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MOST URGENT

Kuldeep Singh Sachdeva
Deputy Director General – TB
Central TB Division
Ministry of Health and Family Welfare
Nirman Bhawan
New Delhi 110 108

2 June 2020

Via e-mail: ddgtb@rntcp.org

Subject: Comments on draft 'National Strategic Plan to End Tuberculosis in India 2020–2025' and request for immediate action to eliminate TB in elephants

Dear Dr Sachdeva,

I am writing from People for the Ethical Treatment of Animals (PETA) India on behalf of our more than 1.5 million members and supporters with reference to our earlier letter, dated 25 May 2018 (**Annexure 1**), regarding the importance of recognising the zoonotic nature of tuberculosis (TB) in the draft "National Strategic Plan to End Tuberculosis in India 2020–2025" in order to eliminate the disease in India.

TB Is Prevalent in Many Species

According to the World Health Organization, "Over 30 new human pathogens have been detected in the last three decades, 75% of which have originated in animals" (**Annexure 2**). With regard to TB, according to the World Organisation for Animal Health, "Cattle are considered to be the major reservoir *M. bovis*, and are the main source of infection for humans. Nevertheless, the disease has been reported in many other domesticated and non-domesticated animals" (**Annexure 3**). Other affected species include elephants as well as animals bred for use by humans, including on factory farms, such as sheep, goats, chickens, and pigs.

According to a recent scientific paper, 7.3% of India's bovine population, an estimated 21.8 million cattle, are infected with TB (**Annexure 4**). In a human study, 12.6% of people in one group were found to be carrying *M bovis* (the virus that causes bovine TB), and consumption of raw milk was found to be a key factor in their infection (**Annexure 5**). A serodiagnostic study indicates that the incidence of TB in sheep is 1.5% (**Annexure 6**). According to another paper, "Tuberculosis in birds is most prevalent in chickens and in wild birds raised in captivity" (**Annexure 7**). In another study, at least 6% of pigs tested positive for TB (**Annexure 8**).

A 2018 evaluation report of captive elephants in Jaipur by the Animal Welfare Board of India (AWBI) – a central government statutory body – revealed that in a rapid serological test, 10% of the elephants checked, who are used for rides and other tourist interactions near Jaipur, were found to be reactive for TB (**Annexure 9**).

A scientific study of 600 elephants in Karnataka, Kerala, and Tamil Nadu published in 2012 found "evidence for high prevalence of asymptomatic *M. tuberculosis* infection in Asian elephants in a captive Indian setting" (**Annexure 10**). A study published in 2013 discovered "two probable cases of cross-species transmission of M. tuberculosis

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between mahouts and captive elephants. First is [a] case of human-to-elephant [transmission] and second is a case of elephant-to-human transmission of M. tuberculosis" (**Annexure 11**). Another paper, published in 2016, states, "There is evidence to suggest cross-species tuberculosis transmission," based on one-time screenings of nearly 800 elephants and their mahouts over a period of three years (**Annexure 12**).

Zoonotic TB is a major occupational hazard for animal handlers, veterinarians, farmers, slaughterhouse workers, and butchers in India. According to the OIE, clinical differentiation of TB caused by *M tuberculosis* is not possible and *M bovis* is responsible for up to 10% of human infections.

Screening of Each Animal for TB Is Impractical, and No Disease Surveillance Is Conducted for Animals

The latest census report released by the Department of Animal Husbandry and Dairying reveals that the livestock population in India grew by 4.6% from 512 million in 2012 to about 536 million in 2019. It is impractical to screen the entire livestock population for TB, and no routine surveillance is conducted anywhere in the country. As a result, the number of animals suffering from TB and the number of humans contracting zoonotic tuberculosis annually might be higher than is currently estimated.

Diagnosis of TB in Animals Is Difficult

According to the OIE, clinical signs of TB in animals are not very distinctive, and therefore it is difficult for veterinarians to make a definitive diagnosis based on these signs alone. The laboratory procedures most commonly used for diagnosis of TB do not differentiate *M tuberculosis* from *M bovis*, leading to underdiagnosis of zoonotic TB. A confirmatory diagnosis of TB in animals can be made only through bacterial culture and identification in the laboratory, and this process can take eight weeks or more. Zoonotic TB in humans is often initially extrapulmonary and may be misdiagnosed. The progress of the disease is slow, taking months or years to reach the fatal stage, and an infected animal can shed the bacteria within the herd before the appearance of clinical signs.

