Thesis Ref. No________

INVESTIGATION ON THE OCCURRENCE AND PATHOLOGY OF PARATUBERCULOSIS (JOHNE'S DISEASE) IN APPARENTLY HEALTHY CATTLE SLAUGHTERED AT ELFORA EXPORT ABATTOIR BISHOFTU, ETHIOPIA

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MASTER PROGRAM IN VETERINARY MICROBIOLOGY

JUNE, 2014

BISHOFTU, ETHIOPIA

INVESTIGATION ON THE OCCURRENCE AND PATHOLOGY OF PARATUBERCULOSIS (JOHNE'S DISEASE) IN APPARENTLY HEALTHY CATTLE SLAUGHTERED AT ELFORA EXPORT ABATTOIR BISHOFTU, 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

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TABLE OF CONTENTS **PAGES**

SIGNED DECLARATION SHEET V

ACKNOWLEDGEMENTS VI

LIST OF TABLES VIII

LIST OF FIGURES IX

LIST OF ANNEXES X

LIST OF ABBREVIATIONS XI

ABSTRACT XIII

1. INTRODUCTION 1

2. LITERATURE REVIEW 4

2.1. The Mycobacteria 4

2.2. The *Mycobacterium avium-intracellulare* complex (MAC) 4

2.3. The *Mycobacterium avium* subsp. *paratuberculosis* (MAP) 5

2.3.1. Phonotypic characteristics 6

2.3.2. Molecular characteristics 6

2.3.3. Types or strains of MAP 7

2.4. Paratuberculosis or Johne's disease (JD) 8

2.4.1. Host range 9

2.4.2. Susceptibility to infection 9

2.4.3. Prevalence and geographical distribution of Johne's disease 10

2.4.4. Economic impact 12

2.4.5. Transmission 13

2.4.6. Pathogenesis and host immune response to MAP 14

2.4.7. Pathology 18

2.4.8. Clinical picture and stages of Johne's disease *20*

2.4.8.1. Clinical picture 20

2.4.8.2. Disease stages 22

2.4.9. MAP and Crohn's disease (CD) a zoonotic potential *25*

3. DIAGNOSTIC METHODS 27

3.1. Direct detection of the causative agent 28

3.1.1. Microscopic examination *28*

3.1.2. Bacteriological culture *29*

3.1.2.1. Conventional culture of MAP on solid media 29

3.1.2.2. Radiometric culture of MAP on liquid media 30

3.1.3. Molecular approaches for paratuberculosis diagnosis *31*

3.1.3.1. Conventional PCR 31

3.1.3.2. Real time PCR 32

3.2. Detection of host response to MAP infection 34

3.2.1. Clinical signs and gross pathology *34*

3.2.2. Histological diagnosis *34*

3.2.2.1. Histopathological diagnosis 34

3.2.2.2. Immunohistochemistry (IHC) 35

3.2.3. Immune based test platforms for MAP diagnostics *36*

3.2.3.1. CMI based diagnostics 36

3.2.3.1.1. Intradermal skin test 36

3.2.3.1.2. Interferon Gamma (IFN–γ) assay 37

3.2.3.2. Serological based diagnostics 38

3.2.3.2.1. Agar gel immunodiffusion test (AGID) 38

3.2.3.2.2. Complement fixation test (CF) 39

3.2.3.2.3. Enzyme linked immunosorbent assay (ELISA) 39

4. CONTROL OF PARATUBERCULOSIS 41

4.1. Vaccination 42

5. MATERIALS AND METHODS 43

5.1. Study area 43

5.2. Study animals 43

5.3. Study design 43

5.4. Sample size determination 44

5.5. Sampling methods 44

5.6. Study methodology 44

5.6.1. Gross examination and tissue sampling 44

5.6.2. Histopathological examination 46

5.6.3. Cultivation and confirmation of MAP from affected tissues 47

5.6.4. Direct smear staining 48

5.7. Data analysis 49

6. RESULTS 50

6.1. Pathology 50

6.1.1. Gross pathological lesions 50

6.1.2. Histopathological lesions 53

6.2. Isolation and characterization of MAP 60

6.3. Direct smear staining from tissue lesion sample 61

6.4. Association between histopathology, tissue culture and acid fast staining 62

7. DISCUSSION 63

8. CONCLUSION AND RECOMENDATIONS 70

9. REFERENCES 72

10. ANNEXES 95

CURRICLUM VITAE (CV) 103

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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ACKNOWLEDGEMENTS

Thanks are expressed to the Mycobacterial infections project under the sub thematic research project entitled "Paratuberculosis and avian tuberculosis in selected rural communities of Ethiopia and their animals" under the AAU thematic research project for source of financial support in my thesis.

I would like to express my deepest gratitude to my academic advisor Dr. Gezahegne Mamo from Addis Ababa University for providing me the chance to work with this sub thematic research project and for his continuous and intellectual advice throughout the study periods, provision of materials, for the financial supports and correction of the manuscript.

Thanks are expresses to Aklilu Lemma Institution of Pathobiology (ALIPB) for the material and technical support I received during the study. I am particularly indebted to Dr. Gobena Ameni from Aklilu Lemma Institute of Pathbiology, My advisor, for providing me the chance to work with this sub thematic research project and in the institute throughout the study periods, provision of materials, for financial supports and correction of the manuscript.

Thanks are also expressed to National Animal Health Diagnostic and investigation Center (NAHDIC) for providing me the chance to work the histopathological portion of my study with the institute. I am particularly indebted to Dr. Asegedech Sirak from National Animal Health, Diagnostic and investigation Center, My advisor, for her kind support, sharing her experience and discussing ideas.

I am grateful to Ato Hailu Getu for his friendship, his tremendous help and for his support in the TB Laboratory of Aklilu Lemma Institute of Pathobiology (ALIPB); I have gained much experience and unreserved assistance while culturing the samples at the institute. I am also grateful to Mr. Adane Worku, Aboma, Mandafero, Dr. Mesfin and Ato Nega for his kind support during my stay at the institute.

I am also grateful to W/ro Ayinalem, Mr. Tewdrose and Mr. Selmone for his kind support in the pathology laboratory of National Animal Health Diagnostic and investigation Center (NAHDIC); I have gained much experience and unreserved assistance while doing the histopatological examination of the tissue samples at the institute.

In addition, I am highly grateful to my family: In particular to Mulumebet Mohammed, Enani Aychew, Mohammed Sulyman and Etenshi Mohammed for their love, encouragement and support during the study period. Thanks also go to the following individuals and friends for their involvement in one way or the other: Dr. Matios Lakew, Dr. Dwit G/mechale, Dawit Alemayehu, Habib Mohammed and others not mentioned here.

Above all, I praise the Almighty God for giving me the courage and strength in my life.

LIST OF TABLES

Table 1: Histopathological criteria and grading of the lesions found in the last part of the ileum and the ileocecal valve 47

Table 2: Different categories of gross pathological lesions observed during sample collection from the abattoir 53

Table 3: Frequency and percentage of Johne's disease positive cases of cattle in different age groups examined by histopathology 54

Table 4: Grading distribution of Johne's disease positive cases in cattle using histopathological examination of ileum 60

Table 5: Frequency and percentage of MAP positive isolates from tissue culture of cattle in different age groups and type of media 61

Table 6: Frequency and percentage of Johne's disease acid fast positive tissue sample of cattle in different age groups 62

Table 7: Linearly weighted kappa coefficients between histopathology, acid fast and MAP tissue culture 62

LIST OF FIGURES

Figure 1: Theoretical global epidemic curve for herd level paratuberculosis in dairy cattle. 11

Figure 2: Gross pathology of ileocaecal valve section from apparently healthy cattle. Congestion of ileocaecal valves with cauliflower-like corrugation and hypertrophy 51

Figure 3: Gross pathology of the distal ileum section from apparently healthy cattle. Corrugation and thickening of mucosa of the ileum with transverse folds 52

Figure 4: Intestine (Ileocecal valve); mononuclear cells infiltration of the mucosa and lamina propria of cattle. 55

Figure 5: Intestine (Ileocecal valve); scattered epithelioid cells in the lamina propria of the villi and between the crypt as nests or cords with large numbers of plasma cells. 55

Figure 6: Intestine (disatal ileum); hyperplasia and replacement of lamina propia crypt with lymphocytes, polymorphs and macrophage.. 56

Figure 7: Intestine (Ileocecal valve); multinucleated giant cells in the sub mucosa of the intestinal section of cattle.. 57

Figure 8: Intestine (Ileocecal valve); Payer's Patches proliferation and extending towards the mucosa. 58

Figure 9: Intestine (Ileocecal valve); Intestine, mucosa villi necrosis surrounded by mononuclear cells. 58

Figure 10: Ileocecal lymph node; presence of mononuclear cell aggregation and cell debris in the cortex surrounded by epitheloid cells and macrophages............................. 59

LIST OF ANNEXES

Annex I: Anti mortem data collection format 95

Annex II: Gross lesion data collection format 96

Annex III: Histopathological technique 97

Annex IV: Media used for isolation and confirmation of MAP from affected tissues... 100

Annex V:Acid fast staining or Ziehl Neelsen's staining methods 102

LIST OF ABBREVIATIONS

AFB	Acid-fast bacilli
AGID	Agar gel immunodiffusion
bp	Base pairs
CD	Crohn's disease
CFT	Complement fixation test
CMI	Cell-mediated immunity
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
FAPs	Fibronectin attachment proteins
GALT	Gut associated lymphoid tissue
HEYM	Herrold"s egg yolk medium
HPC	Hexadecylpyridinium chloride
HRM	High resolutions melt analysis
IBD	Inflammatory bowel disease
IFN-γ	Interferon gamma
IL	Interleukin
INF	Interferon
IS	Insertion sequence
JD	Johne's disease
Kb	Kilo base
LJ	Lowenstein Jensen medium
M	Molar
M.	*Mycobacterium*
MAA	*Mycobacterium avium* subsp. *avium*
MAC	*Mycobacterium avium* complex
MAH	*Mycobacterium avium* subsp. *Hominissuis*
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
MAS	*Mycobacterium avium* subsp. *silvaticum*

MB	Middle brook
MHC	Major Histocompatibility complex
NaOH	Sodium hydroxide
OD	Optical density
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PPD	Purified protein derivate
REA	Restriction endonuclease analysis
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
Se	Sensitivity
Sp	Specificity
Subsp.	Subspecies
Th	T helper
TNF	Tumor necrosis factor
TNF-α	Tumor Necrosis Factor-alpha
ZN	Ziehl-Neelsen staining
µl	Microliter
µM	Micromolar
%	Percentage

ABSTRACT

A cross sectional study was conducted from October 2013 to June 2014 in apparently healthy cattle at Bishoftu ELFORA export abattoir to investigate the occurrence and pathology of paratuberculosis (Johne's disease) and isolation of its causative agent. The occurrence of paratuberculosis was investigated using histopathology, bacteriological culture and acid-fast staining. Of the 400 animals examined a total of 45 tissue samples illustrating gross pathological lesions from the last portion of the small intestine, ileocecal valve and associated lymph nodes were collected. Tissue sections from the 45 ilea and associated lymph nodes were stained with hematoxylin and eosin (H&E). The occurrence of the disease was 17.8 % (95% CI=6.1-29.4) using histopathology. Grading from I-IV of histopathological lesions based on the type and amount of cellular infiltrate revealed that most of the positive cases were in grades I (8.9%) and II (6.7%). 45 tissue samples from ileum and associated lymph nodes were cultured on modified Herrolds egg Yolk and Lowesten-Jensen medium with and without mycobactin J. Results of the culture revealed 11.1% (95% CI=1.6-20.6) were positive and isolated as Mycobacterium avium subsp. paratuberculosis. Isolated colonies were confirmed by mycobactin dependence, ZN staining and their long incubation period. When acid-fast staining of 45 ileum and associated lymph nodes were implemented the occurrence of the disease was 13.3% (95%CI=3%-23.6%). The occurrence of the disease among age group were significantly different using histopathology (P=0.002) and culture (P=0.012). The present study on paratuberculosis in cattle using histopathology, mycobactin J supplemented culture and acid-fast staining revealed the occurrence of paratuberculosis in apparently healty cattle in Ethiopia and it is interesting to note that this is the first study on paratuberculosis in cattle in Ethiopia that showed the occurrence and isolation of its causative agent Mycobacterium avium subsp. paratuberculosis. The results strongly suggest the need to investigate on the distribution and economic significance of the disease at the national level in order to develop rational methods of control strategies.

Keywords: *Acid fast stain, Cattle, Culture, Histopathology, Ileum, Mycobacterium avium subsp. paratuberculosis, paratuberculosis (Johne's disease)*

1. INTRODUCTION

Paratuberculosis or Johne's disease (JD) is a chronic, progressive and infectious granulomatous enteritis which affects ruminants and a wide variety of domestic and wild life species all over the world (Chiodini *et al.,* 1984; Singh *et al.,* 2014). It is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) which is a slow growing mycobactin dependent acid fast bacillus belonging to the genus mycobacterium. The bacterium are very small 0.5 x 1.5 µm rod-shaped organisms and naturally occur as clumps entangled with one another by a network of intracellular filaments (Ayele *et al*., 2001). At the present time, JD is considered one of the most serious diseases affecting the world's cattle industry (Chiodini *et al*., 2012). In 1895, Johne and Frothingham discovered the disease by demonstrating the presence of acid fast bacilli (AFB) in sections of bovine intestine. At the turn of the century, the disease became recognized throughout northern Europe and the USA especially after the successful isolation of the causative agent by F.W. Twort (Harris and Barletta, 2001).

Most cattle with Johne's disease are infected as young calves via fecal oral transmission and *in utero* transmission has also been reported (Whitlock and Buergelt, 1996). In cattle susceptibility to infection appears to be highest in young animals and declines progressively with age (Sergeant, 2003). Young animals less than six months of age are thought to be the most susceptible to infection and older animals are more resistant to infection. Although calves may be exposed to the MAP at younger ages but the clinical presentation of the disease will likely only appear 2-4 years of age after exposure and it is characterized by intermittent diarrhea, emaciation, reduced production and ultimately death (Harris and Barletta, 2001; Mc-Spadden *et al*., 2013). Paratuberculosis induces a significant economic and health problem worldwide especially in the cattle industry. Economic losses occur due to animal culling, lowered milk production, reduced carcass value and poor reproductive performance and are estimated to be about $200 per infected cow per year in herds with at least 10% prevalence (Hailat *et al*., 2012). These production losses have an economic cost of $200-250 million annually to the United States dairy industry (Ott *et al*., 1999).

On average the economic losses caused by bovine paratuberculosis have been estimated to be higher than those for other bovine diseases such as bovine viral diarrhea, enzootic bovine leucosis and neosporosis (Chi *et al.,* 2002). The disease is not only important because of its economic impact in the livestock industry but it is also one of the key suspected causal agents of Crohn's disease of an inflammatory bowel disease of humans and also considered as a potential zoonosis (Stevenson *et al*., 2009). MAP occurs in most parts of the world with an increasing prevalence. Many studies have been done around the globe to determine the prevalence of MAP in cattle as well as in other species (Chiodini *et al*., 1984). In many European countries paratuberculosis has been described as endemic. A recent study critically reviewed published data representing prevalence of MAP in Europe and estimated the overall prevalence to be 20% (Nielsen and Toft, 2009). The true prevalence among cattle was measured in serological studies conducted in France, Germany, Italy and Turkey. It has been suggested that the prevalence of MAP is at least 3-5% in several countries (Munster *et al*., 2012).

In the USA the infection is spread throughout the country and the herd prevalence is strongly associated with herd size. In 1996, the herd level prevalence of bovine paratuberculosis was reported to be 21.6% and within herd prevalence to be 40% as forty percent of herds with more than 300 heads was found to be infected (Thirunavukkarasu *et al*., 2013). A less even distribution of paratuberculosis has been reported in Australia where the infection rate in dairy herd ranges from 9 to 22 % (Manning and Collins, 2001). In contrast to these industrialized countries in most African countries the occurrence, distribution and prevalence of the disease is unknown (OIE, 2008). Not much research documenting paratuberculosis has been done but the occurrence of the diseases is suspected in most countries. There are very few studies carried out to date and only case reports and limited prevalence studies covering small regions in a few countries are available. JD was reported in cattle from Egypt (Salem *et al*., 2005). The disease has also been reported in Kenya, Tanzania and Ugandan.

In Tanzania the overall prevalence of the disease by clinical examination as well as Ziehl-Nielsen staining of faecal smears was estimated to be 1.9% (Okuni, 2013). The existence of MAP infection in Ugandan cattle was only based on a small abattoir study in which paratuberculosis incriminating lesions was 12/100 tissue samples from a local abattoir and a seroprevalence study estimated the prevalence of antibodies to MAP to be 8.8% (Okuni *et al*., 2011). A recent market survey in some areas of Ethiopia indicated that the prevalence of the disease was not known but Temesgen and Gemechu in 1995, reported a single case of paratuberculosis from the country based on history of diarrhea case lasted for two years and clinical examination (Okuni, 2013). Unfortunately these were not confirmed by a specific method. In fact, because of disease nature or the different stages of Johne's disease and some limitations of the diagnostic tests diagnosis of paratuberculosis is challenging where a single effective diagnostic tool has not yet been identified (Stabel and Bannantine, 2005).

No previous study on the occurrence of the disease in cattle and on the isolation and characterization of the causative agent, *Mycobacterium avium* subsp *paratuberculosis* have been done in Ethiopia. Hence, investigation of the occurrence of paratuberculosis with isolation and characterization of its causative agent has paramount importance to assess the impact of the disease (economic and public health) in order to implement feasible preventive and control measures that can reduce the impact of the disease on the livestock sector of Ethiopia.

Therefore, the objectives of this study were:

- ❖ To investigate the occurrence and to describe the pathological lesions of paratuberculosis (JD) in apparently healthy cattle slaughtered at ELFORA export abattoir Bishoftu, Ethiopia.

- ➢ Isolation and characterization of its causative agent *Mycobacterium avium* subsp. *Paratuberculosis* from paratuberculosis lesions in tissues obtained from slaughtered cattle.

2. LITERATURE REVIEW

2.1. The Mycobacteria

Taxonomically mycobacteria belong to a single genus *Mycobacterium* within the family *Mycobacteriaceae* and the order *Actinomycetales* (Cocito *et al*., 1994). Mycobacteria belong to the phylum *Actinobacteria* are characterized by non-motile, non-sporulated, rods shaped, acid alcohol fastness, high genomic content of guanine and cytosine (61-71%) and the presence of long and complex mycolic acids in their cell wall. There were 133 recognized and proposed mycobacterial species and several subspecies (Herthnek, 2009). Analysis of the 16s rRNA genes of mycobacteria has resulted in the division of this genus in two separate clusters. These correspond to the traditional fast growing mycobacteria represented by non pathogenic environmental isolates and the slow growing mycobacteria containing most of the overt pathogens (Harris and Barleta, 2001). Slow growing mycobacteria of importance in veterinary medicine are found in two major complexes of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* complex (MAC) (Eglund, 2002).

2.2. The *Mycobacterium avium-intracellulare* complex (MAC)

The MAC is a large cluster of genotypically and phenotypically related organisms which comprises several species of slow growing mycobacteria that are prevalent in environmental, veterinary and clinical settings (Bradner *et al*., 2013). The MAC includes professional pathogens of birds and livestock and opportunistic pathogens of humans as well as organisms commonly found in soil and water (Behr and Collins, 2010). Traditionally MAC includes two species such as *Mycobacterium avium* and *Mycobacterium intracellulare* (Turenne *et al*., 2007). Recently advances in molecular taxonomy have fuelled the identification of novel species within the MAC, including the *Mycobacterium chimaera, Mycobacterium colombiense, Mycobacterium arosiense, Mycobacterium vulneris, Mycobacterium marseillense, Mycobacterium timonense* and the *Mycobacterium bouchedurhonense* (Coelho *et al*., 2013; Tortoli *et al*., 2004).

