ZOONOTIC TUBERCULOSIS - EVALUATING THE STATUS AND POTENTIAL HAZARDS

V. BHANU REKHA

I.D.No. DPV 10024 (VPH)

DEPARTMENT OF VETERINARY PUBIC HEALTH AND EPIDEMIOLOGY

MADRAS VETERINARY COLLEGE

CHENNAI - 600 007

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CERTIFICATE

This is to certify that the thesis entitled **"ZOONOTIC TUBERCULOSIS-EVALUATING THE STATUS AND POTENTIAL HAZARDS"** submitted in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy in the discipline of **Veterinary Public Health and Epidemiology** to the Tamil Nadu Veterinary and Animal Sciences University, Chennai - 600 051, is a record of bonafide research work carried out by **V.BHANU REKHA** under my guidance and that a part of the thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

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Dr. L. GUNASEELAN
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CHAIRMAN : **Dr. L. GUNASEELAN**

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2. Dr. J. JOHN KIRUBAHARAN

3. Dr. ROBINSON J.J ABRAHAM

EXTERNAL EXAMINER :
DATE :

CURRICULUM VITAE

Name	:	**V. BHANU REKHA**
Date of birth	:	07-01-1971
Major field of specialization	:	Veterinary Public Health and Epidemiology
Minor field of specialization	:	Veterinary Microbiology
Educational status	:	M.V.Sc. in Veterinary Public Health
Awards / Fellowships	:	National Scholarship Scheme Merit Certificate, BCWB cash prize for District First rank in SSLC Board Exam. Recipient of Six University Gold Medals from TANUVAS in B.V.Sc. a) Karumathil Narayana Menon Award (LPM,PAT,MIB,CLM,PMD,MST) b) Rao Sahib Dr.Vinayaga Mudaliar Gold Medal (Animal Husbandry) c) P.K.Mylswamy Goundar prize (Poultry Science) d) Sri P.Lakshminarayan memorial Prize (Veterinary Pathology) e) Western Hatcheries Ltd Gold Medal (Poultry Science) f) Dr.A.G.Wilson Memorial Medal (Meat Hygiene) ICAR Junior Research Fellowship 1995-97 **Dr.G.P.Sen endowment award** by the Association of Public Health Veterinarians **Three Best Poster presentation Award**s in **International Conference**s

Conferences/Seminar/Workshop/ Symposium participated	:	Twenty
Publication of research article	:	Elevan
Marital status	:	Married
Permanent address	:	12, Ganapathy Street, Sithankudi, Pondicherry- 605 013 Mobile: 9443659103 e-mail: vivekabhanu@gmail.com
Membership in professional societies	:	a) Pondicherry State Veterinary Council b) Association of Veterinary Public Health Specialists c) Association of Public Health Veterinarians d) Indian Society for Veterinary Immunology and Biotechnology e) Indian Meat Science Association

Dedicated

to

My Husband

and

Children

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(V.BHANU REKHA)

ABSTRACT

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Title	:	**ZOONOTIC TUBERCULOSIS - EVALUATING THE STATUS AND POTENTIAL HAZARDS**
Name of the student	:	**V.BHANU REKHA**
I.D.NO.	:	**DPV 10024 (VPH)**
Degree for which thesis is submitted	:	**Ph.D., in Veterinary Public Health and Epidemiology**
Chairman	:	**Dr. L. GUNASEELAN, Ph.D.,** **Professor and Head,** **Department of Veterinary Public Health** **and Epidemiology,** **Madras Veterinary College,** **Chennai – 600 007.**
College	:	**Madras Veterinary College, Chennai-7**
University	:	**Tamil Nadu Veterinary and Animal Sciences University, Chennai-51**
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The present study was attempted to identify the status of zoonotic tuberculosis in particular in different species including bovines, ovines and humans. The Postgraduate and Research Institute in Animal Sciences (PGRIAS), TANUVAS, Kattupakkam was the organised dairy unit chosen for the study where regular herd based survey for bovine tuberculosis was conducted and unorganised small holder dairy units sampled included cattle and buffaloes from Large Animal Clinics (LAC), Madras Veterinary College (MVC) Teaching Hospital, Chennai (urban setup) and

representative samples from Dharmapuri and Krishnagiri districts (rural category). The human subjects for the study were chosen from Thiruwatteeswarar hospital for thoracic diseases, Chennai.

The samples for the study included 504 bovine milk, 210 pre-scapular lymph gland (PSLG) fine needle aspirates, 510 post-mortem(PM) tissues, 100 processed milk samples and 357 sera samples from bovines, 35 PM tissues from ovines and 279 human clinical samples which included 269 sputum, five lung aspirates, three lymph gland fine needle aspirates and two urine samples. The diagnostic tests employed were staining for acid fast bacilli (AFB), auramine staining, culture, genus specific polymerase chain reaction (PCR) to identify the Mycobacterium tuberculosis complex (MTBC), multiplex PCR to differentiate *Mycobacterium tuberculosis* and *Mycobacterium bovis,* spoligotyping and sequence analysis of isolates. Risk factors for zoonotic tuberculosis were also analyzed with the database created.

While AFB and auramine staining detected 4.33 per cent and 5.39 per cent respectively in bovines, PCR detected 4.51 per cent positivity on screening of 1224 samples. Ovines showed per cent positivity of 14.29 and 20.00 for AFB and auramine staining respectively while PCR identified 8.57 per cent positivity by screening of 35 samples. The positive percentage was 50.90, 60.57 and 65.95 in human subjects by AFB, auramine staining and PCR respectively and the study population included actually suspected or confirmed cases of tuberculosis.

Out of 504 milk samples tested, 7 (1.39 per cent), 9 (1.79 per cent) and 18 (3.57 per cent) samples were positive for tuberculosis by acid-fast, auramine staining and PCR respectively and of the 210 PSLG aspirate samples tested, 10 (4.76 per cent), 13 (6.19 per cent) and 12 (5.71 per cent) samples were positive for tuberculosis for AFB, auramine staining and PCR respectively. Out of the 510 tissue samples tested, 35 (6.86 per cent), 42 (8.24 per cent) and 23 (4.51 per cent) were positive for tuberculosis for AFB, by auramine staining and PCR respectively.

In a routine slaughter house examination of 2000 slaughtered heads of cattle examined during the study, 15.9 per cent were diagnosed with gross tuberculosis like lesions in various organs and of 100 sheep examined at slaughter, 35 per cent showed

gross tuberculosis like lesions. The humoral antibody response was 4.48 per cent in bovine serum by ELISA in a representative unit of dairy cattle indicating progress in clinical tuberculosis. None of the 100 processed milk samples were found to be positive by PCR.

Comparative evaluation of different tests used for diagnosis of bovine tuberculosis and human tuberculosis by statistical analysis using chi-square test indicated a significant difference between the various tests employed (AFB and auramine staining, auramine staining and PCR and AFB and PCR).

Statistical analysis using chi-square test indicated a highly significant difference between the age groups and also among the species studied *viz*. non- descript animals, cross bred cattle and buffalo using both the ELISA and PCR positivity.

Of the 1224 bovine samples screened by *IS 6110* PCR, 53 were found positive for MTBC organisms. On differentiation by multiplex PCR, 48 (4.24 per cent) of 1224 samples were *M. tuberculosis* and only 5 (0.44 per cent) were *M. bovis.*

Isolation of *M. tuberculosis* and *M. bovis* were attempted on samples positive for AFB or auramine staining or PCR. Totally 70 samples were subjected for culture of which 37 samples were found to show characteristic growth. Of the 37 culture positives, 34 were *M. tuberculosis* and three were *M. bovis*. In the present study there was higher number of isolations of *M. tuberculosis* in animals in comparison to *M. bovis*. Transmission Electron Microscopy (TEM) of an isolate of *M. bovis* was carried out for further confirmation to reveal mycobacteria with corresponding morphology.

All the three tissue isolates of ovines were *M. tuberculosis* and the positive per cent was 8.57. However, one chimpanzee lung nodule sample suspected for tuberculosis tested negative for AFB and auramine staining but positive for both *M. tuberculosis* and *M. bovis* by culture and PCR.

In human subjects suspected and positive for TB, out of 279 samples tested, *M. tuberculosis* and *M. bovis* were observed in 183 (65.59 per cent) and one (0.36 per cent) respectively.

Sequence analysis plot and sequence similarity matrix of nucleotide sequences of hypothetical protein 'Rv1506c' (accession no. Z79701) region of eight *M. tuberculosis* and six *M. bovis* field isolates and genbank isolates had shown the difference in sequences between isolates vary from 0 to 2.3 per cent.

The spoligopatterns of the bovines, ovines and a chimp isolate showed the following lineages: Manu1, U, EA15, EA13 and Beijing. While the lineages from various isolates were almost similar, two of them showed a spoligopattern that did not match with any existing pattern in the database (orphan). The *M. bovis* isolates from human and chimp matched with Bovis 1 type.

The results of the binary logistic regression analysis factors associated the occurrence of tuberculosis infection in human beings with the variables presumed to be the determinants in human beings, *viz.*, association with animals, raw milk consumption, family member with tuberculosis, age, education and socio-economic status were found to be statistically significant when all the data points were considered.

Though the major objective of this study was to identify the extent of *M. bovis* in humans, *M. tuberculosis* infection established was at a higher level in the animal population studied. The occurrence of *M. tuberculosis* is an example of reverse zoonosis and these animals potentially constitute a grave public health hazard as virulent bacilli can be transmitted to humans.

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LIST OF ABBREVIATION

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AFB	Acid-fast bacilli
AFS	Acid-fast staining
BCG	Bacillus Calmette Guerin
bp	Base pair
BTB	Bovine tuberculosis
ºC	Degree Celsius
cfu	Colony forming unit
CMI	Cell mediated immunity
CD	Cluster of Differentiation
DTH	Delayed type hypersensitivity
DNA	Deoxy ribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
gm	Gram
HPC	Hexa-decyl pyridinium chloride
hrs	Hours
IFN-γ	Interferon Gamma
IS	Insertion sequence
LJ	Lowenstein- Jensen
MVC	Madras Veterinary College
MTBC	*Mycobacterium tuberculosis* complex
MTCC	Microbial Type Collection Centre
ml	Millilitre

mm	Millimetre
mg	Milligram
mM	Millimolar
nm	Nanometer
ND	Non Descript
OD	Optical Density
OIE	*Office Internationale des Epizooties*
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PPD	Purified protein derivative
PSLG	Pre-scapular lymph gland
rpm	Revolutions per minute
TAE	Tris acetate EDTA
TB	Tuberculosis
TEM	Transmission Electron Microscopy
µl	Microlitre
%	Per cent
WHO	World Health Organization
χ^2	Chi-square
ZN	Ziehl-Neelsen

INTRODUCTION

1. INTRODUCTION

Tuberculosis (TB) is an important zoonosis caused by bacteria of the Mycobacterium tuberculosis complex (MTBC). There were an estimated 8.8 million cases of human TB resulting in 1.5 million deaths globally in 2010. Though *M. tuberculosis* is the most common cause of tuberculosis in humans, *M. bovis* accounts for 0.5 to 7.2 per cent of human tuberculosis cases in industrialized nations and is estimated to be responsible for 10 to 15 per cent of new cases in the developing world (de la Rua-Domenech, 2006).

The MTBC is composed of eight closely related species (*M. tuberculosis*, *M. bovis*, *M. africanum, M. microti, M. pinnipedii, M. caprae, M. canetti* and *M. mungi*), the latter being a recently described species isolated from banded mongoose (*Mungos mungo*) (Alexander *et al.,* 2010) which are obligate pathogens with more than 99per cent genetic homology (Aryan *et al*, 2010).

M. tuberculosis and *M. bovis* are the most pathogenic, but all the mycobacterial species have been isolated from diseased domestic and/or wild animals. In addition, all are zoonoses, with human infection occurring by ingestion of contaminated milk or meat, or through exposure to infectious aerosols or contamination of open wounds (Alexander *et al.,* 2010). However, regardless of which species in this complex is the cause of disease in humans, the resulting disease is indistinguishable clinically, radiologically, and pathologically. It is important to identify isolates of the MTBC to the species level for epidemiologic and public health considerations and to optimize treatment (O'Reilly and Daborn, 1995).

According to the World Health Organization (WHO, 2011), bovine tuberculosis is a neglected, endemic zoonosis. Since a large proportion of the world's population live in countries in which the control of bovine tuberculosis is limited or absent, there is consensus regarding risks to human health (LoBue *et al.,* 2001). The occurrence of *M. bovis* in the human population is persistent, though insufficiently quantified in developing countries. It is to be expected that the incidence of zoonotic tuberculosis in developing countries is heterogeneously distributed and that the livestock producing rural populations are mostly affected by *M. bovis* infections. However, there are only

very few studies that have investigated the prevalence of zoonotic tuberculosis in developing countries. Although overall the proportion of *M. bovis* causing human tuberculosis is very low compared to *M. tuberculosis*, its potential impact on population groups at the highest risk should not be underestimated (Michel *et al.,* 2010).

The diagnosis of the majority of TB cases in developing countries like India relies on acid-fast staining of sputum or positive cultures of *M. tuberculosis* in conjunction with assessment of clinical symptoms and radiographic evidence (Bock *et al.,* 1996; Tattevin *et al.,* 1999). The most common method employed for detection of *M. tuberculosis* infection is the purified protein derivative (PPD) for the tuberculin skin test, but PPD is a crude and poorly defined mixture of mycobacterial antigens, many of which are shared with proteins from the vaccine strain *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) and from non-tuberculous environmental mycobacteria (Huebner *et al.,* 1993). Therefore, the clinical relevance of the tuberculin test with PPD is not highly reliable (Mustafa, 2002). Although the tuberculin skin test is a simple, inexpensive, robust and widely accepted test developed more than 100 years ago for the diagnosis of bovine TB, the delayed-type hypersensitivity response to the antigen has low sensitivity and specificity. Several new and rapid tests for the diagnosis of TB have been developed in recent years (Brock *et al.,* 2001; Perkins, 2000), but they require specialized laboratories and involve costly equipment, reagents and trained staff.

To control zoonotic TB, it is still necessary to find diagnostic methods that are both more rapid to carry out and more sensitive but which are simpler and less expensive. Differentiation and identification of members of the MTBC should improve the clinical and therapeutic management of TB. This allows for the gathering of epidemiological information pertaining to the prevalence, transmission and geographical distribution of the MTBC, including those members associated with zoonotic TB infection in humans. Also differentiating between members of the MTBC provides the clinician with inherent MTBC specific drug susceptibility profiles to guide appropriate chemotherapy.

Since recent reports have identified TB in humans caused by *M. bovis* in countries officially free from bovine TB (Allix-Be´guec *et al.,* 2010) suggests that the true prevalence of zoonotic TB may be underestimated clinically (Cicero *et al.,* 2009). In addition, recent reports show that *M. bovis* infection in younger patient groups in the Western world are still common, most likely owing to consumption of raw milk (products) in the country of origin (Cadmus *et al.,* 2011). Moreover, zoonotic TB remains a significant threat to human health in developing countries where its prevalence is currently unknown, as differentiation between the members of the MTBC is not routinely performed (Somoskovi *et al.,* 2009).

In developing countries, the conditions for *M. bovis* transmission to humans not only exist unchanged, but the human population has a greater vulnerability due to poverty, HIV and reduced access to health care (Ayele *et al*., 2004). Many developing countries have intensified their livestock production to meet the growing demand for food security, which has led to a higher risk of transmission for both *M. tuberculosis* as well as *M. bovis* at the human–livestock interface. Exposure to aerosol-borne infection with *M. bovis* from cattle remains highest in farmers, veterinary staff and rural and slaughterhouse workers, while in developing countries, ethnicity, cultural and religious practices as well as socio-economic factors have been identified as additional contributors to an increased occurrence of *M. bovis* infections in humans. Differential diagnosis should take priority in control plans in order to enable the optimal use of veterinary intervention as a means to reduce the burden of human disease from an animal source.

The isolation of *M. tuberculosis* from cattle raises a number of questions relating to the role of humans as a source of infection to cattle. It may be speculated that in some countries more cattle contract tuberculosis from humans than vice versa. The possible existence of cattle adapted *M. tuberculosis* strains and subsequent cattle-to cattle and cattle-to-human transmission still needs clarification.

The effective control and elimination strategies have been known for a long time, but the disease appears to be still widely distributed and often neglected in most developing countries. Its public health consequences, although well documented from

the past experiences of industrialized countries, have scarcely been investigated and are still largely ignored in these regions (Tenguria *et al.,* 2011). Because of the animal and public health consequences of *M. bovis*, disease surveillance programs in humans should be considered a priority, especially in areas where risk factors are present. Research is needed to determine when *M. bovis* is of zoonotic importance and what the underlying mechanisms of transmission are. Locally operative risk factors for zoonotic TB should therefore be identified to determine persons at risk and develop appropriate control measures.

Hence a study on zoonotic tuberculosis was proposed with the following objectives:

i. To study the status of tuberculosis in animals and humans in and around Chennai.

ii. To compare and evaluate diagnostic tests for tuberculosis in man and animals.

iii. To differentiate among members of *Mycobacterium tuberculosis* complex by molecular techniques from different samples to assess the status of zoonotic tuberculosis.

iv. To identify risk factors and potential hazards of zoonotic tuberculosis.

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REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Tuberculosis (TB) is an important zoonosis caused by bacteria of the Mycobacterium tuberculosis complex (Awah-Ndukum *et al.,* 2012). Mycobacterium bovis, a member of the *M. tuberculosis* complex, has important socio-economic or public health effects with a potential significant impact on the international trade of animals and animal products (Biet *et al.,* 2005). In some developing countries, a high prevalence of bovine tuberculosis has brought great economic losses and trade restrictions on animal husbandry (Du *et al.,* 2011) and the disease is also a major threat to some endangered species (Lantos *et al.,* 2003). Therefore, the prevention and control of bovine tuberculosis (BTB) has great significance for public and animal health.

2.1. Historical review of tuberculosis

M. tuberculosis was thought to have derived from *M. bovis* (Sreevatsan *et al.,* 1997), but more recent evidence suggests a common ancestor (Brosch *et al.,* 2002). Recent studies suggest that the common ancestor of the *M. tuberculosis* complex emerged from its progenitor perhaps 40,000 years ago in East Africa. Some 10,000–20,000 years later, two independent clades evolved, one resulting in *M. tuberculosis* lineages in humans, while the other spread from humans to animals, resulting in the diversification of its host spectrum and formation of other *M. tuberculosis* complex member species, including *M. bovis* (Wirth *et al.,* 2008). This adaptation to animal hosts probably coincided with the domestication of livestock approximately 13,000 years ago (Michel *et al.,* 2010).

Evidence in the form of skeletal lesions date the occurrence of early documented cases of tuberculosis in both humans and animals to at least 3000 BC (Taylor *et al.,* 2007).

Robert Koch reported in 1882 the isolation of the causative organism of TB. Koch first believed that there was only one type of ‘TB bacillus’. However, work carried out by Theobald Smith the following decade demonstrated that there were small but consistent phenotypic differences between the bacilli normally isolated from human patients and diseased cattle. The final report of the Royal Commission,

published in 1911, concluded that not only could *M. bovis* infect man, but it could also result in clinical disease. Cows' milk was identified as a substantial source of infection, particularly for children (de la Rua-Domenech, 2006).

Emil von Behring and leading paediatricians in the early 20th century thought of human tuberculosis caused by the bovine tubercle bacillus as an infectious disease, which was in many cases acquired in early childhood and could, remain latent before causing pulmonary disease in adults. This hypothesis received new support decades later when the rapid success in combating cattle tuberculosis was not immediately paralleled by a decline of human *M. bovis* cases, especially in adults (Michel *et al.,* 2010).

