or calcified foci (Corpa *et al*., 2000).

Fibrous adhesions between the mesentery and the small intestinal serosa or the capsule of the jejuna lymph nodes, as well as between the serosal surfaces of the small and large intestines may be evident. Markedly thickened intestinal wall of the small intestine with intestinal strictures was also noted. Nodular foci of caseous necrosis, sometimes with mineralization, have often been described in the intestinal mucosa, submucosa, serosa, lymphatics, particularly, in the mesenteric lymph nodes. In addition, transverse folds and an uneven, cobblestone-like surface with hyperaemia, ulceration and fibrinous exudates were also seen in some case of natural paratuberculosis. Single or multiple large white, mineralized foci or nodules were seen in the liver of goats (Lybeck *et al*., 2013). Goats

may also show axonal degeneration of sciatic and brachial plexus nerves, and amyloidosis of the renal glomeruli, adrenals and mammary gland (Barker *et al.,* 1993).

2.8.2. Microscopic lesion

Microscopically, the characteristic lesion is chronic diffuse catarrhal enteritis characterized by hyperplasia of macrophages, lymphocytes, plasma cells, epitheloid and giant Langhans cells (multinucleated) in the lamina propria, intestinal sub–mucosa and cortical and para–cortical region of regional lymph nodes leading to atrophy and fusion of intestinal villi with thickening of the mucosa. In some cases, granulomatous lymphangitis can also be observed. In the lymph nodes, the sub capsular and peritrabecular cortical sinuses contain numerous macrophages. Microscopic observations after ZN show AFB, in clumps or within macrophages (Waller, 2000).

2.8.2.1. Sheep

Two distinct types of pathology are apparent, based on the abundance of mycobacteria and cellular infiltrate. The more common form, known as lepromatous or multibacillary, is characterized by numerous acid-fast MAP, packing the cytoplasm of the many large macrophages that infiltrate the mucosa in all cases, forming extensive, diffuse sheets. Lymphocytes and granulocytes are present in much lower numbers. Occasional multi-nucleate, Langhan's-type giant cells may be seen. These changes cause marked thickening of the intestine. The less common form, known as tuberculoid or paucibacillary, comprises approximately 30 % of cases. Thickening of the intestinal wall is less prominent and may be difficult to distinguish from unaffected gut. It is characterized by a more marked lymphocytic infiltrate with scattered, small focal granulomata and giant cells. Lesions may exhibit caseation, calcification or fibrosis, the resultant nodular lesions being visible macroscopically. Acid-fast bacilli are sparse or undetectable in tuberculoid lesions, and are usually absent from caseous or calcified foci (Perez *et al*., 1996).

2.8.2.2. Goat

In goat the earliest histologic lesions in experimental cases consisted of clusters of epithelioid macrophages and giant cells in the basal regions of ileal and jejunal Peyer's patches at three months after inoculation. Lesions tended to coalesce and had extended into the large intestine by 10 months, wher mucosal ulceration without caseous necrosis was evident. In some cases, nodular foci of caseous necrosis with mineralization have been described in the mucosa, submucosa, serosa, lymphatics, and particularly in the mesenteric lymph nodes, which could be easily confused with signs typical of *M. bovis* or *M. tuberculosis* infection. In advanced cases, the lesions of granulomatous enteritis are similar to those seen in other ruminants, but goats also exhibit lesions in the sciatic and brachial plexus nerves (Clarke, 1997).

2.9. Diagnostic Methods of Paratuberculosis

There are no specific clinical signs, and paratuberculosis must be differentiated from other chronic wasting diseases. After initial phase of infection, a number of different sequelae occur, the outcome of which are related to the ability of the host to mount an effective cell-mediated immune response as well as the dose of the initial infection, as a heavy initial infection is more likely to be overcome than a light one. The predominant early immune response is cell-mediated and antibodies are undetectable. During the late stages of disease, the type of host response correlates with the type of pathology. It is clear, therefore, that no single diagnostic test is adequate. In an individual animal, ante-mortem diagnosis, particularly during the preclinical stages, is unreliable because all available diagnostic tests suffer from poor sensitivity and/or poor specificity. Post-mortem diagnosis is more reliable (Reddacliff and Whittington, 2003).

2.9.1. Direct detection of the causative agent

2.9.1.1. Microscopic examination

Ziehl–Neelsen staining is based on the resistance of the mycobacteria to decolorization by acid alcohol treatment. This method has the advantage of being simple, fast and inexpensive, but has the disadvantage of having low sensitivity and specificity. In smears of tissues (intestines near ileocaecal valve or in the intestinal lymph nodes with gross lesions) visualization of groups of brightly pink colored bacilli within the resident macrophages in the lesions is highly suggestive of paratuberculosis (Singh *et al*., 2014). Finding clumps of acid-fast organisms in smears may indicate infection, but a negative result does not rule out the possibility of MAP infection (Reddacliff and Whittington, 2003).

2.9.1.2. Bacteriological culture

The isolation of MAP from an animal provides the definitive diagnosis of infection with the organism. Although culture is technically difficult and time-consuming to carry out, it is the only test that does not produce false-positive results (100% specific), as the colony material can be further tested for confirmation of the result with molecular methods or with the classical methods to judge growth and morphological characteristics (Whittington *et al.*, 1999). It is an essential step for later application of the standardized molecular typing techniques IS*900* RFLP and PFGE. The major problems associated with the use of different culture protocols and media for the isolation of MAP includes, the long incubation periods required to obtain visible colonies and the failure to obtain MAP isolates in some clinical samples obtained from variety of hosts and of course at different disease stages (de Juan *et al*., 2006).

The faecal culture is widely considered to be the gold standard for the diagnosis of paratuberculosis in live animals. Actually, the faecal culture is able to detect most animals in advanced stages of the disease, but identifies only a few animals in early stages of infection. According to the conditions, sensitivity of faecal culture is 70% for

affected cattle (animals with diarrhoea, chronic weight loss or reduced milk production), 74% for infectious cattle (those that shed MAP at the time of testing with the test under evaluation and thereby they are a risk for transmission of MAP to susceptible herd-mates) and 23–29% for infected cattle (animals carry MAP intracellularly but substantial replication need not take place because the infection can be latent). Moreover, due to the potential pass-through phenomenon, it is theoretically possible that faecal culture testing of non-infected animals on contaminated premises can lead to false-positive reactions (Nielsen & Toft, 2008). Culture could only be regarded as "gold standard" on faecal samples from clinical cases that nearly always are shedding MAP. To be regarded as a general "gold standard" for paratuberculosis, it has to be applied on suitable lymph nodes and intestinal samples (preferably, last part of ileum and adjacent lymph node) (Whittington, 2002).

There are several ways to culture MAP using solid or broth culture methods. The most common solid media in use includes HEYM, LJ medium and Middlebrook 7H10 or 7H11 slants. HEYM is popular in many regions, but a modified LJ medium is preferred in some areas of Europe (NRC, 2003). Because of the long doubling time of MAP, fully automated broth based systems have been used in order to detect MAP growth faster (Schwartz *et al*., 2000; Ellingson *et al*., 2004; Naser *et al*., 2004; Chamberlin and Naser, 2008). However, automated liquid culture systems are prone to higher false positive readings due to cross-contamination for *M. tuberculosis* and *M. avium* culture. In addition, some broth culture systems use reagents that may inhibit MAP growth (Gascoyne-Binzi *et al*., 2001). While the growth rate of MAP is slower on solid media compared to broth based media, a pure isolate can only be obtained from solid media. Liquid media may contain other strains of the same species or other species and therefore for primary isolation of a pure isolate, the use of solid media is preferable. Liquid media is useful once a pure isolate is obtained and contamination can be monitored (Okwumabua *et al*., 2010). *In vitro* cultures of MAP require supplementation of medium with mycobactin J, which is available commercially (Whipple *et al*., 1991).

Culture of MAP from the faeces or tissue of animals such as sheep and goats is less successful due to the strain of MAP that usually infects these animals. The S strains have a slower rate of growth and more than one type of media should be used to isolate all possible types. For instance the combined use of HEYM, LJ and Middlebrook allows detecting 100% of S strains and 98 % of C strains (de Juan *et al*., 2006).

A comparison of culture media suggests that mycobactine J supplemented HEYM and LJ supported the growth of many strains with the exception of some strains (mostly S strains). In addition, more isolates grew on HEYM supplemented with mycobactine J but without sodium pyruvate, while there was no difference on the proportion of isolates on LJ with or without sodium pyruvate. Middlebrook 7H10 and 7H11agar supplemented with mycobactine better supports the growth of all tested ovine, bovine and bison type strains isolates from different countries (Whittington *et al*., 2011).

Sodium pyruvate was reported to stimulate the growth of some isolates of MAP in HEYM or LJ medium supplemented with mycobactine J (Jorgensen, 1982). The addition of sodium pyruvate to the HEYM increased the recovery of isolates by 36 and 30.3% in cattle and goats, respectively (deJuan *et al*., 2006). However, recent studies showed that, the addition of sodium pyruvate in HEYM inhibited the isolation of some sheep strains from Spain while enhancing the growth of cattle isolates (Whittington *et al*., 2011; Dimareli-Malli *et al*., 2013). A preliminary MAP liquid medium (Middlebrook 7H9, 7H12) supplemented with oleic acid, bovine albumin, dextrose and catalase (OADC) may be used to accelerate the detection of MAP and the activation of MAP prior to the culture on solid medium (Sohal *et al*., 2012).

MAP strains have been sowed to grow in media supplemented with 1% ferric ammonium citrate used as a substitute of mycobactin J, and compared to mycobactin supplement media, with observed comparatively greater stimulatory effect of media supplemented with mycobactin J than media supplemented with 1% ferric ammonium citrate. However, the difference was temporary; i.e., visible and maximum growth occurred about 2 weeks earlier on media that contained mycobactin than on media that contained ferric

ammonium citrate; but after 10 weeks of incubation, there usually was no observable difference. It was also reported that, growth was stimulated when ferric ammonium citrate and sodium pyruvate were autoclaved separately and then either combined or added separately to cold media by flooding them on the surface and allowing them to diffuse into the media. When they were autoclaved together before they were added to media, no growth occurred (Merkal and Curran, 1974).

Homuth *et al*. (1998) identified extracellular mycobacterial enzyme (reductase) in MAP, which was capable of mobilizing iron from different sources such as ferric ammonium citrate, ferritin, and transferrin by reduction of the metal. The authors reported, higher enzymatic activity at 37°C and between pH 5 and 10.

Genomic study showed that MAP strains had differential expression of genes involved in iron metabolism when exposed to elevated iron concentrations. Hence, Janagama *et al*. (2009) indicated that, in iron rich laboratory media, iron storage gene was up-regulated by cattle strain, while it was down-regulated by sheep strain, suggesting the defective iron storage pathway leading to iron toxicity in the later strain. Thus, when excess iron is provided in culture media it may contribute to the differential growth characteristics among MAP strains.

The cultural criteria to identify MAP are the slow growth rate, the morphology of the colonies, acid fastness in ZN staining and dependence on mycobactin J (mainly in primary culture) (Quinn *et al*., 2011). Primary isolation of MAP is very difficult and takes long time (at least 6 to 8 weeks) despite supplementation with mycobactin J. On HEYM with mycobactine J colonies appeared usually at about 7 weeks (Whittington *et al*., 2011). The rate of growth of MAP is an important practical consideration in diagnostic laboratories and should be assessed when selecting a culture medium for routine use. It is well recognized that incubation periods for some solid media often need to be prolonged; for example, 70% of S strain isolates took more than 3 months to grow, and 50% took 5 to 7 months on LJ or Middlebrook 7H11 medium (de Juan *et al*., 2006). After 7 weeks of incubation, most MAP colonies on HEYM containing mycobactine J

were whitish pin-point in size; where as an initial cream colored colonies with subsequent changes to whitish color may be seen on 7H10. On 7H11 translucent, white or occasionally yellow colonies were seen. It has been reported that many isolates on LJ media with mycobactine J but with-out sodium pyruvate required prolonged incubation to yield only minute colonies as compared to the same media containing sodium pyruvate (Whittington *et al*., 2011).

When culturing MAP from the environment, animals or humans, the sample must be decontaminated in order to remove faster growing microbial species and allow the slower growing mycobacterial population to be identified. Various decontamination agents have been tried and used over the years, most being used in decontaminating sputum samples for *M. tuberculosis* identification (Whipple *et al*., 1991).

Various chemicals and antibiotics that are selectively toxic to organisms other than MAP has been included in decontamination methods. Hexadecylpyridium chloride (HPC) is probably the most commonly used decontaminant; it is less toxic to MAP than are other commonly used decontaminants. HPC is commonly used in North America, but sodium hydroxide is more common in Europe. Other decontaminants are oxalic acid; sodium hydroxide and oxalic acid followed with neomycin and amphotercin B. The double-incubation method (also called the Cornell method) is commonly used for decontamination. It includes a pre-incubation step with brain-heart infusion medium that initiates germination of bacterial and fungal spores, followed by centrifugation, and then a second step with the addition of antibiotics (amphotercin B, vancomycin, and nalidixic acid) to kill the spores that subsequently germinate. Double-incubation culture method demonstrated a higher sensitivity of detection and that it reduced contamination more effectively than did the conventional sedimentation method (NRC, 2003).

Standard decontamination involves incubation with 0.6–0.75% hexadecyl pyridinium chloride (HPC) or NaOH decontamination for 3 h to overnight (Whipple *et al*., 1991; Reddacliff *et al*., 2010). A study looking at milk samples found that leaving MAP exposed to HPC decontamination for 24 h can kill 45% more cells than decontaminating

for 5 h. Again in milk samples it was demonstrated that there was a 50% reduction in MAP recovered if decontamination occurred at 37 °C rather than room temperature (Gao *et al*., 2005). MAP, like most mycobacterial species, is still susceptible to HPC and is one of the most sensitive; therefore it is imperative that decontamination does not remove too many viable MAP cells while still removing background contamination. However, HPC decontamination does not affect cell numbers from bovine blood after 72 hours (Bower *et al*., 2010).