On the basis of genotypic, phenotypic and growth characteristics, biochemical tests and historical reasons the *Mycobacterium avium* has four subspecies (Coelho *et al*., 2013). These include the *Mycobacterium avium* subsp. *avium* (MAA), the *Mycobacterium avium* subsp. *silvaticum* (MAS), the *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and the *Mycobacterium avium* subsp. *hominissuis* (MAH) (Tortoli *et al*., 2004). All four *Mycobacterium avium* subspecies are capable of infecting a diverse range of hosts and possess a high degree of genetic similarity (Coelho *et al*., 2013). The *Mycobacterium avium* subsp. *avium* is the causative agent of avian tuberculosis in all mammals including humans leading to a disseminated disease among immunocompromised patients. The *Mycobacterium avium* subsp. *silvaticum* is an obligate pathogen of animals causing tuberculosis in birds and a paratuberculosis like syndrome in mammals. The *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease (JD) in ruminants and is also implicated in Crohn's disease (CD) in humans (Salem *et al*., 2013).

2.3. The *Mycobacterium avium* subsp. *paratuberculosis* (MAP)

MAP is the MAC member of greatest importance and is capable of infecting and causing disease in a wide array of animal species including non human primates without the need for co-existent immunosuppressive infections (Shin *et al*., 2010). MAP is a small, gram-positive, facultative intracellular, rod-shaped, acid-fast, fastidious and extremely slow growing mycobactin dependent bacillus of 0.5-1.5µm length with a generation time in cell culture of 1.3 to 4.4 days that causes paratuberculosis or Johne´s disease in cattle (Sweeney, 1996; Clarke, 1997). It is found in clumps entangled with one another by a network of intracellular filaments (Ayele *et al*., 2001). MAP grows in rough circular colonies reaching about 1-2 mm in diameter which are usually found to be off-white or yellow in color depending on the culture medium (Rowe and Grant, 2006). MAP full lineage is with the Superkingdom Bacteria, Phylum Actinobacteria, Class Actinobacteria, Subclass Actinobacteridae, Order Actinomycetales, Family Mycobacteriaceae, Genus *Mycobacterium*, Species *Mycobacterium avium*, Subspecies *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Fernandez-Silva *et al.,* 2012; Vansnick *et al.,* 2004).

2.3.1. Phonotypic characteristics

MAP is one of the slowest growing mycobacterial species and hence primary isolation from specimen requires prolonged culture incubation and can take several months. Unlike most mycobacteria, MAP does not produce an iron chelating mycobactin and therefore isolation of MAP requires the addition of the siderophore mycobactin to culture media (Wells *et al*., 2006). The MAP organism is an obligate intracellular pathogen and the only place it can multiply in nature is in a susceptible host within a macrophage. Out of the host it can survive for extended periods but it is unable to multiply (Pinedo *et al.,* 2008). The organism has a thick waxy cell wall made up of 60 % complex lipids which does not only give it the properties of an acid fast bacilli (AFB) but also creates hydrophobicity and an increased resistance to high temperature (Klanicova *et al.,* 2012). Therefore, MAP is able to survive pasteurized milk, low pH, salt and chemicals such as chlorine (Rowe and Grant, 2006). A recent study described a new spore like morphotype in MAP probably being a mechanism to survive unfavorable conditions such as pasteurization (Lamont *et al.,* 2012).

2.3.2. Molecular characteristics

Recently the complete genome sequence of MAP was reported and approximately 15% or 72.2 kpb of the MAP genome is comprised of repetitive DNA like insertion sequences, multigene families and duplicated housekeeping genes (Li *et al*., 2005). Insertion sequences are small mobile genetic elements containing genes related to transposition functions, causing insertional mutations and chromosomal rearrangements (Motiwala *et al.,* 2006). The first insertion sequence (IS) identified in MAP was the IS900 and was determined to be a unique characteristic of this subspecies (Collins *et al*., 1989). IS*900* elements has a size of 1.45 kbp found in multiple copies of 15 to 20 per genome and provide the diagnostic advantage of improved the sensitivity of MAP detection in PCR procedures. PCR targeting the 5’ end of IS*900* has been considered specific for the identification of MAP and is frequently applied to confirm the presence of the organism in the diagnosis of Johne’s disease (Pinedo *et al.,* 2008).

The closely related insertion sequences IS*901* and IS*902* were discovered subsequently and more recently IS*1245* and IS*1311* have been identified in MAP isolates (Motiwala *et al*., 2006). Moreover, several unique sequences have been also identified for the differentiation of MAP from other subspecies of *M. avium* like the sequence 251–255, ISMav2, Hsp X and the F57 gene (Mobius *et al*., 2008).

2.3.3. Types or strains of MAP

Several typing methods have been used to investigate the genetic diversity of *Mycobacterium avium* subsp. *paratuberculosis* (Motiwala *et al*., 2006). At the present the main MAP strains have been classified into three groups of Type I or sheep (S) strain, Type II or cattle (C) strain and Type III or intermittent strain based on the patterns obtained by the IS*900* restriction fragment length polymorphism (RFLP) and pulsed field gel electrophoresis (PFGE) techniques and on growth characteristics and pigmentation (Pinedo *et al*., 2008; Gwozdz, 2010). Additionally to these methods the use of high resolution melt (HRM) analysis based on real time PCR could also differentiate between Types I, II and III strains (Castellanos *et al*., 2010). In addition to these types one additional type Bison type or Type B apparently a subdivision of the Type C was determined using IS*1311*-PCR/REA and subsequent sequence analysis of the IS*1311* (Fernandez-Silva *et al.,* 2012).

The type I or sheep type (S) strains characterized by an extremely slow and mostly pigmented growth have been mainly described in sheep from different parts of the world (Munster *et al*., 2012). The type II or cattle type (C) strain characterized by non-pigmented slow growth, have been described in a broad host range and are commonly isolated from both domesticated and wildlife species including non-ruminant species and also humans (de Juan *et al*., 2006). Type II is the most common MAP strain type isolated from cattle (Stevenson, 2010). The type III or intermediate type comprising non-pigmented strains with a growth time between type I and type II and have been isolated from sheep from Canada, South Africa and Iceland and from goats and bullfighting cattle from Spain (Munster *et al*., 2012).

2.4. Paratuberculosis or Johne's disease (JD)

Paratuberculosis or Johne's disease (JD) is a chronic, progressive and infectious granulomatous enteritis which affects ruminants and a wide variety of domestic and wild life species all over the world and for which currently there is no known efficient treatment (Singh *et al.,* 2014). It is caused by *Mycobacterium avium* subsp. *Paratuberculosis* (MAP) which is a slow growing mycobactin dependent acid fast bacillus belonging to the genus Mycobacterium (Chiodini *et al*., 1984). At the present time, JD is considered one of the most serious diseases affecting the world's cattle industry due to its significant impact on the global economy (Chiodini *et al*., 2012). The disease is not only important because of its economic impact in the livestock industry but it is also one of the key suspect causal agents of Crohn's disease of an inflammatory bowel disease of humans and also considered as a potential zoonosis (Stevenson *et al*., 2009). In 1895, Johne and Frothingham discovered the disease by demonstrating the presence of acid fast bacilli in sections of bovine intestine (Harris and Barletta, 2001).

In 1906, Professor Bernhard Bang gave the first detailed description of paratuberculosis and he proved it to be an infectious disease distinct from tuberculosis (Munster *et al*., 2012). In 1910, the organism was first isolated and received the name *Mycobacterium enteritidis chronicae pseudotuberculosae bovis johne* (Pinedo *et al.,* 2008). In 1912, the first report of successful isolation and cultivation of the bacterium was published. Later on with regard to the phenotypic characteristics it was referred to as *Mycobacterium paratuberculosis, Mycobacterium johnei, Bacillus paratuberculosis, Bacterium paratuberculosis, Mycobacterium enteritidis and Darmtuberculose* (Rowe and Grant, 2006). By the beginning of the 1990s, after identifying a genome almost identical to that of *M. avium* and its subspecies an opinion suggested by the International Association for Paratuberculosis was that this organism should to be recognized and reclassified as a subspecies of *M. avium* and hence renamed as *M. avium* subsp. *paratuberculosis* (MAP). At the turn of the century, the disease became recognized throughout northern Europe and the USA especially after the successful isolation of the causative agent by F.W. Twort (Harris and Barletta 2001; Salem *et al*., 2013).

2.4.1. Host range

Although paratuberculosis or Johne's disease (JD) mainly affects domestic ruminants like cattle, sheep and goats, but all ruminants are susceptible with this chronic infection and are detected in wildlife, exotic animals residing in zoological gardens and non conventional domestic stock (Chiodini *et al.*, 1984; Eglund, 2002). The occurrence and transmission of MAP between wildlife species and domestic livestock has been reported in supporting the role of wildlife as reservoir hosts for this infection (Stevenson *et al.* 2009). Among the wild species in which MAP has been reported are ruminants such as deer, moose, bighorn sheep, mountain goats, bison, buffalo, camels, antelopes, elk, llamas and yaks as well as non-ruminants such as wild rabbits and their predators including foxes and stoats and primates such as mandrills and macaques indicating a wide host range (Pinedo *et al.*, 2008). MAP has also been shown to replicate within monogastric animals following experimental exposure without developing clinical disease (Chiodini *et al.*, 1984). Examples of monogastric animals susceptible to infection with MAP under experimental conditions include horses, mules, pigs and poultry (Cocito *et al*., 1994).

2.4.2. Susceptibility to infection

Age is the factor most commonly regarded as affecting susceptibility to MAP infection. In cattle susceptibility to infection appears to be highest in young animals and declines progressively with age (Sergeant, 2003). Young animals less than six months of age are thought to be the most susceptible to infection and older animals are more resistant to infection (Hailat *et al*., 2012). Although calves may be exposed to the MAP at younger ages but the clinical presentation of the disease will likely only appear 2-4 years after the exposure (Mc Spadden *et al*., 2013). It has been estimated that approximately one third of calves will develop infections with a single exposure (Chiodini *et al*., 1984). Cattle are generally assumed to be resistant to infection by about one year of age and infection of adult cattle may require much higher infective doses and result in longer incubation periods than is the case with neonatal infection (Whitlock and Buergelt, 1996).

A meta-analysis of infection studies concluded that there was a significant difference in age susceptibility to infection between adults and calves less than 6 months of age and between adults and calves 6-12 months of age. Evidence from experimental infection studies suggest that the proportion of successfully infected calves is expected to be 75% in calves less than 6 months, 50% in calves between 6-12 months and 20% in cattle greater than 12 months of age (Windsor and Whittington, 2010).

2.4.3. Prevalence and geographical distribution of Johne's disease

Paratuberculosis is a worldwide disease affecting both developing as well as developed countries in Europe, North America, South America, Asia, Australia and Africa (Behr and Collins, 2010). However, Sweden and some states in Australia are the only regions of the world that can claim freedom from Johne's disease based upon a reliable disease reporting system and extensive surveys using laboratory tests (Salem *et al*., 2005). Despite the worldwide distribution of Johne's disease the actual prevalence in most countries is unknown. This is primarily due to the low priority given to Johne's disease in some countries as compared to concerns given to other diseases and the difficulty in accurately detecting infected animals (Bakker *et al.*, 2000). The reported prevalence of infected animals by country is at least partially a reflection of the diligence with which veterinarians and animal owners look for the disease (Djonne *et al*., 2005).

Studies from various parts of the world have shown the distribution of MAP from its first report in Europe to dairy herds all over the world demonstrates the rapid spreading of MAP over geographical space and highlights the need for paratuberculosis eradication and control programs (Figure 1) (Munster *et al.,* 2012). As over 50% of dairy cattle herds are infected with MAP it is considered an endemic disease in Europe and North America (Nielsen and Toft, 2009). MAP occurs in most parts of the world with an increasing prevalence and many studies have been done around the globe to determine the prevalence of MAP in cattle as well as in other species (Chiodini *et al*., 1984). A recent study critically reviewed published data representing prevalence of MAP in Europe and estimated the overall prevalence to be 20% (Nielsen and Toft, 2009).

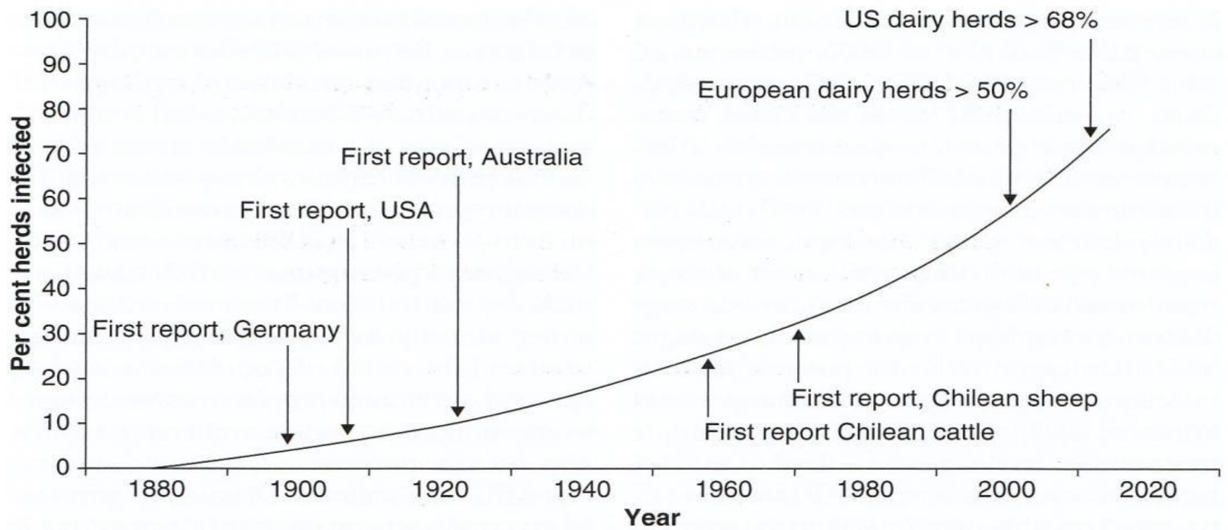

Figure 1: Theoretical global epidemic curve for herd level paratuberculosis in dairy cattle (Behr and Collins, 2010).

The true prevalence among cattle was measured in serological studies conducted in France, Germany, Italy and Turkey. Is has been suggested that the prevalence of MAP is at least 3-5% in several countries (Munster *et al*., 2012). In the USA the infection is spread throughout the country and the herd prevalence is strongly associated with herd size. In 1996 the herd level prevalence of bovine paratuberculosis was reported to be 21.6% and the within herd prevalence to be 40% as forty percent of herds with more than 300 heads were found to be infected (Thirunavukkarasu *et al*., 2013). A less even distribution of paratuberculosis has been reported in Australia where the infection rate in dairy herd ranges from 9 to 22 % (Manning and Collins, 2001).

Paratuberculosis in Africa: In contrast to these industrialized countries in most African countries the occurrence, distribution and prevalence of the disease is unknown (OIE, 2008). Not much research documenting paratuberculosis has been done but the occurrence of the diseases is suspected in most countries. There are very few studies carried out to date and only case reports and limited prevalence studies covering small regions in a few countries are available (Okuni, 2013). JD was reported in cattle from Egypt (Salem *et al*., 2005).

The disease has also been reported in Kenya, Tanzania and Ugandan. In Tanzania the overall prevalence of the disease by clinical examination as well as Ziehl-Nielsen staining of faecal smears was estimated to be 1.9%. The existence of MAP infection in Ugandan cattle was only based on a small abattoir study in which paratuberculosis incriminating lesions was 12/100 tissue samples from a local abattoir and a seroprevalence study estimated the prevalence of antibodies to MAP to be 8.8% (Okuni *et al*., 2011). A recent market survey in some areas of Ethiopia indicated that the prevalence of the disease was not known but Temesgen and Gemechu in 1995, reported a case of paratuberculosis from the country (Okuni, 2013).

2.4.4. Economic impact

JD is considered one of the most widespread infectious disease causing huge economic losses to the ruminant industry worldwide (Salem *et al*., 2013). In the dairy cattle industry for instance economic losses ascribed to JD resulted not only due to the overwhelming expenses of examination and treatment costs but also due to loss of milk production, premature culling, decreased weaning weights in nursing young stock, increased replacement costs and reduced slaughter value and are estimated to be about $200 per infected cow per year in herds with at least 10% prevalence (Lombard, 2011; Over *et al*., 2011; Hailat *et al*., 2012). These production losses have an economic cost of $200-250 million annually to the United States dairy industry (Ott *et al*., 1999).

In the USA it has been estimated that the losses due to paratuberculosis amount to the US dollars 1.5 million annually or approximately 227 US dollars per cow (Manning and Collins, 2001). In addition the economic losses due to bovine Johne's disease (BJD) have been estimated in New England US $15.4 million, in Wisconsin US $52.3 million, in Pennsylvania US $5.4 million and in Australia US $2.1million per year (Verma, 2013). On average the economic losses caused by bovine paratuberculosis have been estimated to be higher than those for other bovine diseases such as bovine viral diarrhea, enzootic bovine leucosis and neosporosis (Chi *et al.,* 2002). The disease also involves losses due to potential limitations in domestic and international trade (NRC, 2003).

2.4.5. Transmission

The introduction of MAP into a population occurs mainly when an infected animal contaminates the pasture with feces containing viable bacteria. Normally animals are most susceptible to infection before birth in prenatal infection or soon after birth in postnatal infection (Salem *et al*., 2013). The postnatal transmission is the most common route through which the fecal oral route especially at early life stage is the main way to contract paratuberculosis in dairy cattle at the individual level (Sweeney, 1996; Clarke, 1997). Neonatal calves are more susceptibility to MAP infection than other groups of age (Windsor and Whittington, 2010). Neonatal calves acquire MAP by direct ingestion of MAP contaminated feces from the manure contaminated teat and udder of the calf´s dam or indirectly via MAP fecal contaminated milk, water, pasture and feedstuff or utensils (Manning and Collins, 2010; Fecteau and Whitlock, 2010). Milk can also be contaminated with MAP due to heterogeneous spread of the bacterium into the milk of infected cows with a higher prevalence of milk infection occurring in heavy fecal shedding cows compared to cows shedding low levels of MAP (Lavers, 2013).

MAP has been identified in colostrum from subclinically infected cows and colostrums has been establish as a risk factor of MAP infection for calves and the practice of feeding pooled colostrum or waste milk from cows has been considered to help the spread of infection to many calves (Fecteau and Whitlock, 2010). Contaminated environments may also be sources from which the bacterium might be ingested (Lavers, 2013). The MAP bacterium percolates slowly through soil and remains on grass and in the upper soil levels representing a risk of ingestion for grazing cattle. The organism can survive for 1 year in the water and sediment of shaded water troughs (Whittington *et al*., 2005). The bacterium has also been detected in biofilms including those present in cattle watering troughs (Lavers, 2013). In addition viable MAP has been detected in settled dust in dairy barns and it has been suggested that bio aerosols may pose a MAP infection risk. Furthermore, a calf infection study indicated that inhalation of aerosolized MAP can result in infection (Eisenberg *et al*., 2011).

Another documented route of infection is the prenatal infection or vertical transmission which descends from mothers to offspring through uterine and placental barriers (Salem *et al*., 2013). From a meta-analysis 9% of fetuses from subclinically infected dams and 39% of clinically affected dams were infected *in utero* with MAP (Whittington and Windsor, 2009). Epidemiologically it has been determined that calves born from seropositive dams to paratuberculosis had 6.6 times more likely to be seropositive compared with calves born from seronegative dams (Aly and Thurmond, 2005). MAP has also been identified in the reproductive tract of infected animals including from the uterine washings from infected cows and in semen of infected bulls. The risk of infection from these sources is suspected to be relatively low and therefore embryo transfer and artificial insemination are not regarded as a significant risk (Sweeney, 1996).