2.2. Etiology

The genus Mycobacterium comprises more than 70 species. Many species of mycobacteria occur in the environment and are rarely associated with disease in humans or animals. The MTBC is composed of eight closely related species (*M. tuberculosis*, *M. bovis*, *M. africanum, M. microti, M. pinnipedii, M. caprae, M. canetti* and *M. mungi*), the latter being a recently described species isolated from banded mongoose (Alexander *et al.,* 2010). These phylogenetically closely related bacteria share more than 99.9 per cent chromosomal identity and they cause tuberculosis with similar pathology in various mammalian hosts (Smith *et al.,* 2006). *M. tuberculosis* and *M. bovis* are the most highly pathogenic, but all the mycobacterial species have been isolated from diseased domestic and/or wild animals. Immunocompromised people are at a greater risk; those with HIV infection are particularly susceptible to mycobacterial infections (Alexander *et al.,* 2010).

Organisms of the Mycobacterium tuberculosis complex, including *M. bovis*, *M. tuberculosis*, *M. africanum*, and *M. microti* are responsible of tuberculosis. The tuberculosis complex organisms are obligate aerobes growing most successfully in tissues with high oxygen content, facultative intracellular pathogens usually infecting mononuclear phagocytes and slow-growing bacteria. The bacteria do not survive exposure to heat, sunlight or dry conditions, and do not replicate outside the host (Angela *et al.,* 2006).

2.3. Prevalence of tuberculosis

2.3.1. Global scenario of zoonotic TB

All the eight closely related species of the MTBC are zoonoses (Alexander *et al.,* 2010), of which *M. tuberculosis* and *M. bovis* are the most highly pathogenic. The subsequent description of *M. bovis* disease in a butcher gave origin to the concept of 'zoonotic tuberculosis' and *M. bovis* is the most frequent cause of zoonotic TB in man. Since then several other members of the MTBC have been shown to be zoonoses, including *M. pinnipedii* (seals), *M. microti* (rodents) and *M. caprae* (cattle) (Ingram *et al.,* 2010).

There were an estimated 8.8 million incident cases of TB (range, 8.5 million - 9.2 million) globally in 2010. Most of the estimated number of cases in 2010 occurred in Asia (59 per cent) and Africa (26 per cent) (WHO, 2011). Worldwide, *M. bovis* TB in humans is clinically indistinguishable from *M. tuberculosis* TB and is estimated to account for 3per cent of all manifestations of human tuberculosis (Cosivi *et al.,* 1998).

In developed countries human TB caused by *M. bovis* accounts for around one per cent of all TB cases and this is achieved by eradicating bovine TB in animals by tuberculin skin testing and slaughter of animals and by reducing the transmission to humans by implementing pasteurisation of milk (Tenguria *et al.,* 2011).

2.3.1.1. Africa

The WHO reported in 1998 that 0.4–10 per cent of sputum isolates from patients in African countries could be *M. bovis*. This is despite the fact that *M. bovis* is more often associated with extrapulmonary disease in humans (Cosivi *et al.,* 1998).

The proportion of human tuberculosis due to *M. bovis* in less developed countries such as Mexico (13.8 per cent), Uganda (6.9 per cent) and Nigeria (5 per cent) and probably reflects consumption of unpasteurised dairy products from diseased animals as well as closer contact with infected bovines. In this setting, the potential impact on human health and food supply recently prompted the World Health

Organization to classify bovine tuberculosis a ‘neglected zoonosis’ (Ingram *et al.,* 2010).

A study in Djibouti showed a low prevalence of *M. bovis* in AIDS patients whereas Ethiopia demonstrated 17 per cent prevalence and Tanzania demonstrated 10 per cent prevalence (Tenguria *et al.,* 2011).

More detailed data including strain characterization have recently been presented and confirm that the occurrence of *M. bovis* in the human population is a persistent, though insufficiently quantified feature in developing countries. The isolation rate of *M. bovis* from symptomatic human patients in specific studies was 6.9per cent in Uganda (Oloya *et al.,* 2008) and 5per cent in Nigeria (Cadmus *et al.,* 2006).

2.3.1.2. America

In Latin America it is estimated that 2 per cent of human pulmonary tuberculosis and 8 per cent of human non-pulmonary tuberculosis is caused by *M. bovis* (Cosivi *et al.,* 1998). However, in some areas of Mexico, this percentage has been calculated to be as high as 14 per cent for human pulmonary tuberculosis (Milian *et al*, 2000).

In the United States of America the proportion of tuberculosis due to *M. bovis* is 1.4 per cent. In Latin America, Argentina described incidence in a main milk region, with a much lower national prevalence ranging from 0.7 per cent to 6.2 per cent (Tenguria *et al.,* 2011).

The isolation rate of *M. bovis* from symptomatic human patients in specific studies was 13.8 per cent in Mexico (Perez-Guerrero *et al.,* 2008) and 2.5 per cent in Latin American countries (de Kantor *et al.,* 2008).

The re-emergence of *M. bovis* in human manifested on the United States–Mexico border has shown the origin of these infections to ingestion of raw-milk products, from Mexican dairy herds. This is demonstrated by a report from New York

City of 35 cases of human *M. bovis* disease detected between 2001 and 2004, almost all of whom were adults born abroad (primarily Mexico) or children of Mexican-born parents. Most of these cases were associated with ingestion of unpasteurized soft cheeses imported from Mexico. Occurrence of *M. bovis* disease by reactivation or primary infection in HIV-infected patients, including, outbreaks of multidrug-resistant (MDR) *M. bovis* strains among hospitalized HIV-infected patients or transmission from patients with infectious pulmonary *M. bovis* disease to immune-competent contacts also appears to occur (Theon *et al.,* 2006).

2.3.1.3. Europe

In the United Kingdom the proportion of tuberculosis due to *M. bovis* is <1 per cent (Ingram *et al.,* 2010). *M. bovis* TB comprised 1.4 per cent of all TB cases in humans in the Netherlands during 1993–2007 (Majoor *et al*, 2011).

2.3.1.4. Australia

Ongoing transmission between cattle and wild species (including the brush tail possum in New Zealand) is thought to explain the significant proportion (2.7 per cent) of disease due to *M. bovis* in New Zealand (Ingram *et al.,* 2010).

2.3.1.5. Asia

The highest prevalence of TB cases is in Asia, where China, India, Bangladesh, Indonesia, and Pakistan collectively make up over 50per cent of the global burden (Mathema *et al*, 2006). Many of the genotypes were identical to patterns from farmed and wild animals (Baker *et al.,* 2006). The isolation rate of *M. bovis* from symptomatic human patients in specific studies was 0.5 per cent in Taiwan (Jou *et al.,* 2008).

Although overall the proportion of *M. bovis* causing human tuberculosis is very low compared to *M. tuberculosis*, its potential impact on population groups at the highest risk should nevertheless not be underestimated (Michel *et al.,* 2010).

2.3.1.6. Indian scenario of zoonotic tuberculosis

India alone accounted for an estimated one quarter (26 per cent) of all TB cases worldwide (WHO, 2011).

There is absence of an accurate and methodical estimation of the contribution of *M. bovis* to the global burden of TB and very few efficient surveys of *M. bovis* diagnosis are available at the national level. There is no variation in clinical and radiological findings between TB caused by *M. tuberculosis* and *M. bovis*. Culture method is expensive and does not promote growth of *M. bovis* because of the use of Lowenstein- Jensen culture media (LJ media) with glycerol and there is no clinical interest in differentiating the causative agent and treatment of TB caused by *M. tuberculosis* or *M. bovis* although *M. bovis* is pyrazinamide resistant. There is a direct correlation between the prevalence of human TB of bovine origin and that of TB in livestock (Cosivi *et al.,* 1998).

Slaughter house examination revealed gross lesions of TB in 3.5 per cent cattle in Ferozepur abattoir (Taylor, 1918), 16 per cent in Ferozepur and Kanpur (Edwards, 1927) and 1.5 per cent in Uttar Pradesh (Dwivedi and Singh, 1966). A total of 15,737 animals when tested for bovine tuberculosis using single intradermal tuberculin test in different parts of Punjab, India, over a 23-year period (1986-2009), 847 animals showed positive reaction with overall prevalence being 5.38 per cent (Sharma *et al*, 2011).

Tuberculin testing of cattle in four organized farms in Karnataka revealed 25.74 per cent positive reactors (Aswathnarayanan *et al.,* 1998) and 16.56 per cent of cattle and buffaloes in Tamil Nadu (Ganesan and Neduchelliyan, 2001). Bovine tuberculosis infection detected by Single Intra dermal test (SID), acid fast staining, polymerase chain reaction (PCR), Bacillus Calmette Guarein- Enzyme Linked Immunosorbent Assay (BCG-ELISA) and PPD-ELISA (Purified protein derivative- ELISA) were 16.66, 7.84, 12.74, 10.78 and 18.62 per cent in organized sector respectively whereas in unorganized sector 7.27, 7.27, 6.36 and 8.18 per cent respectively by AFS, PCR, BCG-ELISA and PPD-ELISA (Selvam, 2009).

Screening of 161 cattle of an organized cattle farm in north India, suspected to be suffering from tuberculosis, culture revealed a total of 54 *M. tuberculosis* complex isolates, of which 40 were identified as *M. bovis* and 14 as *M. tuberculosis* (Srivastava *et al.,* 2008).

2.3.2. Global scenario of BTB in animals

2.3.2.1. Africa

There are several reports about the incidence of BTB in animals in Africa. Infection of buffalo with *M. bovis* has a worldwide distribution and up to 92 per cent prevalence in buffalo herds has been reported from South Africa (Sharma *et al.,* 2011). A study confirmed the presence of *M. bovis* in milk in Tanzania (Kazwala *et al.,* 2006; Cadmus *et al.,* 2006). Over a nine-year period (1995-2003), tuberculous lesions were detected in 0.82 per cent of 385,784 slaughtered cattle in the Douala abattoir (Cameroon) and 0.18 per cent of 45,737 slaughtered cattle in the Bamenda municipal abattoir (Cameroon) (Ndukum *et al.,* 2010). BTB in animals have been reported from 33 of 43 African countries. In central Ethiopia, BTB is endemic in cattle with varying prevalence of 3.5 per cent to 50 per cent, depending on the geographical areas, breeds, and husbandry practices (Aylate *et al.,* 2013).

2.3.2.2. Asia

In few studies based on slaughtered animals the incidences of bovine tuberculosis in cattle and buffalo in Pakistan have been reported to be varying from 2.25 per cent in 1969 to 7.3 per cent in 1989 (Sharma *et al.,* 2011).

2.3.2.3. Tuberculosis in other animals

Although cattle are considered to be the main hosts of *M. bovis*, isolations have been made from many other livestock and wildlife species and transmission to humans constitutes a public health problem (Ayele *et al*. 2004; OIE 2009).

The paper by Mendoza *et al*. (2012) identifying different pathogenic species (*M. bovis* and M. caprae) in sheep flocks raises questions as to the significance of exposure risk from one host species infected with different MTBC species. In Spain, it

is not uncommon for cattle, sheep and goats to graze together, increasing the likelihood of cross-species transmission. It is not clear if the epidemiology of these infections is the same as that of *M. bovis* and the risk of transmission of each pathogen to humans handling livestock remains to be determined.

2.4. Transmission of MTBC

2.4.1. Transmission of *M. bovis* between human and cattle

Bovine tuberculosis is a relevant zoonosis that can spread to humans through inhalation of infectious droplet nuclei and by ingestion of raw milk (Thoen and Barletta, 2005). Transmission by direct contact or droplet transmission is possible among high-risk groups, such as veterinarians and animal keepers, who are in frequent contact with animals (Unea and Mori, 2007).

M. bovis is shed by cattle through respiratory secretions, faeces and milk, and sometimes in the urine, vaginal secretions or semen. Another relevant factor is the known ability of this zoonotic microorganism to establish itself within many types of animal host, including cattle, goats, deer, badgers, many smaller rodent species and even birds of prey (Palmer *et al.,* 2002).

A case of transmission of *M. bovis* from animal to man and back to animal was documented where cattle developed tuberculosis after being exposed to a patient infected with *M. bovis* who was reported to have been exposed and contaminated during childhood and the strain isolated in the cattle and the patient were identical (Fritsche *et al*, 2004).

Isolation of *M. bovis* from humans and suggests transmission between livestock and humans in the pastoral areas of South-East Ethiopia (Gumi *et al.,* 2012)

The widespread co-infection of cattle and wildlife populations with *M. bovis* has raised the question of the risk of *M. bovis* transmission to humans through occupational or recreational exposure to wildlife. Few reports exist with limited

information, but it is generally believed that the risk of direct disease transmission at the wildlife–human interface is probably negligible (Michel *et al.,* 2010).

2.4.2. Transmission of *M. tuberculosis* between human and cattle

Besides *M. bovis*, transmission of *M. tuberculosis* from infected humans to animals and vice versa has been reported (reverse zoonosis) (Fritsche *et al.,* 2004). Among domestic animals, infection with *M. tuberculosis* has been most frequently identified in cattle (Thakur *et al.,* 2012).

Human pulmonary TB transmission to cattle may be bidirectional (Ocepek *et al.,* 2005) as well, as cases of bovine infection were linked to farm workers with genitor-urinary tuberculosis urinating on hay (Collins 2000).

Although human-to-cattle transmission of *M. tuberculosis* has been reported (Ayele *et al*, 2004), the isolation of *M. tuberculosis* from any species especially from cattle other than human is important. *M. tuberculosis* infection has also been reported in a wide range of domestic or wildlife animal species, most frequently living in close contact with humans for long time. Among domestic animals, infection with *M. tuberculosis* has been most frequently identified in cattle (Ocepek *et al*, 2005) with less severe disease than that caused by *M. bovis*.

Similarly, previous studies have reported the isolation of *M. tuberculosis* from lesions in cattle from developing countries. Infection rates of 6.2 per cent and 7.4per cent have been reported in Algeria and Sudan, respectively (Boulahbal *et al.,* 1978; Sulieman and Hamid, 2002), and a recent slaughterhouse study from Ethiopia indicated that around 7–27 per cent of isolates were *M. tuberculosis* (Berg *et al.,* 2009). Isolation of *M. tuberculosis* from livestock suggests transmission between livestock and humans in the pastoral areas of South-East Ethiopia (Gumi *et al.,* 2012)

Infection with *M. tuberculosis* occurs most frequently in animals living in close contact with humans and has therefore been one of the most frequently recorded infectious diseases of captive wildlife. The risk of spillover of *M. tuberculosis* from humans to animals is considered high where tuberculosis in humans continues to be a

great public health concern. This was demonstrated in an 11-year study on *M. tuberculosis* cases in the National Zoological Gardens of South Africa, which indicated that the disease was more frequently transmitted by visitors to animals rather than between animals. *M. tuberculosis* has been encountered as an emerging disease especially in Asian elephants in zoological gardens in the USA but also in their native countries (Michel *et al.,* 2010).

The number of documented cases of *M. tuberculosis* in cattle appears to have increased in recent years (Chen *et al.,* 2009; Srivastava *et al.,* 2008), which may be due to improved diagnosis by molecular tools and/or an actual increase in transmission from humans to cattle in these countries. Many developing countries have intensified their livestock production to meet the growing demand for food security, which has also possibly led to a higher risk of transmission for both *M. tuberculosis* as well as *M. bovis* at the human–livestock interface (Michel *et al.,* 2010).

M. tuberculosis in cattle most frequently produces an acute infection and the infected animals show short term sensitization to tuberculin (Erler *et al.,* 2004). In a study where co-occurrence of *M. bovis* and *M. tuberculosis* existed among the cattle of the dairy farm in Palampur, India, the possibility of transmission of infection by inhalation of bacilli from grass contaminated with infected human urine, faeces, or sputum or an animal attendant or milker with active pulmonary tuberculosis, however could not be ruled out (Thakur *et al.,* 2012).

The pathological changes in cattle do not appear to support disease transmission, since *M. tuberculosis* infection usually does not progress beyond the development of small granulomas in several different lymph nodes (Cousins *et al.,* 2004). On the other hand, Srivastava *et al*. (2008) were able to isolate *M. tuberculosis* from milk samples in India, suggesting transmission to humans is likely to occur through unpasteurised milk. The possible existence of cattle adapted *M. tuberculosis* strains and subsequent cattle-to cattle and cattle-to-human transmission still needs clarification.

On a few occasions, *M. africanum*, another member of the *M. tuberculosis* complex supposed to primarily affect humans, has been implicated in the development of granulomatous lesions in lymph nodes and lungs of cattle and pigs (Rahim *et al.,* 2007) which are indistinguishable from those caused by *M. bovis*. The true prevalence of M. africanum in livestock and its implications on animal and human health remain currently unknown, owing to national control measures being largely based on gross pathological examination of lesions (Michel *et al.,* 2010).

2.5. Pathogenesis

After infection, there is an initial interaction between macrophages and mycobacteria, either bacteria can be killed and eliminated from the host or lie dormant or lead to development of active tuberculosis or reactivate from dormancy at some stage in the future. Bovine tuberculosis in cattle is an infectious, chronic but progressive disease characterised by the formation of typical granulomatous lesions with varying degrees of necrosis, calcification and encapsulation (Michel *et al.,* 2010).

Analysis of 247 naturally PPD+ *M. bovis*-infected cattle revealed that 92per cent of these animals had no clinical signs and the findings also suggested that encapsulation and multinucleated giant cells control *M. bovis* viability, whereas neutrophils may serve as a cellular biomarker of bacterial proliferation during natural infection (Menin *et al.,* 2013).

Although BTB is an important neglected zoonosis which significantly decreases livestock production and impacts public health, little is known about the dynamics of host *M. bovis* interactions and the immune-pathological parameters associated with protective host response in cattle during natural *M. bovis* infection (Menin *et al.,* 2013).

2.6. Immune response

It is well established that *M. bovis* causes a delayed hypersensitivity type (DTH) reaction; T-cell recognition of mycobacterium antigens may be the major immune response to tuberculosis (Medeiros *et al.,* 2010). The DTH response is

regarded as an indicator of a cellular immune response that is consequence of infection and disease due to Mycobacterium species.

Pollock and Neill (2002) measured the role of B-cell responses and reported that these cells induced humoral production only in advanced stages of BTB. This fact was reinforced by Welsh *et al* (2005), who documented the switch from cellular to humoral immune response in all tuberculous animals analyzed. Initially, animals developed strong antimycobacterial cell-mediated immune (CMI) responses. However, as the disease progresses, the cellular response decreases, whereas an increasing humoral response, based on IgG1 antibodies, can be clearly demonstrated. Nevertheless, the humoral response does not seem to be able to control the infection, progression of pathology, and increased bacterial load. Waters *et al*. (2006) reported that experimentally infected cattle also have an early antibody response, with production of IgM- and IgG-specific antibodies.

During the infection of cattle by Mycobacteria cell-mediated immune response and humoral immunity both these two responses are antagonistic (Ritacco *et al.,* 1991). It was established that the dominant immune response to mycobacterial infections in cattle is cellular rather than humoral in nature (Welsh *et al.,* 2005).

The progression of the disease may explain the anergy of some infected cattle to common CMI-based tests. The absence of CMI response in infected animals occurs particularly when the bacterial load is high. In human tuberculosis, lack of skin test reactivity in some individuals has been associated with an absence of lymphocyte homing receptors.

Denis *et al* (2007) hypothesized that anergic cattle are a group of animals in which this anti-inflammatory component has been recruited, preventing the expression of markers of immunity to tuberculosis, such as bovine PPD responsiveness. Recent data in a bovine model of tuberculosis infection suggested there is an appreciable release of Interleukin-10 (IL-10) associated with disease progression, strengthening the involvement of IL-10 in PPD anergy (Medeiros *et al.,* 2010).

Infection with Mycobacteria stimulates a complex array of cellular immune responses, a dominant component of which is a type 1 CD4+ -cell response and the tuberculin test used to diagnose BTB is an in vivo assay of this response (Morrison *et al.,* 2000).

2.7. Diagnosis

Although extensive knowledge on TB is available, the diagnosis and species identification is not a simple matter. But species identification of MTBC organisms is an important tool to know the transmission, surveillance and control of the disease.

In cattle BTB is often diagnosed based on history, clinical findings, results of tuberculin test, and/or necropsy findings, however the definitive diagnosis of bovine tuberculosis is the cultural isolation of *M. bovis* from tissues collected from suspected animals (Wood *et al.,* 1991). Examination of direct smears by AFB and histopathology are initial diagnostic procedures (Theon and Steele, 1995).