After the culture specimen is decontaminated the MAP organisms must be concentrated to enable detection of lower numbers of organisms (to increase the sensitivity of the technique). Centrifugation of supernatant has been reported to increases recovery of MAP from clinical specimens (Whipple *et al*., 1991). Various combinations of speed and duration of centrifugation have been suggested, including 900g (gravity units) for 30 min (Whitlock and Rosenberger, 1990), 1000g for 30 min (Whitlock *et al*., 1989), and 1700g for 20 min (Stabel, 1997). High speed centrifugation resulting in compaction of the pellet, can result in increased contamination rates and creates difficulty in resuspending the pellet (Whipple *et al*., 1991). Comparative study on MAP concentration methods showed that, centrifugation yielded more organisms than did sedimentation, but had higher rates of contamination than did sedimentation (Stabel, 1997).

2.9.2. Detection of host response to MAP infection

2.9.2.1. Gross pathological examination

The gross appearance of the intestines may be indicative. The bright-yellow pigmentation of the ileum and the presence of enormous numbers of acid-fast bacilli in smears from the intestinal erosions or enlarged mesenteric lymph nodes provide confirmation of the diagnosis. In some studies, normal looking samples of intestines and associated lymph nodes were randomly selected and studied at histopathological level and none of the samples proved to be affected with paratuberculosis (Cravel *et al*., 2002; Sivakumar *et al*., 2006). Many other etiologies such as parasites may end to gross lesions similar with

paratuberculosis and minor lesions could easily be missed, hence it would be advisable routinely to search the terminal ileum, ileocaecal junction and the associated lymph nodes microscopically for acid-fast organisms by smear or histopathology (Oryan *et al*., 2008).

2.9.2.2. Histopathological diagnosis

Tissue samples can be obtained from distal portions of the ileum, ileocaecal valve and mesenteric lymph nodes. In most cases, histological examination of intestinal tissues and mesenteric lymph nodes is very important, especially since macroscopic thickening of the intestines or enlargement of the mesenteric lymph nodes are not consistent features (Bannantine *et al*., 2004). The advantage of the histopathological diagnosis is that it allows identifying animals with focal lesions associated with sub-clinical stages, who's fecal and/or milk excretion is insufficient for bacterial culture or PCR (Waller, 2000).

Histopathologic features have been proposed as a good parameter of paratuberculosis diagnosis in sheep (Hilbink *et al*., 1994; Clarke and Little, 1996). Moreover, it has been reported that, histpathologic diagnosis is more sensitive than bacteriological, immunodiagnostic and molecular methods, if appropriate tissue specimen collection is performed, with preferential sampling of the ileocaecal junction, terminal ileum and ileocaecal and ileal mesenteric lymph nodes (Collins, 1996; Perez *et al*., 1996; Gwozdz *et al*., 2000a&b). Although bacteriological culture of feces or intestinal tissue has been commonly used to evaluate serologic test results in cattle this approach does not seem reliable in small ruminants, because the causative organism can be difficult to isolate (Juste *et al*., 1991; Shulaw *et al*., 1993). Culture of these tissues, or analysis by PCR, is helpful if considered in conjunction with histopathology, as it is possible to detect MAP in these tissues in the absence of lesions sufficient to cause disease (Bannantine *et al*., 2004).

2.9.2.3. Immunohistochemistry

This technique uses a MAP-specific antibody marked with enzymes, which allows visualizing the reaction on the enzymatic substrate. The advantage of this method is that

it enables to identify spheroplasts and MAP in tissues. It shows good sensitivity in tissue from sub-clinically infected animals. However, false positive due to cross reaction with other mycobacteria may occur. The sensitivity is usually low as compared with bacterial culture (Martinson *et al*., 2008).

2.9.2.4. Humoral immune response

Enzyme linked immunosorbent assay (ELISA) is, at present, the most sensitive and specific test for serum antibodies to MAP in cattle. Its sensitivity is comparable with that of the complement fixation test (CFT) in clinical cases, but is greater than that of the CFT in sub-clinically infected carriers. The specificity of the ELISA is increased by *M. phlei* absorption of sera. Several absorbed ELISA kits are commercially available. In small ruminants the commercially available ELISA had a specificity of 98.2–99.5% and detected 35–54% of animals with histological evidence of infection (Hope *et al*., 2000). In another study, the estimated specificity of ELISA was 99% and its sensitivity measured against histological results was 21.9% (Sergeant *et al*., 2003). In small ruminants, the sensitivities of ELISA are in the range 16–100%, and the specificities of ELISA are in the range 79– 100% (Nielsen & Toft, 2008).

The agar gel immune-diffusion test (AGID) test is useful for the confirmation of the disease in clinically suspect cattle, sheep and goats. It has been reported that in small ruminants in New Zealand and Australia the AGID offers slightly higher sensitivity and specificity than that obtained by the ELISAs. The reported specificity and sensitivity of the AGID measured against histological results were 99–100% and 38–56%, respectively (Hope *et al*., 2000; Sergeant *et al*., 2003). In experimentally infected goats, ELISA and AGID test were found to be 100% sensitive from 180 and 210 days post infection onward, respectively (Munjal *et al*., 2005).

The specificity of ELISA tests may suffer because of the occurrence of cross reactions. MAP is known to share common antigens with other *Mycobacterium* species, *Nocardia* species and *Corynebacterium* species. Corynebacterial infections may produce cross

reactivity in serologic tests used to diagnose MAP infection. This is of particular concern in goats because caseous lymphadenitis caused by *Corynebacterium pseudotuberculosis* is a common disease in the species. The problem of cross reaction occurs mainly with ELISA and CFT, while the AGID test is not influenced by antibodies to *Corynebacterium pseudotuberculosis* (VanMetre *et al*., 2000).

2.9.2.5. Cellular immune response

Intradermal reaction (IDR) is performed by intradermal inoculation of purified protein derivative (PPD)–J. The skin thickness is measured with a caliper before and 72 hours after inoculation. An increase in skin thickness greater than 2-3 mm is considered positive. IDR has an estimated sensitivity of 54.0% and a specificity of 79.0% (Kalis *et al*., 2003). The advantages are that it is easy to perform in the field, and that there is a chance of early detection of infected animals. The disadvantages are its low sensitivity and its low specificity (due to probable cross-reactions). Modification of johnin and tuberculin tests (double intra-dermal tuberculin /johnin) and intra-venous johnin have also been used for the diagnosis of paratuberculosis in domestic livestock. However, there are complications regarding the interpretation of the results of skin test. The effect of various cut-off values while conducting skin test has not been recorded. Moreover, the test performance may be affected significantly by subtle difference in antigen occurring in several batches of antigen. For this reason research is further required for increasing the skin test's value (Whittington and Sergeant, 2001).

Interferon (IFN)–γ test evaluates the specific production of cytokine, IFN–γ by T lymphocytes after stimulation with purified protein derivative. In animals with the sub-clinical stage, the sensitivity of this test is higher than that of the serological tests, but low in absolute terms (41.0%) (Gwozdz *et al*., 2000a). The advantage of the IFN–γ test is the significant secretion of IFN–γ during the early stages and may be used to detect animals in the sub–clinical stage. However, it has several disadvantages: i) the possible cross-reactions, ii) the need to process the sample quickly since cells must be alive, iii) its high cost and iv) its low sensitivity (Stabel and Whitlock, 2001).

2.9.3. Molecular approaches for paratuberculosis diagnosis

McFadden *et al.* (1987) have identified a sequence, termed IS*900*, which is an insertion sequence specific for MAP. It has been reported that a small number of isolates other than MAP have produced amplified products the same size as expected from MAP. A restriction enzyme digest may be applied to positive IS*900* products to confirm that their sequence is consistent with MAP. The identifications of new DNA sequences considered to be unique to MAP (ISMav2, f57, and ISMap02 sequences), offer additional tools for rapid identification of this organism using the PCR technology (Stabel and Bannantine, 2005). The restriction enzyme analysis of IS*1311*, an insertion sequence common to MAA and MAP can be used to distinguish between these species and for typing of ovine, bovine and bison strains of MAP (Sevilla *et al*., 2005). In recent years, real-time PCR methods have been extensively developed to detect MAP from different specimens (blood, milk, faeces, tissues and environmental samples). However, this molecular tool is greatly influenced by the quality of nucleic acid samples. Therefore, a DNA extraction method that provides a high quality DNA sample and a maximum bacterial DNA recovery is a critical step to use real-time PCR (Park *et al*., 2014).

2.10. Zoonotic Potential of *Mycobacterium avium* subspecies *paratuberculosis*

In humans MAP is incriminated to be involved in the development of CD, a form of inflammatory bowel disease (Sibartie *et al*., 2010). Crohn's disease is an inflammatory disorder of the intestine of unknown cause. The disease is chronic, and patients tend to remit and relapse. Symptoms include general malaise, chronic weight loss, abdominal pain and diarrhea. It is a life-long disease that has no cure. CD usually begins early in life, with peak incidence between the ages of 16 and 25 years, but it can occur in early childhood or later in life. The three most common patterns of disease are the involvement of the terminal ileum and cecum, the ileum alone, and the colon alone (Hermon-Taylor and El-Zaatari, 2004).

The occurrence of CD is not uniform throughout the world. In general, CD is much more common in developed countries than in developing nations. Whether this reflects a true difference in prevalence or is the result of variations in disease diagnosis, definition, recognition and reporting is not clear (Eisen and Sandler, 1994). The highest rates in Europe are found in Sweden (Ekbom *et al*., 1991) and in the United Kingdom, especially Scotland (Kyle, 1992). Disease rates are lower in Mediterranean countries. Prospective studies of European incidence show a variation of 0.9 to 9.5 per 100,000 populations (European Commission, 2000).

Meta-analyses of epidemiological studies concluded that MAP is more often detected in intestinal tissue of CD patients than of healthy volunteers (Feller *et al*., 2007; Abubakar *et al*., 2008). However, MAP is also frequently found in samples from healthy human donors and an etiological role of MAP in human disease has by no means been proven (Fecteau *et al*., 2010). More recently in India, MAP among human population showed higher prevalence in persons who worked with goat herds endemic for Johne's disease and suffered from gastrointestinal problems, than in humans with no history of contact with animals (Singh *et al*., 2011).

2.11. Economic Impact of Paratuberculosis

The actual losses in productivity and profit are difficult to assess, making it likely that the impact of the paratuberculosis is underestimated worldwide (Johnson-Ifearulundu *et al*., 1999). Paratuberculosis costs the USA agriculture and dairy industry more than 250 million dollars annually (Speer *et al*., 2006). It has been assessed that the total losses resulting from paratuberculosis reached 12–15 % of the total value of economic production, while the decrease in milk and meat production or both was calculated to be 6–19 % (Paolicchii *et al*., 2003).

Ovine paratuberculosis affects the financial performance of individual producers through its biological effect on production and regulatory restrictions. Anecdotal evidence suggests that, paratuberculosis adversely affects productivity primarily through increased

animal mortality, reduced lambing percentages, reduced wool cut and possibly decreased fiber quality. Of these, increased mortality is considered the most serious, with annual losses of up to 15% reported, although mortality can vary considerably between infected flocks and rates above 10% are uncommon (Eppleston and Simpson, 1999). As no efficient treatment is known, the disease leads to economic losses due to relevant decrease in milk production, costs in the diagnosis and in disease control, the early culling of affected animals and the low carcass value at slaughter (Wiszniewska and Szteyn, 2002).

Paratuberculosis is spread most effectively through the movement of infected animals that contaminate a new environment, thus setting the stage for widespread exposure of more animals. MAP also can be isolated from semen and embryos from affected breeding stock, although the effectiveness of this route of transmission is unknown. Consequently, it has serious implications for domestic and international trade of live animals and germplasm. The identification of MAP in animal products also can affect trade, especially if a link between MAP and CD is demonstrated conclusively (OIE, 2014 b&c).

2.12. Control Strategies of Paratuberculosis

Of the serious obstacle in paratuberculosis eradication programs is the lack of universally accepted and sensitive enough tests to detect infected animals in the earliest stages of the disease. The best test is the one that detects faecal shedders of MAP, which are animals in more advanced stage of the disease, and more likely to transmit the infection to their calves *in utero* or through their milk (Collins, 1994). Therefore, effective disease control programmes depend on a clear understanding of the sources of infection and the routes of transmission and early detection of infected animals, thereby allowing removal of carrier individuals from the herd (Pavlik *et al*., 2000).

MAP is more or less resistant to chemotherapeutic agents *in vitro* and treatment of infected animals is not successful. Although treatment may result in clinical

improvement, and in some cases remission, animals continue to shed MAP in faeces and, upon withdrawal of chemotherapy, clinical disease recurs (Chiodini, 1991).

Paratuberculosis control programme is time consuming and economically relatively costly, hence prevention of a herd or flock from new infection is the first option to be adopted. This practice could be achieved by maintaining the disease free status of a herd or flock and stock replacement animals. Maintaining the paratuberculosis free status of a farm by a closed herd or flock system or introduction of animals from a tested negative herd or flock combined with a careful feeding of all animals is an essential step to reduce the risk of new infection (Kennedy *et al*., 2001).

Various procedures and preparations have been used for the disinfection of different components of the environment such as liquid manure. The application of wastewater management using chlorine-based disinfectants have been tested and used. However, the procedures which have been applied thus far have not been 100% effective and have failed to kill of all MAP cells in the environment (Whan *et al*., 2001).