2.4.6. Pathogenesis and host immune response to MAP

Ingested MAP bacteria enter the intestinal wall of ruminants through the small intestine mucosa primarily in the region of the ileum, via M cells which is a specialized epithelial cell that lacks the brush border microvilli, digestive enzymes and surface mucus commonly associated with enterocytes residing in the Peyers Patches (Momotani *et al*., 1988). These are part of the gut associated lymphoid tissue (GALT) which normally allows the development of healthy gut flora while defending against enteric pathogens (Stabel, 2010). MAP takes advantage of these cells to enter the gut mucosal barrier and this depends mainly upon the expression of bacterial major membrane proteins and on the binding of fibronectin or both (Bannantine *et al*., 2003). MAP expresses fibronectin attachment proteins (FAPs) and fibronectin bound to these receptors can in turn bind to integrins on M cells and mediate the uptake of MAP (Woo and Czuprynski, 2008).

Intact and degraded MAP is transported in vacuoles across the M cells to macrophages or dendritic cells in subepithelial and intraepithelial areas of the Peyers Patches and in the adjacent lamina propria and is soon phagocytosed by macrophage which is its natural host cell (Sigurethardottir *et al.*, 2004). The intestinal macrophage is the target cells for MAP infection. Macrophages are known to have several receptors that are involved in the

uptake of mycobacteria and receptors like the complement receptors of CR1, CR3 and CR4, immunoglobulin receptors FcR, the mannose receptor and scavenger receptors aids in the opsonization and phagocytosis of the bacteria (Woo and Czuprynski, 2008). After their uptake MAP organisms are subject to the killing and degradation activities of the macrophage (Stabel, 2010).

However, as an intracellular organism MAP has the ability to survive in the macrophage (Fernandez-Silva *et al.,* 2012). A mechanism intensely studied in mycobacteria macrophage interactions is the ability of these organisms to prevent phagolysosomal fusion and acidification of the phagosomal compartments in which they reside (Dupont, 2002). Evasion of and resistance to macrophage killing mechanism are prerequisite for the persistence and multiplication of mycobacteria and the development of disease (Clarke, 1997). An effect of the prevention of phagolysosomal fusion appears to be the sequestration of phagosomes from the MHC class II compartment resulting in the prevention of peptides binding to MHC class II molecules for subsequent presentation to CD4+ T-cells (Dupont, 2002). Inhibition of the expression of MHC class molecules may correspond with the absence of T-cell activation in the very early stages of infection and may serve to attenuate the immune response allowing the organism to proliferate in infected cells (Juste *et al*., 1994).

In this situation the immune response is thought to remain unstimulated until contact with antigen is initiated by routes other than through the gut. This may occur through recirculation of infected macrophages or subcutaneous vaccination (Juste *et al*., 1994). Using these mechanisms mycobacteria are thus able to persist within the macrophages at the site of entry and are also transported to the draining lymph nodes. T-cells are eventually activated and induce inflammatory responses which serve to contain the pathogen at infected sites (Dupont, 2002). As host cell mediated immunity emerges intracellular killing of the pathogen occurs by activated macrophages. Activated macrophages are the main effecter cells involved in killing of intracellular MAP and the proinflammatory cytokine interferon gamma (IFN-γ) plays an important role for activation of macrophages (Stabel, 2000).

Once the MAP infected macrophage is activated the phagosome containing MAP is fused with lysosomes containing bactericidal agents and MAP is degraded into smaller fragments or peptides. Major histocompatibility complex (MHC) molecules are surface proteins with a peptide binding groove that can bind a variety of different antigens. The MHC molecules bind the foreign peptide in an intracellular location and transport it to the cell surface where it in combination with a ligand can be recognized by T cells (Mikkelsen *et al*., 2011). The activated macrophage produce interleukin-1 (IL-1) a cellular messenger that along MAP infected macrophages present antigens associated with major histocompatibility complex class I and II on the cell surface to CD8+ and CD4+ T cells, activates T lymphocytes and other cytokines such as Tumor Necrosis Factor (TNF)-α and Interleukin (IL)-2. Subsequently activated T cells produce IL-2 which results in clonal expansion of specific CD8+ cytolytic T cells and CD4+ T helper cell populations (Stabel, 2010).

CD4+ T helper (Th) cell populations can differentiate into either Th1 or Th2 subpopulations based on the nature of the antigen presented (Stabel, 2000). The Th1 lymphocyte population produces IL-2, TNF-β and Interferon (INF)-γ cytokines which direct cell mediated immune function. In contrast the Th2 subpopulation of lymphocytes is responsible for induction of humoral immune function via cytokines IL-4, IL-5, IL-6 and IL-10 (Stabel, 2000; Stabel, 2010). The differentiation of CD4+T-cell population is skewed towards a Th1 T-cell subpopulation in the early stages of MAP infection characterized by the secretion of the Th-1-associated cytokines including INF-γ (Stabel, 2010). IFN-γ also induces the secretion of IL-12 by antigen presenting cells (APCs) which results in Th1 induction through a paracrine pathway and it also works to directly complement the Th1 polarization through an autocrine mechanism that does not involve IL-12 (Mikkelsen *et al*., 2011).

It appears that CD4+ T cell (Th1) is the primary source of IFN-γ in the early stages of paratuberculosis infection however CD8+ T cell and γδ T cell can also produce IFN-γ (Fernandez-Silva *et al.,* 2012). Regardless of T cell phenotype CD4+, CD8+ or CD4- and CD8- γδ T cells and IFN-γ appears to be the most critical cytokine for controlling

mycobacterial infections (Stabel, 2010). Production of IFN-g has been recognized as a key step in resistance against mycobacterial diseases in general and it may provide a means to help monitor early infection in some animals. In some cows the cellular immune response has been shown to be able to control the infection with the cow's never developing clinical signs but remaining subclinically infected for life (Tiwari *et al.,* 2006).

In those animals in which the cellular immunity is unable to control the disease a detectable humoral immune response will develop along with increased shedding of bacteria (Tiwari *et al.,* 2006). Typically the organism proliferates slowly in the ileal mucosa and regional lymph nodes. The concentration of MAP in the intestinal mucosa and lumen increases. In an attempt to contain the infection and to recruit inflammatory cells cytokines are released that forms granulomatous lesions in the intestine which is the hallmark characteristic of MAP infection. If the host is unable to contain the infection through granuloma formation then MAP continues to proliferate and the host continues to recruit mononuclear phagocytes from peripheral circulation to the inflammatory foci as a result the granulomatous lesion expands (Chiodini, 1996).

Late infection: Although the granulomatous inflammation contains the MAP infection for a period of time eventually the infection overtakes the immune response (Sweeney, 2011). This appears to happen at the same time as the cell mediated immunity wanes intracellular mycobacteria proliferate and the humoral immune response takes over and at this point infection progresses more rapidly and disease associated with multibacillary lesions develops (Sweeney, 2011; Stabel, 2000). As the immune response starts to lose control of the infection MAP is shed in increasing quantities in the feces and the MAP organism spreads to other tissues in the body (Lavers, 2013). However, antibody effecter mechanisms are of little use against the remaining intracellular mycobacteria and thus clinical cases with multibacillary lesions tend to have high serum antibody concentrations (Clarke, 1997).

The progression of paratuberculosis from a subclinical to clinical state is associated with a switch from Th1 to Th2 immune response. The mechanism responsible for the loss or reduction of type 1-like response is unknown, but it has been related to undefined host genetic factors, to the constant exposure of immune cells to antigen released from infected macrophages or to the development of antigen specific or general regulatory cell populations (Coussens, 2004). The production of Th2 regulatory cytokines, IL-4, IL-5 and IL-10 supports a humoral immune response characterized by the expansion of B lymphocytes, immunoglobulin secretion, an influx of inflammatory cells in the site of infection, an upregulation of IL-10 and control of Th1-mediated responses in which IL-4 and IL-10 play specific roles in the suppression of IFN-γ production by CD4+ Th1 cells (Stabel *et al*., 2007; Stabel, 2010).

Humoral responses in paratuberculosis seem to show an inverse relationship with cell-mediated responses in cattle and sheep (Chiodini *et al.,* 1984). Serum antibody concentrations usually rise later than CMI in the course of an infection and can be detected by the complement fixation test (CFT), agar gel immunodiffusion (AGID) test or enzyme-linked immunoabsorbent assay (ELISA) (Clarke, 1997). Immunoglobulin (Ig) G, IgM and weak IgA responses are found in the serum of chronically infected cattle. In the same way immunehistological labeling of bovine intestine also showed an increase IgG+ and IgM+ cells but not IgA+ cells. Local antibody provokes immediate hypersensitivity reactions and histamine release and is a major cause of the enteritic lesions (Clarke, 1997; Fernandez-Silva *et al.,* 2012).

2.4.7. Pathology

Gross pathological changes may vary depending on the stage and species involved (Manning and Collins, 2001). Common findings at post mortem in advanced cases include pathology associated with cachexia, there is wasting with gelatinous atrophy of fat depots and serous effusion into body cavities (Salem *et al*., 2013). In cattle the main lesions of Johne's disease are usually confined to the intestine and associated lymph nodes. Thickening of the intestinal wall up to three or four times normal thickness with

corrugation of the mucosa is characteristic. In the intestine lesions were most prominent in the distal ileum and usually stopped abruptly at the ileocaecal valve. In only a few cases were large intestinal lesions noted and no lesions were found in the duodenum (Clarke, 1997; Tiwari *et al.,* 2006). The ileum is thickened and doughy when handled and usually the only visible change in the lining is a slight fleshy soft thickening or a faint granularity of the surface with slight congestion (Salem *et al*., 2013).

The ileocecal junction with reddening of the lips of the valve in the early stages to oedema with gross thickening and corrugation later was noted. No ulceration or discontinuity of the mucosal surface occurs (Ayele *et al*., 2001). The lymph nodes are enlarged three to five folds, edematous and soft (Chiodini *et al*., 1984). The afferent lymphatic vessels in the intestinal peritoneum and mesentery may be thickened, convoluted and may contain numerous small 1-4 mm caseous or calcified whitish nodules. Similar nodules or white flecks may be seen on the peritoneal surface of the ileum, the cut surface of the intestinal wall and the mesenteric lymph nodes which are almost enlarged and oedematous (Salem *et al*., 2013). Although all clinical cases present the same picture of a febrile, diarrheagenic and chronic wasting the severity of the signs is unrelated to the extent of the pathology (Corpa *et al*., 2000; Salem *et al*., 2013).

Histopatological lesions of Johne‟s disease are characterized by granulomatous enteritis with aggregations of large macrophages with abundant granular cytoplasm often referred to as epithelioid cells in the intestinal mucosa and submucosa, lymphatics and in the cortex of mesenteric lymph nodes. In the intestines these aggregations of macrophages are accompanied by focal or diffuse infiltration composed of lymphocytes, eosinophils and occasional neutrophils. Multinucleate giant cells are seen in the intestinal mucosa and cortex of the mesenteric lymph nodes of cattle, deer and small ruminants (Gwozdz, 2010). Acid fast bacilli (AFB) are found in the monocyte derived cells in varying numbers (Dennis *et al*., 2011). A wide spectrum of histopathological lesions have been reported based on the extent, distribution, severity and characteristics of the cellular infiltrates and the number of Acid fast bacilli in different species (Okuni *et al*., 2013).

The histopathological lesions of JD have been classified either as multibacillary or paucibacillary based on the number of bacilli in the granulomas or as mild, moderate and marked depending on the severity (Dennis *et al*., 2011). It has also been classified as diffuse, multifocal or focal based on the extent of granulomas and lastly as tuberculoid or lepramatous based on the proportion of macrophages and lymphocytes and the number of acid fast bacilli infiltrating the lesions (Balseiro *et al*., 2008). These lesion spectra provide a variety of fingerprints by which the disease may be recognized (Okuni *et al*., 2013). A single classification that is able to include all the different types of the lesions is difficult to achieve because the diversity of the lesions has been attributed by many factors such as the different strains of the organism or different stages of the disease and the immunological or genetic status of the host (Gollnick *et al*., 2007; Okuni *et al*., 2013).

Histopathological changes based on the severity of lesions and the degrees of MAP infiltration of tissues differentiate MAP infection into two categories, a paucibacillary or tuberculoid and a multibacillary or lepromatous form (Clarke and Little, 1996). The paucibacillary or tuberculoid form is characterized by lymphocytic reaction with few or no acid fast bacteria. Conversely, the multibacillary or lepromatous form is characterized by large numbers of macrophages and epitheloid cells together with large numbers of acid fast bacteria (Clarke, 1997). This indicates separate immunopathological mechanisms may be involved in the development of inflammatory lesions. Differences in the host immune response is associated with the different forms of MAP with a predominant cell mediated immune response are characteristically present in animals with a paucibacillary form of infection. Conversely, the multibacillary form is associated with a stronger humoral response and weaker cellular immunity (Clarke, 1997).

2.4.8. Clinical picture and stages of Johne's disease

2.4.8.1. Clinical picture

In cattle the main symptoms of clinical paratuberculosis are diarrhea and wasting. Signs of illness are rarely demonstrated before 2 years of age and most cases are seen in 2 to 4

year old animals. Animals exposed later in life are less likely to develop the disease in which some animals become resistant, never develop lesions or shed the organism and have no signs of the disease. Later on with high or repeated infective doses rapid replication of the organism occurs in these animals leading to the development of lesions and shedding of the organism (Fecteau *et al.,* 2010). Poor nutrition, stress related to transport, lactation, parturition and immunosuppression by agents like bovine viral diarrhea virus (BVD) have been proposed as accelerating or precipitating in the onset of the clinical phase of infection (Corn *et al*., 2010).

Animal illness occurs in a one at a time fashion in the herd which often does not unduly alarm the producer. So it may come as no surprise that many cattle producers even some with infected herds have no or little awareness of JD. The initial phase can be elusive and may be limited to weight loss, decreased milk production and roughening of the hair coat. Typically the organism proliferates slowly in the ileal mucosa and regional lymph nodes (Salem *et al*., 2013). Developed lesions in the intestinal wall gradually result in malabsorption syndrome that means nutrient absorption is retarded in which animals begin to demonstrate intermittent diarrhea. The physiological mechanism for the development of diarrhea was thought to be related to antigen antibody reactions in the intestine with subsequent release of histamine (Tiwari *et al*., 2006).

Occasionally several weeks later after the onset of diarrhea a soft swelling in the submandibular jaw area known as bottle jaw or intermandibular edema may develop due to protein loss from the bloodstream into the digestive tract (Manning and Collins, 2001). Later edema may disappear and thirst increases as a result of fluid loss from diarrhea. Generally animals have no fever and continue to demonstrate normal appetite while feces is watery, homogeneous and without offensive odor, blood, epithelial debris or mucus. JD is progressive in which affected animals become increasingly emaciated and usually die as the result of dehydration and severe cachexia (Woodbury *et al*., 2008; Corn *et al*., 2010).

2.4.8.2. Disease stages

The cardinal clinical sign of paratuberculosis in cattle is the chronic progressive weight loss with chronic or intermittent diarrhea (Fernandez-Silva *et al.,* 2012). The progression of JD is often very slow and affected animals do not show clinical symptoms until the final stages of the disease. Because of this a diagnosis of the disease is very difficult and is often overlooked. In many cases animals may be infected and spread the infection to other animals in the herd years before a diagnosis is made (Bradner, *et al.,* 2013**).** The progression of MAP infection from asymptomatic infection to clinical disease can be subdivided into four stages (Whitlock and Buergelt, 1996). Whitlock and Buergelt, (1996) proposed the following four categories of the disease differentiated according to the severity of clinical signs, shedding of bacteria into the environment and the possibility of infection to be detected using laboratory methods (Fecteau and Whitlock, 2010).

Stage 1 or silent infection: In this stage animals typically exhibit no overt evidence of infection with MAP and typically found in calves, heifers, most immature young stock that are below two years of age and in animals that are adult having exposure to MAP with limited doses which present no clinical signs but are possibility of shedding infectious organisms which is undetectable with any diagnostic test (Singh *et al.,* 2014). During this silent stage of MAP infection macroscopic alterations of affected tissue are rare and usually seen in animals which have been experimentally infected (Clarke, 1997). However, microgranulomas in the intestine and in the lymph nodes may be found via histology (Wu *et al*., 2007). In this phase the infection can spread to adjacent lymph nodes or eventually into disseminated infection (Fecteau and Whitlock, 2010).

Infected cattle seem identical to non infected herd mates regarding growth, weight gain and outward appearance (Clarke, 1997). Infected animals such as calves and young livestock may shed the organism at levels below the threshold of detection and therefore, there is no effective diagnostic test that can detect infection in this stage (Salem *et al*., 2013). The only way to detect infected cattle at this early stage is only by post mortem evaluation of the agent by culture of multiple intestinal tissues or histopathology analysis

(Sweeney *et al*., 2006). Other diagnostic tests such as johnin skin testing and gamma interferon tests that utilize the cell mediated response (CMI) have also been used to detect this stage of the disease. However, there are common antigens between MAP and other environmental *Mycobacterium* species resulting in low specificity (Sp) for these tests making them ineffective as routine screening tests (Tiwari *et al*., 2006).

Stage 2 or the subclinical disease: It includes subclinical carrier adults where there is no evidence of the disease. Bacterial shedding is intermittent and animals may be classified as positive or negative by tests like fecal culture (Salem *et al*., 2013). MAP persists and multiplies in subepithelial macrophages leading to a chronic transmural inflammatory reaction (Manning and Collins, 2001). Some of these infected cattle may be detected by fecal culture and subsequently removed from the herd. However, focal lesions, variable rates of disease progression and shedding and dilution of organisms in large volumes of intestinal content result in intermittent detection of fecal shedding (Tiwari *et al*., 2006). Therefore, other infected animals test negative by using current fecal culture techniques yet they may be shedding low numbers of organisms in the manure which contaminate the environment and pose a threat to other animals on the farm (Fecteau and Whitlock, 2010).

Some animals may have detectable antibodies to MAP, an altered cellular immune response like increase IFN-γ or both particularly if they are getting close to entering the next stage of the disease which is the clinical phase and may be prone to other diseases such as mastitis and infertility (Fernandez-Silva *et al.,* 2012). However, MAP fecal shedding usually occurs before a detectable antibody response (Whitlock and Buergelt, 1996). An unknown proportion of stage 2 animals progress slowly to clinical disease but because so most cattle are culled from the herd for reasons unrelated to partuberculosis such as infertility, mastitis, lameness or reduced milk production and before clinical signs typical of paratuberculosis are never recognized by the owner or herd veterinarian and the magnitude of the MAP infection within a herd can be obscured (Fecteau and Whitlock, 2010).

Stage 3 or clinical disease: Initial clinical signs follow a prolonged incubation period of 2 to 10 years depending on the exposure level and the capacity of an animal to fight the infection (Tiwari *et al*., 2006). Clinical disease may be precipitated by parturition, lactation or other stresses (Fernandez-Silva *et al.,* 2012). The first apparent sign is gradual weight loss despite a normal or occasionally an increased appetite. During a period of 3 to 6 months concurrent with the weight loss the manure consistency becomes more fluid. The diarrhea may be persistent or intermittent at first with periods of normal manure consistency. Thirst is usually increased and milk production is decreased. At the same time appetite and vital signs of heart rate, respiratory rate and temperature remain normal (Tiwari *et al.,* 2006; Salem *et al*., 2013).

Clinical symptoms are seen in older cows starting at an age of three years and more. Prominent is also the thickening of the wall of the intestine and corrugation of the intestinal epithelium (Munster *et al.,* 2012). Animals shed the bacilli in large amount in billions and have high antibody titers so most animals at this stage have a positive fecal culture and have increased serum antibody levels detectable by the commercial enzyme linked immunosorbant assay (ELISA) and agar gel immunodiffusion (AGID) test. It is estimated that only 10% to 15% of infected animals survive to this stage of infection because they are often culled due to reduced productivity earlier in the subclinical stage (Tiwari *et al.,* 2006)**.** These animals have a higher frequency of transmitting MAP *in utero* and have a higher frequency of MAP isolated from milk (Fecteau and Whitlock, 2010).