2.7.1. Clinical examination

The cornerstone of TB control in cattle and other species is the accurate detection and removal of animals infected with *M. bovis*. Infection of cattle with this organism is usually chronic and can remain subclinical for a long period. Importantly, infected cattle can become infectious long before they exhibit any obvious clinical signs or lesions typical of TB detectable even with the most careful veterinary examination (de la Rua-Domenech *et al.,* 2006).

Bovine tuberculosis naturally develops as a chronic progressive lung tuberculosis disease which is comparable to the human clinical pattern (Romero *et al.,* 1999). The clinical signs generally expected only in the final stages of TB infection which include weight loss and respiratory abnormalities.

Even if present, the clinical signs of TB in cattle are not pathognomonic. As a result, effective ante mortem surveillance for bovine TB must primarily rely on the detection of infected cattle at an early stage by the use of sensitive immunodiagnostic tests (de la Rua-Domenech *et al.,* 2006).

Despite the absence of clinical signs of BTB, the majority of infected cattle displayed high frequency and severity of the BTB-lesions in the lung (68.6 per cent) as well as pulmonary-associated lymph nodes (Menin *et al.,* 2013).

2.7.1.1. Choice of samples for TB diagnosis

Analyzing samples which included blood, fine needle aspirates prescapular lymph gland (PSLG), milk, pharyngeal swab, rectal pinch and faecal sample from 161 cattle suffering from tuberculosis, culture revealed a total of 54 *M. tuberculosis* complex isolates. Among the samples analyzed, PSLG was found to be most suitable specimen for isolation of *M. tuberculosis* complex from cattle and is thus of diagnostic importance (Srivastava *et al.,* 2008). It is recommended that, as a minimum, pooled lymph node samples from the head and thorax be cultured when no visible lesions are detected at post-mortem examination (OIE, 2009).

In a study screening of blood, nasal mucus, and milk from 50 cattle free of tuberculosis for TB revealed, all 3 sample types from a single infected animal were never positive. Nevertheless, nasal mucus was the sample with the highest number of positive cases. This is probably because tuberculosis infection occurs in mainly by the respiratory route and thoracic lesions are much more frequent than those located in either the mesenteric region or in the mammary glands (Romero *et al.,* 1999).

In India, Srivastava and others (2008) isolated the bacterium from six out of 154 (3.9 per cent) milk samples; from these samples, four were from SICCT-negative test cows. Bacteriological culture and PCR applied in parallel enhanced the efficacy of direct diagnosis of tuberculosis and PCR for the detection of M bovis DNA in milk samples has been more commonly reported (Zarden *et al.,* 2013)

2.7.2. Post-mortem examination in bovine tuberculosis

Much of the information that is currently available on pathogenesis of tuberculosis has been derived from infections and pathology generated in the experimental models, which is not typical of natural infections, either in terms of distribution or severity of lesions thus leading to misconception (Pollock *et al*. 2006).

Documentation of such information could contribute to better understanding of disease transmission and diagnosis and eventually to proper designing of control strategies both in animals and humans.

A study by Biffa *et al.,* (2012) described the severity of pathology and associated factors in Ethiopian cattle naturally exposed to bovine tuberculosis. Cattle with characteristic TB-like lesions to describe severity of pathology and factors associated with it. Severity of pathology was determined based upon gross lesion characteristics, distribution and presence/absence of viable mycobacteria. The study showed that TB-like lesions and *M. bovis* were more frequently observed in lungs and respiratory lymph nodes. Mammary lesions yielded significant proportion of *M. bovis* upon culturing. Intestinal lesions were the second most frequently encountered pathology; upon culturing, however, the tissue specimens yielded the lowest proportion of *M. bovis* isolates.

Lymph nodes have been found to be more commonly infected than other tissues. In the head region, mandibular, parotid and retropharyngeal lymph nodes, in thorax, mediastinal and tracheobranchial, in abdomen, liver, spleen, mesenteric lymph nodes, mesentery and kidney have to be examined for the presence of tuberculous lesions (Hope *et al.,* 2005).

Detection of tuberculous lesion through abattoir inspection was 5 per cent by Mekibeb *et al*. (2013), 4.2 per cent by Demelash *et al*. (2009) and 15.3 per cent by Seid (2007) at the same slaughter house in Ethiopia. The discrepancy of findings with the present study might be due to methodology (clinical examination expertise and frequency of collection of lesions of non-tuberculous origin), time period, and possible difference in the origin of the cattle and their local BTB burden.

2.7.3. Delayed hypersensitivity test (DTH)

The tuberculin skin test measures the DTH response. Three major types of tuberculin tests in use are caudal fold test, single intra-dermal test in the neck region and comparative intra-dermal cervical test.

The tuberculin skin test is a simple, inexpensive, robust and widely accepted test that was developed more than 100 years ago for the diagnosis of BTB. A phenomenon known as desensitisation has been reported when a second tuberculin skin test has been applied to *M. bovis* infected or sensitised cattle within a short period that may lead to a false-negative response (Buddle *et al.,* 2011).

The standard method for detection of BTB is the tuberculin test, which involves the intradermal injection of bovine tuberculin purified protein derivative (PPD) and the subsequent detection of swelling (delayed hypersensitivity) at the site of injection 72 hours later. This may be performed using bovine tuberculin alone or as a comparative test using avian and bovine tuberculins. The tuberculin test is usually performed on the mid-neck, but the test can also be performed in the caudal fold of the tail. The skin of the neck is more sensitive to tuberculin than the skin of the caudal fold. To compensate for this difference, higher doses of tuberculin may be used in the caudal fold (OIE, 2009).

Some of the main deficiencies of the test are its inability to differentiate between distinct species of the *M. tuberculosis* complex, failure to distinguish between latent stages of infection and disease and failure to distinguish vaccinated and infected individuals. In addition, anergy, exposure to environmental Mycobacteria and operator errors can lead to false results (de la Rua-Domenech *et al.,* 2006).

The limitations for skin test and cell-mediated immunity based tests include delayed-type hypersensitivity response to the antigen has low sensitivity and specificity, i.e it is not possible to determine if the response to PPD is attributable to exposure to *M. bovis*, M. avium subsp. paratuberculosis, or environmental mycobacteria, e.g., Mycobacterium avium subsp. avium, there is a predominant CMI response during the early phases of infection with low bacillary loads which may be reduced or absent in animals at the advanced stages of disease with high bacterial loads i.e the animals become anergic to CMI-based tests, animals need to be handled twice over a 72-h period to obtain results of the test, other CMI-based tests are expensive and require trained staff to perform the tests and interpret the results and false positives

may result from exposure to environmental mycobacteria and/or vaccination with BCG (Koo *et al.,* 2005).

However, at late disease stages, the cell-mediated immune response can wane as opposed to a generally increasing humoral immune response and the tuberculin skin test or Bovigam1 tests can therefore give false negative results (de la Rua-Domenech *et al.,* 2006) This is of importance for the diagnosis of bovine tuberculosis in settings where no or little disease control measures are applied and where the percentage of late stage diseased animals is believed to be high.

Common causes of false-negative in the caudal fold test for bovine tuberculosis includes very early *M. bovis* infection, generalised *M. bovis* infection contributing to anergy, stress associated with poor nutrition, transportation, parturition or change in hierarchy in dairy herds, concurrent viral infection, use of immunosuppressive drugs and test administered incorrectly. Whereas, the causes for false positive include exposure to environmental mycobacterial species, including members of the M. avium complex, infection with M. avium subsp. paratuberculosis, vaccination against Johne's disease, infection with skin tuberculosis (unidentified acid-fast mycobacteria), infection with some non-mycobacterial bacterial species (Buddle *et al.,* 2011).

Several modifications of the test are now being used to improve specificity by altering the cut-off or using specific antigens present in virulent mycobacteria such as the 6 kDa early secreted antigenic target (ESAT-6) and 10 kDa culture filtrate protein (CFP-10). While antibody based tests generally lack sensitivity, as high levels of antibodies tend to occur late in the disease process, they may have unique desirable properties such as the ability to be used as a cow-side test. The use of these new ancillary tests in association with skin-testing will improve the detection of *M. bovis*-infected cattle and reduce the unnecessary slaughter of false-positive reactors (Buddle *et al.,* 2011).

2.7.4. Blood-based laboratory tests

Besides the classical intradermal tuberculin test, a number of blood tests have been used. Due to the cost and the more complex nature of laboratory-based assays, they are usually used as ancillary tests to maximise the detection of infected animals

(parallel testing), or to confirm or negate the results of an intra-dermal skin test (serial testing). There is also evidence that when an infected animal is skin tested, an enhanced blood test can occur during the following week. This allows for better separation of in-vitro blood test responses leading to greater test accuracy. The gamma-interferon assay and the lymphocyte proliferation assay measure cellular immunity, while the ELISA measures humoral immunity (de la Rua-Domenech *et al.,* 2006).

2.7.4.1. Interferon-gamma assay

The more recently developed Bovigam1 test for cattle and its analog in humans, the QuantiFERON1-Tb-test, detect the production of interferon-gamma in (in vitro) stimulated blood samples. Applied in both standard commercial and customised test formats this assay has contributed significantly to the improved detection of early *M. bovis* infection in cattle as well as an increasing number of wildlife species (Michel *et al.,* 2010). Recent improvements of the test include the use of two *M. tuberculosis* complex specific antigens, ESAT-6 and CFP-10, which has resulted in increased test specificity (Buddle *et al.,* 2011). In contrast to the tuberculin skin test, the interferon-gamma test could also be used to differentiate between infected and vaccinated individuals. However, interferon-gamma release assays have not been found to assist significantly in diagnosing human tuberculosis in countries with a high endemic latent tuberculosis infection and high HIV prevalence (Barth *et al.,* 2008). For diagnosis in animals in developing countries, the test also appears to be impractical as it requires sophisticated laboratory equipment and the need to quickly process the blood samples after collection (de la Rua-Domenech *et al.,* 2006).

2.7.4.2. Enzyme linked immune-sorbent assay (ELISA)

The ELISA appears to be the most suitable of the antibody-detection tests and can be a complement, rather than an alternative, to tests based on cellular immunity. It may be helpful in anergic cattle and deer. An advantage of the ELISA is its simplicity, but sensitivity is limited mostly because of the late and irregular development of the humoral immune response in cattle during the course of the disease. Specificity is also

poor in cattle when complex antigens such as tuberculin or *M. bovis* culture filtrates are used. However, a comparison of antibody levels to PPD-B and PPD-A has been shown to be useful in increasing specificity in the ELISA. Improvement may be possible by using a combination of different antigens, including proteins (e.g. MPB70 and MPB83, which are specific but lack sensitivity) (OIE, 2009). Moreover, in M.-bovis-infected animals, an anamestic rise has been described, resulting in better ELISA results 2–8 weeks after a routine tuberculin skin test (Lyashchenko *et al.,* 2004).

2.7.4.2.1. ELISA in animals

An ELISA with MPB70, a major antigen of Mycobacterium bovis using bulk tank milk samples revealed ELISA might be a potential efficient screening test for bovine TB of dairy cows (Jeon *et al.,* 2010). The kinetics of antibody production in Mycobacterium bovis-infected cattle revealed MPB83-specific immunoglobulin M (IgM) was detected prior to MPB83-specific IgG detection; however, early IgM responses rapidly waned, suggesting a benefit of tests that detect both IgM- and IgG-specific antibodies. The VetTB STAT-PAK test detected responses in sera from 60 per cent (15/25) of the animals by 7 weeks after challenge and detected responses in 96 per cent (24/25) of the animals by 18 weeks (Waters *et al.,* 2006).

2.7.4.2.2. ELISA in humans

A study on IgG and IgM antibody responses in human using ELISA with extracted antigens from the *M. tuberculosis* strain H37Ra suggested its potential application for the diagnosis of mycobacterial infections in cases where suspicion is high in combination with other clinical criteria (Nath *et al.,* 2011).

2.7.4.3. Advantages and difficulties in blood based assays

In developing countries, serological tests, which are based on the detection of the humoral immune response, may be of particular use. Unfortunately, to date, no satisfactory serological test is available. Some of the problems related to the development of serological tests for tuberculosis diagnosis include the observed highly

variable antibody responses between individuals to mycobacterial antigens and antigenic variation between mycobacterial strains (Michel *et al.,* 2010).

Another recently developed serological test for animals is based on antibody detection using fluorescence polarisation but has shown variable effectiveness in different settings (Ngandolo *et al.,* 2009). Serological assays, enzyme-linked immunosorbent Assay (EIA), an immunochromatographic assay (ICGA), and a latex bead agglutination assay (LBAA) utilizing a synthetic peptide derived from early secretory antigenic target 6 (ESAT6-p) for early bovine TB detection and a recombinant major secreted immunogenic protein (rMPB70) for detection of the advanced stages of bTB, can be used with the skin test to determine the status of disease and reduce the frequency of misdiagnosis of animals free of bovine TB (Koo *et al.,* 2005). Comparision of the response induced by QuantiFERON-TB Gold antigens to that obtained with the intradermal comparative tuberculin test and BOVIGAM assay showed that the QuantiFERON-TB Gold technique used in humans could also be applied for the diagnosis of TB infection in cattle.

2.7.5. Antigen detection methods

More direct methods for tuberculosis diagnosis are based on the isolation or detection of the bacterium in sputum samples or biopsies (mostly in humans) or at postmortem, from tuberculous organ lesions (generally in animals).

2.7.5.1. Acid fast staining (AFS)

In spite of the numerous diagnostic methods, the early diagnosis of active tuberculosis still depends on the presence of acid-fast bacilli (AFB) in stained smears. The specificity of AFB microscopy is high but its sensitivity varies. The sensitivity of AFB microscopy is influenced by diverse factors such as the prevalence of tuberculosis, the quality and number of specimens, the staining technique, the experience of the microbiologist, etc (Avkanoguz *et al.,* 2011). AFB smear requires 10^5 AFB/ ml of sample for recognition by direct microscopy whereas culture detects as few as 10-100 colony forming unit (cfu)/ml (Isaac, 2013).

2.7.5.1.1. Acid-fast staining in Animals

The presence of Mycobacteria in a given sample can be assessed by Ziehl-Neelsen staining followed by light microscopy. AFB was detected in ZN-stained smears from 33.3per cent (11/33) of suspected animals. Fourteen (14) of 94 smears were positive which includes 4 from lung samples and 10 from lymph nodes, mainly bronchial and mediastinal, however, the number of AFB detected per smear was very low (Isaac, 2013).

2.7.5.1.2. Acid-fast staining in Humans

A modified Ziehl-Neelsen (ZN) stain, involving cytospin slides with Triton processing, requiring only 0.5 ml of cerebrospinal fluid specimens for detection of extracellular *M. tuberculosis* and intracellular *M. tuberculosis* in the neutrophils, monocytes, and lymphocytes clearly was found to be more effective and sensitive than the conventional ZN stain, providing clinicians a convenient yet powerful tool for rapidly diagnosing tuberculous meningitis (Chen *et al.,* 2012).

A modified microscopy for acid-fast bacilli to diagnose TB using small membrane filters (SMFs) after special processing and prefiltration had the sensitivity of 89per cent using fluorescence microscopy (FM) was 89per cent whereas the sensitivity of routine FM of centrifuged specimens was 60per cent or that of direct sputum smears was 56per cent (Fennelly *et al.,* 2012).

2.7.5.2. Fluorescent staining

The presence of Mycobacteria in a given sample can be assessed by auramine O staining and fluorescence microscopy. As for the staining techniques, the sensitivity of fluorochrome staining is higher than that of carbol-fuchsin methods such as Erlich-Ziehl-Neelsen (EZN) or Kinyoun, which are the most commonly used techniques. Fluorochrome microscopes are more expensive than conventional microscopes. However, recently developed lower cost light-emitting diode (LED) fluorochrome microscopes are expected to decrease the cost burden resulting from the use of traditional fluorochrome microscopes. These new microscopes are scheduled to be

comprehensively evaluated for routine use in high-burden countries (Avkanoguz *et al.,* 2011).

Fluorescence microscopy has been shown in numerous studies to be at least 10per cent more sensitive than traditional light microscopy. The lower limit of detection for auramine staining was 10^4cfu/ml (Hendry *et al.,* 2009).

Recent work with *M. tuberculosis* suggests that the auramine O staining technique may be more sensitive and specific than Ziehl-Neelsen staining (Marais *et al.,* 2008). However, microscopic detection of Mycobacteria shows a generally low sensitivity (from 50 to 70per cent) for human sputum samples. This is mainly due to the requirement of a high bacterial load for microscopy, which is particularly problematical in humans with HIV or in nonpulmonary tuberculosis. A much higher sensitivity can be achieved by prior culture of the bacteria.

2.7.5.3. Culture

Culture is still regarded as the gold standard for tuberculosis diagnosis despite certain deficiencies. For example, the yield of culturable (quantities) of bacteria from blood, urine, lavage and cerebrospinal fluid is very low. Bacterial culture is also time consuming and does by itself not allow the differentiation between distinct mycobacterial species However, in many cases, culture is a prerequisite for further testing and characterization of Mycobacteria (Michel *et al.,* 2010).

To process specimens for culture, the tissue is first homogenised using a mortar and pestle, stomacher or blender, followed by decontamination with either detergent (such as 0.375–0.75per cent hexa-decyl pyridinium chloride (HPC), an alkali (2–4per cent sodium hydroxide) or an acid (5per cent oxalic acid). The alkali or acid mixture is shaken for 10–15 minutes at room temperature and then neutralised. Neutralisation is not required when using HPC. The suspension is centrifuged, the supernatant is discarded, and the sediment is used for culture and microscopic examination. It is recommended that, as a minimum, pooled lymph node samples from the head and thorax be cultured when no visible lesions are detected in tuberculin or interferon test positive animals at post-mortem examination (OIE, 2009).

For primary isolation, the sediment is usually inoculated on to a set of solid egg-based media, such as Lowenstein–Jensen, Coletsos base or Stonebrinks; these media should contain either pyruvate or pyruvate and glycerol. An agar-based medium such as Middlebrook 7H10 or 7H11 or blood based agar medium may also be used. Cultures are incubated for a minimum of 8 weeks (and preferably for 10–12 weeks) at 37°C with or without CO_2.

2.7.5.3.1. Differentiation of *M. tuberculosis* and *M. bovis* by culture and biochemical tests

Characteristic growth patterns and colonial morphology can provide a presumptive diagnosis of *M. bovis* and *M. tuberculosis*; however every isolate needs to be confirmed. Isolates can be identified by determining traditional cultural and biochemical properties.

Phenotypic characteristics used for the discrimination between isolates of *M. tuberculosis* and *M. bovis* include eugonic growth for the former and dysgonic growth for the latter. *M. tuberculosis* isolates are aerobic, growth enhanced by glycerol, no growth with pyruvate, reduce nitrates, produce niacin, no sensitivity to thiophene-2-carboxylic acid hydrazide (TCH) and isonicotinic acid hydrazide (INH), nicotinamidase positive and not resistant to pyrazinamide (PZA).

On a suitable pyruvate-based solid medium, colonies of *M. bovis* are smooth and off-white (buff) in colour. The organism grows slowly at 37°C, but does not grow at 22°C or 45°C. *M. bovis* organisms are microaerophilic, no growth with glycerol, growth enhanced by pyruvate, no nitrate reduction, no niacin production, sensitivity to thiophene-2-carboxylic acid hydrazide (TCH) and isonicotinic acid hydrazide (INH), nicotinamidase negative and natural resistance to pyrazinamide (PZA). It is a microaerophillic and nonchromogenic bacterium (de la Rua-Domenech, 2006 ; OIE, 2009).

2.7.5.3.2. BACTEC culture technique

Novel methods, such as the radiometric BACTEC culture technique and the commercially available deoxyribonucleic acid (DNA) probes have considerably reduced the time needed for culture and identification of mycobacteria. A study carried out to compare one such automated system (BACTEC MGIT 960TM system) with conventional culture method (L.J.) for the isolation of Mycobacteria revealed the recovery rate was 82.8per cent and75.9per cent, the mean time for detection of Mycobacteria was 17.8 ± 0.9 days and 46.5 ± 0.4 days and the contamination rate was 6.9 per cent and 10.3 per cent for BACTEC MGIT 960 TM system and Lowenstein-Jensen medium, respectively (Mohamed *et al.,* 2011).