Vaccination with MAP, with or without adjuvant, has provided practical benefits in the control of paratuberculosis in sheep. Although vaccination does not prevent infection, it reduces the number of bacteria that are excreted, as well as the number of sheep that develop pathology and clinical illness. However, it is important to recognize that some vaccinated sheep may shed large numbers of bacteria in their faeces and, therefore, vaccination must be used in conjunction with other managemental interventions (Emery and Whittington, 2004). Moreover, revaccination is not advised, since this causes severe local reactions in sensitized animals. As vaccination causes allergy to avian and mammalian tuberculin and antibodies detectable by several tests, including the CFT for paratuberculosis cannot be used in flocks that require certification for export (Sharp, 2007).

Even though it was once thought that goats behaved like sheep regarding tuberculosis and, thus, were considered relatively resistant to paratuberculosis, goats are highly

susceptible to both tuberculosis and paratuberculosis. In spite of that, vaccination against paratuberculosis in goats has been applied with the same criteria as in sheep. This has caused a large amount of confusion in some regions, where both infections are present, because it has been possible neither to evaluate vaccine efficacy properly nor control tuberculosis efficiently. Goat vaccination against paratuberculosis seems currently in use in Spain, the Netherlands, France, Norway and India (Juste and Perez, 2011).

3. MATERIALS AND METHODS

3.1. Study Areas

The study was conducted from November 2014 to June 2015 at Bishoftu ELFORA export abattoir, Mojo modern export abattoir and Organic export abattoir located in East Shoa Zone of Oromia Regional State. Bishoftu ELFORA export abattoir is located in Bishoftu town at 47 kilometers Southeast of Addis Ababa. The area is located at 9°N latitude, 40°E longitudes and at an altitude of 1850 meters above sea level in central high lands of Ethiopia. Mojo modern and Organic export abattoirs are located in Mojo town at 73 Kilometres South East of Addis Ababa. The area has a latitude and longitude of 8°N and 39°5E, respectively with an elevation between 1788 meters and 1825 meters (NMSA, 2003). In ELFORA export abattoir, on average 100 heads sheep and 600 heads of goats were slaughtered four days per week for export. In Mojo modern and Organic export abattoirs on average 1500 heads of goats were slaughtered daily while sheep were slaughtered occasionally.

3.2. Study Animals

The study was carried out in sheep and goats slaughtered in the selected abattoirs. Animals were considered for the study regardless of sex, body condition, origin and breed. Animals in age group of below 12 months were not considered for gross pathological observation according to Hailat *et al*. (2010) to increase the sensitivity of diagnostic tests. Animals in all slaughter houses were apparently healthy males of 1year to four years old, found in good body condition. In all export abattoirs sheep for gross pathological examination were Black-head Ogaden breed coming from Borana and Somali districts, while goats were mainly coming from Arbamich, Afar, Bale and Konso districts of the country.

3.3. Study Design

A cross sectional study was carried out from November 2014 to June 2015 to estimate the prevalence of paratuberculosis and characterize the causative agents in apparently healthy sheep and goats slaughtered at the selected export abattoirs. Before slaughter, information was recorded concerning animal's age, sex, health status and origin in appropriate format (Appendix I). Sheep and goats age determination was done according to Abebe and Yami (2008), as described in appendix II. Tissue samples from paratuberculosis suspected gross lesions, particularly terminal ileum, ileocaecal valve, ileocaecal lymph nodes and mesenteric lymph nodes were collected and subjected for bacteriological culture, ZN staining techniques and histopathological examination.

3.4. Sampling Method and Sample Size Estimation

The export abattoirs were purposively selected for this study based on availability and accessibility of study animals. Animals were selected using systematic random sampling method and all animals fulfilling the inclusion criteria were considered for the study. Since there was no previous prevalence study with paratuberculosis on sheep and goats in Ethiopia, sample size was calculated by considering 50% expected prevalence, 95% confidence interval and 5% required precision, and the formula for estimation of sample size is given below as follows:

$$n = \frac{1.96^2 \text{ X } P_{exp} \text{X } (1-P_{exp})}{d^2}$$ …….. Thrusfield (2007)

Where n= required sample size, d= desired absolute precision, P_{exp}= expected prevalence

Thus, the calculated sample size was 384 from each species. However, due to the availability of goats for sampling sample size of goats was doubled in order to increase the precision of the study. Accordingly, total of 1152 animals (384 sheep and 768 goats) were considered for the study.

3.5. Gross Examination, Sample Collection and Transportation

All procedures of gross lesion examination, sample collection and transportation were performed according to the protocol of OIE (2014d). After slaughter, at the point of removal of the gastrointestinal tract of each selected animal, identification tag was attached to the caecum which matches each animal identification number. Samples from 384 sheep and 768 goats were examined for gross lesions associated with paratuberculosis. Complete gross examination of the gastrointestinal tract was performed with emphasis of distal portion of ileum, ileocaecal valve, cecum, colon and associated lymph nodes. The intestines were opened to expose the mucosa and held up to the light to visually inspect for thickening, corrugation, hemorrhage and hyperemia. In addition the mesenteric lymph nodes were examined from the presence of lesions visually and incised for any pathological changes. Information on gross characteristics of tissues and organs of each animal was recorded in appropriate format (Appendix III). In animals where there were lesions, portions of terminal ileum, ileocaecal valve and respective lymph node tissues were collected using a sterile universal bottle containing physiological saline solution. To avoid contamination, faeces and other contents were rinsed using water (used in the abattoirs) from portions of intestinal tract prior to sample collection. Each tissue sample intended for bacterial culture was immediately placed in a cold box and transported to AAU, CVMA bacteriology laboratory. Samples for culture were kept at minus 20 °C until needed. For histopathological examination, fresh tissue samples were trimmed to a smaller size of 4 mm to 1 cm thickness and fixed in 10% buffered formalin and kept at room temperature until processed.

3.6. Laboratory Methods and Procedures

3.6.1. Culture media

Tissue samples with characteristic gross lesions of paratuberculosis were subjected to culture on HEYM and LJ medium prepared on screw capped tube according to protocol of OIE (2014d) (Appendix IV).

3.6.2. Tissue digestion and decontamination

Tissue specimens were prepared for culture at College of Veterinary Medicine and Agriculture, Microbiology laboratory in level 2 biological safety cabinet. Approximately 4 grams of scrapped intestinal mucosa and lymph node parenchyma of tissue lesions were chopped using sterile blades and manually homogenized with a mortar and pestle. Physiological saline solution (0.85%) was added to homogenized tissue samples and prepared for decontamination. The tissue homogenates were subjected to decontamination using NaOH according to Mohammed (2014) and oxalic acid according to Storset *et al*. (2001) with slight modification.

Briefly, in the NaOH method, tissue decontamination was achieved by mixing 6 ml of tissue homogenate and equal volume of 4% NaOH in a 15 ml centrifuge tube. The mixture was vigorously shaked for 15 minutes at room temperature. The suspension was then centrifuged at 3,000 ×g for 15 minutes. By removing the supernatant fluid, the sediment was neutralized with 2N HCl using phenol red as an indicator. Neutralization was achieved when color of the solution was changed from red to persistent yellow. Finally, the sediment was mixed to resuspend and used for media inoculation.

In the oxalic acid method, decontamination was achieved by mixing 4 ml of tissue homogenate and equal volume of 5 % oxalic acid in a 15 ml centrifuge tube. The mixture was vortexed and let to stand for 30 minutes with occasional shaking. Sterile normal saline solution was then added to 14 ml mark on the centrifuge tube. The suspension was centrifuged at 3,000 ×g for 15 minutes. By removing the supernatant fluid, the sediment was neutralized with 4% NaOH using phenol red as an indicator. Neutralization was achieved when color of the solution became persistent pale pink. Finally, the sediment was mixed to resuspend and used for media inoculation.

3.6.3. Inoculation of culture media

Media inoculation and culture result follow up was performed according to the recommendation of OIE (2014d). 0.1 ml of sediment was dispensed onto each slant of

four tubes of HEYM of which the two tubes were supplemented with 1% ferric ammonium citrate and sodium pyruvate whereas the other two tubes were not supplemented with ferric ammonium citrate and used as a control. The sediment was also cultured onto each slant of four tubes of LJ of which the two tubes were supplemented with 1% ferric ammonium citrate and sodium pyruvate whereas the other two tubes were prepared without ferric ammonium citrate and used as a control. The inoculated tubes were kept inclined with loosened screw to facilitate the evaporation of excess moisture for one week. After one week the tubes were placed vertical with tightened screw and incubated aerobically at 37°C for 16 weeks. The slants were observed for visible colonies weekly from the sixth week onwards. Cultures with no evidence of growth after 16 weeks were considered as negative or failure to grow. A culture was considered positive when small colonies of about 2mm diameter/larger were seen in culture media supplemented with ferric ammonium citrate six week onwards and showed a characteristic appearance in ZN stained smear.

3.6.4. Direct smear staining

In parallel to culturing, smears from sediments of inoculums were stained by ZN staining method according to Quinn *et al*. (2011) (Appendix V) and examined microscopically at 100 x magnifications for acid-fast organisms that have the morphological characteristics of MAP. The findings were registered as positive when the bacteria appeared as acid fast rods in clumps or dispersed.

3.6.5. Histopathology

For histopathological examination the formalin-fixed tissue samples were processed by routine methods as described by Luna (1968) (Appendix VI). Sections of 4-5 μm was cut and stained with hematoxylin and eosin (HE) staining and examined histopathologically. The hematoxylin and eosin stained sections were observed under 4×, 10× and 40× objectives of a microscope and lesions were recorded. The intestinal tissue sections were considered positive for paratuberculosis when there were diffuse or focally aggregated

macrophage, lymphocytes and epithelioid cells infiltrating the lamina propia of the villi and between crypts, Peyer's patches of ileum and ileocaecal valve and draining lymph nodes. Histopathologic lesions were graded according to the classification system proposed by Perez *et al*. (1996) (Appendix VII) and Corpa *et al*. (2000) (Appendix VIII) for sheep and goats, respectively.

3.7. Data Analysis

Data from gross examination and laboratory results were entered into MS excel 2010 spread sheets. The occurrence of the disease based on gross lesion, histopathological lesions, ZN staining and tissue culture was determined by using frequency distribution. Chi-square or Fisher's exact tests were performed using STATA 11.0 (Stata Corp LP, College Station, TX, USA) to evaluate the differences in distributions of gross lesions between age groups, origin and species. Agreement between the three diagnostics used was calculated with a linearly weighted kappa coefficient (Brenner and Kliebsch, 1996). P-value ≤ 0.05 was considered significant. Multivariable logistic regression analyses were performed to quantify crude and adjusted effects of pre-specified risk factors on gross lesion development. A P-value ≤ 0.05 was considered statistically significant. In cases of estimating the effect of different risk factors in terms of odds ratio (OR) with corresponding 95% confidence interval, statistical significance was assumed if the confidence interval did not include one among its values.

4. RESULTS

4.1. Prevalence of Paratuberculosis Based on Gross Pathology

In the present study from 1,152 examined sheep and goats, gross lesions consistent with paratuberculosis were seen in 3.99% (95% CI=2.9-5.1) animals. Out of 384 examined sheep, gross lesions consistent with paratuberculosis were recorded in 5.21% animals (95% CI=2.8-7.4). Whereas, gross lesions corresponding with paratuberculosis were seen in 3.4% (26/768) (95% CI=2.1-4.7) of goats. There was no statistically significant difference (P>0.05) on prevalence between species and age groups. However, the prevalence was higher in animals aged ≥ 2 years (4.56%) than younger (3.48 %). In both sheep and goats there was no statistically significant (P>0.05) difference on the occurrence of paratuberculosis between age groups and origin of animals. Higher prevalence was found in age group of ≥ 2 years than younger animals and in animals coming from Borana areas (6.2%) than those coming from Somali (2.1%) (Table 1).

Table 1: Association of different risk factors to gross pathological examination of sheep and goat for paratuberculosis

Animal	Variable	N	n (%)	$\chi 2$	P-value
Sheep	Age				
	1 - 2 years	214	8 (3.7)		
	≥2 years	170	12 (7.1)	2.1158	0.146
	Origin				
	Borana	289	18 (6.2)		
	Somali	95	2 (2.1)	2.4619*	0.181
Goat	Age				
	1 - 2 years	390	13 (3.3)		
	≥2 years	378	13 (3.4)	0.0066	0.935
Sheep and goat	Age				
	1 - 2 years	604	21 (3.48)		
	≥2 years	548	25 (4.56)	0.8827	0.347
Over all	Sheep	384	20 (5.2)		
	Goat	768	26 (3.4)	2.219	0.136
	Total	**1,152**	**46 (3.99)**		

N=total animals examined; n=total animals positive; *= the value is Fisher's exact test

Multivariable logistic regression analysis (Table 2) showed that older animals (≥2 years) had 1.3 times the odds of being positive on gross examination of tissue for the diagnosis of paratuberculosis as than younger (adjusted OR=1.3; CI =0.73-2.39). In addition, the odds of gross lesion was 0.32 times lower in sheep originated from Somali than those of Borana origin (adjusted OR = 0.32; CI=0.07-1.42).

Table 2: Multivariate logistic regression analysis of sheep and goats showed gross pathology in relation with host-related risk factors

Variable	N	n	Crude odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Age				
1 - 2 years	604	21	1	1
≥2 years	548	25	1.8 (0.72-4.54)	1.3 (0.73-2.39)
Origin (Sheep)				
Borana	95	2	1	1
Somali	289	18	0.35 (0.08-1.55)	0.32 (0.07-1.42)
Animal				
Sheep	384	20	1	-
Goat	768	26	0.64 (0.35-1.16)	-

N=total animals examined; n=total animals positive

Gross pathologies were observed in ileocaecal valve, ileocaecal lymph node and mesenteric lymph node of all (100%) paratuberculosis suspected sheep (Table 3) and goats (Table 4). Gross lesions of terminal ileum were recorded in 90% of sheep and 65.4% of goats, while in colon gross lesion was recorded in 10 % and 7.7% of sheep and goats respectively. In sheep the most frequently observed gross lesion in ileocaecal valve was mucosal thickening (Figure1), while some ileocaecal valve became thickened and hyperemic (Figure 2). Moreover, the most frequently observed gross lesion in the lymph nodes was oedematous swelling of the parenchyma (Table 3).