The fourth stage or advanced stage of clinical paratuberculosis: Comprises animals that rapidly progress from the stage III with rapid condition deteriorated characterized by severe weight loss despite of normal or increased appetite and profuse diarrhea. The developing chronic enteritis is responsible for emaciation, reduced milk production, reduced fertility and premature culling of dairy cattle (Chiodini *et al*., 1984). Due to the intestinal damage animals develop hypoproteinemia which can lead to edema especially in the submandibular region known as bottle jaw (Munster *et al.,* 2012).

At this stage most animals are culled from the herd in an earlier stage before this time due to the chronic or intermittent diarrhea, decreased milk production and/or weight loss (Tiwari *et al*., 2006). Most animals are sent to slaughter at this point and may not pass inspection for human consumption. Otherwise, death occurs as result of dehydration and cachexia (Fecteau and Whitlock, 2010). In this stage culture of the agent, molecular biology techniques of PCR, ELISA and histopathology all are positive for the majority of animals tested. The gastrointestinal tract is the preferential location to sample in order to isolate the agent but dissemination of MAP throughout the tissues can occur (Pinedo *et al.,* 2008). Although the organism can sometimes be cultured from sites distant from the gastrointestinal tract extra intestinal lesions are rarely detected. When extra intestinal lesions are present the liver, other parts of the intestinal tract and the lymph nodes are the most common sites (Fecteau and Whitlock, 2010).

2.4.9. MAP and Crohn's disease (CD) a zoonotic potential

The hypothesis postulating that there is a causal link between the organism MAP and the human disease *Crohn's disease* (CD) and has been first suggested in 1913 (Munster *et al*., 2012). Since then despite almost 100 years of investigation debate still occurs and the responsibility of MAP as the etiological agent of CD is not widely accepted. There is a controversy regarding the potential zoonotic role of MAP in human CD and its importance as a public health issue (Mendoza *et al*., 2009). Public health issues have been raised about the transmission of MAP from animal products and the potential for subsequent infection and perhaps disease. However, a number of researchers have asserted that the organism is the primary cause of human CD citing clinical similarities between JD in ruminants and CD in humans and Crohn's disease is thought by some to be linked to MAP (Mann and Saeed, 2012).

CD is a rare chronic inflammatory disease appearing in the last part of the small intestine, but also manifesting itself in other parts of the gastrointestinal tract (Munster *et al*., 2012). Although the cause of Crohn's disease in man remains unknown it is likely to be due to a combination of genetic predisposition, an abnormal immune response and

environmental factors. A long standing controversy about whether MAP plays a role in Crohn's disease in humans is still not resolved and the demonstration of a link would have enormous implications on human health (Vansnick *et al*., 2004). MAP may play a role as a public health issue and should be introduced as a potential zoonotic agent to national surveillance programs (Munster *et al*., 2012).

Potential vehicles of transmission of MAP from cattle to humans are different food products with animal origin. MAP was isolated from dairy products such as milk, cheese and soft curd cheese (Smith *et al*., 2011). It is known that MAP can survive pasteurization of milk and it has been found in water supplies indicating that humans are at least exposed to the MAP bacteria (Grant *et al.,* 2002). Due to contamination with MAP by dissemination into the tissue or fecal contamination of the carcass meat may be also a possible route of exposure of MAP to humans (Munster *et al*., 2012). In any case reviews and meta-analysis from independent scientists, scientific associations and from governmental organizations have concluded that with the current scientific evidence the role of MAP as the causal agent of CD cannot be refuted nor denied. Nonetheless, this relation of causal or coincidental should not be ignored taking into account the detection of MAP in food, water and environment that could represent permanent sources of exposure of MAP to humans (Mendoza *et al*., 2009).

3. DIAGNOSTIC METHODS

The first sign of JD is only conspicuous to the animal owner which is either the unreasoned loss of body conditions and productivity or the development of sudden diarrhea which does not respond to any kind of antidiarrheal therapy. Subsequently confirmation of these clinical complaints has to be done through the accurate and proper diagnosis of the disease. Various laboratory diagnostic techniques have been evolved for the diagnosis of JD (Salem *et al*., 2013). The evaluation of diagnostic tests performance is usually done by comparing the ability of the test to detect infected and healthy animals based on a gold standard or a benchmark test that could identify animals as truly infected or none infected. Generally diagnostic tests for JD have a high specificity while the sensitivity varies due to the biology of this slowly progressing chronic disease usually less than 50 % (Salem *et al*., 2013). Diagnostic tests for paratuberculosis (JD) are divided into two categories, those that detect the organism and those that assess the host response to infection (Pinedo *et al.,* 2008).

The first category includes acid-fast stain on fecal smear, bacteriologic culture from fecal or tissue specimens and polymerase chain reaction test (PCR). The second category, detection of host response includes clinical signs in combination with gross and microscopic pathology and detection of immune response to infection which comprise cellular immune response and humoral or antibody response to MAP (NRC, 2003). Several other diagnostic methods have been suggested and used for the detection of MAP. However, many of them lack in specificity and sensitivity due to the slow progress of infection and the different stages of immune response. Considering the complex pathobiology of MAP it is impossible for any method to perform well during all stages of the disease (Munster *et al*., 2012). This is of special importance during the earlier stages of infection where a single effective diagnostic tool has not yet been identified (Stabel and Bannantine, 2005). These limitations demand to adequately define the purpose of diagnosis in order to apply the most appropriate diagnostic procedure (Fernandez-Silva *et al.,* 2012).

3.1. Direct detection of the causative agent

The best way of diagnosing JD is to detect the bacterium that causes the infection. Direct detection of MAP is usually performed by microscopic examination of stained smears, by isolating the entire organism with culture methods and by detecting the organism's genetic material (Salem *et al*., 2013).

3.1.1. Microscopic examination

The microscopic examination of fecal smears is a rapid and economical way of obtaining a diagnosis which is used to screen feces for acid fast staining microorganisms. The most common staining technique used to identify acid fast bacteria is the Ziehl-Neelsen stain (ZN) in which the bacteria are stained bright red and stand out clearly against a blue background (Palomino, 2007). The technique is based on the fact that due to the mycolic acids in their cell wall mycobacteria retain the primary stain or carbol fuchsin after exposure to a decolorizing acidic alcohol. A counter stain or methylene blue is usually employed to color the background flora and to highlight the red stained mycobacteria (Vansnick *et al*., 2004). Ziehl-Neelsen staining technique is usually performed as a primary test giving a preliminary diagnosis to veterinarians especially after the development of the primary complaints reported by the farmers (Salem *et al*., 2013).

Ziehl-Neelsen staining is also used to detect the bacteria in tissue samples through an impression smear made from ileum, mesenteric lymph nodes or other specimens (Pinedo *et al.,* 2008). A presumptive diagnosis of paratuberculosis in feces or tissue can be made if clumps of three or more organisms of small 0.5-1.5 μm strongly acid-fast bacilli are found (Manning, 2001). This method has the advantage of being simple, fast and inexpensive but has the disadvantage of having low sensitivity and specificity. In cases of severe diarrhea MAP concentration decreases relative to the amount of feces thus increasing the likelihood of false negative results. A similar condition occurs in animals with subclinical paratuberculosis where there is low rate of fecal excretion (Singh *et al*., 2014).

3.1.2. Bacteriological culture

Culturing of MAP from feces, milk and tissues is the most reliable method of detecting infected animals and has been the basis of partuberculosis diagnosis for a century and remains one of the most widely used diagnostic test (Collins, 1996). There are several culture methods which vary with respect to media, sample type and sample processing protocols. The cultivation of MAP is always performed using special media supplemented with mycobactin J. Since MAP organisms are vastly outnumbered by other bacteria or fungi in faecal and intestinal tissue specimens the successful isolation of the target organism depends on efficient inactivation of the undesirable microbes (Gwozdz, 2010). However, decontamination of the specimen to selectively kill faster growing non mycobacterial organisms and concentration of organisms before inoculation of the medium is required (NRC, 2003). It is also recommended to supplement the media with an antibiotic mixture like vancomycin, amphotericin and nalidixic acid to create additional selectivity against non mycobacterial overgrowth (Munster *et al*., 2012). There are two techniques for the isolation of MAP, the conventional culture on solid media and liquid media culture (Gwozdz, 2010).

3.1.2.1. Conventional culture of MAP on solid media

Conventional culture of MAP on a more or less selective growth medium is time consuming of 12 to 32 weeks and requiring several months of incubation especially for samples with low MAP load. Most laboratories use Herrold's egg yolk medium (HEYM). Other commonly used media are modified Lowenstein Jensen medium (LJ) and Middle brook (MB) 7H10 agar (Vansnick *et al.,* 2004). Both media are supplemented with egg yolk and mycobactin. Modified Middlebrook 7H10 medium supports the growth of both ovine and bovine strains whereas Herrold's egg yolk medium (HEYM) primarily supports the growth of the bovine strains (Gwozdz, 2010). Lowenstein-Jensen (LJ) medium with mycobactin is also suitable for the isolation of ovine strains and in some cases performs better than MB 7H10 medium. Other media such as Dubos medium and Watson Reid medium are rarely used (de Juan *et al.,* 2006).

The decontamination procedure is an essential step in all used culture protocols. At present there are two basic decontamination protocols in use accompanying the conventional culture method on egg based solid media. One method uses the oxalic acid and sodium hydroxide (NaOH) decontamination protocol followed by Lowenstein Jenssen medium (LJ) supplied with mycobactin J (MJ) and another method uses hexadecylpyridinium chloride (HPC) decontamination protocol in combination with Herrold's egg yolk medium (HEYM) supplied also with MJ (HEYM-MJ) (Salem *et al*., 2013). Sodium pyruvate was reported to stimulate the growth of MAP and is included in both the LJ and the HEYM (Munster *et al*., 2012). The addition of sodium pyruvate to HEY may inhibit the growth of some isolates but in most cases substantially increases the recovery rate and number and size of colonies (de Juan *et al.,* 2006). Primary colonies of MAP on solid media may be expected to appear any time from 5 weeks to 6 months after inoculation. The sheep strains grow less well than the cattle strains and primary cultures on solid media should not be discarded as negative without prolonged incubation for up to 6-8 months (Gwozdz, 2010).

3.1.2.2. Radiometric culture of MAP on liquid media

Another more rapid and sensitive technique for the isolation of MAP is the use of a radiometric based broth media where the bacterial growth is detected radiometrically (Salem *et al*., 2013). The most commonly used radiometric system is the BACTEC system of Becton Dickinson. In this system a liquid culture medium (BACTEC 12B) containing an isotopically labeled nutrient source of 14CO_2 labeled palmitic acid is used. The BACTEC apparatus detects the 14C labeled CO_2 that is produced by the metabolization of the labeled palmitic acid. For radiometric MAP culture egg yolk suspension and mycobactin J need to be added to the BACTEC 12B vials (Vansnick *et al.,* 2004). This system is used in only a few laboratories because the equipment is expensive and requires the use and disposal of radioactive waste (Grant *et al.*, 2003). The main advantage of this method is that it can detect positive samples sooner than conventional culture of 12-16 weeks. In addition it can recover isolates from a wide variety of animal species including the slow growing sheep isolates (Salem *et al*., 2013).

3.1.3. Molecular approaches for paratuberculosis diagnosis

3.1.3.1. Conventional PCR

The invention of the polymerase chain reaction (PCR) earned Kary Mullis shared Nobel Prize in Chemistry in 1993, as the technique soon became immensely important in the field of molecular biology (Mullis and Faloona, 1987). The method of PCR relies on the enzymatically driven and temperature controlled exponential amplification of a specific target sequence in the template DNA extracted from the sample. Two short target specific DNA fragments or oligonucleotides (primers) are key components to enable selective and repeated amplification. Elongation occurs by acting Taq polymerase originally isolated from the thermophilic bacterium *Thermus aquaticus* (Munster *et al*., 2012). The introduction of PCR technique as a powerful tool in the microbiological diagnosis of different pathogens has led to its extensive application in the diagnosis of JD. PCR methods which have the theoretical potential to detect one single gene copy have made the rapid identification of MAP in clinical samples possible by reducing detection time (Motiwala *et al*., 2004; Rajeev *et al*., 2005).

The most important break-through in the diagnostic of paratuberculosis was the identification of a repetitive insertion sequence IS900 which is specific for MAP (Green *et al*., 1989). In 1989, a novel DNA insertion sequence (IS*900*) in MAP was reported (Eglund, 2002). IS*900* is present in multiple copies (15-20) in MAP genome and consists of 1,451 base pairs (bp) of which 66% is G + C showing a degree of target sequence specificity (Green *et al*., 1989). The multicopy nature of the sequence makes it an ideal target sequence for the detection of MAP. Due to a booster effect caused by a multiple number of target sequences a higher level of sensitivity is achieved than what can be achieved with single copy genes as targets (Munster *et al*., 2012). The characterization of the *IS900* insertion sequence in the MAP genome has enabled the specific identification of the bacterial DNA by the polymerase chain reaction (PCR) (Singh *et al*., 2014).

The discovery of the MAP specific genetic element IS900 and the use of PCR based techniques have greatly improved the diagnosis of Johne's disease (Harris and Barletta, 2001). Several polymerase chain reaction (PCR) assays targeting the IS900 region have been developed for the detection of MAP DNA (Djonne *et al.,* 2003). So far the IS900 is commonly used as an abundant reference marker for the molecular detection of MAP. Consequently, different IS900-based PCR assays such as conventional, seminested and nested PCR have been described for the detection of MAP in feces, milk, tissues, semen and human intestinal tissues without primary culture or in cases when MAP has not been possible to cultivate or isolate (Herthnek *et al*., 2006; Mobius *et al*., 2008).

All of the PCR probes used commercially today are based upon identifying IS900 with in the MAP genome and although specificity of IS*900* based PCR is considered nearly 100%. However, mycobacteria other than MAP have been found to carry IS*900*-like elements with nucleotide sequences that are up to 94% identical to the nucleotide sequence of MAP IS*900* (Englund *et al*., 2002). Some PCR systems that target IS*900* also can give false positive results with DNA from mycobacteria other than MAP and with from other types of organisms (Mobius *et al*., 2008). In response to the uncertainty about the specificity of PCR systems that target IS*900* for identification of MAP several PCR assays using primers specific molecular target sequences other than IS*900* for use in MAP identification systems have been developed such as ISMap02, IS*Mav2*, hspX, locus 255 and F57(Rajeev *et al*., 2005).

3.1.3.2. Real time PCR

PCR is a fast and specific method and are generally used as a qualitative method to evaluate biological samples. In the last few years IS*900* based PCR was developed for diagnosis of paratuberculosis and has become popular alternative to culture. For identity confirmation of cultured colonies the sensitivity of conventional PCR is sufficient but for direct PCR on clinical samples however, higher sensitivity is usually required. Furthermore specificity is also questionable (Manning and Collins, 2001). Therefore specificity as well as sensitivity of the conventional PCR assay must be enhanced by

using modifications of the PCR and additional methods such as real–time or quantitative PCR (rtPCR) (Herthnek, 2009).

A wide variety of quantitative rtPCRs are applied to determine pathogen load, measure the response to therapeutic agents and characterize gene expression. In comparison to conventional PCR, rtPCR possesses several advantages such as ability for quantification, high sample throughput, high speed, less handling of PCR products and subsequently decreased risk of false positive results caused by amplicon carry over (Munster *et al*., 2012). In real time PCR the instrument monitors the reaction progress throughout every temperature cycle by measuring the light emitted from a fluorophore incorporated with the DNA product. One was based on the detection of SYBR green bound to PCR products and the second more specific method detected the cleavage of a fluorogenic or TaqMan probe bound to a target sequence during primer extension phase (NACMCF, 2010). SYBR green I is a dye that binds to all double stranded DNA (dsDNA) molecules regardless of sequence when SYBR green I dye intercalates into dsDNA its fluorescence increases significantly (Bolske and Herthnek, 2010).

During the different stages of PCR the intensity of this fluorescence will vary depending on the amount of dsDNA present in the sample (Rasmussen, 2001). In TaqMan, a specific probe sequence with attached fluorescent reporter dye and quencher dye hybridizes to the target sequence near one of the primers. When the probe is intact the quencher dye is close enough to the reporter dye to suppress the reporter fluorescent signal. During PCR the 5 prime nuclease activity of the polymerase cleaves the hydrolysis probe separating the reporter and quencher (Fernandez-Silva *et al.,* 2012). Novel primers and probes that amplify small fragments which is less than 80 bp of the MAP specific insertion sequence IS*900* were designed. Both the SYBR green and TaqMan assays are able to detect 3 to 4 fragments of DNA extracted from MAP strain ATCC19698 of 0.6 to 0.8 cells per assay. Both SYBR Green and TaqMan assays were highly specific for the detection of MAP (Pinedo *et al*., 2008).

3.2. Detection of host response to MAP infection

3.2.1. Clinical signs and gross pathology

Clinical signs appear in the stage III of paratuberculosis and include, gradual weight loss in spite of a normal appetite. With the progress of disease manure consistency becomes more fluid and diarrhea may be intermittent. Serum and biochemical changes include low concentration of total protein, albumin, triglycerides and cholesterol (Pinedo *et al*., 2008) Muscle enzymes levels increase as a result of muscle wasting. However these changes are not specific enough to be useful as diagnostic tests. During stage IV animals become increasingly lethargic, weak and emaciated. Most animals are culled before this stage because of reduced milk production or severe weight loss. Intermandibular edema, cachexia and persistent diarrhea characterize the terminal stage (Whitlock and Buergelt, 1996).

Gross lesions are confined to the terminal portion of the small intestine and associated lymph nodes. Thickening, corrugation and hyperaemia of the intestinal mucosa, Lymph nodes are enlarged and edematous and subserosal lymphatics appear tortuous, dilated and thickened (Pinedo *et al*., 2008). Although gross lesions can lead to a correct diagnosis of the disease in advanced stages in some cases even animals with advanced disease may lack such lesions. Moreover, in early stages of the disease the lesions may be so subtle as to escape recognition. Many cases of the disease are missed until the infection reaches significant prevalence (Okuni *et al*., 2013).

3.2.2. Histological diagnosis

3.2.2.1. Histopathological diagnosis

Clinical signs and gross pathology will provide an indication of the presence of JD in advanced cases. However, a definitive diagnosis requires histopathological examination. Histopathological detection of Johnes disease is largely confined to animals submitted for

necropsy (Whitlock and Buergelt, 1996). Tissue samples can be obtained from distal portions of the ileum, ileocecal valve, mesenteric lymph nodes and biopsy or scraping of the rectal mucosa are the appropriate tissues for examination (Singh *et al*., 2014). Tissue samples collected during post mortem examination are preserved in 10% buffered neutral formalin, embedded in paraffin, sectioned at 5µm and stained with haematoxylin and eosin (H&E) and Ziehl Neelsen (ZN) (Cousins *et al*., 2002).

From a single animal each Ziehl Neelsen stained section of tissue with cellular changes indicative of Johne‟s disease should be examined under oil immersion for a minimum of 5 minutes before reporting no evidence of acid fast organisms (Gwozdz, 2010). A positive diagnosis of lesions consistent with MAP infection is indicated if in any one section of one or more single giant cells and/or one or more accumulations of three epithelioid macrophages are observed in the intestinal lamina propria and/or lymph node cortex with the presence of at least one acid fast bacillus morphologically consistent with MAP (Pinedo *et al*., 2008). A finding suggestive of MAP infection is indicated if in any one section of two single Langhans giant cells and/or two accumulations of three epithelioid macrophages in the intestinal lamina propria and/or lymph node cortex are observed without the detection of an acid-fast bacillus (Gwozdz, 2010). The advantage of the histopathological diagnosis is that it allows to identify animals with focal lesions associated with subclinical stages whose fecal and/or milk excretion is insufficient for bacterial culture or PCR (Singh *et al*., 2014).