TB Treatment for Animals Is Ineffective and Costly, and the Law Mandates Euthanasia of Affected Animals

Misdiagnosis of zoonotic TB because of extrapulmonary infection often results in a delay in initiating treatment. According to the OIE, "Zoonotic TB poses challenges for patient treatment and recovery. *M. bovis* is naturally resistant to pyrazinamide, one of the four medications used in the standard first-line anti-TB treatment regimen. As most healthcare providers initiate treatment without drug susceptibility testing, patients with zoonotic TB may receive inadequate treatment." Treatment of tuberculosis in any species is a time-consuming and expensive process, often complicated by patient non-adherence. Although The Prevention and Control of Infectious and Contagious Diseases in Animals Act, 2009, mandates the euthanasia of chickens who test positive for *M paratuberculosis* and cattle infected with *M bovis*, for cultural and religious reasons, such disease-control practices are rarely observed.

Prevention and Control of TB in Animals Is Impossible

There is no vaccination protocol for preventing TB in animals. The *M bovis* Bacille Calmette-Guérin (BCG) vaccine used in humans for nearly a century does not provide complete protection against TB in other animals, and animals vaccinated with BCG may show a false positive reaction during the tuberculin skin test, making it difficult to

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detect infected animals. Since the diagnosis of TB in animals is difficult and no surveillance system exists, the movement of undetected infected animals plays a key role in the spread of the disease, making it impossible to eliminate TB in animals.

Prevalence of Reverse Zoonosis

In countries where many humans come into close contact with animals, like India, *M tuberculosis* is also transmitted from humans to animals. In a study conducted in northern India, eight out of 30 lung tissue samples collected from bovines on farms confirmed that the animals were infected with *M tuberculosis*, indicating possible human-to-cattle transmission and adaptation of this organism in bovine tissues. A paper published in 2017 following confirmation that TB had been found in three wild Asian elephants in southern India stated that "tuberculosis may be spilling over from humans (reverse zoonosis) and emerging in wild elephants".

Action Required

1. The draft "National Strategic Plan to End Tuberculosis in India 2020–2025" currently overlooks the prevalence of TB in animal species bred, reared, and used for human purposes and its significance regarding zoonosis and reverse zoonosis. The current draft is focused only on addressing the outcomes of zoonotic TB in humans by strengthening laboratory and diagnostic systems, which doesn't address the cause of the issue. In light of the evidence presented above – and the lessons offered by the COVID-19 pandemic, an outbreak of another disease that originated in animals – in order to eliminate TB in India, the strategic plan must include stringent action to address the transmission risk posed by factory farms, wet markets, and slaughterhouses.
2. In its 7 May 2014 judgment in the matter of *Animal Welfare Board of India v A Nagaraja and Others*, the Honourable Supreme Court of India noted, "Entertainment, exhibition or amusement do not fall under … exempted categories [under Section 11 of The Prevention of Cruelty to Animal Act, 1960] and cannot be claimed as a matter of right under the doctrine of necessity." In order to eliminate TB in elephants – and thereby protect both the animals and the humans they come into contact with, including tourists, handlers, and the general public, from the disease – we request that the Central Tuberculosis Division advise the Ministry of Fisheries, Animal Husbandry & Dairying to prohibit the exhibition and training of elephants for performances, including joy rides and other human-animal interactions – since these are not considered a necessity under the law.

I can be contacted on 9910817382 or at ManilalV@petaindia.org.

Thank you for your time and attention to this urgent matter.

Kind regards,

Dr Manilal Valliyate
CEO

cc: Preeti Sudan, Secretary, Ministry of Health and Family Welfare (secyhfw@nic.in)
Professor Rajiv Garg, Director General of Health Services, Ministry of Health and Family Welfare (dghs@nic.in)

ANNEXURE - 1

PEOPLE FOR THE ETHICAL TREATMENT OF ANIMALS

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MOST URGENT

Devesh Gupta, Addl DDG
Central Tuberculosis Division
Room No 533-C
Ministry of Health & Family Welfare
Government of India
Nirman Bhawan
New Delhi 110 011

11 June 2018

Subject: Tuberculosis-infected elephants forced to interact with tourists in Jaipur pose a grave threat to public health

Dear Mr Gupta,

I am writing to you from People for the Ethical Treatment of Animals (PETA) India on behalf of our more than 1 million members and supporters to urge you to direct the Government of Rajasthan to bring an immediate end to interactions between humans and elephants infected with tuberculosis (TB) as well as those who have not yet been tested for TB. We also ask that you provide the infected elephants with immediate veterinary care in order to prevent any further spread of this zoonotic disease, which can be transmitted from animals to humans.