Although biochemical techniques also allow the differentiation between distinct mycobacterial species, these methods are very laborious, time consuming and appear to be erroneous (Michel *et al.,* 2010).

2.7.5.4. Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) based techniques are indispensable for the accurate differentiation of mycobacterial species and molecular epidemiological investigations of tuberculosis transmission.

2.7.5.4.1. PCR for MTBC

Rapid identification of isolates to the level of *M. tuberculosis* complex can be made by Gen Probe TB complex DNA probe or polymerase chain reaction (PCR) targeting 16S–23S rRNA, the insertion sequences *IS6110* and IS1081, and genes coding for *M. tuberculosis*-complex-specific proteins, such as MPB70 and the 38 kDa antigen b have been used (OIE, 2009).

Several PCR systems have been developed for the detection of species belonging to the *M. tuberculosis* complex (MTBC). The most commonly used system is based on primers that amplify segments of the *IS6110* element, particularly targeting the 123-bp and 245-bp fragments. A genus specific PCR targeting *IS 6110* detected *M. bovis*-infected animals reducing the time for diagnosis to 2 days, providing the ability to detect the presence of *M. bovis* in samples even when organisms have become

nonviable for culture or when there is an overgrowth by other mycobacteria. Although the test was not able to distinguish between different members of the *M. tuberculosis* complex, all of the positive samples from bovine sources could be assumed to be *M. bovis*, since other members of the *M. tuberculosis* complex have never been isolated from cattle specimens in that laboratory (LiéBana *et al.,* 1995).

2.7.5.4.2. PCR for *M. bovis*

Specific identification of an isolate as *M. bovis* can be made using PCR targeting a mutation at nucleotide positions 285 in the oxyR gene,169 in the pncA gene, 675/756/1311/1410 and 1450 of the gyrB gene and presence/absence of RDs (Regions of Difference) (OIE, 2009).

Another PCR system that has yielded successful identification of *M. bovis* isolates is focused on the amplification of a 500-bp DNA fragment in the RvD1Rv2031c genomic sequence (Figueiredo *et al.,* 2012).

2.7.5.4.3. PCR for differentiation of *M. tuberculosis* and *M. bovis*

A multiplex PCR based on the 12.7-kb region was reported as an excellent test for rapid and easy definitive identification *M. bovis* or *M. tuberculosis*, as it can be expected that the 12.7-kb fragment polymorphism is likely to be the most stable genetic difference between these two pathogens (Bakshi *et al.,* 2005). The utility of the hupB gene-based nested PCR assay (N-PCR) was reported for direct detection of *M. tuberculosis* and/or *M. bovis* in bovine samples (Mishra *et al.,* 2005).

2.7.5.4.4. Disadvantages of PCR

PCR can be used for any sample material in theory, but has some problems of its own, e.g. certain samples may contain PCR inhibitors, which could lead to false negative results. Conversely, the generation of a vast number of DNA amplicons can quickly give rise to false positive results. Moreover, the technique requires a relatively sophisticated laboratory and well trained technicians (Michel *et al.,* 2010).

2.7.5.5. Loop-mediated isothermal amplification method (LAMP)

The loop-mediated isothermal amplification method, a novel technique for amplification of nucleic acid is a recently developed method that has several advantages, including rapidity, high sensitivity, ease of application and low cost (Neonakis *et al.,* 2011).

LAMP has several advantages as compared to the conventional PCR-based methods like reactions are performed at a fixed temperature (60–65° C) that saves time and convenient as no purchase of expensive instruments is needed. This assay is a highly specific reaction since it uses six different primers recognizing eight distinct regions on the target sequence also results in a highly efficient synthesis of amplified products, which is at least 50 times higher than the one obtained by classical PCR reactions in identical volumes. Simultaneous production of high amounts of insoluble salt of magnesium pyrophosphate as by-product enables the visual detection of positive reactions, simply by observing the resulting white turbidity by the naked eye avoiding the need for costly instruments for the time-consuming post-amplification detection step (Aryan *et al.,* 2010).

2.7.5.6. Spoligotyping

Spoligotyping is a PCR-based technique method is simple, rapid, robust and economical mean for typing *M. tuberculosis* complex and is a candidate for use in resource-poor situations. Spoligotyping will identify *M. bovis* isolates and provide some molecular-typing information on the isolate that is of epidemiological value (Kamerbeek *et al.,* 1997). However, the differentiating power of spoligotyping is less than *IS6110* typing when high copy number strains are being analyzed (Barnes and Cave, 2003).

2.7.5.7. Restriction-fragment-length polymorphism (RFLP)

Several different molecular methods have been made available for epidemiological and evolutionary studies as a result of comparative genomic studies. *IS6110* is a novel mycobacterial insertion element formed the basis of a reproducible

genotyping technique. Its standard approach to genotyping *M. tuberculosis* isolate was based on restriction-fragment-length polymorphism (RFLP) analysis. The copy number in IS1081, an alternative molecular marker is lower than that of *IS6110*, which limits its use in epidemiological studies (Kanduma *et al.,* 2003). Although there are numerous copies of PGRS (polymorphic GC-rich sequences) repetitive element in the *M. tuberculosis* complex, the difficulties in computerizing the analysis of PGRS fingerprints due to its complexity in patterns limits the wide use (Yang *et al.,* 2000).

2.7.5.8. Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat (MIRU-VNTR)

Various studies demonstrated the importance of MIRU-VNTR method for tracking epidemiological key events, such as transmission or relapse and provide non ambiguous data (Mazars *et al.,* 2001), and are highly portable between different laboratories.

2.7.5.9. Random amplification of polymorphic domains (RAPD)

RAPD is a PCR-based method used for genotyping *M. tuberculosis* (Palitta *et al.,* 1993). A combination of typing methods based on more rapid and slower molecular clacks maybe able to exactly differentiate between the contributions of remote and recent transmission in clustering of infected patient and controlling outbreaks (Asgharzadeh and Kafil, 2007).

2.8. Comparison of different methods

2.8.1. Comparision of SIT, CFT and SICCT

Comparative studies have suggested that when bovine PPD is injected in the skin of the neck, as described in the SIT, the test is more sensitive than when injected in the caudal fold of the tail, as in the CFT. However, the CFT is more cost-effective and practical for the yarding conditions that exist for cattle in the Southern Hemisphere and North America. The SICCT is more specifi c than the CFT and SIT, as responses to avian and bovine PPD are compared, but the SICCT is less sensitive, as small

responses to bovine PPD are not recorded as positive, and prior exposure to M. avium species may mask infection due to *M. bovis* (Buddle *et al.,* 2011).

2.8.2. Comparison of Interferon-gamma assay and Caudal fold skin test

Practical advantages of the interferon- gamma test over the caudal fold skin test for bovine tuberculosis includes high sensitivity, detects *M. bovis* infection earlier than the tuberculin skin test, only one visit to the farm required, eliminates potential operator bias in reading skin tests, no delay in repeat testing and defined antigens can be used to differentiate other mycobacterial infections. Whereas the disadvantages include non-specific responses in animals <15 weeks old, blood is perishable; should be transported at between 10–26°C, relatively high cost of laboratory-based test and accurate identification required on blood tubes; potential for human error.

2.8.3. Comparison of culture to other diagnostic procedures (PCR, microscopy, ELISA)

Comparison of culture to other diagnostic procedures by examining ninety-four tissue specimens from 33 animals with granulomatous lesions in slaughterhouses in the Mejia canton – Ecuador during 2007 and 2008 displayed a lower sensitivity, with 56.5per cent for PCR, and 43.5per cent for direct microscopy and histopathology; however, the specificity was higher (94.4per cent for PCR and microscopy, and 97.2per cent for histopathology (Proano-Perez *et al.,* 2011).

In a study conducted by Du *et al.,* (2011) in northeast and northwest China in cattle, the concordance between antibody against bTB detected by use of the indirect ELISA, and positive results of isolation of *M. bovis* on culture was 52.8per cent (38/72) whereas the concordance between positive results for TST and MTBC was only 4.0per cent (43/1067).

2.9. Risk factors in tuberculosis

2.9.1. Risk factors in animals

Risk factors contributing to difficulties in controlling bovine tuberculosis in cattle across continents can have their origin at farm level. In animals, age, cattle, breed

and behaviour can have a significant influence (Menzies and Neill, 2000). Genetic variability and age-associated factors have been shown to potentially be involved in susceptibility to *M. bovis* infection (Driscoll *et al.,* 2011). Nutritional deficiencies (Griffin *et al.,* 1993) and immunological dysfunction in cattle may enhance bovine tuberculosis infection. However, host independent factors are considered more important in most cases and include, amongst others, production types and management practices, cattle movement, existence of a wildlife reservoir, and possibly strain related differences and survival of *M. bovis* (Michel *et al.,* 2010).

2.9.1.1. Breed

A study in Ethiopia by Biffa *et al*, (2012) showed that female and exotic breed were at a relatively higher risk of developing severe tuberculosis and TB pathology was more severe in cattle raised under large-scale farming. Isolation of *M. bovis* from mammary and muscle tissues implies a potential threat of zoonotic transmission, where raw milk and raw beef constitute a customary dietary regimen.

Studies performed also identified the animals' breed as a risk factor for a positive skin test. In 1998, a cross-sectional study carried out in Africa in intensive dairy farm animals of Eritrea suggested that imported breeds, used to improve the dairy industry in tropical areas, may be less resistant to bTB compared to indigenous breeds, e.g. zebu. The difference of susceptibility between breeds is likely to be related to differences in management: imported dairy animals are generally kept under intensive conditions (Humblet *et al.,* 2009).

2.9.1.2. Age

One of the main individual risk factors identified by numerous studies in both developed and developing countries is the age of animals. The duration of exposure increases with age; older animals are more likely to have been exposed than younger ones. Animals might get infected at a young age, but only express the disease clinically when they are adults (Griffin *et al.,* 1996)

2.9.1.3. Sex

Male cattle are mostly used as oxen, which are kept longer in the herd than females. Due to this particular longevity, it is more probable that they get in contact with infected cattle from other affected herds and in turn get infected; this would imply that between-herd contact is a major source of BTB transmission (Kazwala *et al*, 2001). From 2006 to 2007, a cross-sectional study on 1,470 animals in Uganda revealed significantly more females positive to the skin test than males (Inangolet *et al*, 2008). Gender-linked factors are probably related to management practices or behavioural habits; males and females are managed differently, both in developed and developing countries. In developed countries, dairy cows usually reach an older age than males because of their role in calving and milk production.

2.9.1.4. Body Condition Scoring (BCS)

Animals suffering from a clinically advanced BTB often present a low BCS as a result of the long-lasting pathological process (Humblet *et al.,* 2009).

2.9.1.5. Co-infections, Heredity and other factors

The role of co-infection in cattle with diseases highly prevalent in some areas (e.g., trypanosomiasis, fasciolosis, chronic contagious bronchopneumonia), nutritional challenges and the genetic role of different cattle breeds to BTB susceptibility. Studies examining association between helminth infection indicators and tuberculosis disease demonstrated that helminths may impair immunity against mycobacterial infections (Méndez-Samperio, 2012).

Apart from the above risk factors at the animal level also include genetic resistance and susceptibility to BTB, vertical and pseudo-vertical transmission and auto-contamination.

2.9.1.6. Herd-level risk factors

Herd-level risk factors include history of BTB outbreak in the herd and human antecedent of tuberculosis in the household. Herd size, type of cattle industry or enterprise, management, intensity of the farming system and housing of cattle, manure,

feeding, supplementary feeding and feed storage, cattle-to-cattle transmission via the faeco-oral route, lack of performance of diagnostic tests, intradermal tuberculin test frequency (reduced opportunity of detection), reduced human handling and contact with veterinary services, introduction of cattle in a herd: purchase, movements of animals, contact between animals, culling rate, other domestic species, wildlife and environmental persistence of *M. bovis* and influence of climate.

Risk factors at the region/country level includes BTB prevalence and antecedents in the region/country, contiguity with other BTB restricted herds, international trade and trans-border movements of cattle and migration – Globalisation (Humblet *et al.,* 2009).

2.9.2. Risk factors in humans

The current TB epidemic in human is being sustained and fuelled by two important factors: the human immunodeficiency virus (HIV) infection and its association with active TB disease and increasing resistance of Mycobacterium tuberculosis strains to the most effective (first-line) anti-TB drugs (WHO, 2009). Other contributing factors include population expansion, poor case detection and cure rates in impoverished countries, active transmission in overcrowded hospitals, prisons, and other public places, migration of individuals from high-incidence countries due to wars or famine, drug abuse, social decay, and homelessness (Ahmad, 2011).

In humans, risk factors for mycobaterial infections, being especially well described for *M. tuberculosis* (Collins and Kaufmann, 2001) include the intensity of exposure, age, immune system, HIV coinfection, genetic factor, vaccination status and also socio-economic factors. Occupational groups especially farmers or abattoir workers as well as veterinarians, are generally infected with *M. bovis* by the respiratory route through aerosols from infected cattle and develop typical pulmonary tuberculosis. Reactivation occurs under stress or in old age, since mycobacteria in a latent state may become subject to less stringent control by host systems (Moda *et al.,* 1996).

2.9.3. Risk factors in zoonotic tuberculosis

2.9.3.1. Close physical contact

Sharing of the same households by tuberculosis patient and reactor cattle suggest transmission of Mycobacterium species between cattle and their owners. Cattle owners and non cattle owners had 5 per cent, 3 per cent tuberculosis patients in their house, respectively (Girmay *et al.,* 2012).

2.9.3.2. Food hygiene practices

BTB is a principal zoonotic problem, transmitted to human primarily through consumption of raw meat, milk, and other products obtained from infected cattle (Mekibeb *et al.,* 2013). Consumption of milk contaminated by *M. bovis* has long been regarded as the principal mode of TB transmission from animals to humans. In regions where bovine TB is common and uncontrolled, milkborne infection is the principal cause of cervical lymphadenopathy (scrofula) and abdominal and other forms of nonpulmonary TB. Although proper food hygiene practices could play a major role in controlling these forms of TB, such practices are often difficult to institute in developing countries. Both *M. bovis* and *M. tuberculosis* have also been found in milk samples. Thus, serious public health implications of potentially contaminated milk and milk products should not be underestimated (Cosivi *et al.,* 1998).

2.10. Control

In general, with the exception of a few countries with a wildlife reservoir of *M. bovis*, the prevalence of bovine tuberculosis has reached very low levels, in most developed countries (Michel *et al.,* 2010). The situation is profoundly different in developing countries, which are in general unable to apply expensive test-and-slaughter schemes for the control of animal tuberculosis. Although in parts of the Latin American and Caribbean countries there has been significant progress in bovine tuberculosis control and infection rates under 1per cent have been reported for 30 per cent of the region's cattle, 70 per cent of cattle are kept in areas where rates of infection are higher and where herd prevalence of up to 56 per cent have been reported (de Kantor and Ritacco, 2006; de Kantor *et al.,* 2008). On the African continent, more than

80 per cent of the human population co-exists with cattle in the absence of any organized control of bovine tuberculosis (Cosivi *et al.,* 1998).

TB control plans should include specific policies and valid analytical system to ensure the prompt identification, triage, and referral or management of animal with suspected or confirmed TB disease, and measures should be taken to minimise the risk for transmission of organisms of the *M. tuberculosis* complex (Angela *et al.,* 2006). The unsuccessful eradication plans may be related to the relaxation of cattle testing and to spillover of infection to badgers, deer and farm cats and dogs (Hancox, 2002).

Although animal test-and-slaughter schemes have successfully reduced the prevalence of bovine tuberculosis in most industrialized countries, such expensive control programmes have been increasingly questioned considering their economic burden and increasing opposition by farmers (Torgerson and Torgerson, 2009; Bennett, 2009). Most importantly, in the mainly rural livestock producing areas of developing countries, bovine tuberculosis can have devastating impacts on the livelihood of millions of the world's most vulnerable communities as the disease compromises their sustainable food supply, income and social status (WHO, 2006).

The breakthrough in the eradication of bovine and zoonotic tuberculosis in developed countries was achieved through mandated tuberculin testing of livestock and removal of positive reactors and compulsory pasteurisation of milk. As a result of these rigorous and expensive control efforts, the risk of contracting zoonotic tuberculosis has become extremely low in developed countries over the past few decades (Michel *et al.,* 2010).

Human-to-human transmission of *M. bovis* as well as concurrent infection with *M. tuberculosis* is rare and quite probably occurs mostly in unusual cases. Where the opportunity exists, transmission of *M. bovis* from humans back to cattle can occur and may, under these circumstances, complicate efforts to control bovine tuberculosis in cattle (Michel *et al.,* 2010).

Immuno-suppression due to HIV-infection is a known complication in humans affected by *M. tuberculosis* and has recently emerged as an aggravating factor in *M.*

bovis infection in humans at the livestock–human interface, mainly in nosocomial outbreaks. Some of these were caused by multidrug-resistant *M. bovis* strains and caused complications in hospitalized HIV-infected patients (Cobo *et al.,* 2001).

The epidemiology of bovine TB is well understood and effective control and elimination strategies have been known for a long time, the disease is still widely distributed and often neglected in most developing countries. Its public health consequences, although well documented from past experiences of industrialized countries, have scarcely been investigated and are still largely ignored in these regions (Tenguria *et al.,* 2011).

2.11. Future directions for zoonotic tuberculosis

The consequences of *M. bovis* presence in animal-human-interface prompt a fruitful disease surveillance programs in humans especially in areas where risk factors are present. The increase of TB in such areas calls for stronger intersectoral collaboration between the medical and veterinary professions to assess and evaluate the scale of the problem, mostly when zoonotic TB could represent a significant risk, e.g. in rural communities and in the workplace. Research is needed to determine when *M. bovis* is of zoonotic importance and what the underlying mechanisms of transmission are. Locally operative risk factors for zoonotic TB should therefore be identified to determine persons at risk and develop appropriate control measures. International cooperation in all aspects of zoonotic TB remains essential in the fight against this disease (Tenguria *et al.,* 2011).

The simultaneous study of mycobacteria in humans and livestock allow relating transmission risks. It demonstrates an added value of a ‘‘One Health’’ approach of closer cooperation of human and animal health sectors (Gumi *et al.,* 2012).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Location of study

The subjects for the study included for sampling were bovines, ovines and humans. While bovine sampling was done in organised and unorganised small holder dairy units in urban and rural setting with further sampling from Perambur slaughter house which included bovine and ovine species, the human subjects for the study were chosen from Thiruwatteeswarar hospital for thoracic diseases, Chennai.

The Postgraduate and Research Institute in Animal Sciences (PGRIAS), TANUVAS, Kattupakkam is the organised dairy unit chosen for the study where regular herd based survey for bovine tuberculosis (BTB) is conducted and the unorganised small holder dairy units sampled were from Large Animal Clinics (LAC), Madras Veterinary College (MVC) Teaching Hospital, Chennai (urban) and a representative samples from Dharmapuri and Krishnagiri districts (rural).

3.1.2. Samples for the study

The samples from study group in the above locations were both ante-mortem and post-mortem. The bovine samples were collected from apparently healthy animals and animals with tuberculosis like lesions or emaciated conditions at post-mortem (PM). The samples included 504 milk samples, 210 pre-scapular lymph gland (PSLG) fine needle aspirates, 510 PM tissue samples and 357 serum samples from bovines and 35 tissue samples from ovines.

In the human subjects, 279 samples including 269 sputum samples, five lung aspirates, three lymph gland fine needle aspirates and two urine sample were collected from suspected and confirmed cases of tuberculosis.

3.1.3. Diagnostic support materials

3.1.3.1. Biologicals

Reference strains

The reference strains used in this study are given below:

Strain details	Source
Mycobacterium tuberculosis (MTCC No 300)	Microbial Type Collection Centre (MTCC), Chandigarh, India.
Mycobacterium microti (MTCC No 1727)	
Mycobacterium kansasii (MTCC No 3058)	
Mycobacterium bovis BCG vaccine strain	Serum Institute of India, Pune

The strains were tested for their purity by morphological and biochemical characteristics.