3.2.2.2. Immunohistochemistry (IHC)

Immunohistochemical techniques provide an adjunct to standard histological methods for diagnosis of partuberculosis (Gwozdz *et al*., 2000). This technique uses a MAP specific antibody marked with enzymes which allows visualizing the reaction on the enzymatic substrate (Singh *et al*., 2014). The advantage of this method is that it enables to identify spheroplasts and MAP in tissues. It shows a good sensitivity in tissue from subclinically infected animals. However, false positive results due to cross reaction with other mycobacteria may occur (Singh *et al*., 2014; Martinson *et al*., 2008).

3.2.3. Immune based test platforms for MAP diagnostics

The true state of infection can often only be established through culture of multiple tissues. However, bacterial growth is slow with test results being available only after months of incubation and up to 100 tissues may be required to establish the infection status of an animal (Whitlock *et al*., 1996). Further, shedding of MAP at detectable levels in faeces is irregular (Nielsen and Toft, 2008). Therefore immune based diagnostic tests are relevant alternatives to faecal culture and several tests measuring either specific CMI or antibodies have been developed (Mikkelsen *et al*., 2011).

3.2.3.1. CMI based diagnostics

A cell-mediated immune (CMI) response are the first and strongest host response to mycobacterial infections and are useful for early detection of MAP infection (Robbe-Austerman *et al*., 2007). It is now well recognized that immunological resistance and the earliest specific immunological responses to MAP infection depend on cell mediated immune mechanisms. There are two tests available for the detection of CMI response to MAP infection which can be performed in vivo or in vitro using skin testing for delayed type hypersensitivity and measuring interferon gamma (IFN-γ) release after stimulation of peripheral blood mononuclear cells with specific antigens (Kalis *et al.,* 2003).

3.2.3.1.1. Intradermal skin test

A well known CMI test for early diagnostic testing of MAP was limited to the intradermal skin test. This test evluates the delayed type hypersensitivity response 72 hours after intradermal injection of purified protein derivative (PPD) and is an indication of the cell mediated response of the animal (Singh *et al*., 2014). PPDs are crude undefined extracts of mycobacterium antigens of different origin such as MAP PPDj or Johnin, *M. avium* subsp. *avium* (MAA) or PPDa or *M. bovis* or PPDb (Mikkelsen *et al.*, 2011). It is prepared by growing MAP in liquid culture, autoclaving it and centrifuging the sample to precipitate the solid particles. The liquid supernatant is called PPDj

(Bannantine *et al.*, 2010). In cattle this is injected under the caudal skin fold and a few days later the injection site is examined for the presence of inflammation indicating type I cell mediated hypersensitivity. The skin thickness is measured with a caliper before and 72 hours after inoculation and an increase in skin thickness greater than 2–3 mm is considered positive (Mikkelsen *et al.*, 2011; Singh *et al*., 2014).

Development a positive reaction is an indicative of prior exposure to antigen or MAP infection. Since this test detects a cell mediated immune response it is useful for the detection of the early stages of MAP infection (Kalis *et al*., 2003). The advantage of this test is that it is quick, cheap and easy. However, In spite of successful application of the skin test in the control of bovine tuberculosis it is only occasionally used in the control of paratuberculosis because its specificity has been reported to be low. However, problems with antigenic cross reactivity of environmental mycobacteria have recently precluded its use as a diagnostic tool for paratuberculosis (Collins, 2003). Another drawback of the skin test is that each individual animal needs to be restrained twice within a period of 72 hours first for the application of the test and later for the reading of the results. As a consequence the skin test has to some extent been replaced by an *in vitro* CMI assay measuring secreted IFN-γ in response to an antigen (Mikkelsen *et al.*, 2011).

3.2.3.1.2. Interferon Gamma (IFN–γ) assay

The introduction of the in vitro IFN-γ assay has replaced the skin test using the CMI response in the diagnosis of MAP infection (Antognoli *et al*., 2007). It is a laboratory test originally developed for the diagnosis of bovine tuberculosis but also available for the diagnosis of paratuberculosis (Kalis *et al*., 2003). This test evaluates the specific production of cytokine IFN–γ by T lymphocytes after stimulation with PPD. The IFN-γ assay requires only one intervention for blood collection. Whole blood samples are cultured with MAP antigens in a proliferation assay and released IFN-γ is measured in the supernatant by ELISA. Similar to the skin test the IFN-γ MAP assay is based on PPD as antigen for stimulation and will therefore inherently have the same specificity problems (Mikkelsen *et al.*, 2011).

Furthermore, large scale use of the IFN-γ assay for routine diagnostics is limited by the short timeframe of less than 24 hours allowed from blood sampling at the farm to stimulation with antigens in the laboratory. Finally, fluctuating IFN-γ responses to PPDs in calves younger than 15 months puts another limitation of the usefulness of the IFN- γ assay (Mikkelsen *et al.*, 2011). In animals with the subclinical stage the sensitivity of this test is higher than that of the serological tests (Singh *et al*., 2014).

3.2.3.2. Serological based diagnostics

An immunological response to MAP infection can be detected by measuring host antibody production and given that antibody response occurs late in the course of infection so the pathbiology of paratuberculosis limits the ability of tests for serum antibodies to detect animals in the early stages of infection (Pinedo *et al*., 2008). Several diagnostic assays have been developed to detect antibodies in either serum or milk. These are the complement fixation (CF) test, the agar gel immunodiffusion (AGID) test and ELISA which can be very sensitive when antibody production is at its highest as in the late stages of disease but because the sensitivity of antibody detection tests increases with the progression of disease actual clinical signs may be evident before a positive test result is achieved (Collins, 1996) .

3.2.3.2.1. Agar gel immunodiffusion test (AGID)

The AGID test has been used successfully in control programes of JD in cattle, sheep and goats and was developed as a quick confirmatory test for animals showing clinical manifestations of JD (Tiwari *et al*., 2006). AGID test is based on the precipitation of immune complexes formed by the antibodies of infected animals with a soluble antigen from a protoplasmic extract of MAP in a gel matrix of agar. It is a simple, fast and relatively inexpensive method but has low sensitivity in the early stage of the disease but becomes more useful in later stages (Singh *et al*., 2014). Although the test's sensitivity is lower than that of the ELISA its relative low cost makes it a very attractive test especially in small ruminants (Woodbury *et al*., 2008).

3.2.3.2.2. Complement fixation test (CF)

The complement fixation test was one of the first serological tests developed for the diagnosis of Johne's disease and has been the standard test used for cattle for many years (Munster *et al*., 2012). This assay detects complement fixing antibodies to MAP. First complement in serum samples is inactivated by heating. The serum is subsequently mixed with a known amount of MAP antigen and complement and the antibodies in the serum are allowed to fix the complement. In MAP negative samples the complement remains free in the serum and can be detected with a second antibody antigen system (Vansnick *et al.*, 2004). The CF test works well on clinically suspect animals but lacks in both sensitivity and specificity. Therefore, the test is not applied for control purposes (Singh *et al*., 2014). Nevertheless, the CFT test is still the only test demanded for the import or export of cattle with in Europe (Bottcher and Gangl, 2004).

3.2.3.2.3. Enzyme linked immunosorbent assay (ELISA)

ELISAs are serological tests that indirectly detect MAP infection by assaying humoral antibody responses in the host. The first ELISA detecting antibodies against MAP was developed 1978 (Munster *et al.,* 2012). The ELISA has a significantly higher sensitivity and specificity than that obtained by CF test or AGID. ELISA tests are commonly used on both serum and milk samples however, these assays are often limited by the issues of specificity and the fact that a variable immunological response is seen during different stages of infection and low sensitivities particularly in the subclinical stage of infection (Nielsen and Toft, 2008). Despite the clearly noticeable low figures of sensitivity and specificity ELISA is considered as the method of choice for the detection of JD positive herds. This is due to the suitability of samples collection, rapid laboratory turnaround time, low cost and possibility to test a large number of samples in a short time (Collins, 2011).Several ELISA kits for bovine paratuberculosis based on serum antibody detection have become available and some companies have adopted this technology for milk samples (Salem *et al.,* 2013).

Most ELISA tests in current use are modifications of the method developed by Yokomizo *et al*. (1983) who developed an ELISA for MAP detection in cattle sera. The aim was to minimize the nonspecific reactions caused by IgM by measuring only IgG1 against a bacterial protoplasmic antigen. Three years later the same group reported that pre absorption treatment of sera with *Mycobacterium phlei* increased the specificity of the ELISA test by removal of cross-reacting antibodies (Pinedo *et al*., 2008). At present ELISA test kits or services are commercially available from a number of sources and sensitivity and specificity of ELISA for MAP detection have been described in numerous reports (NRC, 2003; Vansnick *et al.*, 2004). Multiple antibody ELISAs have been evaluated, and Se and Sp estimates vary greatly within and between tests (Nielsen and Toft, 2008).

Reports on the sensitivity of the ELISA are contradictory as they depend on the standard used for fecal culture, tissue culture, etc to calculate the relative sensitivity (Eda *et al*., 2006). The ELISA has a sensitivity rising from almost zero at the early stages of the disease up to 75 % when the animal reaches the clinical stage. Sensitivity of ELISA is the highest for animals with lepromatous lesions, those with clinical symptoms or those that shed large number of bacteria (Fernandez-Silva *et al.,* 2012). The use of ELISA in longstanding infected herds reveals reasonable sensitivity as the antibodies become more abundant in later stages of the disease. On the contrary, the test yields a very low sensitivity in young recently infected animals and therefore, it is not trusted to detect the disease in a farm where the spread of infection has just begun (Salem *et al.,* 2013). Regardless of these disadvantages ELISA testing of sera is still the method of choice for epidemiological studies and herd-based diagnosis (Nielsen and Toft, 2008).

4. CONTROL OF PARATUBERCULOSIS

Control of paratuberculosis is based on the fact that if no control measures are taken the prevalence of Johne´s disease will further increase and seriously affecting the quality of domestic livestock (Fernandez-Silva *et al.,* 2012). In addition the economic impact of paratuberculosis on production and reproduction, the suspected but unconfirmed causal relationship between MAP and Crohn´s disease and the detection of relative small quantities of MAP in food and water are sufficient reasons to support control programs to improve animal health and welfare and to reduce the exposure of the food chain to MAP under a precautionary principle (Bakker, 2010). Due to the economic and potential zoontic risk posed by MAP control programs to stop the spread of this disease have been initiated by several countries. However, effective control of JD remains practically unachievable. Factors like faecal shedding of the organisms by subclinically infected animals, lack of reliable diagnostic tools with high specificity and sensitivity and absence of suitable vaccines are some issues that hamper the effective control of JD (Thirunavukkarasu *et al*., 2013).

Currently most of the practices to control JD rely on management interventions designed for limiting the introduction as well as transmission of the pathogen. The factors at the herd level include strength of herd and their replacement, common source of water and manger, common housing, manure contamination of feed and water as well as calves, washing of udder before milking and type of housing (Ott *et al*., 1999; Pence *et al*., 2003). However, control programs for paratuberculosis have been based on different diagnostic and intervention approaches depending on the objectives pursued at regional or national scale (Bakker, 2010). Although control measures like the test and cull strategy are practiced especially for cattle in countries where bovine tuberculosis is a problem and drug therapy has been researched using *in vitro* studies but vaccination is still the primary mode of controlling MAP infection (Harris and Barletta, 2001; Thirunavukkarasu *et al*., 2013).

4.1. Vaccination

Although cost effective and considered as the most effective way in controlling paratuberculosis and further it has been suggested that vaccination may initiate the ending of the huge problem of paratuberculosis throughout the world perhaps marking the difference between doing nothing and making progress towards control globally (Singh *et al.,* 2014). The first reported vaccination against MAP was reported by Vallee and Rinjard as early as 1926. Since then a number of several vaccine types such as kill whole cell based, live attenuated whole cell based and improved whole cell based have been developed for both ovine and bovine JD and have demonstrated to be effective on cellular and humoral immune response, highly profitable and effective to reduce MAP shedding (Rosseels and Huygen, 2008). However, vaccination to control bovine paratuberculosis has been relatively little used in the frame of control programs compared to sheep and goats mainly due to reasons regarding interference with skin testing for bovine tuberculosis, failure to prevent infection and the presence of large lesions at the inoculation site (de Lisle, 2010).

The current trend is towards developing subunit vaccines to aid in the control of JD (Shin *et al.,* 2005). DNA vaccines can offer an alternative approach that may be safer and elicit more protective responses. A genomic DNA expression library was generated and subdivided into pools of clones to determine DNA vaccine efficacy by immunizing mice via gene gun delivery and challenging them with live virulent MAP (Pinedo *et al.,* 2008). DNA vaccines are also being researched mainly because the characterization of protective mycobacterial antigens has been greatly facilitated by the analysis of immune responses induced after DNA vaccination (Thirunavukkarasu *et al*., 2013). Also the use of lipid conjugated antigens and lipid based adjuvants are also gaining importance. Experimental immunizations carried out with the purified mycobacterial glycolipid, glucose monomycolate (GMM) obtained from mycobacterium tuberculosis in cattle found that GMM was potent in generating a specific T cell response without inducing B cell responses and hence could be a likely candidate for a model antigen for vaccination purposes (Nguyen *et al.* 2009).

5. MATERIALS AND METHODS

5.1. Study area

The study was conducted from October 2013 to June 2014 at ELFORA export abattoir Bishoftu, Ethiopia. ELFORA export abattoir is found at 47 kilometers Southeast of Addis Ababa in East Shoa Zone of Oromia Regional State. Currently the abattoir is one of the most facilitated modern export abattoirs in Ethiopia and is exporting meat of small ruminants though cattle are slaughtered for local market. During the study on average 200-250 cattle were slaughtered per weeks based on local market needs.

5.2. Study animals

Animals used for the study were those cattle which were ready to be slaughtered at Bishoftu ELFORA export abattoir. The majority of animals in this study were local breed of apparently healthy cattle raised in an extensive farming system. The study animals were only males of different body condition and age groups. The ages of the animals ranged from 4 years to 12 years. Ages of the animals were determined by based on dental eruption; accordingly, animals were categorized as into four groups of $\leq$ 4, 4-6 years, 6-8 years and >8 years (Dreier *et al*., 2006).

5.3. Study design

A cross-sectional abattoir-based study was conducted in ELFORA export abattoir Bishoftu. Tissue samples that had characteristics gross pathological lesion of paratuberculosis were collected and the samples were investigated using histopathology, bacteriological tissue culture and Acid-fast staining (AFS) methods.

5.4. Sample size determination

The sample size required for this study was determined using the formula given by Thrusfield, (2007). Since there was no previous study conducted on paratuberclosis in cattle in the present study area or in Ethiopia 50% expected prevalence was considered with 95% confidence interval and 5% required precision and the number of animals sampled was determined using the formula

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

Where n= required sample size, d= desired absolute precision, P_{exp}= expected prevalence (50%).

Thus, the calculated sample size was 384. However, in order to increase the precision of the study a total of 400 animals were examined.

5.5. Sampling methods

A systematic random sampling procedure was used to pick every fifth animal slaughtered during the visit. The slaughterhouses were visited 4 days per week to collect the samples. Before slaughter information about the animals regarding age and body condition of the cattle were recorded.

5.6. Study methodology

5.6.1. Gross examination and tissue sampling

Immediately after the animal was stunned and hanged the animals were identified with numbered ear tag that allowed each animal to be followed through the slaughter chain of the abattoir to track the carcass to the evisceration area. After slaughter at the point of

removal of the gastrointestinal tract of each study animal on the slaughter chain an identification tag was attached to the caecum and the number of the tag was matched and recorded against the animal's ear tag. Samples from 400 animals were thoroughly examined for any paratuberculosis suspected gross pathological lesions. Complete gross examination was performed with emphasis of the digestive system of small and large intestines and regional lymph nodes by selecting the caecum and tracing it to the ileocaecal valve and then arranging the small intestine so that the distal small intestine, associated mesenteric lymph nodes, ileocaecal valve, ileocaecal lymph nodes and caecum were visible.

Significant gross lesions including information about mucosal corrugation, thickening and hyperemia of the intestine were recorded. Furthermore, regional lymph node size, shape and color were carefully described. Any other significant gross pathological findings were also recorded. Tissues with gross pathological changes were sampled and tissues from the last portion of their ilea, ileocecal valves and respective lymph nodes which is the primary areas of tissue involvement in paratuberculosis s disease process were collected from slaughtered cattle. Tissues lesion samples of ilea and associated lymph nodes were trimmed to a smaller size of 4 mm to 1 cm thickness fixed in 10% buffered formalin and subjected to histopathological examination at National Animal Health, Diagnostic and Investigation Center (NAHDIC).

Similarly, fresh tissue lesion samples from ilea and associated lymph nodes were collected and transported in cold-chain to Aklilu Lemma Institute of Pathobiology (ALIPB) TB laboratory for direct smear by acid fast staining method and mycobacterial culture. Samples for culture were kept at minus 20 °C until needed. Corresponding to each sample all the necessary anti-morteum and post morteum information were collected and registered on data collection format (Annex I and II).

5.6.2. Histopathological examination

For histopathological examination, the formalin fixed tissue samples from the distal ileum, ileo-cecal valve (ICV) and assciated lymph nodes were processed by routine methods as described by Bancroft and Stevens (1990). Sections of 4-5 µm were cut using microtome and stained with hematoxylin and eosin (H&E) method (Annex III). The hematoxylin and eosin (H&E) stained sections from 45 ilea and the associated lymph nodes (45) were observed under 4×, 10×, and 40× objectives of a microscope and pathological lesions were recorded. The lesions were classified and graded into grades of I, II, III and IV, according to the type and amount of cellular infiltrates of lymphocytes, macrophages and epithelioid cells.

The intestinal tissue sections were considered positive for paratuberculosis when there were macrophage infiltrations or epithelioid cells obvious in the lamina propia of the villi and between crypts and there was involvement of the Peyer's patches as demonstrated by the presence of starry sky macrophages/ tingible body macrophages or micro-granuloma (Hailat *et al*., 2010). The grading criteria of tissue lesions that were used are shown in Table 1. Therefore, grades I was considered suspected while grades II, III and VI were considered positive.

Table 1: Histopathological criteria and grading of the lesions found in the distal part of the ileum and the ileocecal valve

Finding	Grade
Absent or very few macrophages and lymphocytes without apparent thickening of the intestinal mucosa	Negative
Many lymphocytes with some Macrophages with occasional or no epithelioid cells	Grade I
In addition to the previous criteria presence of many macrophages with an increased number of lymphocytes, few scattered epithelioid cells and moderate Peyer's patches proliferation and crypts replacement	Grade II
In addition to criteria of previous grade, observation of a prominent number of epithelioid cells in nests or scattered and sever Peyer's patches proliferation and crypts replacement	Grade III
Presence of multinucleated giant cells with or without epithelioid cells within typical granulomatous lesion.	Grade VI

Source: Hailat *et al*. (2012)

5.6.3. Cultivation and confirmation of MAP from affected tissues

Tissue samples with characteristic gross pathological lesions of paratuberculosis were subjected to culture on modified Herrold's egg yolk medium (MHEM) and Lowenstein Jensen medium (LJ) with addition of mycobactin J according to OIE protocol (OIE, 2008) (Annex IV). Tissue specimens collected from the slaughtered cattle were processed and prepared for culture at the Aklilu Lemma Institute of Pathbiology (ALIPB) in a biological safety cabinet. Tissue samples of approximately 3-5 grams of scrapped intestinal mucosa and lymph node parenchyma were dissected using sterile blades and manually homogenized using a mortar and pestle. This was followed by decontamination by shaking the homogenate in an equal volume of 4% NaOH for 10-15 minutes at room temperature and neutralized with 1% (0.1N) HCl using phenol red as an indicator.