The findings of an April 2018 evaluation report on captive elephants in Jaipur by the Animal Welfare Board of India (AWBI), a statutory body operating under the Ministry of Environment, Forest and Climate Change, revealed that, ten out of 91 captive elephants in Jaipur used for joy rides and other forms of tourist interactions are infected with TB (**Annexure 1**). The AWBI evaluation team included experts, such as senior veterinarians of the Rajasthan Forest and Animal Husbandry departments and other veterinary and wildlife experts. TB can be transmitted to tourists, animal handlers, and the general public. The post-mortem reports for four elephants who died within a period of five months in 2017 indicate that most of the animals had been suffering from respiratory diseases – including possibly TB – and had heavy internal loads of parasites. The AWBI report also states that elephants in Jaipur are particularly at risk of contracting TB because of routine transport within the city and routine interactions with tourists that may expose them to infected humans or elephants as well as because of stress factors, including painful restraining methods, extreme confinement, variable water quality, an inconsistent food supply, and poor nutrition.

We hail the vision of the Honourable Prime Minister for aiming to eliminate TB in India by 2025, which is five years ahead of the global target of 2030. However, the *National Strategic Plan for Tuberculosis Elimination 2017–2025* does not, unfortunately, account for the zoonotic nature of TB transmission or for the prevalence of the disease in animals. Since it's impossible to achieve the complete eradication of the disease in humans without also eradicating it in animals, it's imperative that TB surveillance be conducted in animals, too. It's also necessary that action be taken under the provisions of the law for the treatment, prevention, and control of the disease in animals.

This is the basic concept of the One Health Initiative, which has been supported and promoted by prominent international organisations such as the World Health Organisation, the Food and Agriculture Organisation of the United Nations, and the World Organisation for Animal Health.

As per a *Hindustan Times* news report dated 24 March 2018 (**Annexure 2**), the experts at the Indian Veterinary Research Institute believe that the prevalence of TB infection is as high as 15 per cent in buffaloes and cows in this country. This news report further states that according to recent research, approximately 9 per cent of all TB infections are transmitted from other animals to humans and that the percentage is higher in TB cases among children.

The study "A Cross-Sectional Study on Prevalence of Bovine Tuberculosis in Indian and Crossbred Cattle in Gangetic Delta Region of West Bengal, India" by Ratan Das *et al.* (**Annexure 3**) concludes, "The findings indicate a higher prevalence of BTB in exotic crossbred animals in Gangetic delta and variation in breed susceptibility, thereby suggesting an urgent review of the present policy on adopting national crossbreeding program and implementation of 'One Health' approach."

An Indian study titled "Serodiagnosis of Tuberculosis in Asian Elephants (*Elephas maximus*) in Southern India: A Latent Class Analysis" by Shalu Verma-Kumar *et al.* (**Annexure 4**) concludes as follows: "Our results provide evidence for high prevalence of asymptomatic *M. tuberculosis* infection in Asian elephants in a captive Indian setting."

An Indian study titled "Prevalence of Zoonotic Tuberculosis and Associated Risk Factors in Central Indian Populations" by Prachi R Bapat *et al.* (**Annexure 5**) concludes that "[b]ased on the requisite inclusion criteria, we recruited a total of 301 [human] participants whose blood samples were subjected to polymerase chain reaction-based detection and differentiation of *Mycobacterium bovis* and *Mycobacterium tuberculosis*. *M. bovis* was detected in 11.4%, 8.9%, and 12.6% of the recruited participants belonging to three distinct population groups (Groups A, B, and C, respectively)".

Under the Prevention and Control of Infectious and Contagious Diseases in Animals Act, 2009 (Annexure 6), paratuberculosis, the disease caused by the *Mycobacterium avium* subspecies *Paratuberculosis* (*M. paratuberculosis*), and bovine tuberculosis, the disease in cattle caused by *Mycobacterium bovis*, are notifiable and state governments are vested with the responsibility to take necessary and immediate action under the provisions of the law.