Media

i. Lowenstein Jensen (LJ) Medium Base - M162

ii. Gruft Mycobacterial supplement - FD053

3.1.3.2. Reagents, chemicals and glasswares

Stains

i. Acid-fast staining (Ziehl-Neelsen staining) kit (HiMedia Laboratories Pvt. Ltd., Mumbai, India)

ii. Auramine staining (RNTCP Manual, 2010)

iii. Phospho-tungstic acid one per cent (Negative stain)

Decontaminants and chemicals

i. One per cent Cetyl Pyridinium chloride (CPC) solution

ii. Four per cent Sodium hydroxide

iii. Polyethylene glycol (PEG) 200

iv. 2M Pottasium hydroxide

Glasswares and plasticwares

Standard disposable glasswares and plasticwares were used in this study.

3.1.3.3. Primers

Oligonucleotide primers used in this study are listed below:

Primer sequence (5' – 3')	Product size	References
IS 6110		
IS41 (F) 5'-CCT GCG AGC GTA GGC GT-3'		LiéBana *et al.,* 1995
IS43 (R) 5'-TCA GCC GCG TCC ACG CC-3'	317 kb	
Hypothetical protein 'Rv1506c'		
CSB1CommonFP-(5'-TTC CGA ATC CCT TGT GA-3')		Bakshi *et al.,* 2005
CSB2 *M. bovis* RP- (5'-GGA GAG CGC CGT TGT A-3')	168 bp.	
CSB3-*M. tuberculosis* RP-(5'-AGT CGC CGT GGC TTC TCT TTT A-3')	262 bp.	
Multiplex PCR for both genus and species of MTBC		Verma *et al.,* 2011
IS 6110 F: 5' GAC CAC GAC CGA AGA ATC CGC TG3'		
IS 6110 R: 5' CGG ACA GGC CGA GTT TGG TCA TC3'	445 bp	
12.7 kb common F: 5' CAC CCC GAT GAT CTT CTG TT 3'	389 bp	
12.7 kb R1 *M. tuberculosis* : 5' GCC AGT TTG CAT TGC TAT T 3'	823 bp	
12.7 kb R2 *M. bovis*: 5' GAC CCG CTG ATC AAA GGT AT 3'		

The above mentioned primers were custom synthesised and obtained from Sigma-Aldrich, Bangalore, India.

3.1.2.4. **Spoligotyping**

Direct locus- DRa (0.2 µmol/µl) and DRb (0.2 µmol/µl) primers were used for spoligotyping. (Kamerbeek *et al.,* 1997).

3.1.2.5. ELISA kit

The recombinant antigen Mycobacterium bovis antibody test kit (IDEXX kit, IDEXX laboratories, USA) designed to detect the presence of antibody to *M. bovis* in bovine serum and plasma samples was used in this study.

3.1.2.6. Risk analysis

An interview schedule was designed to collect details in human subjects for risk assessment in tuberculosis (Annexure 1).

3.2. METHODS

3.2.1. Collection and processing of samples

3.2.1.1. Milk

About 10 ml of milk was collected from all the lactating quarters of each animal in the study group in a single sterile vial aseptically and stored at - 20°C until processing.

3.2.1.2.Processed milk

Pasteurized and/or boiled milk samples from vendor shops in Chennai city were also collected (about 50 ml each) and stored at - 20°C until processing.

3.2.1.3.Lymph node biopsy

Lymph node aspirates were collected using a 16 Gauge needle from PSLG (Plate 1) and stored at - 20°C for further processing.

3.2.1.4. Tissues

PM tissue samples included lungs, spleen, liver, mesentery or bronchial lymph nodes with evidence of tuberculous lesions and from emaciated animals. They were collected using triple packaging system consisting of an inner watertight primary screw capped container, water tight secondary container (sealable plastic bag) and a tertiary robust outer container (icebox).

3.2.1.5.Human subjects

Sputum samples, lung aspirates, fine needle aspirates and urine samples from humans were collected in sterile containers.

3.2.1.6. Sample processing

The milk, tissue, PSLG and sputum samples were processed as per standard methods described (OIE, 2009). The suspension was decontaminated by adding double the volume of 1per cent CPC solution and centrifuged at 5000 rpm for 20 minutes. Sediment was used for smear, culture and DNA extraction. Smears were also made from tissue impressions or culture isolates or from fine needle aspiration biopsy contents.

3.2.1.7. Sera

Bovine blood samples were collected using vaccutainer and sera separated stored at - 20°C until processing testing.

3.2.2. Staining

3.2.2.1. Acid-fast staining

The tissue impression smears, smears from milk samples, lymph node biopsy smears and human sample smears were stained by acid fast staining as per the standard protocol (RNTCP Manual, 2010).

3.2.2.2. Auramine staining

The smears made from various samples described above were stained by auramine stain solutions as per the recommended protocol (RNTCP Manual, 2010).

3.2.3. Isolation and identification

The sediment after initial processing of samples were inoculated on to two slants of LJ medium with Gruft Mycobacterial supplement, one with glycerol and the other with pyruvate without glycerol (RNTCP Manual, 2009). All the LJ media slants were incubated at 37°C. Slants were examined for colony growth after 2 weeks and at weekly intervals during the incubation period. Typical colonies of *M. tuberculosis* are rough, buff, tough, non-pigmented (cream coloured) and slow- growers, i. e. Colonies appearing one to two weeks after inoculation. Typical colonies of *M. bovis* are smooth and off-white (buff) in colour. When visible growth was evident, the characteristic colonies were examined by staining for initial identification and then by PCR for confirmation.

For Transmission Electron microscopy (TEM) image, the culture of *M. bovis* was suspended in phosphate buffer saline. One drop of culture mixed with one drop of 1per cent Phospho-tungstic acid and loaded on 3mm diameter copper grid. This was viewed under TEM (Technai10, Philips, Netherlands)

3.2.4. Polymerase chain reaction (PCR)

3.2.4.1.DNA Extraction

DNA was extracted from tissue samples, milk, lymph node biopsy and human clinical samples using DNeasy Blood and Tissue Kit (QIAGEN, USA) as per the manufacturer′s instructions.

For processed milk samples, DNA was isolated by alkaline lysis polyethylene glycol method (AL-PEG) (Chomczynski and Rymaszewski, 2006). In this method to 100ul of processed milk, 500ul of AL -PEG reagent (60g PEG 200 + 0.93ml 2M KOH + 39 ml water) was added and incubated at 60^0C for 10 mins. From this 2-3 µl of supernatant was used as a template in PCR.

3.2.4.2.MTBC specific PCR

Polymerase chain reaction was performed targeting Insertion Sequence 6110 (*IS6110*) gene specific to MTBC (Lie'Bana *et al.,* 1995). The cycling conditions are as follows:

cycling conditions

Initial denaturation	94°C for 5 minutes
Denaturation	94 °C for 1 minute
Annealing	68°C for 2 minutes
Extension	72°C for 1 minutes
Repeated for 30 cycles	
Final extension	72°C for 7 minutes

3.2.4.3. Multiplex PCR to differentiate *M. tuberculosis* and *M. bovis*

Multiplex PCR was performed on the samples as per the method of Bakshi *et al* (2005).

PCR cycling conditions

Initial denaturation	94°C for 5 minutes
Denaturation	94 °C for 1 minute
Annealing	52.3°C for 1.30 minutes
Extension	72°C for 1 minutes
Repeated for 30 cycles	
Final extension	72°C for 5 minutes

3.2.4.4.Multiplex PCR to identify both MTBC and differentiation of *M. tuberculosis* and *M. bovis*

Multiplex PCR was performed as per the method of Verma *et al*. (2011) with DNA extracted from tissue samples, milk, lymph node biopsy and human clinical samples.

PCR cycling conditions

Initial denaturation	95°C for 5 minutes
Denaturation	94 °C for 1 minute
Annealing	54°C for 1 minute
Extension	72°C for 1 minutes
Repeated for 30 cycles	
Final extension	72°C for 7 minutes

3.2.4.5.Agarose gel electrophoresis

The amplified products were subjected to agarose gel electrophoresis on a 1.2 per cent gel and viewed under gel documentation system for the bands of desired molecular weight.

3.2.5. Purification and sequencing of PCR products for sequence analysis

The PCR products of the MTBC isolates were gel purified using HiYield Gel purification Kit (Real Genomics, USA) as per manufacturer's instructions. The purified product was eluted with 15 µl of nuclease free water. Finally the purified products of CSB1, CSB2 and CSB3 were checked in agarose gel electrophoresis and sequenced in an automated sequencer (ABI Genetic Analyzer, USA) using Big Dye terminator kit (ABI). Both the forward and reverse sequencing were performed. The *M.*

tuberculosis isolates were characterized by sequence analysis using two sets of primers CSB1 and CSB3 and *M. bovis* isolates were characterized by sequence analysis using two sets of primers CSB1 and CSB2. Sequence analysis was performed using MEGA 5.2 software (Tamura *et al.,* 2011) by comparing the *M. tuberculosis* and *M. bovis* field isolates with other sequences available in GenBank.

3.2.6. Spoligotyping - In vitro amplification of spacer DNA by PCR

A PCR-based method to simultaneously detect and type MTBC was performed on the field isolates. Amplification of the spacers was accomplished by using the primers DRa and DRb, which enable to amplify the whole DR (Direct Repeat) region.

DNA of *M. tuberculosis* strain H37Rv and *M. bovis* BCG P3 were used as positive controls and water as negative control. PCR was performed as follows:

Initial denaturation	94°C for 3 minutes
Denaturation	94 °C for 1 minute
Annealing	55°C for 1 minutes
Extension	72°C for 30 seconds
Repeated for 25 cycles	
Final extension	72°C for 7 minutes

Hybridization of the biotin-labeled PCR products to the immobillized spacer-oligos on the membrane was done (Kamerbeek *et al.,* 1997) that represent spacers of known sequence. The presence of spacers is visualized on film as black squares after incubation with streptavidin-peroxidase and the isolates were typed by 'SPOTCLUST' (http://www.rpi.edu/_bennek/EpiResearch).

3.2.6. ELISA test procedure

ELISA was performed and the results were calculated as per the manufacturer's directions.

3.2.7. Data management and statistical analysis

Data have been entered in to MS office 2007 Excel spread sheet, coded and analysed by SPSS version 17. Comparison of different diagnostic tests and risk factors, Chi-square test, kappa statistics, sensitivity and specificity were carried out as per Thrusfield (2005).

3.2.8. Risk assessment

The interview schedule was used for collection of details on demography of human cases, animal association and consumption of raw milk.

To analyse the factors associated with occurrence of tuberculosis infection in human beings binary logistic model was fitted. IBM_SPSS_Statistics version 20 software was used for the analysis.

The following logit model was postulated.

$$\ln (p_i / (1- p_i)) = \beta_o + \beta_1X_1+ \beta_2 X_2+ \beta_3X_3 + \beta_4X_4 + \beta_5X_5 + \beta_6X_6 + \beta_7X_7 + \beta_8X8 +\beta_9X_9 +\beta_{10}X_{10} +\beta_{11}X_{11}$$

Where,

p_i = Probability of occurrence of tuberculosis in human beings.

$(1- p_i)$ = Probability of non-occurrence of tuberculosis in human beings

β_0 = Constant term

β_i's = Coefficients

X_i = Determinant factors

Variables used in binary logistic fit for factors affecting the occurrence of tuberculosis infection in human beings are given below:

X_i - Variables

A - Constant

X_1 - Animal association (Yes = 1, No = 0)

X_2 - Raw milk consumption (Yes = 1, No = 0)

X_3 - Family members with tuberculosis (Yes = 1, No = 0)

X_4 - Age (<20 yrs = 0, 20-40 yrs = 1, 40 -60 yrs = 2, 60 yrs above = 3)

X_5 - Education (No formal education = 0, Primary = 1, Secondary = 2, Tertiary = 3

X_6 - Socio economic status (Lower = 0, Lower middle = 1, Upper middle and Upper = 2

RESULTS

4. RESULTS

This study attempted to identify the status of zoonotic tuberculosis in particular in different species including bovines, ovines and humans. The diagnostic tests employed were staining for acid fast bacilli (AFB), auramine staining, culture, genus specific polymerase chain reaction (PCR) to identify the Mycobacterium tuberculosis complex (MTBC) and multiplex PCR to differentiate Mycobacterium tuberculosis and Mycobacterium bovis including spoligotyping and sequence analysis of isolates. Risk factors for zoonotic tuberculosis were also analyzed with database.

4.1. Overall status of tuberculosis (TB) in the study population

The overall prevalence of tuberculosis infection in bovines, ovines and humans from various samples is presented in Table 1.

While AFB and auramine staining detected 4.33 per cent and 5.39 per cent respectively in bovines, PCR detected 4.51 per cent positivity. Ovines showed per cent positivity of 14.29 and 20.00 for AFB and auramine staining respectively while PCR identified 8.57 per cent positivity. The positive percentage was 50.90, 60.57 and 65.95 in human subjects by AFB, auramine staining and PCR respectively.

In a routine slaughter house examination of 2000 slaughtered heads of cattle examined during the study, 15.9 per cent were diagnosed with gross tuberculous like lesions (Table 2a) in various organs (Table 2b) and of 100 sheep examined at slaughter 35 per cent showed gross tuberculous like lesions.

The prevalence established for humoral antibody response was 4.48 per cent (16/357) in bovine serum (Plate 2).

4.2. Utility of samples in establishing status of bovine tuberculosis (BTB) in different systems

In this study, bovines randomly selected were sampled and included milk, as collection was non-invasive and easier for screening large number of animals and

Table 1: Status of Tuberculosis in the study population

Test	Species tested		
	Bovine N=1132 (% positivity)	**Ovine N=35 (% positivity)**	**Human N=279 (% positivity)**
AFB staining	49 (4.33)	5 (14.29)	142(50.90)
Auramine staining	61 (5.39)	7 (20.00)	169(60.57)
IS 6110	53 (4.51)	3 (8.57)	184(65.95)

Table 2a - Slaughter house study of bovines at Post-mortem examination

Total no. of bovines selected for study N = 2000	**AFB staining**	**Auramine staining**	**PCR**
Tuberculosis like lesions 318 (15.9)	24(1.2)	29(1.45)	18(0.9)
Emaciation 192 (9.6)	11(0.55)	13(0.65)	5(0.25)
No of bovines samples collected from 510 (25.5)	35(1.75)	42(2.1)	23(1.15)

(Number in parenthesis indicates per cent to total)

Table 2b - Prevalence of bovine tuberculosis based on post-mortem lesions in viscera

Kind of Tissue (N=318)	Tuberculosis like lesion	AFB staining	Auramine staining	PCR
Bronchial LN	48 (15.09)	4 (1.26)	5 (1.57)	3 (0.94)
Lung	104 (32.7)	7 (2.2)	8 (2.51)	6 (1.89)
Liver	36 (11.32)	2 (0.62)	2 0.62)	1 (0.31)
Multiple organ involvement (lung, bronhial LN/liver)	84 (26.42)	9 (2.83)	11 (3.45)	7 (2.20)
Mesentry	10 (3.15)	1 (0.31)	1 (0.31)	-
Spleen	23 (7.23)	-	-	-
Other organs (heart, mammary gland)	18 (5.66)	1 (0.31)	2 (0.62)	1 (0.31)
Total	318	24 (7.55)	29 (9.11)	18 (5.66)

(Number in parenthesis indicates per cent to total)

fine needle aspirates of pre-scapular lymph gland (PSLG), as it is considered an ideal ante-mortem sampling procedure to identify the infection status in animals.

Tissue samples were collected during post mortem examination of slaughtered bovines from organs with tuberculosis like lesions and lymph glands and organs of emaciated animals. The positivity of milk, fine needle aspirates of pre-scapular lymph gland (PSLG) and tissue samples (1224) from different systems viz. organized sector, unorganized sector and slaughter house is presented in Table 3a and 3b.

Out of 504 milk samples tested, 7 (1.39 per cent), 9 (1.79 per cent) and 18 (3.57 per cent) samples were positive for tuberculosis by acid-fast and auramine staining and PCR respectively. Out of the 210 PSLG aspirate samples tested, 10 (4.76 per cent), 13 (6.19 per cent) and 12 (5.71 per cent) samples were positive for tuberculosis by AFB, auramine staining and PCR respectively Out of the 510 tissue samples tested, 35 (6.86 per cent), 42(8.24 per cent) and 23(4.51 per cent) were positive for tuberculosis by AFB, auramine staining and PCR respectively (Table 3a). None of the 100 processed milk samples were found to be positive by PCR.

4.3.Status of bovine tuberculosis in different systems and demographics in bovines

4.3.1. Serological status by ELISA

The Enzyme Linked Immunosorbent Assay **(**IDEXX ELISA) was employed with 357 serum samples to identify the serological status of BTB in different systems, age groups and species of bovines (Table 4).

Statistical analysis revealed no significant difference ($P>0.05$), in the prevalence of tuberculosis between organized, unorganized sectors and slaughter house sample surveyed.

Table 3a -Utility of samples in establishing status of BTB in different systems

Samples surveyed		Origin of samples			Total (per cent)
		Organized Sector(N)	Unorganized Sector(N)	Slaughter house(N)	
Milk (N=504)	**AFB staining**	1(54)	6(450)	-	7(1.39)
	Auramine staining	1(54)	8(450)	-	9(1.79)
	PCR	0(54)	18(450)	-	18(3.57)
PSLG (N=210)	**AFB staining**	2(12)	8(198)	-	10(4.76)
	Auramine staining	2(12)	11(198)	-	13(6.19)
	PCR	0(12)	12(198)	-	12(5.71)
PM tissues (N=510)	**AFB staining**	2(3)	-	33(507)	35(6.86)
	Auramine staining	2(3)	-	40(507)	42(8.24)
	PCR	1(3)	-	22(507)	23(4.51)

Table 3b – Bovines sampled in the study

Study subjects	Milk	Both milk and PSLG samples	PSLG	PM tissues	Total
No of Bovines In the study	412	92	118	510	1132
No of samples from bovines in the study	504	210		510	1224

Table 4– Identification of the status of BTB in different systems, age and breed of bovines by ELISA

System	ELISA		
	Positive (%)	**Total**	**Chi-square test**
Organized sector	3(4.17)	72	NS
Unorganized sector	11(6.01)	183	
Slaughter house	2(1.96)	102	
Total	16(4.48)	357	
Age (years)			
≤ 5	2 (1.51)	132	4.31* P< 0.05
>5	14 (6.22)	225	
Total	16 (4.48)	357	
Bovines			
Non descript	2 (1.2)	171	10.92** P< 0.01
Cross bred	13 (9.6)	149	
Buffaloes	1 (2.8)	37	
Total	16 (4.48)	357	

However, on comparison of age groups, statistical analysis using chi-square test indicated a significant ($P<0.05$) difference and a highly significant ($P<0.01$) difference in the sero-positivity amongst the species studied viz. non- descript animals, cross bred cattle and buffalo (Table 4).

4.3.2. Polymerase chain reaction (PCR)

PCR was performed for the detection of MTBC organisms. Since molecular diagnosis is considered more specific to establish the presence of MTBC, the PCR results were attributed to analyze the status of bovine tuberculosis.

Statistical analysis revealed no difference ($P>0.05$), in the prevalence of tuberculosis between organized sector, unorganized sector and slaughter house samples and also body condition scoring of the animals (Table 5).

Statistical analysis using chi-square test indicated a highly significant ($P<0.01$) difference between the age groups and also among the species viz. non- descript animals, cross bred cattle and buffalo using the PCR positivity.

4.3.3. Post mortem examination

On detailed post mortem examination of 2000 cattle during the study period, 510 tissue samples were collected. Of this, three were from organized dairy unit and remaining 507 were from animals slaughtered in an abattoir. The sampling was from predominantly emaciated animals showing tuberculosis like lesions (Plates 3a and 3b). Out of 2000 animals, 318 (15.9 per cent) animals showed tuberculosis like lesions and 192 (9.6 per cent) were emaciated. Of the 100 sheep carcasses examined post-mortem lesions of tuberculosis were noticed in 35 (Plate 3c and 3d).