Table 3: Gross lesions and their distribution in different tissues and organs of paratuberculosis suspected sheep

Observed gross lesion	Frequency (%) of lesion in				
	ICV	ICLND	MLND	Distal ileum	Colon
Mucosal thickening	12 (60)	-	-	12 (60)	-
		-	-		-
Mucosal thickening and hyperemia	8 (40)	-	-	6 (30)	2 (10)
Oedematous swelling	-	12 (60)	15 (75)		-
Oedematous swelling and hyperemia	-	8 (40)	5 (25)		-
Total	20 (100)	20 (100)	20 (100)	18 (90)	2 (10)

ICLND=ileocaecal lymph node; ICV=ileocaecal valve; MLND=Mesenteric lymph node

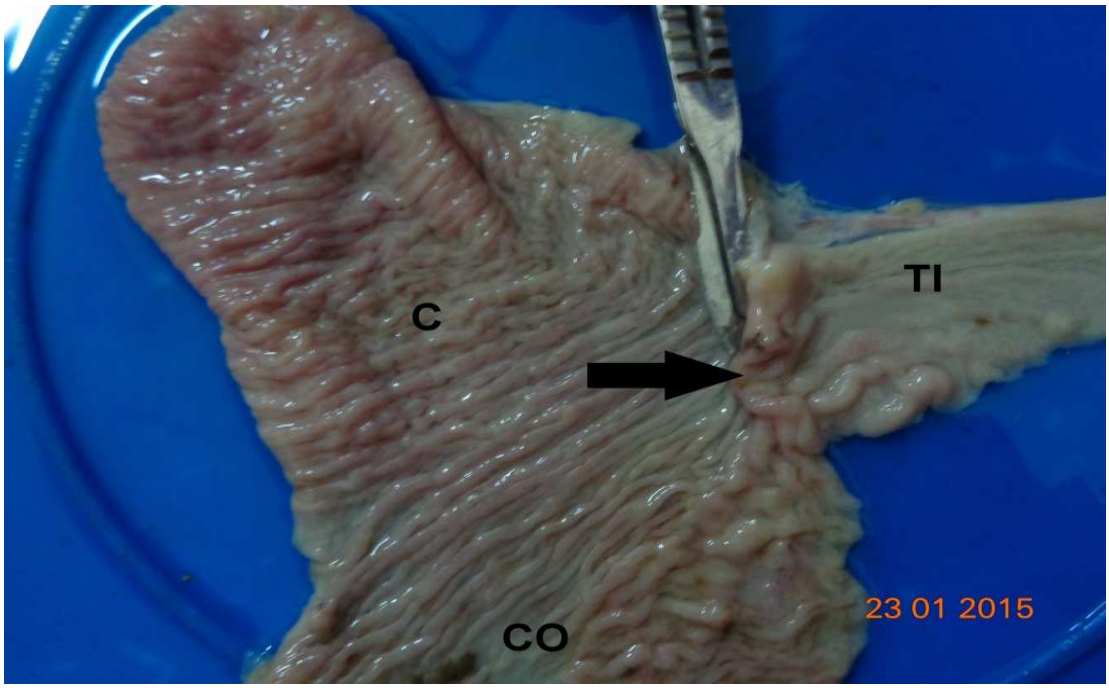

Figure 1: Gross thickening of ileocaecal valve (arrow head) and terminal ileum (TI) from apparently healthy sheep. C (cecum); CO (colon)

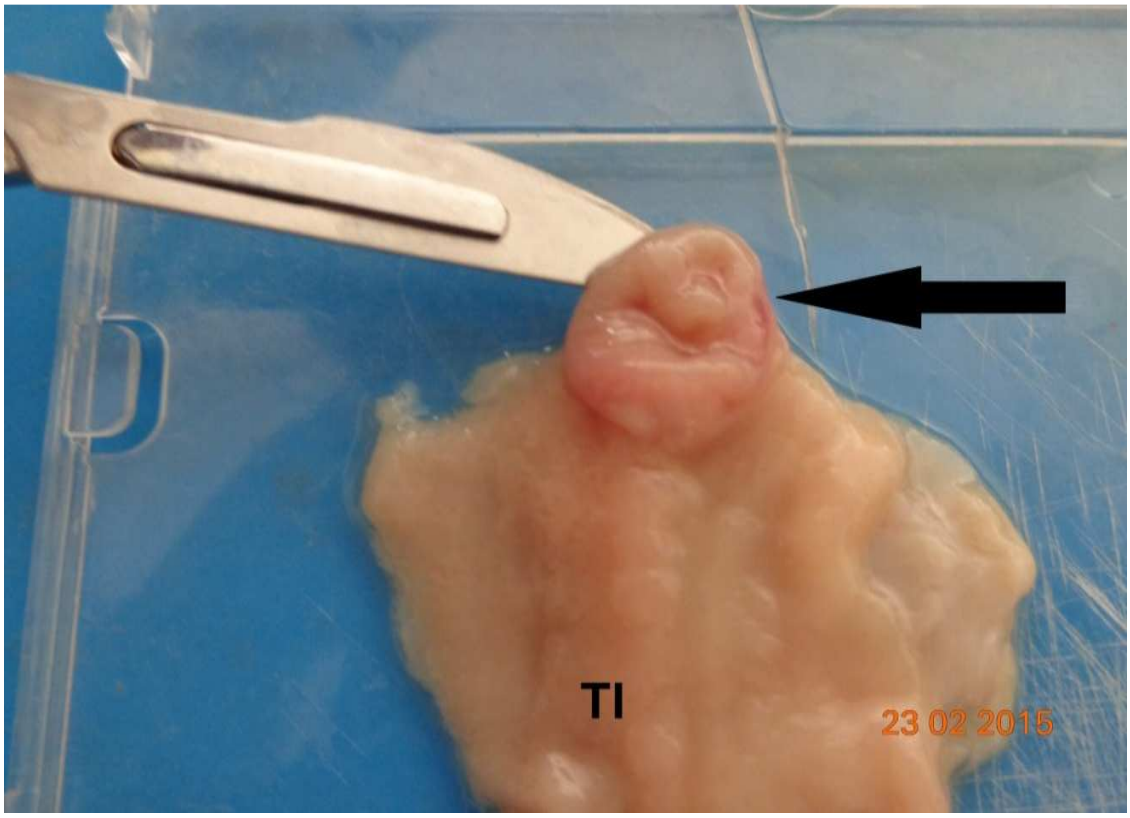

Figure 2: Protrusion and hyperemia of ileocaecal valve (arrow head) and thickening of the terminal ileum (TI) in apparently healthy sheep

In goat gross lesions observed in the mucosa of distal ileum and ileocaecal valve were thickening, hyperemia and petechial hemorrhage (Figure 3B). The most frequently observed gross lesion in the lymph nodes was oedematous swelling of the parenchyma and lymph nodes were enlarged about twice the normal size (Figure 3A). In few cases hyperemia of the cut surface was recorded (Table 4).

Table 4: Gross lesions and their distribution in different tissues and organs from paratuberculosis suspected goats

Observed gross lesion	Frequency (%) of lesion in				
	ICV	ICLND	MLND	Distal ileum	Colon
Mucosal thickening	11 (42.3)			16 (61.5)	
Mucosal thickening & hyperemia	6 (23.1)			1(3.9)	
Mucosal thickening & petechial hemorrhage	9 (34.6)				2 (7.7)
Oedematous swelling		25 (96.1)	26 (100)		
Oedematous swelling & hyperemia		1(3.9)			
Total	26 (100)	26 (100)	26 (100)	17 (65.4)	2 (7.7)

ICLND=ileocaecal lymph node; ICV=ileocaecal valve; MLND=mesenteric lymph node

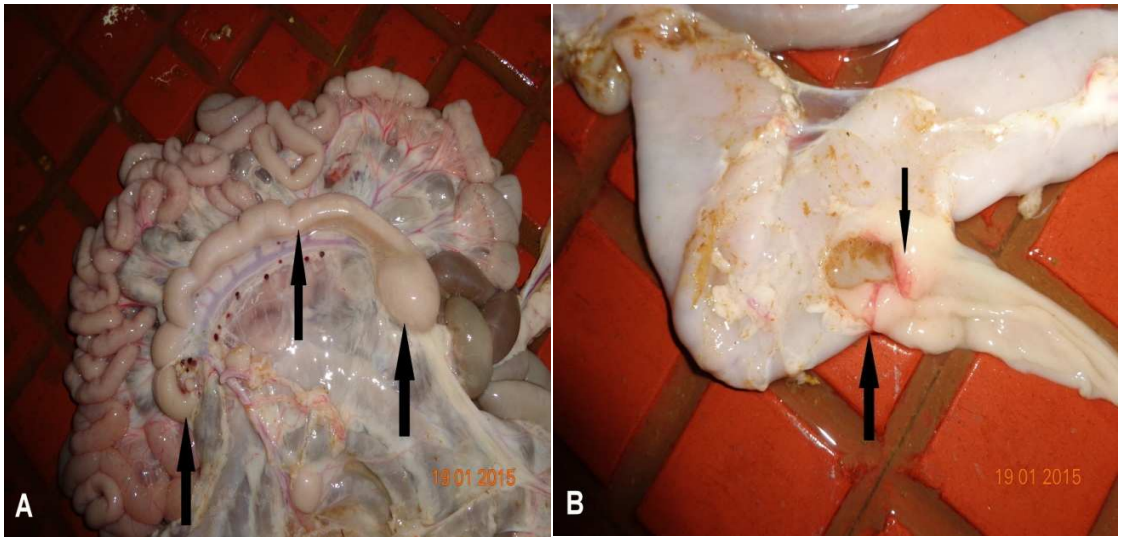

Figure 3: Gross pathological findings in apparently healthy goat (A) Edematous swelling of entire mesenteric lymph nodes (arrow head) (B) thickening and petechial hemorrhage of ileocaecal valve (arrow head) from the same animal

4.2. Microscopic Characteristics of Lesions Associated With Paratuberculosis

Hematoxylin and eosin staining of tissue sections from 13 paratuberculosis suspected sheep showed that, 11 (84.62%) had histological lesions compatible with paratuberculosis. In goat out of 13 tissue samples, 10 (76.9%) had histological lesions consistent with paratuberculosis (Table 5).

Table 5: Occurrence of histological lesions consistent with paratuberculosis in sheep and goats of different age groups

Animal	Age/year	Number of samples tested	Number of positive (%)
Sheep	1 - 2 years	5	4 (80)
	≥2 years	8	7 (87.5)
	Total	13	11 (84.6)
Goat	1 - 2 years	6	5(83.3)
	≥2 years	7	5(71.4)
	Total	13	10 (76.9)

The lamina propria and submucosa of the ileum and ileocaecal valve were diffusely infiltrated with mononuclear cells consisting primarily of lymphocytes and a few numbers of scattered macrophages, epithelioid cells and plasma cells. Cellular infiltrates were often seen in the lamina propria of a villus, in the dome and within or near organized lymphoid tissue of ileum and ICV (Figures 4 and 5). In some sections the villi in ileum and ileocaecal valve exhibited villous distortion and thickening due to infiltrations by mononuclear cells (Figure 6). Payer's patches lymphoid hyperplasia and proliferation by neutrophils, epithelioid cells and some plasma cells with extension towards the lamina proporia was noted in ileum and ileocaecal valve tissue sections (Figure 7).