It's imperative to note that the 10 captive elephants in Jaipur who tested positive for TB, as well as those who haven't been tested for TB, pose a potential threat to humans, including tourists, handlers, the general public, and other animals who interact with them.

Since the Central Tuberculosis Division, under the Directorate General of Health Services, Ministry of Health & Family Welfare, is responsible for effective implementation of the *National Strategic Plan for Tuberculosis Elimination 2017–2025*, we request that you issue directives to the Government of Rajasthan to quarantine

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the 10 TB-infected animals immediately, provide for their veterinary care, and ensure that they are not used for rides until they are declared medically fit by a team of qualified veterinarians after a medical examination. We also ask that you direct that the elephants who have not yet been screened for TB be subjected to such testing immediately.

I can be contacted on 9910397382 or at NikunjS@petaindia.org .

Thank you for your time and attention to this urgent matter.

Kind regards,

Nikunj Sharma
Associate Director of Policy
PETA India

cc:

1. The Honourable Narendra Modi, Prime Minister of India
2. The Honourable Jagat Prakash Nadda, Minister of Health & Family Welfare
3. Smt Preeti Sudan, Secretary, Ministry of Health & Family Welfare

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ANNEXURE - 2

1. Scope and purpose

Zoonoses are defined as those diseases and infections naturally transmitted between people and vertebrate animals. There are three classes as follows: a) endemic zoonoses which are present in many places and affect many people and animals; b) epidemic zoonoses which are sporadic in temporal and spatial distribution; and c) emerging and re-emerging zoonoses which are newly appearing in a population or have existed previously but are rapidly increasing in incidence or geographical range. Examples of the latter include Rift Valley fever, SARS, pandemic influenza H1N1 2009, Yellow fever, Avian Influenza (H5N1) and (H7N9), West Nile virus and the Middle East respiratory syndrome coronavirus (MERS-CoV) reported in the recent past.

It is estimated that, globally, about one billion cases of illness and millions of death occur every year from zoonoses. Some 60% of emerging infectious diseases that are reported globally are zoonoses. Over 30 new human pathogens have been detected in the last three decades, 75% of which have originated in animals [1]. The emerging zoonoses are a growing public health threat in the Eastern Mediterranean Region of WHO. In last two decades, emerging zoonotic diseases have been reported from 18 out of 22 countries in the region, often, with explosive outbreaks and high fatalities never seen in any other WHO region [2]. The recent emergence of MERS-CoV exemplifies that the occurrence of these infections are unpredictable as they originate from animals, often these infections are caused by novel viruses and are only detected when outbreaks occur. The Eastern Mediterranean Region of WHO remains particularly prone to zoonotic infections owing to large number of people in the region living in close proximity to animals, increased volume of international trade, including trans-boundary mass population and livestock movement within neighbouring countries. As the region remains at the cross road of repeated outbreaks from emerging infectious diseases, international travel to and from the region either due to tourism, business or religious reasons, globalization and variable levels of health systems capacity to early detect epidemics have been identified as significant risk factors for emergence and rapid international spread of infectious diseases with zoonotic origin. Varying levels of surveillance and response capacity of the countries at the animal-human interface have often exacerbated these outbreaks.These zoonotic infections are also a concern to global health security owing to its ability to rapidly spread internationally due to global connectivity and proliferation of trade, including trans-boundary movement of animals. The emerging zoonoses have also economic consequences due to loss of animal trade, travel and loss of economic opportunities for the people through loss of livestock.

Given the scale and burden of emerging zoonotic infections in the region, this paper will highlight the (i) growing public health threats of emerging zoonotic infections in the region; (ii) challenges in controlling these infections; and (iii) underpin a strategic approach for predicting, detecting and controlling these infections through an integrated and interdisciplinary approach between the animal and human health sectors.