Among those with tuberculosis like lesions 24 (1.2 per cent), 29 (0.25 per cent) and 18 (0.9 per cent) were positive by acid-fast, auramine staining and PCR respectively. In the emaciated group 11 (0.55 per cent), 13 (0.65 per cent) and 5 (0.9 per cent) by acid-fast, auramine staining and PCR respectively were positive (Table 2a).

Table 5 – Identification of the status of BTB in different systems, age, breed and BCS of bovines by PCR

System	PCR		
	Positive (%)	**Total**	**Chi-square test**
OrganizedSector	1(1.47)	68	NS
Unorganized sector	30(5.4)	556	
Slaughter house	22(4.33)	508	
Total	53(4.68)	1132	
Age (years)			
≤ 5	9(2.33)	387	7.32** P< 0.01
>5	44(5.91)	745	
Total	53(4.68)	1132	
Bovines			
Non descript	10(1.82)	550	24.27** P< 0.01
Cross bred	36(8.55)	421	
Buffaloes	7(4.35)	161	
Total	53(4.68)	1132	
BCS (Body condition score)			
Poor	19(1.82)	311	NS
Medium	29(8.55)	625	
Good	5(4.35)	196	
Total	53(4.68)	1132	

It was observed that lesion in lungs (32.7 per cent) were found to be at a much higher level in comparison to other tissues like lymph nodes (15.09 per cent), liver (11.32 per cent), multiple organ involvement (26.42 per cent), mesentry (3.15 per cent), spleen (7.23 per cent) and other organs (5.66 per cent) (Table 2b).

Efficiency of antigen detection tests for bovine tuberculosis

4.3.4. Acid fast staining

Milk smears from 504 animals and fine needle aspirates of PSLG smears from 210 animals were subjected to acid-fast staining, of which seven (1.39 per cent) of milk and 10 (4.76 per cent) of PSLG aspirates were positive for mycobacteria. Acid-fast staining was also conducted on 510 tissue impression smears from slaughter house animals of which, 35 (6.86 per cent) were positive for mycobacteria (Table 1). Acid-fast were detected in 5 (14.29 per cent) and 142 (50.90 per cent) of 35 ovine and 279 human samples respectively (Plate 4a and 4b).

4.3.5. Auramine staining

Auramine staining was performed on 504 milk smears, 210 PSLG fine needle aspiration smears and 510 tissue impression smears. Mycobacteria were detected in nine milk smears (1.79 per cent), 13 PSLG fine needle aspirate smears (6.19 per cent) and 42 tissue impression smears (8.24 per cent) (Table 1). Auramine staining detected fluorescent bacilli in 7 (20.00 per cent) of 35 ovine samples and 169 (60.57 per cent) of 279 human samples (Plate 5a and 5b).

4.3.6. Culture

Isolation of *M. tuberculosis* and *M. bovis* were attempted on samples positive by AFB or auramine staining or PCR (Plate 6). Totally 70 samples were subjected for culture of which 37 samples were found to show growth which included 12 from milk, 11 from PSLG aspirates and 15 from tissue samples (Plate 6). Transmission Electron Microscopy (TEM) of an isolate of *M. bovis* (Plate 7) was carried out for further confirmation.

Polymerase chain reaction (PCR)

PCR was performed on DNA extracted from 504 milk samples, 210 PSLG fine needle aspirates and 510 tissue samples. The *IS6110* genus specific PCR detected positives in 18 milk samples (3.57 per cent), 14 PSLG fine needle aspirations (6.67 per cent) and 23 tissue samples (4.51 per cent) (Table 1) (Plate 8) of bovines while 3 (8.57 per cent) of 35 ovine and 184 (65.95 per cent) of 279 human samples were positive by PCR.

4.5. Comparative evaluation of different tests

4.5.1. Comparative evaluation of different tests of bovine tuberculosis

4.5.1.1. Comparison of acid-fast staining and Auramine staining

A total of 1224 samples from bovines were screened for tuberculosis by the two staining protocols, acid-fast and auramine staining. While auramine staining detected maximum number of positive samples (64), acid-fast detected only 52 as positive (Table 6).

On comparison of auramine staining with acid-fast staining, the former showed a sensitivity of 100 per cent and specificity of 98.98 per cent. Out of 52 samples detected as positive by acid-fast staining, no sample was negative by auramine staining and of 64 samples positive by auramine staining, 12 samples were negative by acid-fast staining. The concordance between these tests was 99.02 per cent with a kappa value-0.89 (Table 6).

Statistical analysis using chi-square test indicated a highly significant ($P<0.01$) difference between the tests (Table 6).

4.5.1.2. Comparison of acid-fast staining and PCR

On comparison of AFB with PCR on bovine samples, there was a sensitivity of 83.02 per cent and specificity of 99.49 per cent for AFB. Out of 53 samples detected as positive by PCR, nine samples were negative by AFB staining. Of the 52

Table 6 - Comparison of acid-fast and auramine staining in BTB

Test		Auramine staining		Total	Chi-square test	Sensitivity (%)	Specificity (%)	Concordance	Kappa value
		Positive	Negative						
AFB staining	Positive	52	0	52	**984.32****	100.00	98.98	99.02	0.8915
	Negative	12	1160	1172					
Total		64	1160	1224					

Table 7 - Comparison between acid-fast staining and PCR in BTB

Test		PCR		Total	Chi-squaretest	Sensitivity (%)	Specificity (%)	Concordance	Kappa value
		Positive	Negative						
AFB staining	Positive	44	6	50	**880.95****	83.02	99.49	98.77	0.8480
	Negative	9	1165	1174					

samples detected as positive by AFB staining, six samples were negative by PCR. The concordance between these tests was 98.77 per cent with a kappa value-0.85 (Table 7).

Statistical analysis using chi-square test indicated a highly significant ($P<0.01$) difference between the tests (Table 7).

4.5.1.3. Comparison of auramine staining and PCR

A comparison of PCR with auramine staining showed a sensitivity of 88.68 per cent and specificity of 98.72 per cent. Out of 53 samples detected as positive by PCR, 6 samples were negative by auramine staining. Of the 64 samples detected as positive by auramine staining, 15 samples were negative by PCR. The concordance between these tests was 98.28 per cent with a kappa value-0.8084 (Table 8).

Statistical analysis using chi-square test indicated a highly significant ($P<0.01$) difference between the tests (Table 8).

4.5.2. Comparative evaluation of different tuberculosis diagnostic tests in human subjects

4.5.2.1. Comparison of acid-fast staining and auramine staining

A total of 279 samples from human subjects were screened for tuberculosis by the above staining protocols (AFB and auramine staining). Auramine staining detected maximum number of positive samples (169), whereas, AFB detected only 142 as positive (Table 9).

On comparison of auramine staining with AFB staining, auramine staining showed a sensitivity of 82.84 per cent and specificity of 97.87 per cent. Out of 142 samples detected as positive by AFB staining, two samples were negative by auramine staining. Of 169 samples positive by auramine staining, 29 samples were negative by acid-fast staining. The concordance between these tests was 88.21 per cent with a kappa value-0.7588 (Table 9).

Table 8- Comparison of auramine staining and PCR in BTB

Test		PCR		Total	Chi-square test	Sensitivity (%)	Specificity (%)	Concordance	Kappa value
		Positive	Negative						
Auramine staining	Positive	47	15	62	**805.42****	88.68	98.72	98.28	0.8084
	Negative	6	1156	1162					
Total		53	1171	1224					

Table 9 - Comparison of acid-fast with auramine staining in TB of human samples

Test		Auraminestaining		Total	Chi-square test	Sensitivity (%)	Specificity (%)	Concordance	Kappa value
		Positive	Negative						
AFB Staining	Positive	140	2	142	158.41**	82.84	97.87	88.21	0.7588
	Negative	29	108	137					
Total		169	110	279					

Statistical analysis using chi-square test indicated a highly significant (P<0.01) difference between the tests (Table 9).

4.5.2.2. Comparison of acid-fast staining and PCR

PCR on comparison with acid-fast staining on human samples, showed a sensitivity of 76.09 per cent and specificity of 97.89 per cent. Out of 184 samples detected as positive by PCR, 44 samples were negative by acid-fast staining. Of the 142 samples detected as positive by acid-fast staining, two samples were negative by PCR. The concordance between these tests was 83.51 per cent with a kappa value-0.67 (Table 10).

Statistical analysis using chi-square test indicated a highly significant (P<0.01) difference between the tests (Table 10).

4.5.2.3. Comparison of auramine staining and PCR

On comparison of PCR with auramine staining, PCR showed a sensitivity of 90.22 per cent and specificity of 96.84 per cent. Out of 184 samples detected as positive by PCR, 18 samples were negative by auramine staining. Of the 169 samples detected as positive by auramine staining, 3 samples were negative by PCR. The concordance between these tests was 92.47 per cent with a kappa value-0.8386 (Table 11).

Statistical analysis using chi-square test indicated a highly significant (P<0.01) difference between the tests (Table 11)

4.6. Status of zoonotic tuberculosis

4.6.1. Status of zoonotic tuberculosis in animals

Differentiation of MTBC organisms by PCR had shown that of the 53 isolates from bovines, 48 were *M. tuberculosis* and only 5 were *M. bovis* whereas by culture of the 37 positives, 34 were *M. tuberculosis* and three *M. bovis*. The prevalence of *M. tuberculosis* was found to be 4.24 per cent and *M. bovis* was 0.44

Table 10 - Comparison of acid-fast staining with PCR in TB of human samples

Test		PCR		Total	Chi-square test	Sensitivity (%)	Specificity (%)	Concordance	Kappa value
		Positive	Negative						
AFB staining	Positive	140	2	142	137.21**	76.09	97.89	83.51	0.6684
	Negative	44	93	137					
Total		184	95	279					

Table 11 - Comparison of auramine staining with PCR in TB of human samples

Test		PCR		Total	Chi-square test	Sensitivity (%)	Specificity (%)	Concordance	Kappa value
		Positive	Negative						
Auramine staining	Positive	166	3	169	198.84**	90.22	96.84	92.47	0.8386
	Negative	18	92	110					
Total		184	95	279					

per cent in the bovine population (Table 12). All the three tissue isolates of ovines were also *M. tuberculosis* and the positive per cent was 8.57.

However, one chimpanzee lung nodule sample suspected for tuberculosis tested negative for AFB and auramine staining but positive for both *M. tuberculosis* and *M. bovis* by culture and PCR (Plate 11).

4.6.2. Status of zoonotic tuberculosis in humans

Differentiation of MTBC organisms by PCR and culture had shown that none of the human samples were positive for *M. bovis* except one fine needle aspiration biopsy sample which was acid- fast bacilli positive but culture negative and when subjected for *IS6110* PCR and multiplex PCR, it was positive for *M. bovis* (Plate 11). In humans out of 279 samples tested, *M. tuberculosis* and *M. bovis* were 183 (65.59 per cent) and one (0.36 per cent) respectively.

4.7. Sequence of events of *M. bovis* infection in a dairy cow

A cross bred cow in an organized dairy unit positive by *M. bovis* ELISA on herd examination for tuberculosis was followed up to understand the sequence of events in *M. bovis* infection since tuberculin test positive (Plate 12)

The skin test has shown that the animal was tuberculosis positive in June 2012 and the weight records between April 2012 and 2013 February showed progressive decline. ELISA was performed in July 2012 and again on September 2012 and on both occasions the animal was positive for BTB by ELISA.

Along with serum for ELISA, milk and PSLG aspirates were also collected and subjected for AFB, auramine staining, culture and PCR. None of the antigen detection method was able to detect MTBC organisms.

The cow died in quarantine in June, 2013. The post-mortem tissue samples included lung and lymph nodes subjected for acid-fast staining, auramine staining, culture and PCR were positive for *M. bovis*.

Table 12 - Status of zoonotic tuberculosis by Multiplex PCR

Test	Species tested		
	Bovine N=1132 (% positivity)	**Ovine N=35 (% positivity)**	**Human N=279 (% positivity)**
Mycobacterium tuberculosis	48 (4.24)	3 (8.57)	183 (65.59)
Mycobacteriumbovis	5 (0.44)	0 (0)	1(0.36)
Total	53 (4.68)	3 (8.57)	184(65.95)

The cow positive by skin testing with purified protein derivative (PPD) elicited a cell mediated immune response followed by progressive loss in body weight and an ELISA positive for humoral response as clinical progression of disease with a final confirmation on post-mortem tissue samples.

4.8. Sequencing and phylogenetic analysis

4.8.1. Nucleotide sequence of hypothetical protein region of *M. tuberculosis* and *M. bovis* field isolates

Nucleotide sequences of hypothetical protein 'Rv1506c' (accession no. Z79701) region of eight *M. tuberculosis* and six *M. bovis* field isolates are given in sequence identity plot (Figure. 12). Few field isolates were selected representing all the sample origin including three *M. tuberculosis* bovine milk isolates, two *M. tuberculosis* bovine tissue isolates, one *M. tuberculosis* chimp tissue isolate and two *M. tuberculosis* ovine tissue isolates. Among six sequenced *M. bovis* field isolates four were from bovine tissue origin, one from chimp tissue and one from human fine needle lymph node aspiration biopsy.

4.8.2. Sequence analysis

Different sequences of *M. tuberculosis* and *M. bovis* had been aligned using ClustalW with the software MEGA 5 (Tamura *et al.,* 2011) and similarity matrix had been constructed with Bioedit software to analyze the identity with the existing isolates in the genebank database.

Sequence identity plot of hypothetical protein 'Rv1506c' region of Sequence *M. tuberculosis* and *M. bovis* field isolates had shown similarity between them and with other existing isolates in genbank (Table 13a and 13b).

Sequence identity matrix of *M. tuberculosis* field isolates showed similarity varied from 100 to 97.7 per cent whereas *M. bovis* field isolates showed 100 per cent similarity between them and genbank isolates (Table 13c and 13d).

Table 13a – Sequence identity plot of hypothetical protein ‘Rv1506c’ region of *M. tuberculosis*

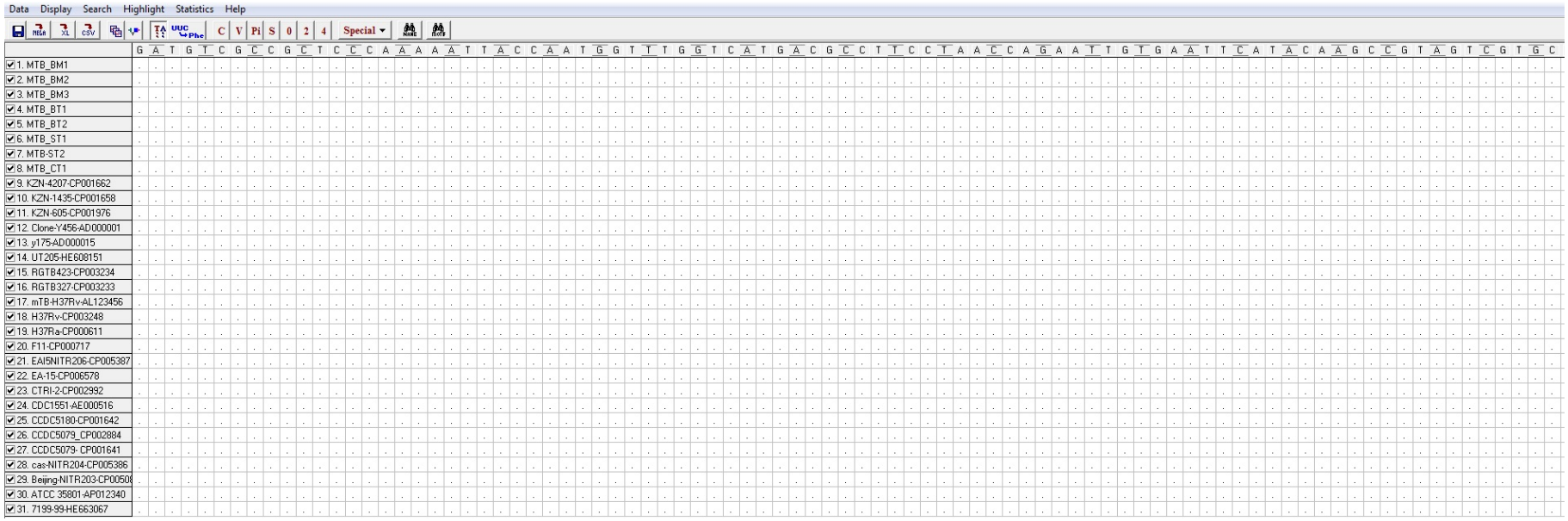

Table 13b – Sequence identity plot of hypothetical protein 'Rv1506c' region of *M. bovis*

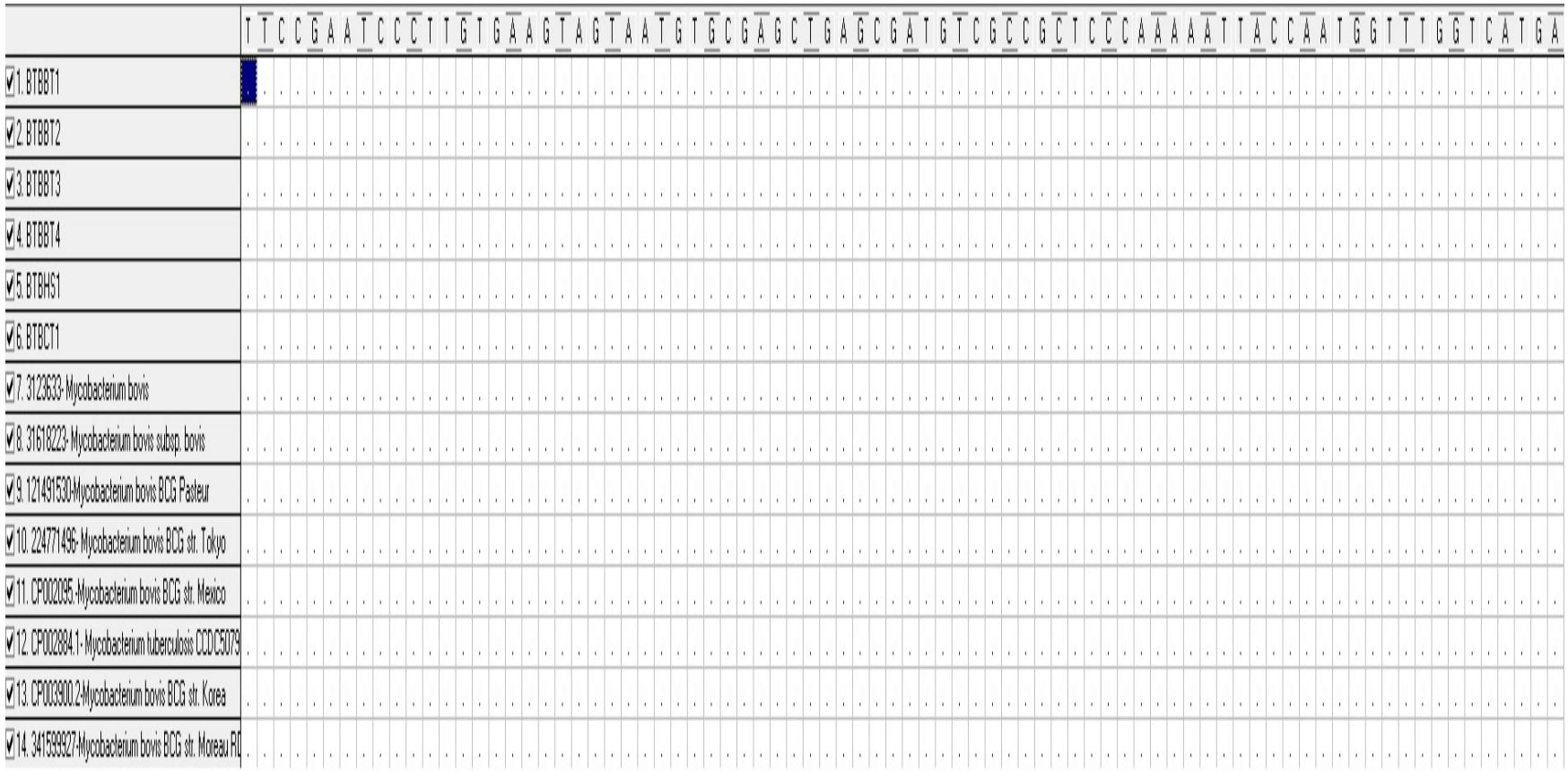

Table 13c – Sequence similarity matrix table of hypothetical protein ‘Rv1506c’ region of *M. tuberculosis*