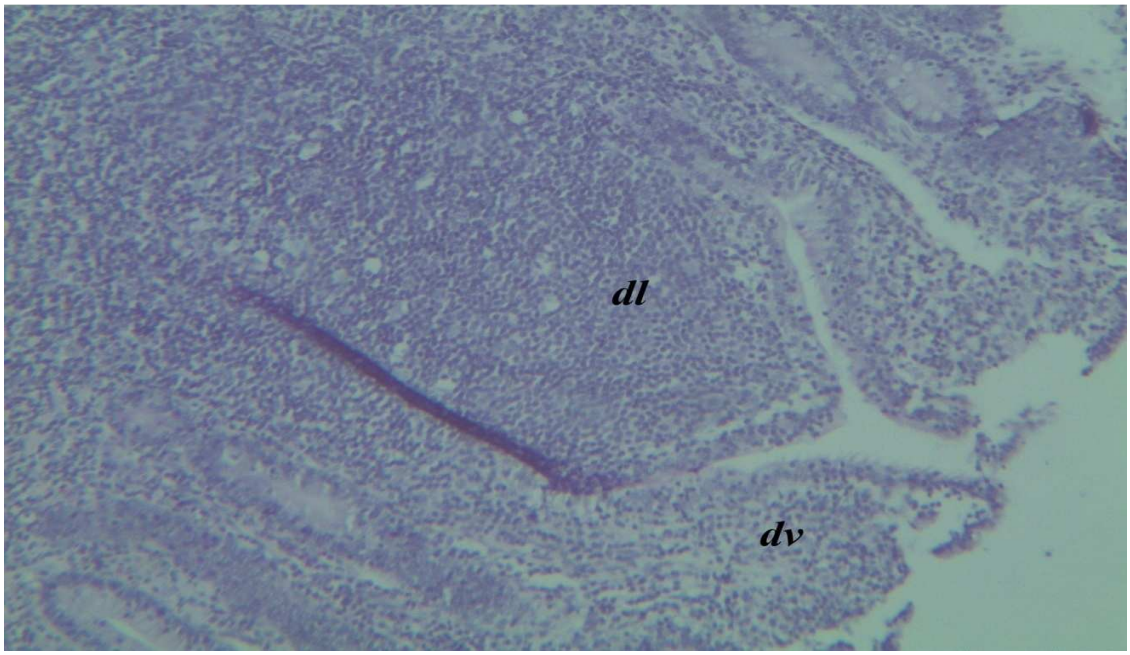

Figure 4: Ileum. Diffuse granulomatous infiltrates in lamina propria of a villus (dv) and in the dome (dl). HE staining (10x)

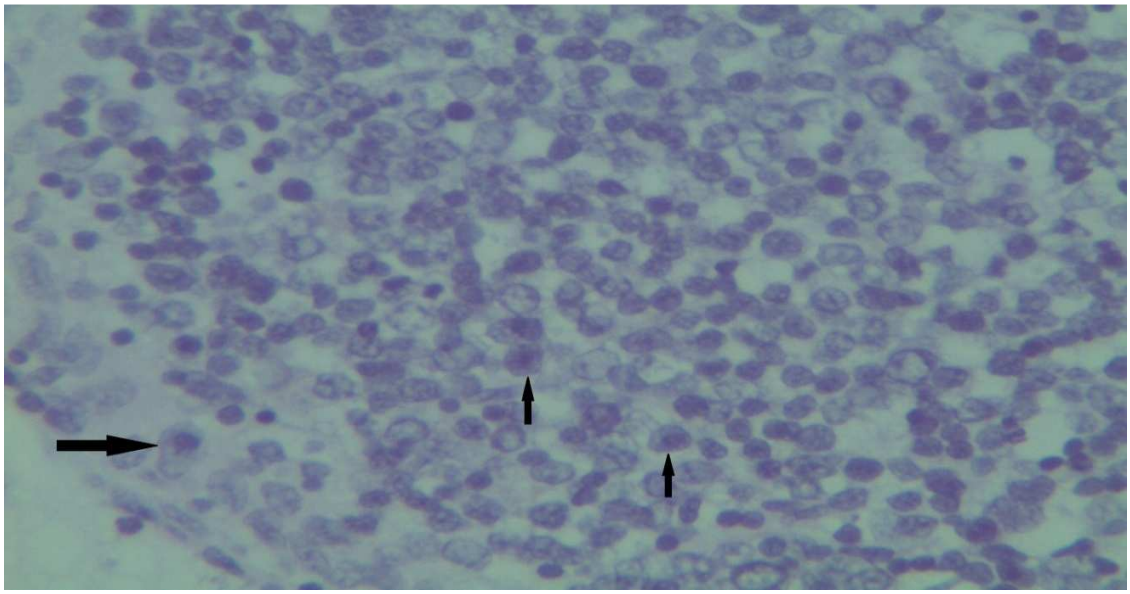

Figure 5: Higher magnification of figure 4. Granulomatous infiltrate formed mainly by lymphocytes and occasional macrophages (small arrow) and epithelioid cells (large arrow) scattered among them (40x)

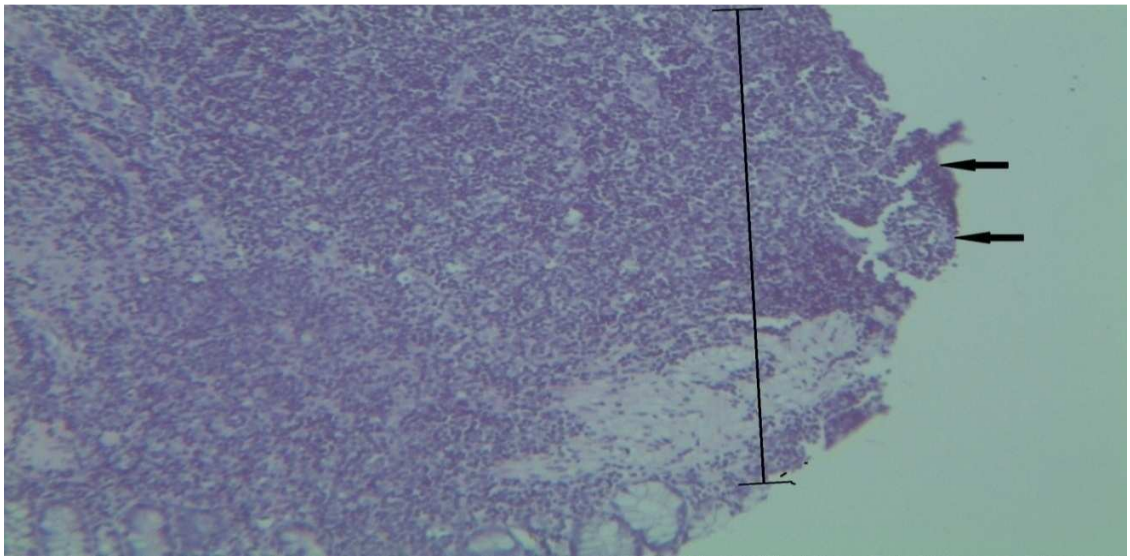

Figure 6: Ileocaecal valve. Intestinal villi infiltrated with inflammatory cells, causing shortening (arrow) and thickening (bar) of the villi. HE staining (10x)

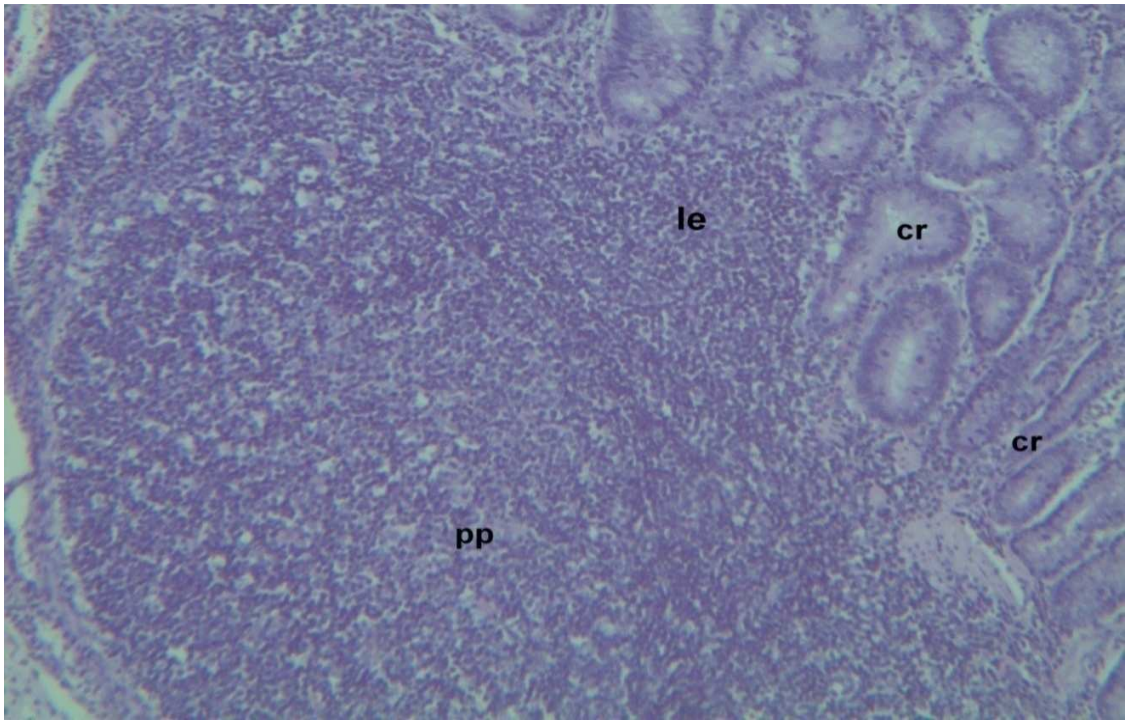

Figure 7: Ileocaecal valve. Payer's Patches (pp) proliferation and extension of infiltrates (le) towards the mucosa causing crypt (cr) replacement. HE staining (10x)

Two types of lesion were observed in the lymph nodes. The first type consisted of diffuse infiltration of lymphocytes, macrophages and epithelioid cells with notable expansion towards paracortex. The second type was focal areas of necrosis in the cortical and paracortical regions surrounded by lymphocytes, macrophages and some epithelioid cells (Figures 8 and 9).

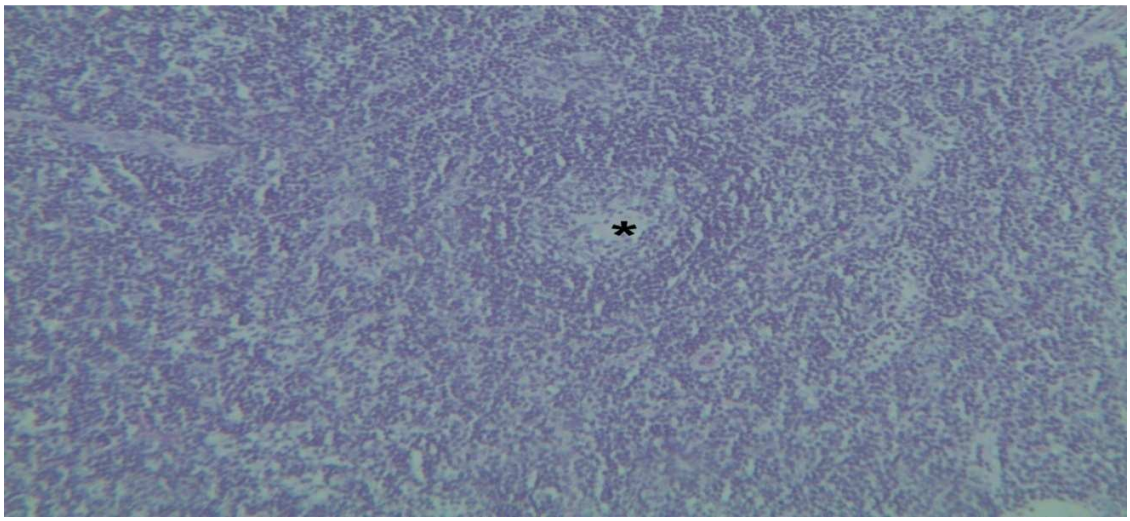

Figure 8: Ileocaecal lymph node (Goat). Small area of necrosis (asterisk) located in the cortex. HE staining (10x)

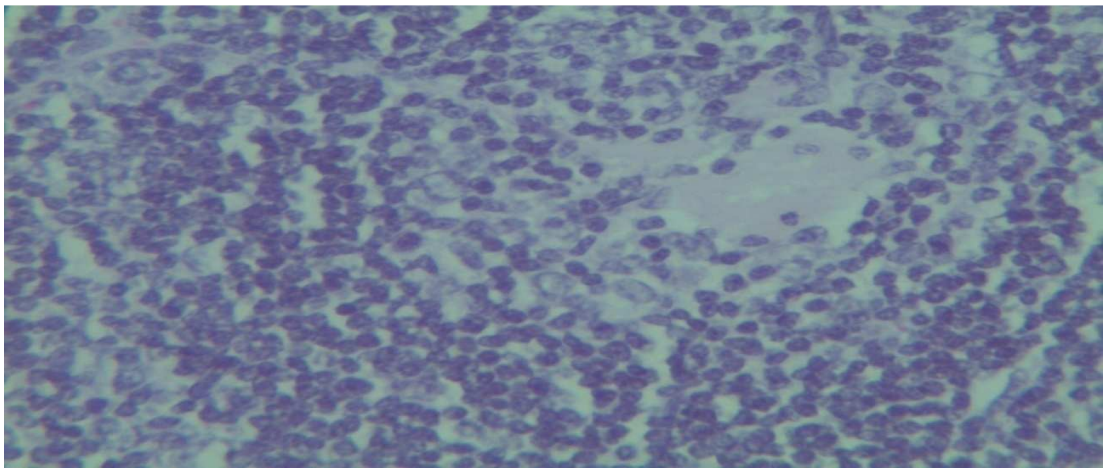

Figure 9: Higher magnification of figure 8: Macrophages and epithelioid cells are surrounded by many lymphocytes (40x)

Varieties of lesions and their frequency on different tissues are presented in table 6. The result showed that, 11 (100%) of terminal ileum/ileocaecal valve from sheep and 10 (100%) of the same tissue from goat were diffusely infiltrated mainly with lymphocytes and scanty number macrophages and/or epithelioid cells. In both species lesions were seen in areas of payer's patches and areas not associated with payer's patches. In lymph nodes, the most commonly seen histological lesion was diffuse lymphocytic infiltration of cortical and paracortical area with scanty number macrophages and/or epithelioid cells.

Table 6: Types of microscopic lesions and their distribution in different tissue parts of sheep and goats

Microscopic lesion	Tissue section	Parts	Number (%)	
			Sheep	Goat
Diffuse lymphocytic infiltration accompanied with few macrophages /epithelioid cells	TI/ ICV	Areas of PP	11 (100)	10 (100)
		None PP	11 (100)	10 (100)
	LND	Cortex	7(63.6)	7(70)
		Para cortex	2 (18.2)	0
Necrotic foci surrounded by lymphocytes, macrophages and epithelioid cells	TI/ ICV	Areas of PP	0	0
		None PP	0	0
	LND	Cortex	2 (18.2)	2 (20)
		Para cortex	0	1 (10)

ICV=ileocaecal valve; LND=lymph node; PP= payer's patches; TI=terminal ileum

Grading of paratuberculosis using histopathology revealed that, all lesions observed were categorized under grade 3c for sheep and diffuse lymphocytic type for goat, as diffuse granulomatous infiltrations composed of mainly lymphocytes and few macrophages/epithelioid cells were found in all cases.