Neutralization was achieved when the colour of the solution turned from purple to yellow. The suspension was then centrifuged 3,000 × for 15 minutes, the supernatant discarded and sediment was used for culture. The sediment was cultured onto each slant of four tubes of modified Herrold's egg yolk medium (MHEM) of which the two tubes were prepared and supplemented with mycobactin J and sodium pyruvate and the other two tubes were prepared without the addition of mycobactin J but with the addition of sodium pyruvate and used as a control. The sediment was also cultured onto each slant of four tubes of Lowenstein Jensen (LJ) medium with mycobactin J. The two tubes were supplemented with mycobactin J of which one was enriched with glycerol and the other with pyruvate and the other two tubes were used as a control and prepared with out mycobactin J but one tube was enriched with glycerol and the other one with pyruvate.

The tubes were kept inclined with loosened screw to facilitate the evaporation of excess moisture and inoculums fluids for one week. After one week the tubes were placed vertical with tightened screw and incubated aerobically at 37°C for 16 weeks or until macroscopic growth was observed while they were examined on a regular basis for macroscopic growth. Cultures were first examined after 8th weeks of inoculation and subsequently every week up to 16 weeks for the presence of any growth. No evidence of bacterial growth after this incubation period was considered as a negative result. A culture was considered positive when white spot colonies were seen (Hailat *et al*., 2010). Growths of MAP colonies were isolated and confirmed by mycobactin J dependency, Ziehl Neelsen (ZN) staining and its long incubation period (Okuni *et al*., 2013).

5.6.4. Direct smear staining

Scrapings of the intestinal mucosa and lymph node parenchyma were carried out using scalpel blade and smears were done on microscopic slides. Samples were taken especially from the ileo-cecal valve and associated lymph nodes parenchyma. Slides were dried by air, fixed with methanol (100%) and stained by Ziehl Neelsen (ZN) staining method according to Coles (1986). Stained slides were observed using oil immersion of 100 x objectives of a microscope. Each slide was examined for 30 minutes in order to make

very high the chance to detect acid acid-fast bacilli. The findings were registered according to the bacteria appearance in which observation of bacteria's in clumps taken as positive while if no acid-fast bacilli were observed the sample was regarded as negative (Annex V).

5.7. Data analysis

Data from gross examination and laboratory results were entered into MS excel 2010 spread sheets. The occurrence of the disease was determined by using frequency distribution. Differences in distributions of histopathological lesions, tissue culture and acid fast staining results between age groups were evaluated using Fisher's exact tests. Agreement between the three diagnostics used was calculated with a linearly weighted kappa coefficient (Brenner and Kliebsch, 1996). Analyses were performed using STATA 11.0 (Stata Corp LP, College Station, TX, USA). A P-value ≤ 0.05 was considered significant.

6. RESULTS

6.1. Pathology

6.1.1. Gross pathological lesions

In the present study when the intestines and the corresponding lymph nodes of 400 animals were examined grossly in the slaughterhouses a variety of gross lesions were observed in 45 (11.3%, 95% CI=8.1-14.4) animals in the last portion of the small intestine, ileocecal valve and corresponding lymph nodes (Table 2). The intestinal walls were found thickened at various locations predominantly near the ileocaecal junction or valve. The lesions involving the lips of the ileoceacal valve included hyperaemia, swelling and protrusion. In 1.5% of the cases the swelling had a cauliflower like appearance (figure 2). Congestion and thickening of the ileocaecal valve without cauliflower like appearance was also found in 2.5% of the cases.

Other lesions observed in the ileocaecal junction were necrosis associated with haemorrhages, nodules in the mucosa of the ilea with hyperaemia. In 1.5% of the examined animals the distal intestinal segments mainly ileum showed thickening of the mucosa with corrugation in which transverse folds were observed which is a pathogenomonic sign of paratuberculosis disease (figure 3). No lesions were observed in intestine segments other than the ileum. Lesions in the lymph nodes were oedema, hyperaemia and nodular hyperplasia. In many of the cases the ileal and ileocecal mesenteric lymph nodes were congested, enlarged over the normal size and they were connected with each other and appeared as cords. There were no gross lesions in specimens from 355 animals sampled (88.8%).

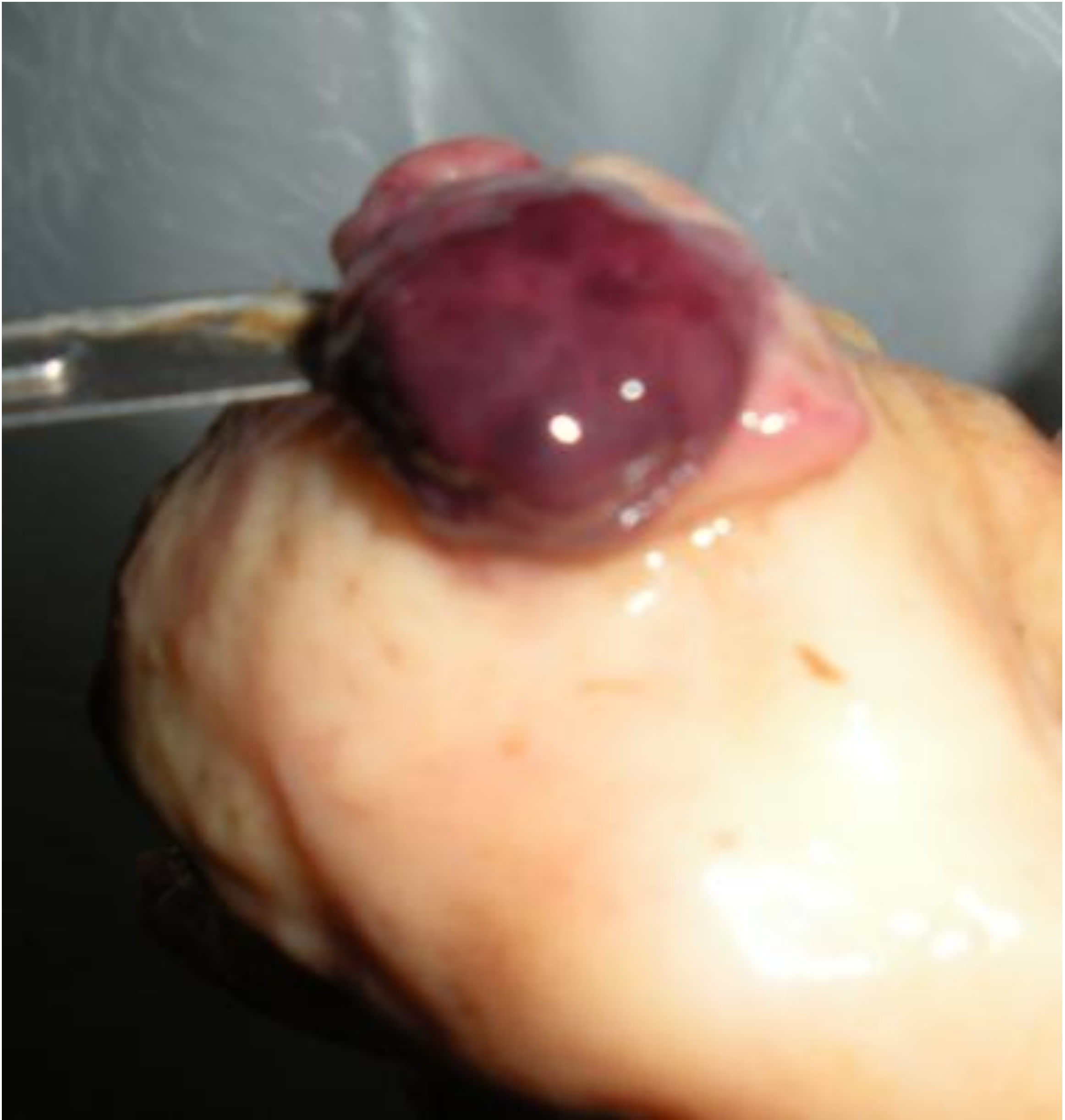

Figure 2: Gross pathology of ileocaecal valve section from apparently healthy cattle. Congestion of ileocaecal valves with cauliflower-like corrugation and hypertrophy

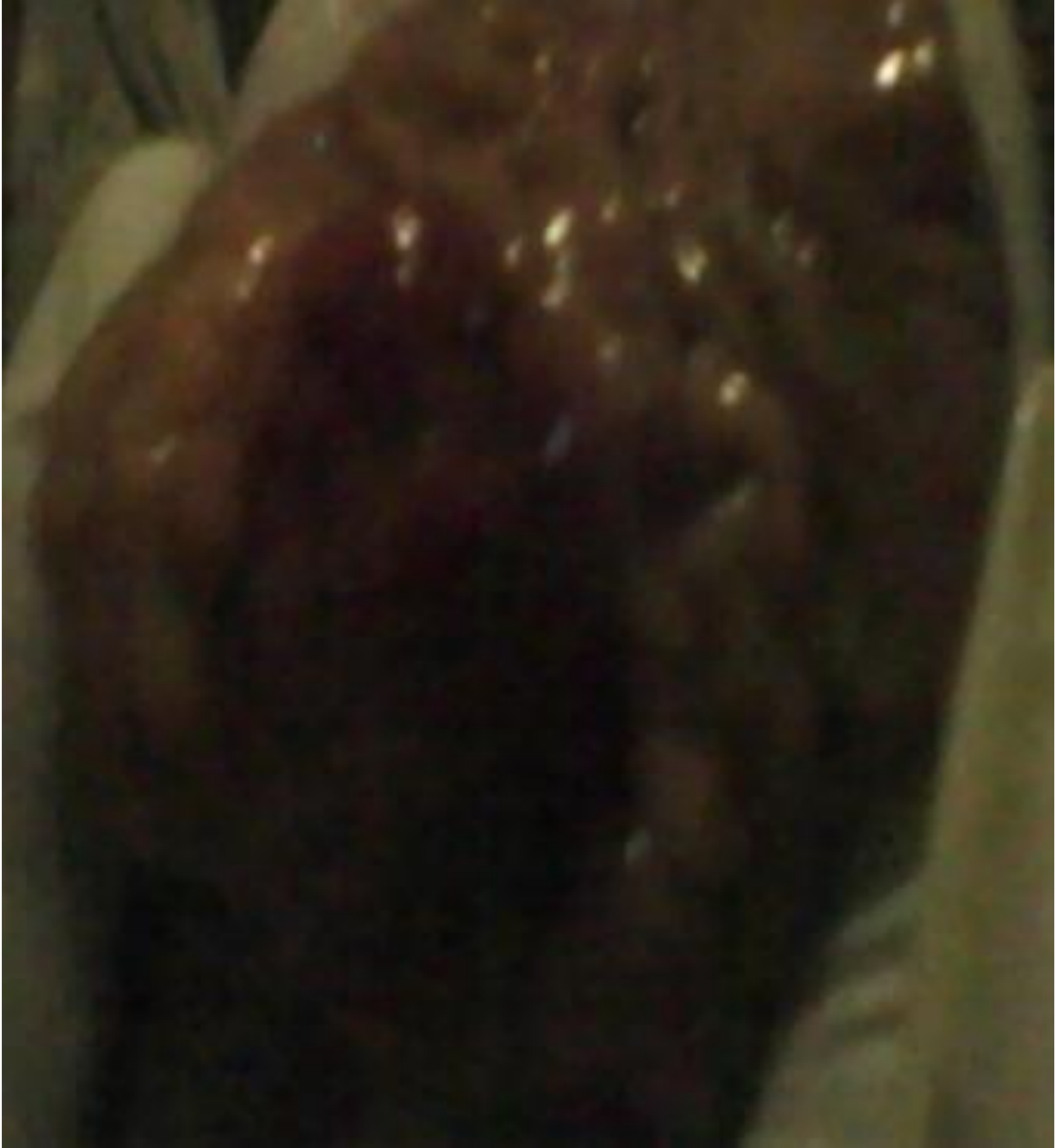

Figure 3: Gross pathology of the distal ileum section from apparently healthy cattle. Corrugation and thickening of mucosa of the ileum with transverse folds

Table 2: Different categories of gross pathological lesions observed during sample collection from the abattoir

Type of lesion	Number	% of the total
Corrugation and thickening of mucosa of the ileum	6	1.5
Congestion and thickening of ileocaecal valves with cauliflower like corrugation and hypertrophy	6	1.5
Congestion and thickening of ileocaecal valve with no cauliflower appearance	11	2.8
Swelling and prolapse of ileocaecal valves into the caecum	30	7.5
Presence of small nodules in the mucosa of ileocaecal valve	9	2.3
Hyperaemia of the lips of the ileoceacal valve	29	7.2
Haemorrhages in the mucosa of the ileum	10	2.5
Hyperemia on the mucosa of the ileum	11	2.8
Necrosis and haemorrhages on the ileocaecal valve	1	0.3
Oedema of lymph nodes	23	5.8
enlargement of lymph nodes	28	7.0
Nodular hyperplasia of lymph node	2	0.5
Congestion of lymph nodes	23	5.8
connection of the lymph nodes with each other	19	4.8
No lesion observed	355	88.75%

6.1.2. Histopathological lesions

Histopathological examination of the hematoxylin and eosin (H&E) tissue sections from the 45 intestinal ileum and associated lymph node samples revealed that 17.8 % (95% CI=6.1-29.4) had histopathological lesions consistent with JD (Table 3). The four age groups were compared for the presence of paratuberculosis positive histopathological lesions as indicated in (table 3). Histopthologicaly positive lesions of 25% and 75% were

found in animal ≤ 4 years and 4-6 years old age groups respectively but a higher proportion of histopathological positive lesions were observed in cattle with the age group of 4-6years of age. There was a significance difference among the age groups of cattle with positive paratuberculosis histopathological lesion (F=11.568, p=0.002)

Table 3: Frequency and percentage of Johne's disease positive cases of cattle in different age groups examined by histopathology

Age group (years)	Sample tested	Sample positive	(%)	Fishers exact	P-value
<4	5	2	25		
4-6	15	6	75	11.568	P=0.02
6-8	17	0	0		
>8	8	0	0		
Total	45	8	100		

The histopathological lesions were variable ranging from mild to sever thickening and congestion of the mucosa due to inflammatory cell infiltrations. The lamina propria and the submucosa of the ileum were infiltrated with mononuclear cells consisting primarily of lymphocytes with a few numbers of macrophages and plasma cells (Figures 4). Variable degrees of eosinophilic infiltrates were present in some of the examined intestinal section. Occasionally a number of dead and degenerated nematodes were observed. Occasional numbers of epithelioid cells were also seen. They were present either as a scattered form in the lamina propria of the villi and between the crypts or as nests or cords with large numbers of plasma cells (Figure 5). Infiltration of the lamina propria with lymphocytes and plasma cells between the crypts were also noted. Hyperplasia and replacement of lamina propia crypts with infiltration by lymphocytes and macrophages were noted in some tissue sections (Figure 6).

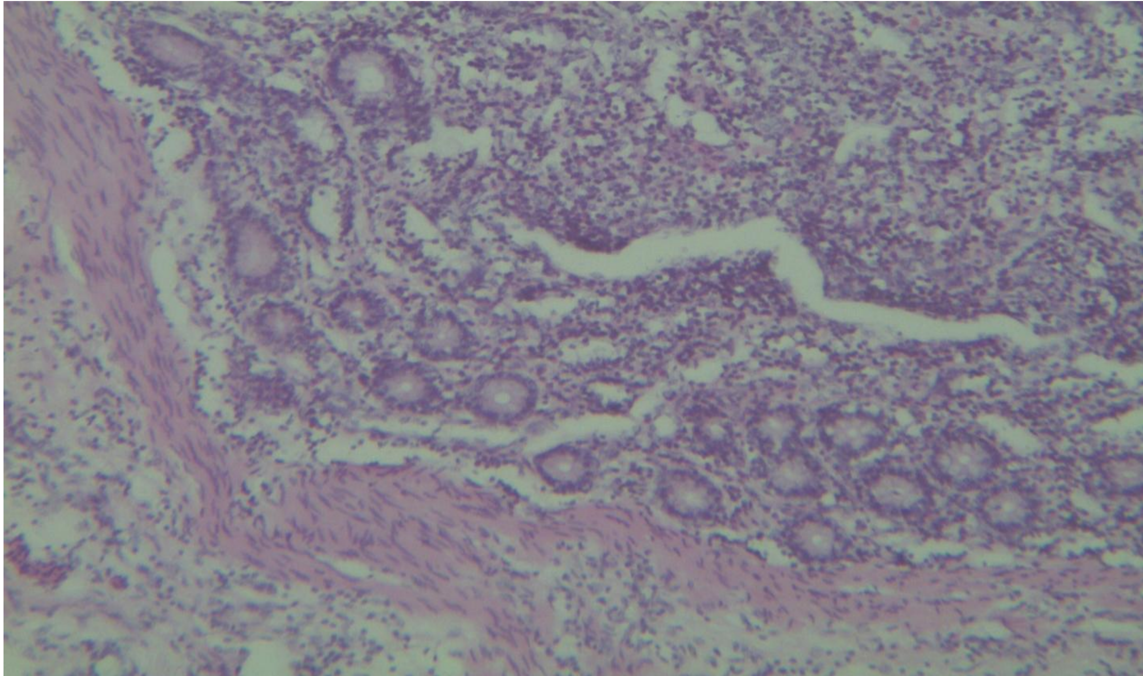

Figure 4: Intestine (Ileocecal valve); mononuclear cells infiltration of the mucosa and lamina propria of cattle (H&E, x10).

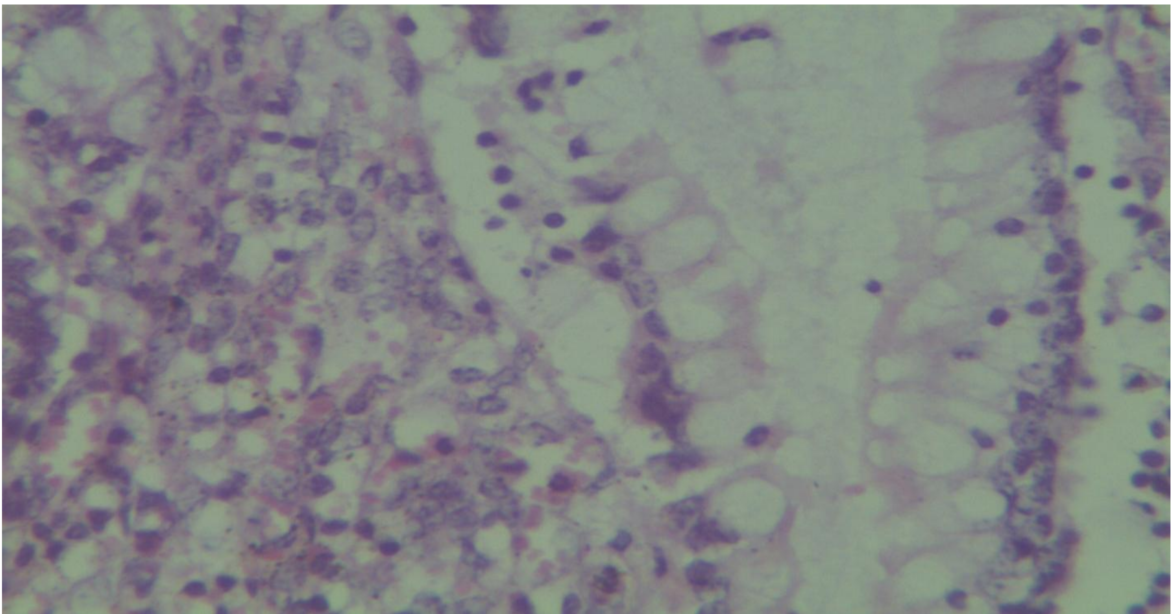

Figure 5: Intestine (Ileocecal valve); scattered epithelioid cells in the lamina propria of the villi and between the crypt as nests or cords with large numbers of plasma cells (H&E, x40).