2. Current situation in the Region

The exact extent of the burden of zoonotic diseases in the Eastern Mediterranean Region of WHO is not known. While endemic zoonoses like brucellosis, anthrax and rabies have not been eliminated from the countries, the region continues to witness both sporadic and epidemic occurrence of emerging zoonoses (Table-1). The region has recently seen outbreaks from Yellow fever in Sudan [3], Chikungunya in Yemen [4], West Nile fever in Tunisia [5] and Q fever in Afghanistan [6, 7] and Iraq [8]. The region is home to a number of arbo and filoviruses. While outbreaks from Crimean –Congo haemorrhagic fever (CCHF) occur periodically in Afghanistan [9], Iran [10] and Pakistan [11-14], as a seasonal surge, nosocomial outbreaks of the disease have been reported in recent years in Iraq [12], United Arab Emirates [14] and Sudan [16-18] in the region. The viral haemorrhagic fevers are perpetual risks in the region. The Ebola Haemorrhagic Fever in Sudan in 2004 was the only viral haemorrhagic fever seen the region which is caused by a filovirus [18]. The sudden expansion of Rift Valley fever, endemic in sub-Saharan Africa along the animal trade routes to Yemen [19], Saudi Arabia [20-21] and Sudan [22] is a characteristic example of geographic expansion of emerging zoonoses in the region.

The highly pathogenic avian influenza spread rapidly through the Eastern Mediterranean Region in 2006 with large epizootics reported in a number of countries while human infections have occurred in Djibouti [23], Iraq [24], Pakistan [25] and Egypt [26]. The avian influenza is now presumed entrenched in Egypt with a low level of transmission throughout the year [27]. In 2009, the influenza A (H1N1)pdm 09 of swine origin affected all the countries in the region [28]. Other emerging zoonotic diseases have occurred in the region whose occurrence is rare but still can cause high morbidities. These include Monkey pox [29], Sandfly fever [30] and Plague [31]. As illustrated by the Alkhurma virus, this region is also home to newly emerging pathogens from zoonotic origin [32-34]. As if to remind the region that emerging zoonoses can occur anywhere anytime and that no country is immune to the threats of these diseases, human infection with a novel coronavirus (MERS-CoV) occurred in the region in 2012 [35-36] that rapidly focused global attention on this new virus [40]. Till date, the affected countries for MERS-CoV in the region are Egypt, Iran (Islamic Republic of), Jordan, Kingdom of Saudi Arabia, Kuwait, Lebanon, Oman, Qatar, Tunisia, United Arab Emirates and Yemen.

All countries in the region are at risk from these diseases, and cross-border outbreaks occur frequently. But countries in the region, often plagued by complex emergency situations and often characterized by a failure or inability to effectively address the emergence of new diseases or the re-emergence of endemic ones are probably at greatest risk. Lack of effective control programmes for zoonoses, limited inter-sectoral collaboration between the human and animal health sector with little consensus on the roles and responsibilities of each sector and low priority given to zoonoses are attributed as significant factors for high burden and emergence of repeated zoonotic infections in the region, often with explosive outbreaks. .

Certain disease amplifiers like population movement, fragmented health systems, weak response and laboratory diagnostic capacity and disruption of routine public health services in crisis affected countries have also contributed significantly to the surge of emerging zoonoses in the region.

3. Why do the zoonotic infections matter

An observation of the trend of these zoonotic diseases is that new pathogens from animals particularly viruses remain unpredictable and continue to emerge and spread across the countries. The diseases are also a concern to global health owing to their epidemic potential, high case fatality ratio and the absence of specific treatment and vaccines available to control the spread of most of these zoonotic diseases (with the exception of the yellow fever vaccine) As the world is increasingly inter-connected, emerging zoonoses in one country can potentially constitute a threat to global health security. Ultimately, however, zoonoses matter not just because they are so common, but because they cause morbidity and mortality, high burden on health systems but more importantly, it causes significant economic loses to the countries by way of losing animal trade, travel as well as loss of economic opportunities for the people through loss of livestock.

Therefore, zoonotic diseases can have devastating impact with severe economic consequences for the countries through loss of trade, tourism and consumer confidence. Just to give an example how zoonosis can adversely impact the economy of a country- the emergence of SARS in 2003 has the cost the world economy over USD 50 billion on account of medical treatment and loss of revenue associated with abrupt halt of tourism industry. Another example is that during the outbreak of RVF in Kenya, each household lost on average US$500 as a result of low productivity and cost related to RVF disease control.

4. Challenges of managing zoonotic infections

Globalization has resulted in the unparalleled passage of people, animals and goods across national borders, which in turn has fuelled the international spread of zoonotic infections. Many of the zoonoses are trans-boundary diseases, they spread across borders from their origin, to impact on trade, commerce, tourism and consumer confidence with devastating economic consequences.