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
	Seq->	MTB_BM1	MTB_BM2	MTB_BM3	MTB_BT1	MTB_BT2	MTB_ST1	MTB-ST2	MTB_CT1	KZN-4207-	KZN-1435-	KZN-605-(	Clone-Y45	y175-AD0(	UT205-HE(	RGTB423-(	RGTB327-(	mTB-H37R	H37Rv-CP(	H37Ra-CP(	F11-CP00(	EAI5NITR2	EA-15-CP(	CTRI-2-CP(	CDC1551-/	CCDC5180	CCDC5079	CCDC5079	cas-NITR2	Beijing-NI	ATCC 358(	7199-99-H
1	MTB_BM1	ID	0.996	0.992	0.984	0.992	0.996	0.973	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992
2	MTB_BM2	0.996	ID	0.996	0.981	0.988	1	0.977	0.988	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996
3	MTB_BM3	0.992	0.996	ID	0.981	0.992	0.996	0.977	0.988	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992	0.992
4	MTB_BT1	0.984	0.981	0.981	ID	0.988	0.981	0.973	0.992	0.981	0.981	0.981	0.981	0.981	0.981	0.981	0.981	0.981	0.981	0.981	0.981	0.981	0.981	0.981	0.981	0.981	0.981	0.981	0.981	0.981	0.981	0.981
5	MTB_BT2	0.992	0.988	0.992	0.988	ID	0.988	0.977	0.996	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988
6	MTB_ST1	0.996	1	0.996	0.981	0.988	ID	0.977	0.988	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996	0.996
7	MTB-ST2	0.973	0.977	0.977	0.973	0.977	0.977	ID	0.981	0.977	0.977	0.977	0.977	0.977	0.977	0.977	0.977	0.977	0.977	0.977	0.977	0.977	0.977	0.977	0.977	0.977	0.977	0.977	0.977	0.977	0.977	0.977
8	MTB_CT1	0.992	0.988	0.988	0.992	0.996	0.988	0.981	ID	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988	0.988
9	KZN-4207-	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	ID	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
10	KZN-1435-	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	ID	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
11	KZN-605-(	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	ID	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
12	Clone-Y45	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	ID	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
13	y175-AD0(	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	ID	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
14	UT205-HE(	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	ID	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
15	RGTB423-(	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	1	ID	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
16	RGTB327-(	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	1	1	ID	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
17	mTB-H37R	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	1	1	1	ID	1	1	1	1	1	1	1	1	1	1	1	1	1	1
18	H37Rv-CP(	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	1	1	1	1	ID	1	1	1	1	1	1	1	1	1	1	1	1	1
19	H37Ra-CP(	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	1	1	1	1	1	ID	1	1	1	1	1	1	1	1	1	1	1	1
20	F11-CP00(	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	1	1	1	1	1	1	ID	1	1	1	1	1	1	1	1	1	1	1
21	EAI5NITR2	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	1	1	1	1	1	1	1	ID	1	1	1	1	1	1	1	1	1	1
22	EA-15-CP(	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	1	1	1	1	1	1	1	1	ID	1	1	1	1	1	1	1	1	1
23	CTRI-2-CP(	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	1	1	1	1	1	1	1	1	1	ID	1	1	1	1	1	1	1	1
24	CDC1551-/	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	ID	1	1	1	1	1	1	1
25	CCDC5180	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	ID	1	1	1	1	1	1
26	CCDC5079	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	ID	1	1	1	1	1
27	CCDC5079	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	ID	1	1	1	1
28	cas-NITR2	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	ID	1	1	1
29	Beijing-NI	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	ID	1	1
30	ATCC 358(	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	ID	1
31	7199-99-H	0.992	0.996	0.992	0.981	0.988	0.996	0.977	0.988	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	ID

Table 13d – Sequence similarity matrix table of hypothetical protein 'Rv1506c' region of *M. bovis*

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Seq->	BTBBT1	BTBBT2	BTBBT3	BTBBT4	BTBHS1	BTBCT1	3123633- N	31618223-	12149153C	224771496	CP002095.	CP002884.	CP003900.	341599927
1	BTBBT1	ID	1	1	1	1	1	1	1	1	1	1	1	1	1
2	BTBBT2	1	ID	1	1	1	1	1	1	1	1	1	1	1	1
3	BTBBT3	1	1	ID	1	1	1	1	1	1	1	1	1	1	1
4	BTBBT4	1	1	1	ID	1	1	1	1	1	1	1	1	1	1
5	BTBHS1	1	1	1	1	ID	1	1	1	1	1	1	1	1	1
6	BTBCT1	1	1	1	1	1	ID	1	1	1	1	1	1	1	1
7	3123633- N	1	1	1	1	1	1	ID	1	1	1	1	1	1	1
8	31618223-	1	1	1	1	1	1	1	ID	1	1	1	1	1	1
9	12149153C	1	1	1	1	1	1	1	1	ID	1	1	1	1	1
10	224771496	1	1	1	1	1	1	1	1	1	ID	1	1	1	1
11	CP002095.	1	1	1	1	1	1	1	1	1	1	ID	1	1	1
12	CP002884.	1	1	1	1	1	1	1	1	1	1	1	ID	1	1
13	CP003900.	1	1	1	1	1	1	1	1	1	1	1	1	ID	1
14	341599927	1	1	1	1	1	1	1	1	1	1	1	1	1	ID

4.9. Spoligotyping

Select isolates genetically identified by culture and PCR as of the MTBC were spoligotyped for further strain characterization. Spoligotype patterns of all MTBC isolates were analysed using SPOTCLUST (http://www.rpi.edu/_bennek/EpiResearch).

M. tuberculosis strains isolated in this study from bovines belonged to the Manu 1, U, EA15, EA13 and Beijing lineages, where one of the ovine isolates matched with Manu 1. One bovine isolate and one ovine isolate did not match with any of the *M. tuberculosis* types reported in human. The *M. bovis* isolates from human and Chimp matched with Bovis 1 type (Plate 13).

4.10. Risk factors

The results of the binary logistic regression analysis factors associated with occurrence of tuberculosis infection in human beings in the variables presumed to be the determinants in human beings, *viz*., association with animals, raw milk consumption, family member with tuberculosis, age, education and socio-economic status were found to be statistically significant when all the data points were considered (Table 14a).

The reliability of the usage of the logit model for the correct prediction of these factors affecting the occurrence of tuberculosis infection in human beings was tested by comparing the observed and predicted values. The percentage of correct prediction of factors affecting the occurrence of tuberculosis infection in all 279 human beings was 71.48 per cent which indicates that the model was successful in predicting the factors 71.48 per cent correctly (Table 14b).

Table 14a - Risk factors affecting the occurrence of TB in human beings

X_i	Variables	Co-efficient B	S.E	Wald statistic	Significance
X_1	Animal association	1.592	0.653	5.953	0.015*
X_2	Raw milk consumption	4.863	1.261	14.863	0.000**
X_3	Family members with TB	-1.670	0.591	7.985	0.005**
X_4	Age	-0.562	0.249	5.083	0.024*
X_5	Education	-1.905	0.582	10.708	0.001**
X_6	Socio economic status	-9.040	3.096	8.526	0.004**
Chi-square value- 71.481					
Dependant variable- Occurrence of tuberculosis infection					
** - P < 0.01, * - P < 0.05					

Table 14b - Predictable percent of values

Observed			Predicted		
			TB		Percentage Correct
			No	Yes	
Step 1	TB	No	38	41	48.1
		Yes	9	142	94.0
	Overall Percentage				78.3

The cut value is .500

DISCUSSION

5. DISCUSSION

Amongst the various zoonoses tuberculosis is considered important since both Mycobacterium tuberculosis and Mycobacterium bovis, members of Mycobacterium tuberculosis complex (MTBC) are major causes and highly pathogenic, infecting many animal species which are likely to be the source of infection for humans.

This study attempts to summarize available epidemiological information, examine risk factors that can influence occurrence of zoonotic tuberculosis employing standard conventional protocols including molecular techniques.

5.1. Overall status of tuberculosis in the study population

The geographical distribution of tuberculosis in animals and human populations tend to vary and reported to be slightly higher in developing countries. While *M. tuberculosis* is known to be the cause of human tuberculosis, the other member of MTBC, *M. bovis* is important in cattle (Ayele *et al.,* 2004).

Animal tuberculosis is a public health concern as it bears a zoonotic potential (Renwick *et al.,* 2007) and in some countries, *M. bovis* may account for 5 to 10per cent of human tuberculosis cases (de Kantor *et al.,* 2008). In this study, status of tuberculosis in the study subjects (Bovines, Ovines and Humans) relied on laboratory testing of 1224 samples and confirmation to detect the infected animals.

In this study, the overall status of tuberculosis by the standard protocols including slaughter house surveys ranged from 4.33 to 5.39 in bovines (Table 1), while in the human subjects, who were actually suspected or confirmed cases of tuberculosis it ranged between 50 - 65 per cent (Table 1).

Overall the presence and extent of bovine tuberculosis in the developing world has been poorly investigated. However, the prevalence levels are in agreement with the observations in this study (Demelash *et al.,* 2009, Mekibeb *et al*, 2013, Srivastava *et al*, 2008). There is increasing evidence that *M. bovis* infections become

more significant as zoonosis (Shitaye *et al* 2007; Moda *et al* 1996; Cosivi *et al* 1998) and alternatively *M. tuberculosis* of humans has also been isolated from animals (Cadmus *et al* 2006; Jenkins *et al* 2011). The primary objective in this study was to identify zoonotic tuberculosis due to *M. bovis* and evaluate the extent of the potential sources of *M. bovis* infections in humans and an attempt to identify the overall status of tuberculosis in animals and humans and analyse the prevalence based on the merits and demerits of the various protocols employed including the differentiation of MTBC members involved.

Test for humoral responses employing the ELISA revealed a sero prevalence of 4.48 percent in the study population of bovines (Table 4). The ELISA system in the study was used as an ancillary in vitro assay for bovine tuberculosis though it is not universally adopted as a reliable and cost-effective sero-diagnostic test for routine diagnosis tuberculosis in cattle, other farm animals and humans (Cousins, 2001). However, it is stated that a certain proportion of anergic cattle can be detected by serological ELISA always (Placket *et al* 1989; Yearsley *et al* 1998). Further tuberculosis being a spectral disease in cattle, there is a balance of host immune response shifting from predominantly cell-mediated to humoral as the disease progresses (Rittaco *et al* 1991; Pollock and Neill 2002; Vordermeier *et al* 2004; Welsh *et al* 2005) and hence ELISAproved to be an ideal tool in this study to correlate with the pathogenic progress.

5.2. Utility of samples in establishing status of bovine tuberculosis in different systems

Investigations have pointed out the risk of human infection through consumption of unpasteurized or raw milk (Cotter *et al*, 1996; Bonsu *et al* 2000). Milk was the choice of sample to identify its potential as a route of transmission of zoonotic tuberculosis. Further, it is a non-invasive and easy method to collect and process the samples for large scale screening. While Srivatsava *et al* (2008) pointed out the usefulness of milk sample in skin test negative animals, Zarden *et al* (2013) highlighted that it is a sample of choice for bacteriological culture.

But among the samples analysed, PSLG is recommended by Prasad *et al* (2005) as the most suitable specimen for a confirmatory diagnosis particularly for isolation purposes (Table 3a). However, lymph glands, pus, liver, lung and milk can also show relatively higher isolation (Shah *et al.,* 2002; Ayele *et al.,* 2004; Grange *et al.,* 1996).

During our survey, macroscopic lesions were found in lung, liver, mediastinum, spleen and heart while Teklul *et al.,* (2004) found 84 percent visible lesions in lungs, Tadayon *et al.,* (2008) observed no gross lesion suggestive of bovine tuberculosis in the lungs and Cleaveland *et al.,* (2007) mainly found lesions in the GI tract. Hence in this study, the samples were collected based on the distribution of tuberculous-like lesion in various organs to increase the possibility of a confirmation.

The processed milk samples were negative by polymerase chain reaction (PCR) for the presence of MTBC organisms, which indicates the samples may be actually negative or the AL-PEG method has to be standardized further to prove its utility for DNA extraction from milk samples for Mycobacterium species.

5.3. Status of bovine tuberculosis in different systems and demographics in bovines

The positive results of the study were plotted against certain demographic profiles of the study population focusing on animals. The results of sero-positivity by ELISA, PCR and slaughter house surveys were examined against different system of rearing, age and breed.

A highly significant difference was established in all the assays (ELISA, PCR and Post mortem) in relation to age group and species of bovines.

Higher percentage (6.22 per cent) of animals in the age group above 5 years was sero-positive compared with the group which were 5 years and below (1.51 per cent) (Table 4). This could be due to a relatively long time stay in the herd for older animals that facilitates infection and subsequent advancement of the disease. Results

of our study are in agreement with the findings of Lightbody *et al*. (2000) suggesting the advantage of ELISA in identifying animals in advanced stages of bovine tuberculosis.

A higher proportion of tuberculosis was observed by PCR in animals above 5 years (Table 5) and this is again in agreement to several findings (Mekibeb *et al*, 2013; Regassa *et al* 2010; Dinka and Duressa, 2011; Khan *et al*, 2008). Older animals are likely to have a waning protective capability (O'Reilly and Daborn 1995) with increased possibilities of encountering *M. bovis* over a longer period (Barwinek and Taylor, 1996).

Differences in the seropositivity among different breeds included in the present study were compared and out of 357 animals, 1.2 per cent, 9.6 per cent and 2.8 per cent of animals in the category of non-descripts, Jersey crosses and buffaloes respectively were positive by ELISA (Table 4).

Genetic resistance is to be conclusively demonstrated in case of bovine tuberculosis, however Zebu type of cattle are thought to be much more resistant compared to cross-bred cattle (Vordermeier *et al*, 2012) as was observed in this study where non-descripts showed a lower prevalence compared to cross-breds (Table 4 and 5). The possible reason attributed is restricted movement of cross-bred cattle.

There was no significant difference in ELISA and PCR in relation to system of rearing (Table 3a, 4 and 5) and in PCR with respect to body condition scoring (Table 5).

The results of ELISA showed that a total of 16 samples (4.48 per cent) including three from the organized and 11 from unorganized dairy units were positive (Table 4). The reason for higher number of positive animals in the unorganized set up was confinement of animals in small areas as these were small holder dairy units which possibly contributed to the transmission of BTB between herd mates. Selvam (2009) similarly employing the i-ELISA reported 8.5 per cent (BCG-ELISA) and 13 per cent (PPD-ELISA) sero-positivity from the same organized group using the two different ELISA systems.

The absence of significant difference in infection rates despite differences in body condition scores in the study is consistent with previous reports from Ethiopia (Gumi *et al.,* 2011) and Pakistan (Khan *et al.,* 2008) and this may indicate the presence of other confounding factors in the environment such as over-crowding, poor ventilation and undernourishment.

In the post mortem examination of slaughtered bovines, 15.9 percentage of animals exhibited tuberculous-like lesion in various organs (Table 2a). Post mortem is a regular surveillance method for identifying the status of bovine tuberculosis and routine abattoir inspections combined with detailed abattoir meat inspections have been widely applied in surveillance programmes (Aylate *et al.,* 2013; Mekibeb *et al*., 2013; Pereza *et al* 2011). The percent positivity of tuberculosis-like lesions in this study has been found to be quite high in comparison to several other abattoir inspections (Aylate *et al.,* 2013; Mekibeb *et al*., 2013). The distribution of tuberculosis-like lesions in this study was higher in the lungs which were in agreement with several similar surveys (Aylate *et al.,* 2013; Tigre *et al.,* 2010; Regassa *et al* 2010). This is more likely since organs rich in reticulo-endothelial tissue particularly the lungs and associated lymph nodes are most often affected with tuberculosis like lesions as suggested by Corner (1994).

5.4. Efficiency of antigen detection tests for bovine tuberculosis

5.4.1. Acid-fast staining

Staining by microscopy is known to have low sensitivity as it requires 5000 to 10000 bacilli per ml of sample to yield a positive result (Pereza *et al*, 2011) and this explains the low prevalence level in bovine samples. In ovine samples, the reason for high prevalence could be attributed to less sample size. In addition, microscopic detection of mycobacterium is insensitive since it does not permit the identification of mycobacterial species.

5.4.2. Auramine staining:

Auramine staining is considered to increase the sensitivity and decrease the time for sputum smear examination compared to Ziehl-Neelsen and light

microscopy (Steingart *et al.,* 2006). Hence such low cost light-emitting diode (LED) microscopes have facilitated the expansion of fluorescent microscopy into a cost effective exercise in human tuberculosis diagnosis. This study has employed the auramine staining and found it to be quite superior to Ziehl-Neelsen staining possibly because the lower limit of detection for auramine staining is 10^4 cfu per ml (Hendry *et al.,* 2009) as against acid fast bacilli (AFB) which requires 10^5 cfu per ml of sample (Isaac, 2013).

5.4.3. Culture

Culture is still considered as the gold standard for confirmation of mycobacteria though it does not allow differentiation between distinct mycobacterial species and it is time consuming. Hence in the present study samples positive by staining and PCR were inoculated on Lowenstein-Jensen slants and observed for growth for a minimum of 8 weeks.

Out of 70 samples, 37 (52 percent) were positive by culture. This positivity is slightly on the higher side compared to Mekibeb *et al.* (2013) and Gumi *et al*. (2012). As AFB and PCR positive specimens were alone employed for isolation and it was expected that a higher percentage should have been isolated. But the lower level of positivity by culture in this study can be attributed to non-viable bacteria which could have been detected by PCR. Further, overgrowth with other non-tuberculous environmental mycobacteria could have suppressed the MTBC organism in culture leading to lower isolation.

Transmission electron microscopy (TEM) is a very useful method to investigate detailed ultrastructure of mycobacterial species. When suspended in phosphate buffered saline as done in this study TEM image of *M. bovis* showed a rod shaped bacilli, 2.5 µm long and 0.6 µm wide and an envelope consist of inner layer, middle layer, outer layer and plasma membrane. This data is consistent with the TEM images of Uenishi *et al*. (2009) and according to the previous reports, these might correspond to peptidoglycan, arabinogalactan and mycolic acid, and outer layers (Morioka *et al.,* 1987; Bloom, 1994; Klegerman *et al.,* 1996; Devadoss *et al.,* 1991; Hoffmann *et al.,* 2008).

5.4.4. PCR

PCR based techniques targeting regions of mycobacterial genome such as *IS 6110* have proved to be useful at the genus level for MTBC identification and PCR has gained acceptance because of its enhanced sensitivity (Sankar *et al.,* 2011; El Tigani *et al.,* 2013). However, targeting one genome sequence *IS 6110* may not be sufficient for specifying the MTBC species. The tuberculosis status identified by the PCR was 4.1 percent in bovines and 8.5 percent in ovine samples in this study. These results could really highlight the prevalence status of tuberculosis in these animals. Contrary to this de Araújo *et al*. (2005) and Pereza *et al*. (2011) found a lower detection rate by PCR in suspected animals and they attribute this to different factors that influence nucleic acid amplification such as insufficient quantity of bacilli or presence of inhibitors.

Human tuberculosis predominantly is caused by *M. tuberculosis*, but infection due to *M. bovis* is being increasingly documented (Bouvet *et al.,* 1993). PCR has now become an ideal tool to differentiate *M. tuberculosis* from *M. bovis*. The present study employed *IS 6110* for genus specific diagnosis and a multiplex PCR (Shah *et al.*, 2002) to differentiate *M. bovis* from *M. tuberculosis*.

In animals, the *IS 6110* results ranged between 4.51 and 8.57 percent, while the multiplex PCR differentiated *M. tuberculosis* from *M. bovis*. It was observed that in majority of animal samples, more percentage of *M. tuberculosis* and not *M. bovis* as anticipated was observed (Table 12). This multiplex PCR is a rapid and easy differential technique for identification and differentiation of *M. tuberculosis* and *M. bovis*. This PCR assay was also employed on human subjects and here *M. tuberculosis* positivity was higher.