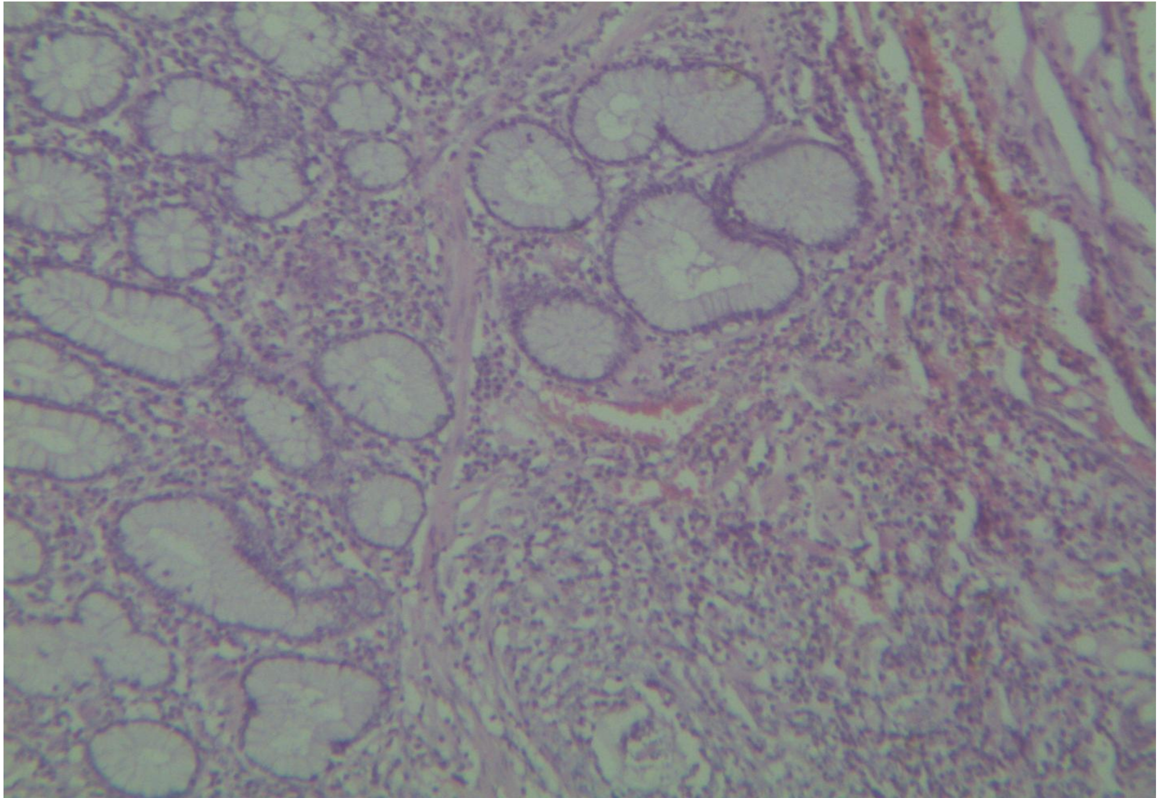

Figure 6: Intestine (disatal ileum); hyperplasia and replacement of lamina propia crypt with lymphocytes, polymorphs and macrophage (H&Ex10).

Multinucleated giant cells were rarely seen in the affected tissues which were scattered through the submucosa of the intestinal sections (Figure 7). In some cases infiltration of the submucosa with neutrophils and lymphocytes associated with areas of necrosis which were changed in to granulomatous reaction were also seen in the intestinal sections. Presence of haemorrhage and congestion in the lamina propia and submucosa of the ileum were also noted in some of the intestinal tissue section.

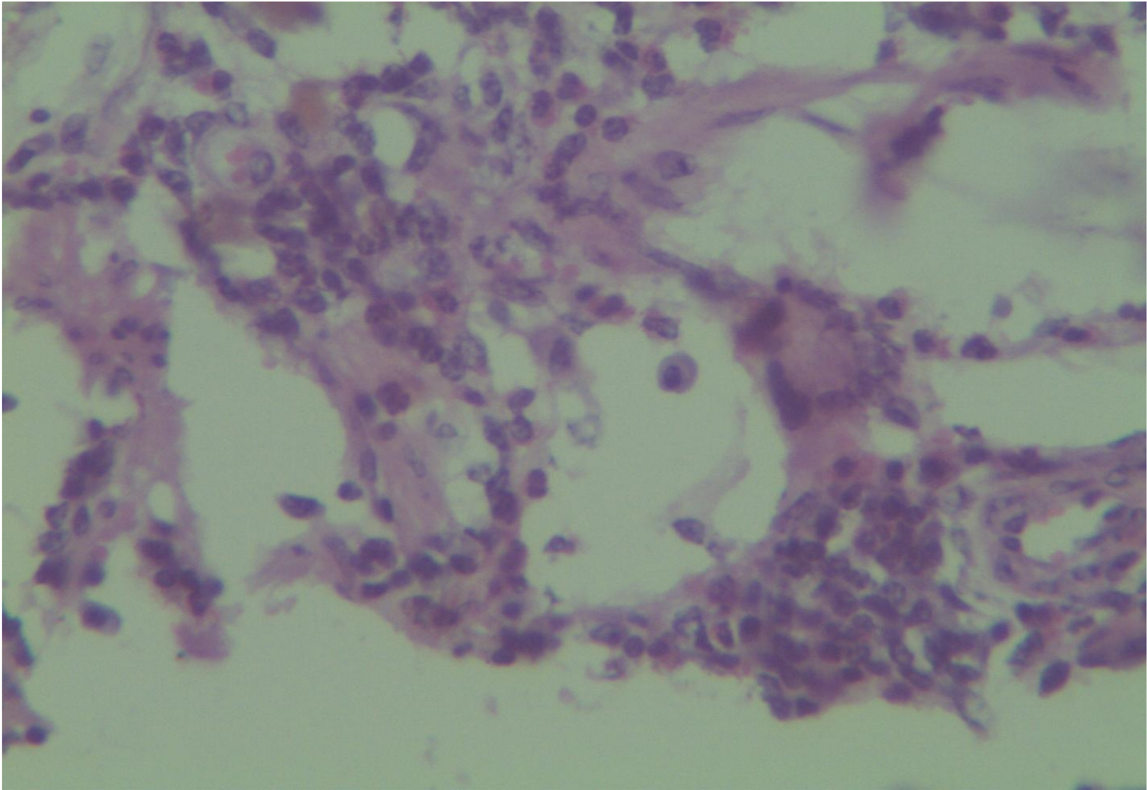

Figure 7: Intestine (Ileocecal valve); multinucleated giant cells in the sub mucosa of the intestinal section of cattle (H&E x 40).

Payer's patches lymphoid hyperplasia and proliferation by inflammatory cells of neutrophils, epithelioid cells, plasma cells and some polymorphs which were extending towards the mucosa was noted in some of the tissue sections (Figure 8). The villi exhibited different changes including villous distortion and thickening by inflammatory cell infiltrations and disruption of the normal architecture of the villi. Other significant villous changes were villi necrosis surounded by mononuclear cells (Figure 9). The lymph node changes were presence of necrotic area in the cortex surrounded by mononuclear cells and infiltration of the subcapsular area with lymphocytes and macrophages. In some affected lymph nodes polymorpoohnuclear cell aggregation and cell debris in the cortex surrounded by epitheloid cells and macrophages were evident (Figure 10). Expansion of the paracortex area by cell infiltration was also noted.

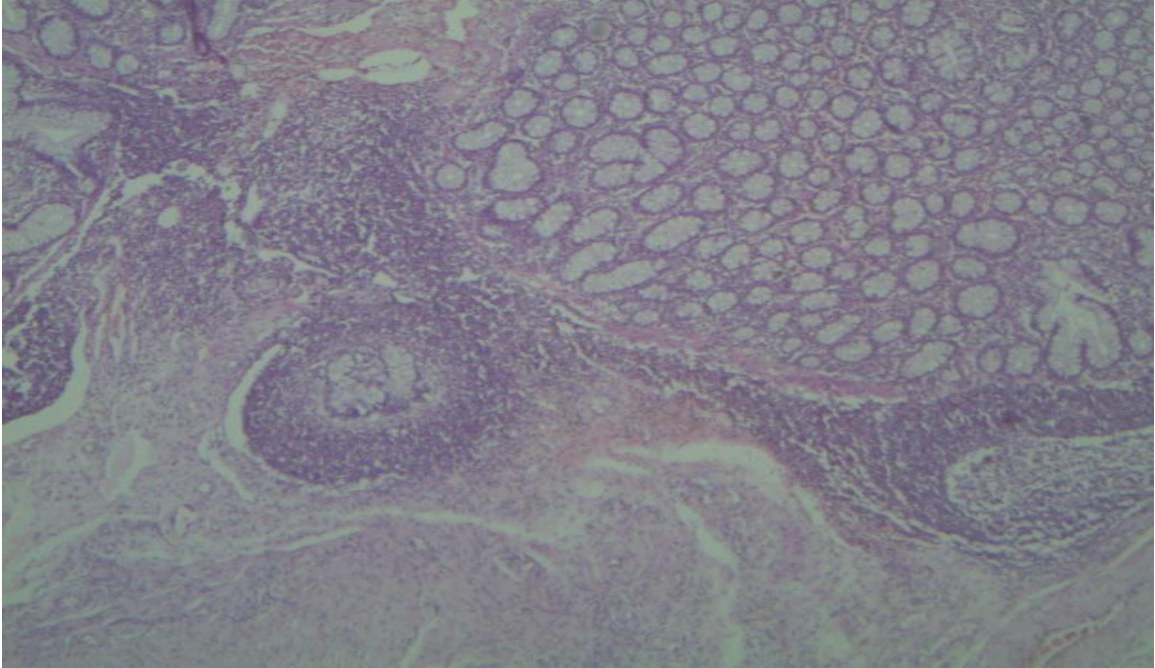

Figure 8: Intestine (Ileocecal valve); Payer's Patches proliferation and extending towards the mucosa (H&E x4).

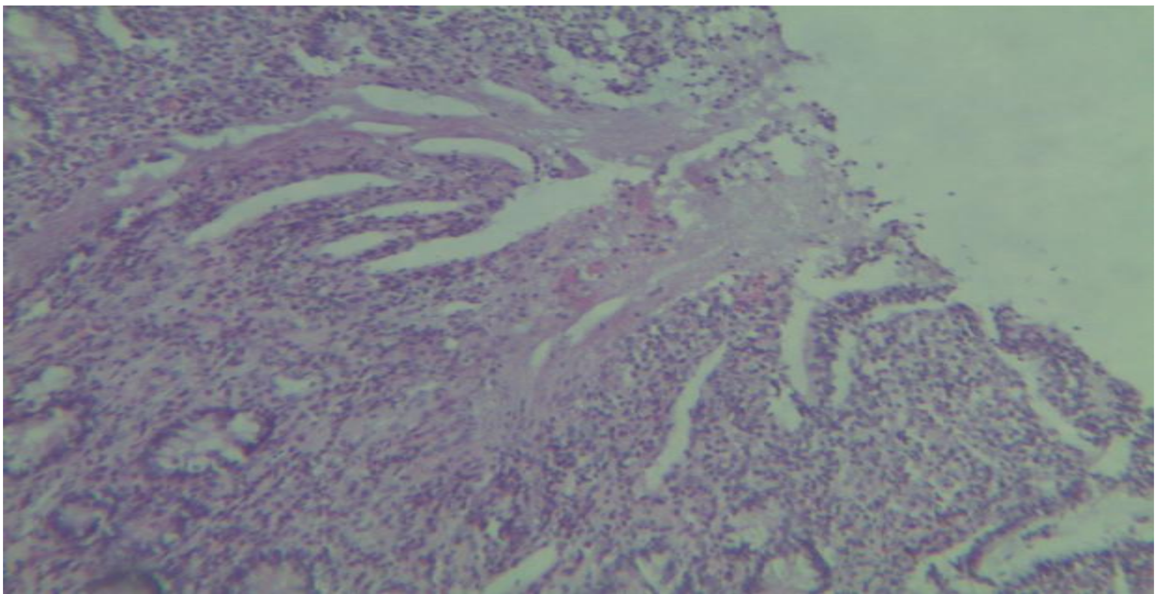

Figure 9: Intestine (Ileocecal valve); Intestine, mucosa villi necrosis surrounded by mononuclear cells (H&E x10).

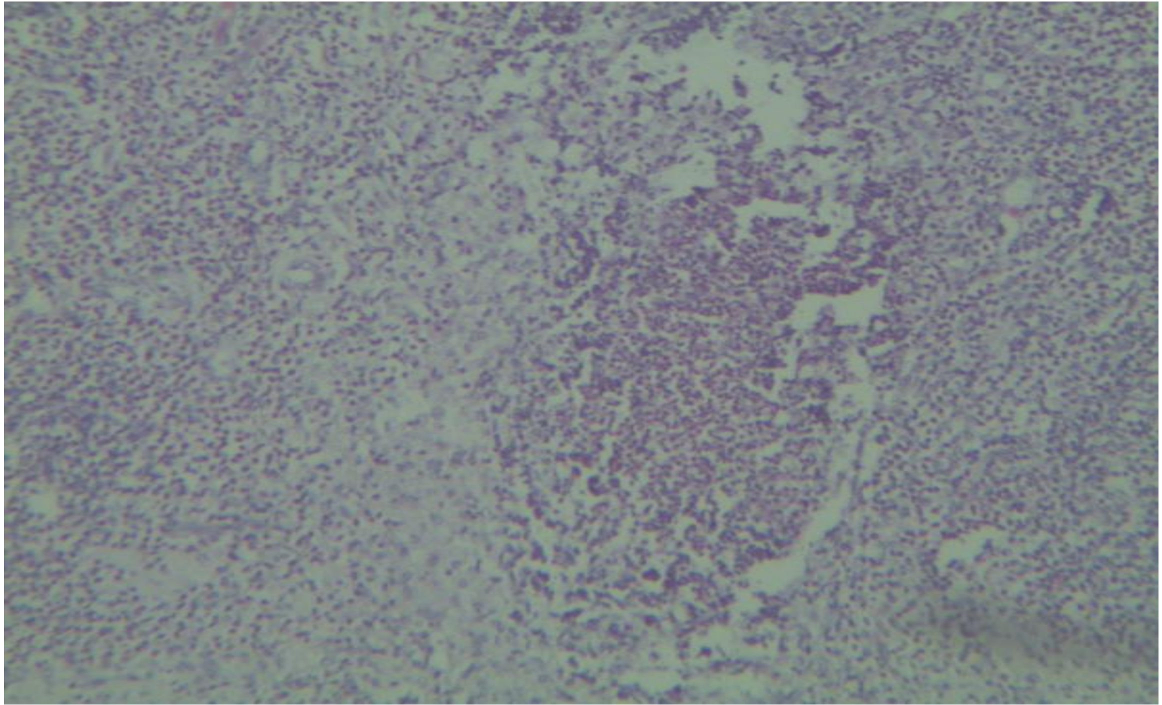

Figure 10: Ileocecal lymph node; presence of mononuclear cell aggregation and cell debris in the cortex surrounded by epitheloid cells and macrophages (H&E x40).

Histopathological lesion grading distribution of Johne's disease positive cases in cattle using histopathological examination of ileum was shown in (Table 4). A higher number of positive cases 4(8.9%) were in grade I and 3 (6.7%) were in grade II representing tissue reaction with prominent lymphocytes infiltration with macrophages accompanied with some epithelioid cells. While no case (0%) was in grade IV. Only 1 (2.2%) grade III lesion was observed representing tissue reaction with multinucleated giant cells with epithelioid cells. In our study cattle which were in grade I considered as suspected (6.7%) for JD, while cattle which were in grades II, III and IV were considered positive (11.1%) for the disease.

Table 4: Grading distribution of Johne's disease positive cases in cattle using histopathological examination of ileum

Sample tested	Histopathology grades			
	I (%)	II (%)	III (%)	IV (%)
45	2(4.4%)	4(8.9%)	2(4.4%)	0(0%)

6.2. Isolation and characterization of MAP

Culture results after 16 weeks incubation period from gross JD suspected intestine and lymph node lesion samples of cattle with respect to age groups and type of media were shown in table 5. Intestinal and lymph node scrapings were taken, prepared and cultured on modified Herrold's egg yolk medium (HEYM) and Lowenstein Jensen medium (LJ) with and without mycobactin J, incubated at 37^0 C for 16 weeks. Samples were examined starting from 8 weeks after inoculation of the media every week. On the 16th week incubation all the cultures were examined for the detection of growing bacteria and in few cultures with mycobactin J white spots scattered on the media were observed and from each of them smear was taken and stained by ZN stain. Out of 45 samples inoculated 5 (11.1%) (95% CI= 1.6-20.6) were positive for MAP Table 5.

From the positive samples 4(8.89%) were grown on modified Herrold's egg yolk medium (HEYM) supplemented with mycobactin J and 1(2.22%) was grown in Lowenstein Jensen medium (LJ) supplemented with mycobactin J. The colonies were characterized by small white spots with irregular border. Smears from all of the growth colonies showed acid fast bacilli in clump form which were similar in appearance to that made from tissue smears. No growth was observed in those media without mycobactin J supplement. The four age groups were compared for the presence of MAP positive

growth as indicated in table 5. All positive growth (100%) of MAP was observed from tissue lesions collected from 4-6 years old. There was a significant difference in the occurrence of disease with growth of MAP in different age group (F=8.025; P=0.012)

Table 5: Frequency and percentage of MAP positive isolates from tissue culture of cattle in different age groups and type of media

Age group (years)	Sample tested	Sample positive (%)			Fishers exact	p-value
		MHEYM	LJ	Total		
<4	5	0(0%)	0(0%)	0(0%)		
4-6	15	4(26.7%)	1(6.7%)	5(100%)	0.025	p=0.012
6-8	17	0(0%)	0(0%)	0(0%)		
>8	8	0(0%)	0(0%)	0(0%)		
Total	45	4(8.9%)	1(2.22)	5(100%)		

MHEN= Modified Herrold's egg yolk medium, LJ= Lowenstein Jensen medium

6.3. Direct smear staining from tissue lesion sample

Analysis of the results pertaining to the acid fast staining of direct smears from the 45 intestinal and associated lymph node tissue samples 13.3% (95% CI=3%-13.6%) were revealed acid-fast bacilli (Table 6). The four age groups were compared for the presence of acid fast bacilli as indicated in table 6. 66.7% positive acid fast bacilli were found in animal with age of 4-6 years old and 33.3% positive acid fast bacilli were found in animal with age of 6-8 years old. There was no significant difference in the occurrence of the disease with showing acid fast bacilli in different age group (F=3.1; P=0.349) of animals.

Table 6: Frequency and percentage of Johne's disease acid fast positive tissue sample of cattle in different age groups

Age group (years)	Sample tested	Sample positive	(%)	Fishers exact	P-value
<4	5	0	0		
4-6	15	4	66.7	3.1	P=0.349
6-8	17	2	33.3		
>8	8	0	0		
Total	45	6	100		

6.4. Association between histopathology, tissue culture and acid fast staining

The highest agreement was between tissue culture and histopathology 99.3% and their Kappa value were 0.733 indicates a good agreement between culture and histopathology but kappa values of tissue culture and acid fast staining were 0.483 indicating moderate agreement. Histopathology and acid fast staining methods were <0.4 indicating fair agreement between the two diagnostic methods (Table 7).

Table 7: Linearly weighted kappa coefficients between histopathology, acid fast and MAP tissue culture

	Agreement	Kappa
Tissue culture-histopathology	99.3	0.733
Tissue culture-acid fast staining	88.9	0.483
Histopathology-acid fast staining	82.2	0.326

CI= Confidence interval

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).