4.3. Direct Smear Staining Result

Ziehl-Neelsen staining of tissue samples from sheep and goats revealed AFB in 80% of sheep and 53.8% of goats tissue suspected of paratuberculosis. In sheep the frequency of AFB was slightly higher in animal with age of 1 year, where as in goat higher frequency was recorded in animals of 2 years old and above than 1 year (Table 7).

Table 7: Frequency and percentage of acid fast positive tissue samples from sheep and goats suspected of paratuberculosis

Animal	Age/year	Number of samples tested	Number of positive (%)
Sheep	1 - 2	8	7 (87.5)
	≥2	12	9 (75)
	Total	20	16 (80)
Goat	1 - 2	13	5(38.5)
	≥2	13	9 (69.2)
	Total	26	14 (53.8)

4.4. Culture Result

Culture of paratuberculosis suspected pooled tissue samples from 20 sheep and 26 goats showed growth on 16^{th} week of incubation in 2 (10%) and 1 (3.8%) of ferric ammonium citrate supplemented media inoculated with sheep and goat samples respectively (Table 8). No growth was detected in the same media without ferric ammonium citrate. In one of solid media inoculated with sheep sample, pinpoint whitish colonies with regular boarder were visible on the surface of slants (Figure 10A), while minute colonies were seen on the other media. Microscopic examination of the stained smears from sheep isolates revealed long, thin and strong AFB in clump (Figure 10B).

Table 8: Growth characteristics of isolates on different media

Isolate	Source	Time to appear/week on				Colony characteristics
		HEYM[a]	HEYM[b]	LJ[a]	LJ[b]	
1	Sheep	-	14	9	14	Pinpoint, whitish, non mucoid
2	Goat	6	8	-		Pinpoint, whitish, non mucoid
3	Sheep	-	-	-	8	Minute, whitish, non mucoid

a= decontamination using NaOH; b= decontamination using oxalic acid

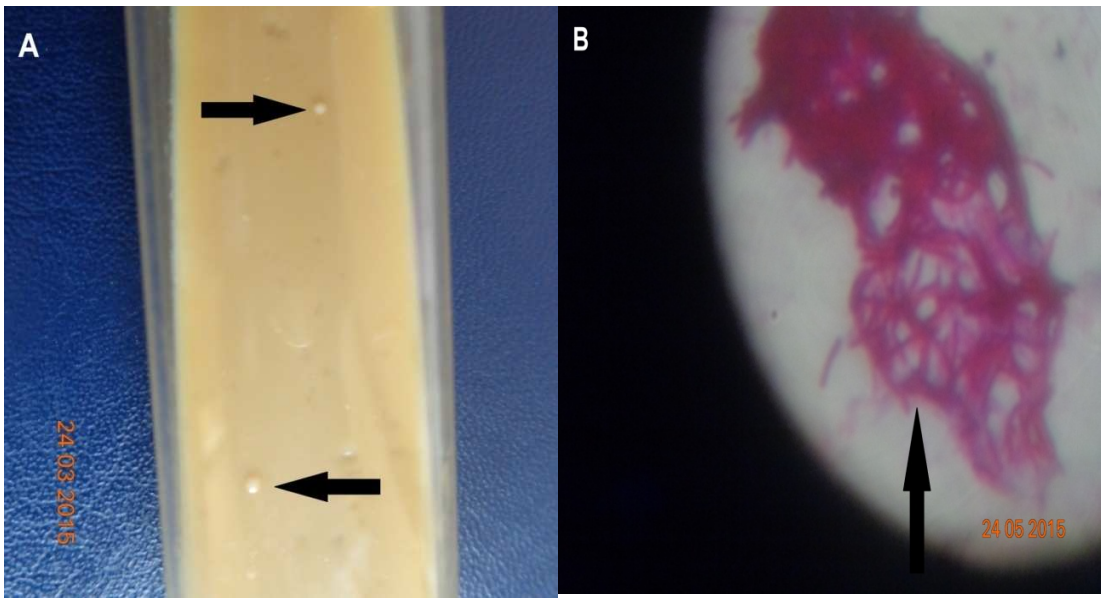

Figure 10: Characteristics of MAP isolated from sheep: (A) Whitish pin point colonies (arrow head) on Lowenstein Jensen media and (B) ZN staining of smear prepared from colonies grown on (A) revealing AFB in clump

In media inoculated with goat sample, the colony was initially whitish, small and with regular border but after additional 8 weeks of incubation it becomes larger and rough (Figure 11A). Short and wider bacilli were seen in smears prepared from goat isolates (Figure 11B).

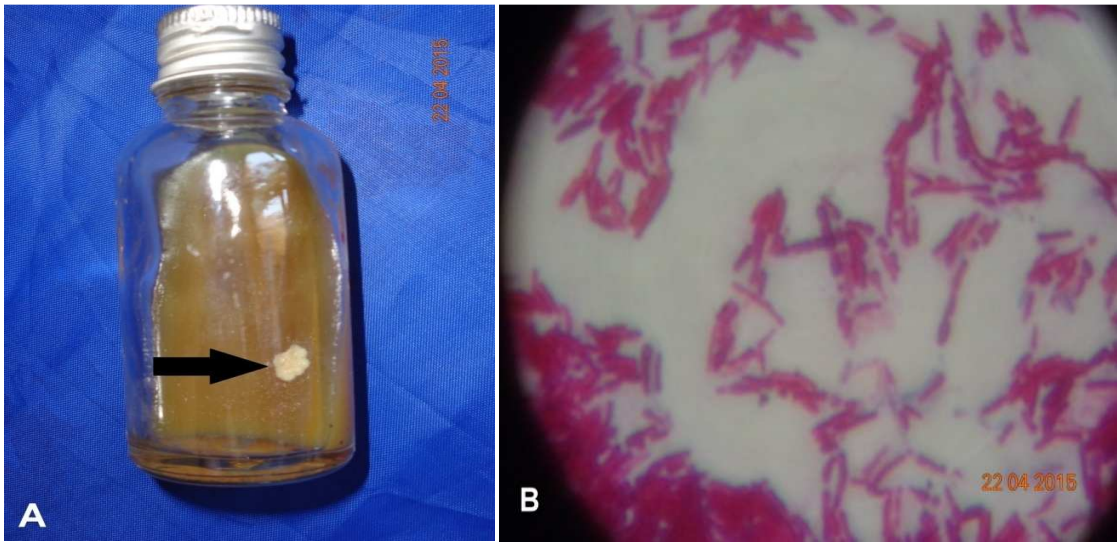

Figure 11: Characteristics of MAP isolated from goat: (A) Whitish large colony (arrow head) on HEYM media and (B) ZN staining of smear prepared from colonies grown on (A) revealing AFB in clump

4.5. Relationship Between Diagnostic Tests

In the present study highest agreement (80.77%) was observed between histopathology and ZN staining, which had Kappa value of 0.3299, indicating fair agreement between them. Poor agreement between tissue culture and histopathology as well as tissue culture and ZN staining was recorded (Table 9).

Table 9: Linearly weighted kappa coefficients between histopathology, ZN staining and culture

Diagnostic tests	Agreement	Kappa
Histopathology-ZN staining	80.77%	0.3299
Tissue culture-histopathology	30.77%	0.0602
Tissue culture-ZN staining	41.3%	0.0717

Interpretation of agreement: poor (κ=0.00-0.20), fair (κ=0.21-0.40), moderate (κ=0.41-0.60), good (κ=0.61-0.80) and excellent (κ=0.81-1.00)

5. DISCUSSION

In Ethiopia, the occurrence of paratuberculosis in livestock based on isolation of the causative agent and pathological study is limited to cattle. So far there are no published documents on the occurrence of the disease in sheep and goat. The present study reported the occurrence of paratuberculosis on sheep and goat based on gross pathological examination, ZN staining, histopathological examination and isolation of MAP for the first time in the country. Moreover, because the aim of the present study was to diagnose the occurrence of subclinical paratuberculosis on sheep and goats, gross pathological observation of intestines and associated lymph nodes was performed in apparently healthy sheep and goats slaughtered in the selected export abattoirs located in central part of the country. Gross pathological findings were confirmed using ZN staining, histopathological examination and isolation of MAP. The confirmation of isolates based on PCR technique is ongoing and will be published up on completion.

In the present study the last portion of ileum, ileocaecal valve, ileocaecal and mesenteric lymph nodes and adjacent portions of intestines were examined grossly and used for laboratory examination. Previous pathological studies on sheep and goat paratuberculosis have demonstrated the importance of tissue sample selection from different sites. In sheep, Perez *et al*. (1996), Oryan *et al*. (2008) and Hailat *et al*. (2010) indicated that, different portions of small and large intestine and associated lymph nodes should be examined with the terminal ileum, ileocaecal valve and ileocaecal lymph node as the first site to be selected. In addition, Corpa *et al*. (2000), Valheim *et al*. (2002), Lybec *et al*. (2013) and Kruger *et al*. (2015) indicated similar sites to be examined in diagnosing paratuberculosis in goats.

5.1. Prevalence of Paratuberculosis Based on Gross Pathology

In the present study, up on examination of intestine and corresponding lymph nodes, gross lesions consistence with paratuberculosis were found in 5.2% and 3.4% of sheep

and goats respectively. In sheep the overall gross lesion based prevalence obtained was lower than the previous reports from Jordan but higher than reports from Iran. Thus, in Jordan 74.8% prevalence was reported by Hailat *et al*. (2010), while in Iran a prevalence of 3.42% was reported by Oryan *et al*. (2008). In support of the present study, Mohammed (2014) reported the occurrence of paratuberculosis in 11.2% of apparently healthy cattle in Ethiopia.

All paratuberculosis suspected sheep and goats showed mild thickening of ileocaecal valve and edematous swelling of ileocaecal and mesenteric lymph nodes. In addition, in few cases gross lesions were observed in colon. This was in agreement with the findings of Perez *et al.* (1992), Clarke and Little (1996), Perez *et al.* (1996) and Oryan *et al*. (2008), who reported the frequent involvement of terminal ileum, ileocaecal valve, and associated lymph nodes and with occasional extension of lesions in to colon in natural cases of ovine paratuberculosis. In agreement with the present finding, Fodstad and Gunnarsson (1979) and Collins *et al.* (1984) reported mildly thickened wall of terminal intestine and enlarged, oedematous regional lymph nodes in majority of natural caprine paratuberculosis while Oryan *et al*. (2008) and Hailat *et al*. (2010) reported severe thickness and corrugation of the ileum and ileocaecal valve in 33.3% and 6.4% of sheep respectively, which was not consistent feature of all cases in the present study.

In the present study gross lesions were observed in 46 (100%) of ileocaecal valve and associated lymph nodes of sheep and goats. Gross lesion of terminal ileum was seen in 90% and 65.4% of sheep and goats respectively. In agreement with the present finding, many studies showed that ileocaecal valve is more frequently involved in the development of lesion in sheep (Carrigan and Seaman, 1990; Clarke and Little, 1996; Perez *et al.*, 1996) and goats (Corpa *et al*., 2000; Valheim *et al*., 2002; Lybec *et al*., 2013) as compared to ileum.

In the present study statistically insignificant (P=0.146) higher prevalence of paratuberculosis was recorded in sheep of 2 years and above (7.1%) than younger animals (3.7%), while in goat, the prevalence was highly comparable (P=0.935) among

age groups. Overall, the present study showed that older animals (2 years and above) had higher chance of developing gross lesion than younger animals, which is in agreement with other studies. Thus, Hemalatha *et al*. (2013) showed that the outcome of MAP infection was less in sheep found between 1 and 2 years than 2- 5 years old animals. Similarly, Valheim *et al*. (2002) reported higher lesions on animals above 2 years than younger animals.

Several studies described that, the outcomes of exposure to MAP infection depends on size of the infective dose, route of infection, mycobacterial strain virulence, local and systemic immune status and age of the host, host resistance genes affecting antigen presentation and intracellular killing and environmental factors. In their study Perez *et al*. (1996) found that majority of examined sheep aged 1.5 years and above had lesion type which was described as marker of early stage of infection by Juste *et al*. (1994). In addition Karpinski and Zorawski (1975) and Gilmour *et al.* (1978) observed that some experimentally infected sheep failed to show clinical signs and went on to recover from infection, suggesting that the immune response plays an important role in the development of lesion. On the other hand, Larsen *et al*. (1975) indicated that, in young animals sever lesion may not develop possibly due to the presence of protective colostral antibodies and the long incubation period of the organism to produce visible lesion. More recently, Kruger *et al*. (2015) showed that host immune response resulted either in an almost complete local elimination of MAP or an uncontrolled mycobacterial proliferation at 9 to 12 month post inoculation in experimentally infected goats, indicating MAP–host interactions during the clinically inapparent phase of paratuberculosis have a major influence on the eventual outcome of infection with MAP.

Other notable finding of the present study was the relatively lower chance of developing gross lesions in sheep originated from Somali than those originated from Borana. This could be related to variation in the pH of soil from the two areas. Tefera *et al*. (2007) and Dalle *et al*. (2014) showed that the soil pH of Borana lowlands rangelands to be moderately acidic with a range from 5.6-7.2 and mean of 6.3, while the pH of soil in

Somali regional state ranged from neutral to alkaline with a mean value of 8.1 (Vågen *et al*., 2013).