PCR, therefore, allows to identify *M. bovis* or *M. tuberculosis* in any biological specimen without previous culture of human or animal samples (Cousins *et al.,* 1991; Lie´Bana *et al* 1995).

5.5. Comparative efficacy of different tests:

The staining protocols for AFB and auramine staining revealed almost uniform percentage positivity in comparison to PCR with the bovine samples (Table 7,8,10,11), while the ovine samples revealed relatively higher percentage positivity than the PCR. Since the human subjects were from known or suspected tuberculosis cases, the percentage positivity was over 50 percent by all the above testing methods in this study.

Characterisation of mycobacteria with conventional staining protocols based on phenotypic features does not allow for precise diagnosis in contrast to molecular techniques (Cook *et al.,* 2003). Hence PCR which is a very sensitive and specific technique in diagnosis of such fastidious bacteria is found to be more sensitive in the present study in agreement with several other workers (Medeiros *et al.,* 2010; Mishra *et al.,* 2005). The use of PCR allows the identification of specific sequence of MTBC and further differentiation (Medeiros *et al.,* 2010).

Auramine staining is found to be more sensitive than Ziehl-Neelsen for microscopic detection of human sputum smear (Marais *et al.,* 2008) (Table 6, 9).

5.6. Zoonotic tuberculosis

Intensification of animal – human contact provides the basis for possible cross-over and increased risk of transmission both ways (animal-human-animal) of *M. tuberculosis* and *M. bovis* at the interface. This has been clearly observed in this study where a higher percentage of positivity was noted for *M. tuberculosis* infection in animals (Table 12). The occurrence of *M. tuberculosis* is an example of reverse zoonosis (Grange, 2001; Grange and Yates, 1994; Fritsche *et al.,* 2004) and these animals potentially constitute a grave public health hazard as virulent bacilli can be transmitted to humans.

One positive isolate of *M. bovis* in human sample might be a case of BCG adenitis considering the clinical findings, age of the patient and absence of animal association or raw bovine milk consumption.

The mixed infection in chimp in this study indicates primates as an active source of tuberculosis infection for zoo attendants and veterinarians and also animals may pick up infection from humans. This invites a detailed study in more captive animals to know the exact status of tuberculosis in them.

Though the major objective of this study was to identify the extent of *M. bovis* in humans, *M. tuberculosis* infection established was at a higher level in the animal population studied. Similar findings earlier have identified 15 to 28.5 per cent of animals to be infected with *M. tuberculosis* (Prasad *et al.,* 2005; Srivastava *et al.,* 2008; Jenkins *et al.,* 2011). Besides being potential public health hazard as stated above, these animals can also serve to provide an environment for transmission of drug resistant *M. tuberculosis*.

5.7. Sequence of events of *M. bovis* infection in a dairy cow

The pathogenesis of bovine tuberculosis is not as well understood as the pathogenesis of tuberculosis in humans. The current discussion will deal primarily with studies in a dairy cow.

In this case of study though the route of infection is unclear with some exceptions, it is agreed that cattle become infected with *M. bovis* by either the oral or respiratory routes (Neill *et al.,* 1994). The oral route is most important in calves nursing tuberculous cows, while the respiratory route is most common in cattle in general (Neill *et al.,* 1994). In this study both routes are likely to be possible, judging from the distribution of lesion in this cow on post-mortem. Surveys of tuberculosis cattle in Great Britain (Phillips *et al.,* 2003) revealed that 67 per cent of tuberculosis lesions were within the lungs and pulmonary lymph nodes (tracheobronchial and mediastinal). The conclusion is that the distribution of lesions suggests a respiratory route of infection, as it has been stated that the route of transmission of *M. bovis* can be deduced by the pattern of lesions observed in the infected animal (Biet *et al.,* 2005). However investigations in naturally infected cattle suggest that lesions of the alimentary tract, although not common generally, are more common in temperate climates where conditions favour dissemination and

survival of *M. bovis* on forage, while respiratory tract lesions are more common in arid climates where conditions favour aerosolization of *M. bovis* (Lepper and Pearson, 1973) as was in this case.

During the clinical progression of the disease it was observed that the body condition score was not affected and progressive loss of body weight was observed only a few months before death. This is in agreement to the study by Dohorty *et al*. (1996), where *M. bovis* infected cattle did not show change in body weight and were apparently healthy clinically during their large part of their study.

Studies on field cases of *M. bovis* infection have indicated that cell-mediated immune responses (CMI) predominate early in the infection. This animal was positive for CMI response and high antibody titre against *M. bovis* almost simultaneously, whereas antigen detection was negative when it was in quarantine. Knowledge of the immune responses which develop in cattle following infection with *M. bovis* is essential both to the understanding of disease pathogenesis and to the logical development of immune-dependent tools, such as diagnostic tests (Vordermeier *et al* 2004; Welsh *et al.,* 2005).

Whilst there are certain aspects of *M. bovis* infection that still remain elusive, further studies on the pathogenesis of bovine tuberculosis are advocated to provide a better scientific basis on which to review control and eradication strategies, which are currently less than effective in many regions.

5.8. Sequence analysis

Sequence analysis plot and sequence similarity matrix of *M. tuberculosis* and *M. bovis* field isolates and genbank isolates had shown the difference in sequences between isolates vary from 0 to 2.3 per cent (Table 13a, 13b, 13c and 13d).

As the product sequenced is of minimal size (262 bp and 168 bp) and it is not a coding sequence, this sequence cannot be used for differentiation other than its diagnostic value.

5.9. Spoligotyping

In the present study, a few selected (18) *M. tuberculosis* isolates from bovines, ovines and a chimp were spoligotyped as it is very often used in molecular epidemiology of MTBC as well as in species and strain differentiation.

This mode of differentiation is particularly relevant for phylogeography. Using the technique will help elucidating the extent of spread of MTBC and clear speculations with regards to risk factors.

The spoligopatterns of the bovines, ovines and a chimp isolates showed the following lineages: Manu1, U, EA15, EA13 and Beijing. While the lineages from various isolates were almost similar, two of them showed a spoligopattern that did not match with any existing pattern in the database (orphan) (Plate 13).

This study indicates a spillover of the common spoligotypes from humans to animals which may lead to further dissemination to humans from these animal populations. Bovines, ovines and a chimp *M. tuberculosis* isolates having similar spoligopattern with human types suggests origin from human source.

In a similar such study by Jenkins *et al.,* (2011), *M. tuberculosis* was isolated from a bovine, a pig and a goat and spoligotyped which strongly supported the possibility of human to livestock transmission (Cadmus *et al.,* 2006). Further epidemiological studies using spoligotyping on human and livestock isolates particularly at the animal-human interface will provide great information on the transmission dynamics of *M. tuberculosis* and *M. bovis* infections.

5.10. Risk factors

Risk factors for zoonotic tuberculosis were also analyzed with database created through questionnaire survey. A number of variables were considered to establish risk factors for zoonotic tuberculosis in human beings. An interview schedule of human tuberculosis cases found significant association between tuberculosis and animal association including consumption of raw milk (Table 14a). Though there are

contrasting reports on this association (Ameni *et al*, 2002) not being very significant, Cook *et al.* (1996) and Ameni *et al.* (2002) have identified statistically significant association between human tuberculosis and animal tuberculosis reactors.

Kleeberg (1984) had indicated that a single cow with tuberculous mastitis can excrete enough viable tubercle bacilli to contaminate bulk milk of hundred cows and these organisms can be found in milk products also. Hence raw milk consumption as was observed in many of the tuberculosis patients in this study could possibly provide for an infection source. This observation also highlights that even if there is no direct animal association, the pooling of milk from various sources can also be an important risk factor for consumers.

There was again highly significant association between socio-economic status and tuberculosis infection in this study (Table 14a). However, this cannot be considered a true reflection as many of the patients surveyed in this study were from a lower socio-economic status. Ameni *et al.* (2002) had identified such a similar association linking a larger proportion of human tuberculosis in rural parts, and attributed this association to poor awareness levels of the human subjects in these districts which can be equated to the lower socio-economic status in this study.

Furthermore, detailed studies on such associations are needed to assess and evaluate the level of the problem of zoonotic tuberculosis. Since in this study there appears to be a higher number of isolations of *M. tuberculosis* in animals in comparison to *M. bovis*. The pattern of animal isolates need to be matched with human isolates to establish transmission from humans to animals and vice versa. Until this is established *M. tuberculosis* infection in animals from human sources will remain a hypothesis.

In conclusion, the varied approaches in establishing the significance and impact of MTBC in animal and human subjects studied may provide opportunities to understand whether this is just spill-over phenomenon or holds promise for epidemiologic and / or pathogenic relevance in future.

SUMMARY AND CONCLUSION

6. SUMMARY AND CONCLUSION

The present study was attempted to identify the status of zoonotic tuberculosis in particular, in different species including bovines, ovines and humans. The Postgraduate and Research Institute in Animal Sciences (PGRIAS), TANUVAS, Kattupakkam was the organised dairy unit chosen for the study where regular herd based survey for bovine tuberculosis was conducted and unorganised small holder dairy units sampled included cattle and buffaloes from Large Animal Clinics (LAC), Madras Veterinary College (MVC) Teaching Hospital, Chennai (urban setup) and representative samples from Dharmapuri and Krishnagiri districts (rural category). The human subjects for the study were chosen from Thiruwatteeswarar hospital for thoracic diseases, Chennai.

The bovine samples were collected from apparently healthy animals and animals with tuberculosis like lesions or emaciated condition at post-mortem (PM) or at slaughter. The bovine samples included 504 milk, 210 pre-scapular lymph gland (PSLG) fine needle aspirates and 510 PM tissue samples. 357 sera samples from bovines and 35 tissue samples from ovines and 100 processed milk samples were also screened for MTBC organisms. In the human subjects, 279 samples including 269 sputum, five lung aspirates, three lymph gland fine needle aspirates and two urine samples were collected from suspected and confirmed cases of tuberculosis.

The diagnostic tests employed were staining for acid fast bacilli (AFB), auramine staining, culture, genus specific polymerase chain reaction (PCR) to identify the Mycobacterium tuberculosis complex (MTBC) and multiplex PCR to differentiate *Mycobacterium tuberculosis* and *Mycobacterium bovis* including spoligotyping and sequence analysis of isolates. Risk factors for zoonotic tuberculosis were also analyzed with database created through questionnaire survey.

While AFB and auramine staining detected 4.33 per cent and 5.39 per cent respectively in bovines, PCR detected 4.51 per cent positivity on screening 1224 samples. Ovines showed per cent positivity of 14.29 and 20.00 for AFB and by

auramine staining respectively while PCR identified 8.57 per cent positivity by screening of 35 samples. The positive percentage was 50.90, 60.57 and 65.95 in human subjects for AFB, by auramine staining and PCR respectively and the study population included actually suspected or confirmed cases of tuberculosis. The geographical distribution of tuberculosis in animals and human populations tend to vary and reported to be slightly higher in developing countries. The prevalence levels are in agreement with the global observations.

In a routine slaughter house examination of 2000 slaughtered heads of cattle examined during the study, 15.9 per cent were diagnosed with gross tuberculous like lesions in various organs and of 100 sheep examined at slaughter 35 per cent showed gross tuberculous like lesions.

The humoral antibody response was 4.48 per cent in bovine serum by ELISA in a representative unit of dairy cattle indicating progress in clinical tuberculosis. Results also suggest the advantage of ELISA in identifying animals in advanced stages of bovine tuberculosis as TB is a spectral disease in cattle and there is a balance of host immune response shifting from predominantly cell-mediated to humoral as the disease progresses.

Out of 504 milk samples tested, 7 (1.39 per cent), 9 (1.79 per cent) and 18 (3.57 per cent) samples were positive for tuberculosis by acid-fast, auramine staining and PCR respectively and of the 210 PSLG aspirate samples tested, 10 (4.76 per cent), 13 (6.19 per cent) and 12 (5.71 per cent) samples were positive for tuberculosis for AFB, auramine staining and PCR respectively. Out of the 510 tissue samples tested, 35 (6.86 per cent), 42(8.24 per cent) and 23(4.51 per cent) were positive for tuberculosis for AFB, by auramine staining and PCR respectively. None of the 100 processed milk samples were found to be positive by PCR for any of the MTBC organisms. Milk was the choice of sample as it is a non-invasive and easy method, but among the samples analysed, PSLG is recommended as the most suitable specimen for a confirmatory diagnosis.

Comparative evaluation of different tests used for diagnosis of bovine tuberculosis and human tuberculosis by statistical analysis using chi-square test indicated a significant difference between the tests (AFB and auramine staining, auramine staining and PCR and AFB and PCR).

Auramine staining is considered to increase the sensitivity and decrease the time for sputum smear examination compared to Ziehl-Neelsen. Culture is still considered as the gold standard for confirmation of mycobacteria though it does not allow differentiation between distinct mycobacterial species and it is time consuming. PCR based techniques targeting regions of mycobacterial genome such as *IS 6110* have proved to be useful at the genus level for MTBC identification. It was observed that in majority of animal samples, more percentage of *M. tuberculosis* and not *M. bovis* as anticipated was observed and multiplex PCR is a rapid and easy differential technique for identification and differentiation of *M. tuberculosis* and *M. bovis*. Characterisation of mycobacteria with conventional staining protocols based on phenotypic features does not allow for precise diagnosis in contrast to molecular techniques and hence PCR which is a very sensitive and specific technique in diagnosis of such fastidious bacteria is found to be more sensitive in the present study

Statistical analysis using chi-square test indicated a highly significant difference between the age groups and also among the species studied *viz*. non-descript animals, cross bred cattle and buffalo using both the ELISA and PCR positivity.

On routine post-mortem examination of 2000 bovine carcasses at slaughter/death, 510 samples which included 3 from organized and 507 from slaughter survey were collected from animals with emaciation and tuberculosis like lesions. Out of the 2000 animals, 318 (15.9 per cent) animals showed tuberculosis like lesions and 192 (9.6 per cent) were emaciated.

Among those with tuberculosis like lesions 24 (1.2 per cent), 29 (0.25 per cent) and 18 (0.9 per cent) were positive for AFB, by auramine staining and PCR respectively. In the emaciated group 11 (0.55 per cent), 13 (0.65 per cent) and 5 (0.9

per cent) for AFB, by auramine staining and PCR respectively were positive. Post mortem is a regular surveillance method for identifying the status of bovine tuberculosis and routine abattoir inspections combined with detailed abattoir meat inspections help in surveillance programmes.

It was observed that lesion in lungs (32.7 per cent) were found to be at a much higher level in comparison to other tissues like lymph nodes (15.09 per cent), liver (11.32), multiple organ involvement (26.42 per cent), mesentry (3.15 per cent), spleen (7.23 per cent) and other organs (5.66 per cent). The distribution of tuberculosis-like lesions in the lungs is more likely since organs rich in reticulo-endothelial tissue particularly the lungs and associated lymph nodes are most often affected.

Isolation of *M. tuberculosis* and *M. bovis* were attempted on samples positive for AFB or auramine staining or PCR. Totally 70 samples were subjected for culture of which 37 samples were found to show characteristic growth which included 12 from milk, 11 from PSLG aspirates and 15 from tissue samples. Transmission Electron Microscopy (TEM) of an isolate of *M. bovis* was carried out for further confirmation to reveal mycobacteria with corresponding morphology.

Of the 1224 bovine samples screened by *IS 6110* PCR, 53 were found positive for MTBC organisms. On differentiation by multiplex PCR, 48 (4.24 per cent) of 1224 samples were *M. tuberculosis* and only 5 (0.44 per cent) were *M. bovis*. Of the 37 culture positives, 34 were *M. tuberculosis* and three were *M. bovis*. In the present study, there was higher number of isolations of *M. tuberculosis* in animals in comparison to *M. bovis*. Intensification of animal – human contact provides the basis for possible cross-over and increased risk of transmission both ways (animal-human-animal) of *M. tuberculosis* and *M. bovis* at the interface.

Of the 100 sheep carcasses examined at post-mortem, lesions of tuberculosis were noticed in 35. The three PCR positive tissue isolates of ovines were *M. tuberculosis* and the positive per cent was 8.57. However, one chimpanzee lung nodule sample suspected for tuberculosis tested negative for AFB and auramine staining but positive for both *M. tuberculosis* and *M. bovis* by culture and PCR. The

mixed infection in chimp in this study may suggest primates as an active source of tuberculosis infection for zoo attendants and veterinarians and also other animals are likely to pick up infection from humans. This invites a detailed study in captive animals to understand the exact status of tuberculosis.

In human subjects suspected or positive for TB, out of 279 samples tested, *M. tuberculosis* and *M. bovis* were observed in 183 (65.59 per cent) and one (0.36 per cent) respectively. One positive isolate of *M. bovis* in human sample might be a case of BCG adenitis considering the clinical findings, age of the patient and absence of animal association or raw bovine milk consumption. PCR has therefore now become an ideal tool to differentiate *M. tuberculosis* from *M. bovis*.

A cross bred cow in an organized dairy unit positive by *M. bovis* ELISA on herd examination for tuberculosis was followed up to evaluate the sequence of events in *M. bovis* infection since test positive.

Sequence analysis plot and sequence similarity matrix of nucleotide sequences of hypothetical protein 'Rv1506c' (accession no. Z79701) region of eight *M. tuberculosis* and six *M. bovis* field isolates and genbank isolates had shown the difference in sequences between isolates vary from 0 to 2.3 per cent. This sequence cannot be used for differentiation other than its diagnostic value.

The spoligopatterns of the bovines, ovines and a chimp isolates showed the following lineages: Manu1, U, EA15, EA13 and Beijing. While the lineages from various isolates were almost similar, two of them showed a spoligopattern that did not match with any existing pattern in the database (orphan). The *M. bovis* isolates from human and chimp matched with Bovis 1 type. This study indicates a spillover of the common spoligotypes from humans to animals which may lead to further dissemination to humans from these animal populations.

The results of the binary logistic regression analysis factors associated the occurrence of tuberculosis infection in human beings with the variables presumed to be the determinants in human beings, *viz*., association with animals, raw milk consumption, family member with tuberculosis, age, education and socio-economic status were found to be statistically significant when all the data points were

considered. Detailed studies on such associations are needed to assess and evaluate the level of the problem of zoonotic tuberculosis.

This study attempted to summarize available epidemiological information, examine risk factors that can influence occurrence of zoonotic tuberculosis employing standard conventional protocols including molecular techniques. Though the major objective of this study was to identify the extent of *M. bovis* in humans, *M. tuberculosis* infection established was at a higher level in the animal population studied. The occurrence of *M. tuberculosis* is an example of reverse zoonosis and these animals potentially constitute a grave public health hazard as virulent bacilli can be transmitted to humans. Detailed studies on such associations are needed to assess and evaluate the level of the problem of zoonotic tuberculosis. The varied approaches in establishing the significance and impact of MTBC in animal and human subjects studied may provide opportunities to understand whether this is just spill-over phenomenon or holds promise for epidemiologic and / or pathogenic relevance in future.

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7. REFERENCES

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APPENDIX

APPENDIX - 1

Name : Respondent No:

Locality : Date:

Questionnaire on Zoonotic tuberculosis

Sex : Male/Female

Age : Yrs

Educational status : No formal education / Completed primary /Completed secondary/Tertiary

Occupation :

Socio economic status : Lower/Upper lower/Lower middle/ Upper middle/ Upper

Association with animal :

Animal Species

How long?(yrs)

Activity

Contact time (Occasionally/ routine)

Consumption of raw milk/dairy product?

Any other

Status of illness? (With respect to TB)

Symptoms : Cough/ chest pain/weight loss/fever/night sweats/lymph node swelling/ GI signs/any other

Diagnosis/test with result : Positive/ Negative

Any other family member/ closely associated person with TB?: Yes/No

Usage of corticosteroids or any other immunosuppressive drugs : Yes/No

Immune status :Age/ Pregnancy/ HIV/ Cancer / DM /Hepatic problems/Excess alcohol/Smoking/ Homelessness/Any other ailments