7. DISCUSSION

Paratuberculosis or Johne's disease has gained the attention of many countries in the world because of the economical losses in cattle and small ruminant industry and its potential for zoonotic transmission to human namely known as Crohn's disease (El-Zaatari *et al*., 2001). In Ethiopia, there is no information on the occuraence of paratuberculosis in cattle and so far no isolation of the causative agent and pathological study was carried in livestock of the country. In the present study the occurrence and pathology of paratuberculosis (JD) coupled with isolation of the *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was investigated in apparently healthy cattle between the age of 4 to 12 years of age by using gross and histopathological examination, tissue culture and acid fast staining of direct smear methods. Studies reporting previously on subclinical cases of Johne's disease have demonstrated the importance of tissue sample selection from different sites and it was found that sampling from limited foci can influence the results (Fraser *et al*., 1999; McDonald *et al*., 1999).

Therefore, the selection of tissues from different sites along with the adjacent lymph nodes should be done to confirm the diagnosis of subclinical Johne's disease with the ileocecal valve as the first site to be selected (Hailat *et al*., 2012). In the present study the last portion of the ileum and the ileocecal valve with their adjacent lymph nodes were utilized as the sample site and were used for all the implemented tests in this study. Gross examination of tissue sample in the slaughter houses from 400 subclinical cattle revealed that 45 (11.3%) of them showed a variety of gross lesions that were normally associated with JD especially in the last portion of the small intestine, ileocecal valve and corresponding lymph nodes. The intestinal wall was found variably thickened in its different regions but it was more so around the ileocecal junction such as corrugation of the mucosa, hyperemia of the mucosa and thickening of the ileocaecal valve. Congested, oedematous, enlarged and corded mesenteric lymph nodes around the ileal and ileocecal valve were also evident.

Analysis of the histopathology results in the present study revealed that the disease occurs in apparently healthy cattle slaughtered at Bishoftu ELFORA export abattoir and the occurrance was 17.8% (95% CI=6.1-29.4). This study was the first of its kind in Ethiopia and the results were in agreement with the reports made by Salem *et al*. (2005) 16.7% in Egypt from indeginous cattle by histopathology. The histopathological finding on the occurrence of JD in the present study was higher than the previous study which was conducted by Okuni *et al*. (2013) 4.7% in cattle slaughtered at two abattoirs in Kampala, Uganda. A relative lower prevalence of 11.19% in Spain from cattle and buffaloes by histopathology has been reported (Sikandar *et al*., 2012). However, the histopatholgical result recorded during this study was lower than that of Hailat *et al*. (2010) and (2012) who reported the occurrence of JD 97% and 65% in Jordan from apparently healthy sheep and goats and cattle respectively by histopathology and Hananeh *et al*. (2013) who reported 32% in Jordan from camel by histopathology.

The variation in results reported from different studies may be as a result of the temporal and spatial factors associated with the sample size, the age of the animals and the stage of the disease. The histopathological lesions such as the infiltration of the mucosa and submucosa with lymphocytes, macrophages, epitheloide cells, replacement of the crypts with macrophages, Peyer's Patches proliferation and extending towards the mucosa and the presence of multinuclted giant cells in the present study was in agreement with the reports made by Hailat *et al*. (2012) in Jordan from cattle, Okuni *et al*. (2013) in Uganda from cattle, Hailat *et al*. (2010) in Jordan from small ruminants, Hananeh *et al*. (2013) in Jordan from camel, Perez *et al*. (1996) from sheep, Watkins *et al*. (2002) from sheep and goats.

The grade I lesions observed in the present study represent the earliest signs of infection or the asymptomatic form which was in agreement with the reprts made by Perez *et al.* (1996) and Burrells *et al.* (1998) in ovine experimental studies. The grade II and grade III lesions observed in the present study represent the mild or paucibcillry form of pathology of the disease which was in agreement with Clark and Little (1996) in cattle and sheep. Our histopathological lesion grading based on the type and amount of cellular

infiltration was comprable to the report made by Hailat *et al*. (2012) in apparently healthy cattle from Jordan and Hananeh *et al*. (2013) in apparently healthy camel from Jordan who encountered the same grading.

In the present study to confirm our histopathological finding the same tissue samples with characterstics gross lesion of paratuberclosis were cultured on the conventional culture media of Herrold's egg yolk medium (HEYM) and Lowenstein Jensen medium (LJ) with the addition of mycobactin J and out of the 45 tissue samples that were cultured 5 culture postive colonies were isolated and confirmed as *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Isolation and confirmation of MAP is the definitive diagnostic test for Johne´s disease and it is standard practice to use culture to confirm a presumptive diagnosis in individual animals (Nielsen and Toft, 2008). Based on the isolation and confirmation of MAP colonies from the cultured tissue samples on the mycobactin J supplemented culture media as the definitive and confirmtory test for JD and this study revealed 11.1% (95% CI=1.6-20.6) occurrence in apparently healthy cattle slaughtered at Bishoftu ELFORA export abattoir. This indicates that the occurrence of JD in cattle in the present study area has approached to that of some European countries (Nielsen and Toft, 2009).

The finding in the present study was in agreement with the reports made by Withers (1959) 11% in Britain based on culture of tissue sample from slaughtered cattle, McKenna *et al.* (2004) 8.5%-11.1% in Canada from culled dairy cows, Vazquez *et al*. (2012) 15.3% in Spain from cattle and Kruze *et al* (2006)14.6% in Chile based on fecal culture of goats greater than 2 years old. In agreement with the present study a similar finding was also find in other speces reported by Hailat *et al*. (2010) 11% in Jordan from apparently healthy sheep and goat at abattoir. In the present study all the isolated colonies were confirmed as *Mycobacterium avium* subsp. *paratuberculosis* (MAP) by their dependency to grow on egg based media that contain mycobctin J as a growth promoter, there long incubation period of about four months and all are positive by acid fast staining.

The isolation and confirmation methods of MAP in the present study was in agreement with the reprts made by Whipple *et al*. (1991) reports that the cultivation of MAP was always performed using egg based media supplemented with mycobactin J and use of egg based medium and supplementation with mycobactin J provided the foundation for subsequent procedures for isolation of MAP. OIE (2008) reports that one of the original methods for confirming the presence of MAP was to inoculate several tubes containing mycobactin J and one tube without and colonies characteristic of MAP were present in the tubes with mycobactin J and not in the tube without a diagnosis of paratuberculosis was made. Hendrick *et al*. (2006) reported that a second attribute for confirmation of MAP was its positive reaction to Ziehl-Neelsen or acid fast staining characteristics and its long incubation time of four months. Chiodini *et al*. (1984) also reprted that historically the isolation of a slow growing acid fast positive bacillus from a cow that required exogenous mycobactin for growth was sufficient to identify a culture isolate as MAP.

The cultural finding in the present study was higher than some of the previous studies reported by Smith (1954) 0.5% and 1% in England from sheep and horses during an abattoir survey respectively, Thoen *et al*. (1975) 0.04% from pigs sampled during an abattoir survey. However the findings of the culture result encountered during the present study was lower than the previous studies reported by Okuni *et al.* (2013) 73.3% in Uganda from a tissue sample collected at abattoir from cattle and Kurade *et al*. (2004) 30% in India from a tissue samples of sheep. A relatively higher finding of 18.7% in Spain from a tissue culture collected at abattoir from cattle (Vazquez *et al*., 2009), Smith (1954) 17% in England in abattoir survey from cattle and also Norval (1954) who reported that 20-30% of cattle sent to slaughter had Johne's disease. The variation in occurance of paratuberculsis and the isolation and confirmation of MAP reported from the different studies may be as a result of different factors associated with the effect of the different chemicals used for decontamination, the effect of other contaminating bacilli, the type of lesions, the status of the animal, the disease stage and the type of media used.

In agreement with the present study finding Collins (1996) reported that due to the fastidious nature of the organism as it was very difficult to culture MAP which results in a standard incubation period of 12 to 16 weeks at 37°C. Whittington *et al*. (2003) reported that the long duration of incubation increases the risk of overgrowth by contaminating bacillus and mold species in faecal and intestinal tissue specimens and the successful isolation of MAP from such samples depends on efficient inactivation of these undesirable organisms. Salem *et al*. (2013) and Stabel (1997) reported that chemical decontamination procedure prior to cultivation is an essential step in all culture protocols. However, decontamination of tissue or fecal samples prior to isolation of MAP was a significant problem that interfered with cultivation of the bacterium. Grant *et al*. (2001) and Whittington *et al*. (2003) also reported that used chemicals for decontamination of faster growing microflora kill or decrease the viability of MAP by 70-99%.

In the present study samples were collected from apparently healthy animals and the finding of positive samples indicate that apparently healthy animals can shed the bacteria as suggested earlier by Whitelock *et al*., (2000). Nielsen and Toft (2008) who reported that cultivation and identification of MAP is the definitive diagnostic test for Johne´s disease nevertheless, although culture still remains as the golden standard, its sensitivity lies around 30% in subclinically infected cattle. Ellis *et al.* (1998) mentioned that culture results in cattle and goats depend on the stage of infection and disease prevalence within the herd where the sensitivity is low less than 50% in subclinical cases. Mc Donald *et al*. (1999) suggested that culture results during subclinical phase depend on the limited distribution of intestinal lesion with small number of acid fast organism.

In the present study of the 45 tissue samples that were cultured more growth was observed in Herrold’s egg yolk medium (HEYM) 8.89% (4/45) than Lowenstein-Jensen medium (LJ) 2.22% (1/45) which is in agreement with the report made by Greig (2000) who reported that the sensitivity of culture is depend on the medium used and the bacterial strain. Nielsen *et al*. (2004) also reported that modified herrold’s egg yolk medium (MHEY) supports growth of bovine isolates of MAP better than Lowenstein-Jensen medium (LJ).

In the present study relative to the histopathology result isolation and confirmation of MAP by conventional culture methods were taken a long period of time of four months which was very laborious and time consuming. In agreement with the present finding Eamens *et al.* (2000) also compared five different types of culture method for the bovine strain and suggested the conventional method is the least sensitive and growth can take 12-16 weeks or even some times growth fails. Stable *et al.* (1998) suggested that accurate diagnosis of paratuberculosis is often laborious by culture and may require up to 12 weeks for detection. Vansnick *et al*. (2004) suggested that the conventional culture of MAP on a more or less selective growth medium is time consuming of 12 to 32 weeks and requiring several months of incubation especially for samples with low MAP load. Taylor and El-Zaatari (2004) reported that many strains of MAP cannot grow at all and therefore, the conventional laboratory culture is not a consistently reliable method for detecting or assessing the viability of these difficult pathogens. Menzie (2001) reported that a negative result means failure to grow or that the animal is still in the early stage of the disease.

Analysis of the direct examination of tissue smears that had characteristics gross lesion of paratuberculosis using acid-fast (ZN) staining technique in the present study revealed 13.3 % (95% CI=3%-23.6%). Several studies has reported that acid fast staining was considered as a gold standard for MAP in milk, milk products, tissues and fecal material of small and large ruminants (Khan *et al*., 2010). Confirmation of the presence of the disease is obtained by the finding of acid-fast bacilli with the morphology of *M. johnei* in smears from the ileal mucosa or mesenteric lymph nodes stained by the Zeihl Neelsen staining method. However, acid-fast organisms may be absent even although enteric lesions are present (Martin and Aitken, 1991). The finding in the present study was in agreement with the reports made by Salem *et al*. (2005) 11% in Egypt from apparently healthy cattle and Huchzermeyer and Bastianello (1992) 11.1% smear from ileum and 10.3% from caecum in South Africa from slaughter sheep.

The result of the present finding was higher than some of the studies which were conducted by Yousof-beygi *et al*. (2003) 3.07% in Iran Urmia abattoir from subclinical cattle and Hailat *et al*. (2012) 1% in Jordan from subclinical cattle and Hailat *et al*. (2010) 5% in Jordan from subclinical goats. However, the acid fast staining result in the present study was lower than the previous studies which was conducted by Greig (2000) 57% from clinically affected sheep and goats with histological lesions, Zimmer *et al*. (1999) 49.3% from clinically affected cattle and a relatively higher result reported by Salem *et al*. (2005) 21% in Egypt from diseased cattle and Zimmer *et al*. (1999) 19.3% from subclinically infected cattle and a relatively higher finding from other species were also reported by Hailat *et al*. (2010) 24% in Jordan from subclinical sheep.

In the present study there was a significance differences on the occurance of paratuberculosis with age by histopathology and baacteriolgical culture methods. A statistically significant higher frequency in MAP infection was observed in animals with the age of 4-6 years old than other groups (P histopathology=0.002 and P culture=0.012). This result was agreeable with the observation made by Nielsen and Ersboll (2006) and Vazquez *et al.* (2009). However, there was not a significance differences on the occurance of paratuberculsis with age by acid fast staining method but the susceptible age was found between 4-6 and 6-8 years old which was in agreement with the reports made by Guy *et al.* (1991) and Cetinkaya *et al*. (1996). With regard to diagnostic tests the good agreement between the histopathology and culture indicates that histopathology could be a good predictor of the presence of the lesion and therefore might anticipate subclinical disease. From the present study paratuberculosis (Johne's disease) could be considered as a significant problem in apparently healthy cattle and believe there is a great need for further studies on the epidemiology of the disease in order to develop rational methods of control effective on the Ethiopian livestock population.

8. CONCLUSION AND RECOMENDATIONS

In the present study gross and histopathology examination, bacteriological culture and acid fast staining findings indicates the occurrence of paratuberculosis (Johne's disease) in apparently healthy cattle at ELFORA export abattoir Bishoftu, Ethiopia. It is interesting to note that this is the first study on paratuberculosis (JD) in cattle in the present study area or in Ethiopia and the results strongly suggests the importance of the disease at the national level. Gross and histopathology examination of tissue sample from apparently healthy cattle was the best dignositic method when conducted in slaughter houses for the diagnosis of paratuberculosis in apparently healthy cattle, but it should be confirmed by bcteriological culture for the presence of the disease. However, bacteriological culture by conventional culture media was very laborious and time consuming for subclinical diagnosis of paratuberculosis. The histopathological examination of subclinical cases alone can be considered as an alterntive method for the diagnosis of Johne's disease and can also be used to confirm the disease. In the present study age was observed as a significant risk factor for the occurrence of paratuberclosis in apparently healthy cattle. Based on the age related occurrence obtained from this study it seems appropriate to intensify time testing at age of 4-6 years the in order to maximize the likelihood of detecting infected animals.

Based on the findings, the following recommendations are forwarded:

- ❖ The investigation on the occurrence of the disease at Bishoftu ELFORA export abattoir warrants serious attention by the government and other supporting agencies.

- ❖ Further studies on the epidemiology of the disease should be conducted in order to understand its status, distribution and economic impact on the livestock sector so as to develop appropriate rational methods of control effective on the cattle population of Ethiopia.

- Further resarche on the isolation and characterizatioon of its caustive agent *Mycobacterium avium* subsp. *paratuberculosis* should be conducted by using the radiometric liquid culture media and by molecular technique in cattle and other livestocks

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10. ANNEXES

Annex I: Anti mortem data collection format

No	Animal identification	Breed			sex		Age(yrs.)				Body condition		
		Local	Exotic	Cross	M	F	<4	4-6	6-8	>10	Good	Medim	Poor

Annex II: Gross lesion data collection format

No	Animal identification number	Gastrointestinal tract	Description of tissue sample location	Gross lesion
		Ileum	Distal ileum	
		Ileocecal valve	Ileocecal valve	
		Caecum	Caecum	
		Ileal and ileocecal mesenteric lymph node	Ileal and ileocecal mesenteric lymph node	
		Ileum	Distal ileum	
		Ileocecal valve	Ileocecal valve	
		Caecum	Caecum	
		Ileal and ileocecal mesenteric lymph node	Ileal and ileocecal mesenteric lymph node	

Annex III: 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 56-58°c.
- 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 $45^{o}c \pm 5^{o}c$.
    - 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. Staining: Hematoxylin and eosin stain:

- Make sure if the slides are well dried in the oven at 65°c± 4°c 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

Annex IV: Media used for isolation and confirmation of MAP from affected tissues

Modified Herrold's egg yolk medium with addition of mycobactin J

Composition (g/l): 9.0 g peptone, 4.5 g sodium chloride, 15.3 g Noble agar , 2.7 g beef extract and 4.1g sodium pyruvate

Preparations: By adding 27 ml glycerine and 870 ml distilled water the above ingredients were mixed by using a stir plate to form a suspension. To adjust the PH of the media to 8.1-8.4 a sufficient amount of 1 N NaOH was added. Then autoclaved at 121°C for 25 minutes and allowed to cooled to 56. After the medium has cooled to 56 ^{0}C, 120ml of the prepared egg yolks, 5 ml of sterile malachite green oxalate (2% w/v in distilled water; mix well before removing the 5 ml aliquot), 4 ml of ferric mycobactin J (2 mg mycobactin dissolved in 4 ml 95% ethanol) and 5 ml of 10 mg amphotericin B/ml sterile distilled water were added. After the media have been thoroughly mixed 9 ml aliquots of the medium were dispensed into a sterile 20 mm x 125 mm screw cap tubes. To allow the medium to solidify by the tubes were placed in a slanted position.

2. Lowesten Jenson medium with addition of mycobactin J

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 (625 ml), sodium pyruvate (4 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 121° C for 15 min. After the solution was cooled to 30°C 13.4 ml of a sterile solution of pyruvate (0.4 mg/ml) and 4 ml of ferric mycobactin J (2 mg mycobactin dissolved in 4 ml 95% ethanol) were added. 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 surgical gauze 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 16 mm diameter 3-15 cm were filled with 7 ml of medium which was allowed to solidify at an angle such that the surface of the medium extended three-fourths up the tube. The tubes of medium were incubated at 90^0 C for 2 hr and then stored upright at 4^0 C for a maximum of 1 weeks

Annex V: Acid fast staining or Ziehl Neelsen's staining methods

Composition:

1. Concentrated carbol fuchsin
   - Basic fuchsin 1 gm
   - Absolute alcohol 10 ml
   - Phenol 5% aqueous solution 100 ml
   - Dissolve the dye in alcohol and add phenol solution
2. Acid alcohol
   - Ethyl alcohol (95%) 97 ml
   - Concentrated hydrochloric acid 3 ml
3. Alkaline methylene blue
   - Methylene blue saturated alcoholic solution 30 ml
   - Potassium hydroxide (1% aqueous sol.) 1 ml
   - Distilled water 99 ml
   - Add potassium hydroxide solution in distilled water and mix methylene blue solution and filter.

Procedure:

- Make a smear of on slide and fix it over the flame methanol fixation.
- 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

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  - Dessi Woizero Siheen academic, technical and vocational school grade 11 and
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- Higher education: - Jimma university

Papers produced

- The prevalence and clinical occurrence of Fasciolosis in small ruminant in and around Dessie and Kombolcha in Amhara Region South WolloZone**,** DVM thesis, Jimma University Collage of Agriculture and Veterinary Medicine, 2008.

- Published scientific paper on Risk Factors and Public Health Significance of Cysticercosis in Cattle and Human in Shire Indasilassie District, Northern Ethiopia, published on line: October 10, 2013 by *Advan. Biol. Res.,* **7**(6):282-287.

Work experience

From December 01, 2008 to October 01, 2008: has been working in JICA/ JALIMPS, international NGO in the position Animal Health management Head of the project. The project was implemented in Amhara National Region State South Wollo Zone at Argoba Special Peoples Woreda. The project was the developmental study on the improvement of livelihood through integrated watershed management program.

From December 01, 2008 t0 June, 01, 2012: has been working in Amhara National Region State South Wollo Zone at Argoba Special Peoples Woreda Agricultural Office as Animal quarantine, inspection, health and quality control expert and Animal health service improvement, disease surveillance and control expert

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