Previous studies indicated the effect of soil pH on the clinical picture of paratuberculosis in different countries. Thus, Kopecky (1977) reported higher prevalence of clinical paratuberculosis in animals reared under acidic soil as compared to alkaline soil in USA, France, Netherlands and England. In Australia, Richards (1989) applied lime to pastures to elevate the soil pH in excess of recommended pasture guidelines, and incidence of clinical paratuberculosis decreased. Similar observations have been reported in South Africa by Michel and Basstianello (2000) and in Spain by Reviriego *et al*. (2000) who subsequently proposed an association between acidic soils and a high prevalence of paratuberculosis. In USA, Brooks *et al*. (1984) demonstrated a highly significant correlation between high numbers of MAC and high acidity (low pH) of the corresponding soils, while no correlations or weak correlations were found with other soil characteristics, such as high concentrations of organic matter, high conductivity, or high moisture content. The findings of these studies are biologically supported, as recent molecular studies on pathogenomics of MAP indicated that exposure of MAP in to acidic condition significantly up regulates many genes involved in its survival and virulence as compared to control group (Wu *et al*., 2007b).

Based on gross lesion examination, statistically insignificant ($P>0.05$) higher prevalence was recorded in sheep than goat, which is in agreement with the finding of Sirak (2010), who reported higher prevalence of AFB in sheep as compared to goats slaughtered at abattoir in Jordan. It may be however, unwise to compare directly the differences in the magnitude of outcomes to MAP infection among species and across studies, as it would be more likely related to husbandry factors (Chiodini *et al*., 1984; Merkal, 1984) as well as to age at which the animals were examined and environmental factors (Clark, 1997). Therefore, lack of details on study animals husbandry practices, origin and age in the present and previous studies, makes direct comparisons unlikely.

5.2. Microscopic Characteristics of Lesions Associated With Paratuberculosis

Hematoxylin and eosin staining of tissues from paratuberculosis suspected animals revealed histological lesions consistent with paratuberculosis in 84.6% and 76.9% of sheep and goats respectively. In the present study the finding of 76.9 % positive cases in goats is higher than the report of Kheirandish *et al*. (2009), who reported 65.4% prevalence of paratuberculosis in grossly and clinically suspected goats slaughtered at abattoir. The observed histological lesions such as granulomatous infiltration of intestinal mucosa and submucosa, Peyer's patches proliferation, replacement of the crypts with inflammatory cells and necrotic foci surrounded by lymphocytes, macrophages and epithelioid cells in cortical and paracortical areas of lymph nodes were in agreement with reports of Reddy *et al.* (1984), Perez *et al.* (1992), Clarke and Little (1996), Perez *et al.*(1996), Michel and Basstianello (2000), Oryan *et al*. (2008) and Hailat *et al*. (2010) who conducted histopathological characterization of lesions in natural cases of sheep and goat paratuberculosis. Similarly, Mohammed (2014) reported histological lesions consistent with the present finding in apparently healthy cattle in Ethiopia.

In the present study the observed microscopic lesions of intestinal mucosa in all sheep with paratuberculosis were equivalent with type 3c lesions category by Reddy *et al.* (1984) and Perez *et al*. (1996) in sheep, with diffuse lymphocytic (paucibacillary) by Kheirandish *et al*. (2008) in ovine paratuberculosis and with type I by Hailat *et al.* (2010) in sheep. Moreover, Kurade and Tripathi (2008) reported majority of sheep with paratuberculosis were having type 3c lesions. In addition the present study showed that, microscopic lesion was recorded in all examined lymph nodes, which was in agreement with Reddy *et al.* (1984), Perez *et al*. (1996) and Kurade and Tripathi (2008), who reported frequent involvement of ileocaecal and mesenteric lymph nodes in diffuse lymphocytic lesion. Contrarily, Shulaw *et al.* (1993) and Oryan *et al*. (2008) reported that in animals having type 3c lesion, majority of lymph nodes didn't show histological lesions consistent with paratuberculosis. In the present study no lesions were observed in the mucosa of intestine without finding them in the Peyer's patches, which is agreeable with Perez *et al*. (1996)**.**

In goat the microscopic lesions of intestinal mucosa were equivalent with the diffuse lymphocytic lesion type by Corpa *et al*. (2000), Kheirandish *et al.* (2009) and Lybec *et al*. (2013) in caprine paratuberculosis, whereas it was similar with the type 3c lesions by Reddy *et al.* (1984), Perez *et al*. (1996) and Kurade and Tripathi (2008) in natural cases of ovine paratuberculosis. In agreement with the present finding, Corpa *et al*. (2000), Valheim *et al*. (2002) and Kheirandish *et al*. (2009) reported frequent involvement of lymph nodes of goats having diffuse lymphocytic lesion in their intestine. But their finding, in majority cases of goats, as focal area of caseous necrosis surrounded by lymphocytes, macrophages and epithelioid cells in the cortical areas of lymph nodes with calcification and fibrosis was not agreeable with the present finding, as it was only recorded in 20 % of animals.

In the present study the occurrence of type 3c lesion in all sheep is consistent with the findings of Reddy *et al.* (1984), who reported in all cases (100%) of ovine paratuberculosis. Disagreeably with the present finding, Perez *et al*. (1996) and Hailat *et al*. (2010) reported type 3c lesions in only 20.5%, 11% of sheep respectively. In goat Corpa *et al*. (2000) reported diffuse lymphocytic lesion in only 14.7% of goats with paratuberculosis.

The diversity of lesions observed in flock of sheep or herd of goat with paratuberculosis could be due to difference in the pathogenicity of MAP strains (Stamp and Watt, 1954), or that different lesions represent different stages of the disease (Rajya and Singh, 1961). In light of the immunological and histopathological spectrum of the mycobacterioses, Chiodini *et al*. (1984), Young *et al.* (1990) and Perez *et al*. (1996) indicated that, the range of paratuberculosis lesions were related to host immune response. Thus, type 3c lesion in sheep and diffuse lymphocytic lesion category in goat may represent the host response is insufficient to limit the multiplication of bacilli, but in which there is a strong cellular response. Similarly, Cravel *et al*. (2002) and Sivakumar *et al*. (2006) showed that diffuse lymphocytic infiltration was seen in the early stages of MAP infections and correlated with strong cell mediated immune response. Moreover, Krüger *et al*. (2014) indicated that host-pathogen interaction during the clinically in-apparent phase of

paratuberculosis is complex with sometimes the host and sometimes the pathogen prevailing. As a consequence, morphologically distinct lesions develop during this early phase of infection. Hence, at tissue sites where the local immune response was apparently able to eliminate MAP, there were only few and small foci of epitheloid cells and macrophages embedded in extensive inflammatory and immune cell infiltrates that consisted of T and B lymphocytes as well as plasma cells indicating a mixed immune response.

In the present study, the findings of histological lesion in relation to the clinical picture and gross pathology is in agreement with the findings of Clarke and Little (1996), Perez *et al*. (1996), Navarro *et al*. (1998) and Valhiem *et al*. (2002), who reported higher proportion of lymphocytes to epitheloid macrophages, in relation with mild gross thickening of intestinal mucosa and without clinical manifestation in experimental and natural cases of ovine and caprine paratuberculosis.

5.3. Direct Smear Staining Result

Ziehl-Neelsen staining of tissue samples from sheep and goats revealed characteristics AFB in clumps in 80% of sheep and 53.8% of goats suspected of paratuberculosis. The finding in the present study was higher than the previous abattoir based reports from Jordan, Canada, India and South Africa. Thus, in Jordan 53% and 5% prevalence were reported by Hailat *et al*. (2010) in sheep and goats respectively. While in Canada, South Africa and India prevalence of 7.6%, 50% and 5.4% were reported by Michel and Bastianello (2000), Arsenault *et al*. (2003) and VinodhKumar *et al*. (2013), respectively in sheep.

The absence of AFB in the intestine and lymph nodes does not altogether remove the possibility of paratuberculosis. Since it has been reported that, light microscopic detection of AFB in subclinical cases can be difficult and many factors can play a role in failure of a microbiologist to detect them. Thus, Condron *et al.* (1994), Clarke and Little (1996) and Perez *et al.* (1996)and Michel and Bastianello (2000) indicated that in lesions

confirmed to gave negative result by ZN staining technique, many of them contained either MAP nucleic acid or antigen, or mycobacterial spheroplast structures and such cases often showed specific serological or lymphocytic proliferative responses to MAP antigen up on investigation by PCR, immunological, immunohistochemical, electron microscopical and histological techniques, suggesting sparsity of organisms, a change in morphology, poor sensitivity of staining, or even misdiagnosis contributed to false negative. In addition, the mere demonstration of AFB also does not conclusively indicate that the animal is suffering from paratuberculosis. However, a large number of AFB along with lesions is highly suggestive of ovine paratuberculosis (Hemalatha *et al*., 2013). Agreeably, Singh *et al*. (1998) and Khan *et al*. (2010) indicated that, in smears form intestines, near ileoceccal valve or in the intestinal lymph nodes with gross lesions, visualization of groups of brightly pink colored bacilli is highly suggestive of paratuberculosis. Moreover, Huchzermeyer and Bastianello (1991), Greig (2000) and Sirak (2010) showed that among different diagnostic method applied, high number of samples revealed positive results using direct smear staining while investigating paratuberculosis on slaughtered sheep, suggesting as preferable diagnostic test to indicate the presence of paratuberculosis.

5.4. Cultural Characteristics of Isolates

In the present study to confirm the suspected gross pathological findings, tissue samples were cultured and 10% of sheep and 3.8% of goats were confirmed to harbor MAP. All isolated colonies were confirmed as MAP due to their growth only on media that contain 1% ferric ammonium citrate as a growth promoter, long incubation period (minimum of 6 weeks), colony morphology and appearance of characteristic AFB on stained smears, which was in agreement with the OIE (2014d) protocol on the isolation and confirmation of MAP.

The culture finding in the present study was comparable with previous abattoir based reports from Jordan. Thus, in Jordan 9% and 11% prevalence in apparently healthy sheep

was reported by Sirak (2010) and Hailat *et al*. (2010), respectively. The cultural finding in the present study was higher than reported by VinodhKumar *et al*. (2013), who reported 4.7% prevalence in apparently healthy sheep slaughtered in India. In the present study the culture prevalence of 3.8% in goats is lower than the prevalence report of Sirak (2010), who reported 10% prevalence in apparently health goat slaughtered in Jordan. In Ethiopia, Mohammed (2014) showed 11.1% culture prevalence in apparently healthy cattle slaughtered at abattoir. The difference in the culture based prevalence of paratuberculosis in different areas of the world could be due to spatial, temporal and methodological factors such as variation in the ecology (soil characteristics), circulating strains of MAP, stage of the disease, the age of animals, culture methods, sampling technique and size (NRC, 2003).

From sheep having gross lesion consistent with the present finding, Perez *et al*. (1996) and Juste *et al.* (1991) isolated MAP from 100% and 38% of samples, respectively and Corpa *et al.* (2000) isolated MAP from majority of goat having disuse lymphocytic type of microscopic lesion, which is not in agreement with the present findings. Moreover, in the present study, despite of the larger proportion of tissues revealing AFB and histological lesions consistent with paratuberculosis, MAP was isolated from only three samples. Menzie (2001) explained that a negative result means failure to grow or that the animal is still in the early stage of the disease.

Previous studies showed lower culture rate due to the effect of chemical decontamination (Stabel, 1997; Grant *et al*., 2001; Whittington *et al*., 2003) and when MAP present in low abundance or in spheroplast form without a bacillary cell wall (Hope *et al*., 1996). In addition, variety studies reported that, sheep strains were very difficult to culture, and even culture systems optimized for growing sheep strains are less sensitive than for the detection of cattle strains (Jakobsen *et al.*, 2000; Collins, 2011). In other study deJuan *et al*. (2006) indicated that culture of MAP strains from sheep and goats was less successful due to their slower rate of growth (more than 30% of sheep strains need extension of duration up to 5 to 7 months) and need the combined use of HEYM, LJ and Middlebrook to isolate all possible types of S strains and C strains.

In the present study all of MAP isolates on slants of culture media were visually appeared first from 6 to 14 weeks of incubation period. In agreement with the present finding Collins (1996) and Sirak (2010) reported that growth occurs on 16 weeks and 12-16 weeks of inoculation, respectively. Whittington *et al*. (2011) showed that, growth of MAP isolates was first appeared on 3-14 weeks on LJ media and 6-12 weeks on HEYM. In agreement with the present finding, Stable *et al.* (1998), Hailat *et al*. (2010) and Mohammed (2014) reported that due to the fastidious nature of the organism culture may require up to 12 weeks for detection.

As described earlier by Whittington *et al*. (2011), it may be unwise to make direct comparisons of results between different studies for certain culture media because media with the same name may not be formulated identically across studies. For instance, in the present study ferric ammonium citrate (1%) was used as supplement for the growth of MAP, while ferric mycobactine J was used in most studies. In agreement with the present finding, Whittington *et al*. (2011) revealed that MAP colonies were whitish, small (pinpoint or minute) non-mucoid with a shiny surface and entire edges on HEYM and LJ media. In the present finding the colonial appearance of one goat isolate after prolonged incubation period is comparable with report of Quinn *et al.* (2011), who reported older colonies of MAP become large (reach up to 2 mm) and colonial morphology changes with age from smooth to rough, and from hemispherical to mammilate. In the present study, the morphological characteristics of MAP on ZN stained smear is in agreement with the report of Whittington *et al*. (2011), who showed that morphologies of some MAP isolates grown on HEYM and LJ media appeared relatively short and wider AFB while others were relatively long, slender bacilli in smears stained by a ZN method.

5.5. Relationship Between Diagnostic Tests

With the aim of identifying relationship between diagnostic tests, linearly weighted kappa coefficient showed fair agreement between histopathology and ZN staining, while poor agreement was recorded between culture and histopathology as well as between culture

and ZN staining. The fair agreement between histopathology and ZN staining is comparable with the result of Mohammed (2014), in the investigation of subclinical paratuberculosis in cattle. Meanwhile, Mohammed (2014) reported good agreement between histopathology and culture and moderate agreement between culture and ZN staining, which is not agreeable with the present finding. The poor agreement between culture and other diagnostic tests could be due to the presence of MAP strains, which couldn't be cultivated (Kazda, 2009), or need a prolonged incubation period (Whittington *et al*. 2011) to show visible colonies, or that required a defined media other than presently used, which have nutrients that better support their in vitro growth (deJuan *et al*., 2006; Whittington *et al*., 2011). Generally, in the present study the fair agreement between histopathology and ZN staining indicates histopathology could be a good predictor of the presence of paratuberculosis in sheep and goats having mild gross lesion. In agreement with the present finding, Juste *et al*. (1991), Clarck *et al*. (1996), Perez *et al*. (1996) and Perez *et al*. (1999) indicated that, histopathologic features have been proposed as a good parameter for the diagnosis of MAP infection in sheep and goats, being more sensitive than bacteriologic culture and other methods if appropriate tissue specimen collection is performed.

6. CONCLUSION AND RECOMMENDATIONS

In the present study gross pathological, histopathological, ZN staining and bacteriological culture findings indicated the occurrence of paratuberculosis in apparently healthy sheep and goats in the country. Thus, it is important to note that, paratuberculosis can occur in sheep and goats without clinical manifestations and may be more important in the propagation of infection to the environment and yet be escaping detection, possibly because of harboring sub clinical infection. Despite the presence of gross and microscopic lesions and the detection of AFB, larger proportion of samples failed to show growth on culture media. Moreover, bacteriological culture was very laborious and time consuming. Another notable finding was the statistically insignificant higher probability of developing gross lesion in animals aged 2 years and above than younger. Thus, it seems appropriate to intensify testing time at age of 1 year and above in order to maximize the likelihood of detecting infected animals. Furthermore, the relatively high occurrence of paratuberculosis in sheep originated from Borana than Somali could indicate higher cluster of paratuberculosis in certain geographical area.

Based on the above concluding remarks, the following recommendations are forwarded:-

- Further studies on the epidemiology of sheep and goat paratuberculosis and characterization of the causative agent should be conducted in Ethiopia, so as to assess economic impact and to design feasible control strategies.
- Future research on the investigation of paratuberculosis should be done using improved diagnostic tools which can save both time and energy.
- Comprehensive study should be conducted to rule out risk factors for ovine and caprine paratuberculosis and the possible interaction with other livestock.

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8. APPENDICES

Appendix I: Ante-mortem data collection format

No	Animal identification	Origin	Sex	Age (years)	Helath status
1					
2					

Appendix II: Age estimation for sheep and goat based on dentition

Description	Age
Young without teeth often a new born	New born
With erupted and growing 1st and 2nd pair of milk teeth	1–2 weeks
With erupted and growing 3rd pair of milk teeth	2–3 weeks
With erupted and growing 4th pair of milk teeth	3–4 weeks
With fully grown milk teeth that started to spread out	9 month
The milk teeth have started to wear down, or are fully spread out	12 months
With erupted and growing 1st pair of permanent teeth	14–17 month
With erupted and growing 2nd pair of permanent teeth	18–23 month
With erupted and growing 3rd pair of permanent teeth	24–36 month
With erupted and growing 4th pair of permanent teeth	3–5 years
The four pairs of permanent incisors have started to wear down	4 years
The permanent incisors have worn down and have started to spread out	5 years
Worn down incisors are spread out and few are lost (broken-mouth)	6 years
Most of the incisors have been lost (smooth-mouth) or worn down to the level of dental pad	7 years

Appendix III: Gross lesion data collection format

Animal ID. No.	Gastrointestinal tract portions and lymph nodes	Tissue location	Gross lesion
	Ileum		
	Ileocaecal valve		
	Jejunum		
	Colon		
	Caecum		
	Ileocaecal lymph node		
	Mesenteric lymph node		

Appendix IV: Media preparation for isolation and confirmation of MAP

A. Herrold's egg yolk medium with addition of ferric ammonium citrate

Composition/L: 9 g peptone; 4.5 g sodium chloride; 2.7 g beef extract; 27 ml glycerol; 4.1 g sodium pyruvate; 15.3 g agar; 10 g ferric ammonium citrate; 870 ml distilled water; 120 ml egg yolks ; and 5.1 ml of a 2% aqueous solution of malachite green.

- Measure 9g peptone; 4.5g sodium chloride; 2.7g beef extract; 27ml glycerol; 15.3g agar and dissolve by heating in 700 ml distilled water.
- Autoclave at 121°C for 25 minutes.
- Cool to 56°C and aseptically add sterile egg yolks and sterile malachite green solution.
- Add 10 g of ferric ammonium citrate (in 100 ml distilled water) and 4.1g of sodium pyruvate (in 70 ml of distilled water) are autoclaved separately and then either combined or added separately to cold media
- Add 50 mg chloramphenicol and 100,000 U penicillin
- Blend gently and dispense 9 ml of media into sterile tubes of 20-25 ml volume holding capacity.

B. Lowesten Jenson medium with addition of ferric ammonium citrate

Composition (g/l): Potassium dihydrogen phosphate (1.5 g), magnesium sulfate (310 mg), magnesium citrate (375 mg), L-asparagine (2.25 g), malachite green (12.5 mg), homogenized whole egg (1,000 ml), sodium pyruvate (4 g) and ferric ammonium citrate (16 g)

Preparations: A mixture of 37.5 g of LJ base were dissolved in 600 ml distilled water and 12 ml glycerin and then autoclaved at 121o C for 15 min. After the solution was cooled to 30oC, 4 g sodium pyruvate and 16 g ferric ammonium citrate were added after being autoclaved separately. By soaking eggs in 70% ethanol, aseptically harvesting the contents and mixing them in a sterile blender a whole egg suspension of about 25 eggs

was made The egg homogenate was then filtered through sterile sieve to remove clumps and 1,000 ml was added to warm (not hot) medium with thorough mixing. The total volume of the medium was 1,600 ml. Glass tubes of 20-25 ml holding capacity were filled with 7 ml of medium which was allowed to solidify by incubating at 85 ^{o}C for 1 hr at an angle such that the surface of the medium extended three-fourths up the tube. The tubes of medium were and then stored upright at 4 ^{o}C for a maximum of 1 week.

Appendix V: Ziehl-Neelsen staining method

- Make a smear of on slide and fix it using flame.
- Flood the smear with carbol fuchsin and heat from the below till steam comes out.
- Allow the hot carbol fuchsin to act for 3 to 5 min. Do not boil the stain or allow it to dry on the slide.
- Wash the slide with tap water.
- Decolorize with acid alcohol for about 15-20 seconds until the bacterial smear appears faint pink or colour less. Wash it with tap water.
- Counter stain with methylene blue for about 30 seconds.
- Wash with tap water, blot dry the slide.
- Examine the slide under microscope with oil immersion objective and make a drawing of a field under microscope
- Acid fast bacteria will take pink / red colour while non acid fast stain blue

Appendix VI: Histopathological technique

1. Fixation of tissue by 10% buffered formalin

2. Trimming tissue:

- The intestinal tissue sample were trimmed to fit in to standard histological processing tissue cassettes (5mm thickness) in cross section including their full thickness having all layers of intestine so that lesions required be included or not missed and labeled

3. Tissue processing:

- Place trimmed tissue in tissue cassettes and make sure proper labeling.
- To ensure complete fixation, allow the cassettes to stay immersed in 10% buffered formalin (2 times) for two hours each.
- Dehydrate all extractable water using different concentrations of ethyl alcohol (70% for one hr, 95% for one hr, 100% for one hr, 100% for two hrs, 100% for two hrs).
- Clear the tissue:
- The clearing reagent must be miscible both with ethyl alchol (dehydrant) and paraffin.
- As the dehydrante is removed the tissue clears and becoming translucent
- Xylene is the most widely used clearing agent and is normally applied as xylene I one hour and half, xylene II one hour and half and xylene III two hrs and half hrs.
- Complete removal of the clearing reagent and its substitution by paraffin wax:
- Two paraffin baths: paraffin I two hrs and paraffin II three hrs.
- The melting point of the paraffin should be maintained to 56-58oc.
- Buckets on the processing machine should be filled with the respective chemical to the level that the cassette holding basket should sink completely.

4. Embedding or Blocking:

- Embedded cassettes one at a time to avoid cross contamination.
- Evaluate tissues for volume and shape.
- Make the paraffin mold large enough so that the tissue shall not abut the sides.
- Held the empty mold underneath the paraffin dispenser and half fill with paraffin by pressing the paraffin dispensing button.
- Transfer each piece of tissue to the half-filled mold and beware of the orientation of the tissue placed on the mold organize the tissue in close proximity in the mold
- Label the block with the correct identification code.
- Write the code on a piece of paper and fix the paper in the paraffin towards one side so that one can read it easily when the block is removed from the mold.
- Transfer the mold half-filled with paraffin and containing tissue to the freezer so that the tissues will be held firmly by the hardened paraffin.
- Each piece of tissue must be pressed down to ensure that all areas of the tissue are on the same plane.
- Remove the block from the mold after it has completely cooled.

5: Sectioning embedded tissues:

- Histopathological evaluation of tissues is possible when tissues are sectioned very thinly (one cell layer thick) and stained.
- The thin sectioning is accomplished by use of a microtome.
- Clean the water bath and refill with distilled water.
- Turn on the water bath and allow setting the temperature to 45oc ± 5oc.
- Trim excess paraffin from the blocks by using a knife so that the block will fit securely in the microtome chuck and the respective file drawer.
- Rough cut (face):
- Place a low profile microtome blade into the blade holder.
- Obtain an ice tray from the freezer and make an ice.
- Fine cut(section):

- Fine cut (section) the blocks by using the fine adjustment advance knob. Section at 5 microns
- Make a ribbon of 3-4 continuous sections and float on the water bath by using forceps, a wooden probe or a fine paint brush.
- Pickup the best section using the slide.
- Place the section in the middle of the slide without any of the tissue touching the sides.
- The zone of paraffin adhere the section to the slide by hooking it on the side of the slide.
- Clean all paraffin debris from the water bath with a kim wipe before the next block is sectioned.
- Section all the slides, place in the metal slide basket from back to front inorder, place the basket in the laboratory oven and make it ready for staining.

6. Hematoxylin and eosin stain:

- Make sure if the slides are well dried in the oven at 65oc± 4oc for 10 minutes.
- Dewax the section with xylene 2x (cleansing).
- Hydrate the sections in graded alcohol (100% 2x, 95% 2x, and 75% 2x) for 3 minutes each.
- Rinse with tap water for 5 minutes.
- Immerse the slide in Myers hematoxylin for 10-15 min.
- Wash in running tap water for 15 minutes.
- Counter stain with eosin from 15 sec to 2 min depending on the age of the eosin, and the depth of counterstain desired. For even staining dip slides several times before leaving in the eosin for the desired time.
- Dehydrate the tissue sections in graded alcohols(70% 2x, 95% 2x and 100% 2x) for two minutes each or until excess eosin is removed.
- Clear in xylene, 2x for 2 minutes each (clearing).
- Mount in DPX

Appendix VII: Criteria for grading of microscopic lesions in sheep

Categories	Findings
1	A mild focal aggregate of foamy macrophages, forming small granulomas in the ileal Peyer's patches interfollicular spaces. No visible gross lesions.
2	More extensive lesions in the ileal Peyer's patches, with granulomas extending into the lamin propria mucosa. no visible gross lesions.
3	If granulomatous lesions are affecting the Peyer's patches and the adjacent mucosa, as well as mucosa not associated with lymphoid tissue, it is category 3. This is further classified as 3a, 3b and 3c.
3a	Multifocal large granulomas are in the lamina propria, submucosa, and serosa of the ileum and in draining lymph nodes, with extension of lesions into the jejunum. Grossly visible thickening of the intestinal mucosa is present in this category.
3b	In this category numerous macrophages and a few multinucleate giant cells spread in mosaic-like sheets through the submucosa and lamina propria to create villous fusion and marked thickening of the intestine.
3c	Diffuse granulomatous enteritis, with marked lymphocytic infiltrates within the mucosa (either related/ not to Peyer's patches) and small, well-defined granulomas and multi nucleoid Langhans giant cells scattered throughout the lesions. Few plasma cells are also found. Granulomas and focal areas of giant-cell necrosis are present within mesenteric lymph nodes. The swelling of the intestinal wall is obvious.

Appendix VIII: Criteria for grading of microscopic lesions in goat

Categories	Findings
Focal	Small granulomata formed by macrophages with clear and large nuclei and abundant slightly foamy cytoplasm in different parts of ileum, jejunum and lymph nodes. There is no noticeable alteration of the gross morphology.
Diffuse multibacillary	Diffuse granulomatous enteritis composed of groups of macrophages distributed in a diffuse manner throughout the ileal and jejunal mucosa. In the Peyer's patches, macrophages form well-delineated granulomata or diffusely infiltrating the lymphoid tissue, especially in the interfollicular areas. Diffuse or multifocal granulomatous lymphadenitis in the lymph nodes.
Diffuse lymphocytic	Diffuse granulomatous enteritis. In the intestinal mucosa (sometimes associated with Peyer's patches) predominant inflammatory cells are lymphocytes infiltrating the entire lamina propria. Among the lymphocytes are some macrophages and well-differentiated Langhans giant cells. Macrophages distributed in a diffuse manner in the other intestinal layers and the lymph nodes. Necrotic foci, with or without calcification and variable in size in the submucosa, serosa and lymph nodes.
Diffuse mixed	Diffuse granulomatous enteritis. Infiltrate contained large numbers of lymphocytes and macrophage in the mucosa and serosa of ileum and jejunum and lymph nodes.

Note: In diffuse type of lesion categories, the ileal and jejunal walls are thickened and corrugated. In addition, the mesenteric and ileocaecal lymph nodes are enlarged with oedematous cut surfaces.