The experiences drawn from the region have shown that most of the zoonotic infection outbreaks have occurred in remote areas, making it sometimes impossible to provide public health services to these hard-to-reach populations. The detection and diagnosis of the disease has been considerably delayed due to difficulties in deploying teams for field investigation, lack

of appropriate and safe sample shipment mechanism, lack of appropriate laboratory diagnostic facilities on-site or in-country, and insufficient capacities of the countries to plan, mobilize and implement appropriate control measures in such settings and to monitor the progress of control measures in geographically dispersed areas. The detection of these disease threats require functional sub-national surveillance capacity and therefore the need for investing in strengthening subnational outbreak surveillance and response capacities in the countries frequently affected by these diseases.

Many viral pathogens causing emerging zoonotic infections in humans originated from animals (specifically wildlife) or from products of animal origin. Knowledge of extra-human reservoirs of these pathogens remains essential for understanding the epidemiology and potential control measures of these zoonotic diseases.

There is also inadequate transparency regarding timely reporting of emerging zoonotic infections to WHO or any other international agency that is mandated to investigate and respond appropriately for global health security. Often medical authorities in the countries deny the existence of human cases, making it difficult for these agencies to understand the epidemiology, disease progression and use the opportunity to understand which methods work and do not work for control of these diseases in different settings.

The major limitation in controlling the zoonotic infections in the region include the lack of effective collaboration between the animal and human health sectors under the concept of “One Health” approach, which links the human with the animal health sector integrating the animal and human disease surveillance and response system that could, otherwise have helped controlling the zoonotic infections in animal reservoirs, enable early outbreak detection, and prevent deadly epidemics and pandemics.

The other challenges of prevention and control of zoonotic diseases in Member States include weak surveillance and reporting systems and limited capacity for laboratory diagnosis of emerging zoonotic diseases such as SARS, Ebola, Marburg, and new strains of influenza. Response capacity at the local level is limited in most countries due to lack of awareness, inadequate supplies and human resource quality and quantities. There are limited institutionalized inter-sectoral collaboration mechanisms between the key sectors. There are difficulties in obtaining accurate and current information and reports on zoonotic disease situations because of the inability to obtain quality information from the grassroot level and inadequate communication between Ministries of Health and Agriculture (Veterinary Services). There is also lack of effective community participation and health education on zoonotic diseases in the Region. In addition, there is limited harmonisation of the various regulatory

public health frameworks. Research on new emerging diseases is limited or absent.

In summary, the main challenges are:

4.1 . Organizational

(i) Poor level of awareness among policy and decision-makers about the serious nature of the disease;

(ii) Insufficient information on the burden , trend and risks of zoonotic diseases;

(iii) Inadequate resources and skilled manpower for control of zoonotic diseases;

(iv) Presence of other competitive health priorities often taking precedence;

(v) Lack of transparency of the countries to report emergence or occurrence of zoonotic disease for fear of repercussions;

(vi) Weakness or absence of collaboration and cooperation between the public health , veterinary, agriculture and wildlife sectors;

(vii) Inadequate collaboration and partnerships to harness resources to support the prevention and control programme of zoonotic diseases

(viii) Absence of cross-talk within the health sector between the surveillance, clinical services and laboratory services departments.

(ix) Breakdown of weakness of health infrastructures specially in countries with complex emergencies;

4.2 . Diagnosis and detection

(i) Lack of integration of human and veterinary sector for exchange of epidemiological and laboratory surveillance data of the human and health sectors;

(ii) Weak disease surveillance system and inadequate diagnostic capacities to detect zoonotic infections;

(iii) Difficulties in international transfer of samples for logistic and economic reasons.

(iv) Difficulties in conducting field investigation in remote areas where most of the emerging zoonotic outbreaks occur.

(v) Weak cross-border collaboration, surveillance and information exchange between the countries

(vi) Inadequate community engagement in the zoonotic control programme

4.3 Control and interruption of transmission

(i) Insufficient capacities of countries to plan, mobilize and implement appropriate control measures.

(ii) High probability of nosocomial transmission of some of the newly emerging zoonoses in health-care settings;

(iii) Poor application of strict barrier nursing and other appropriate infection control measures in

health-care facilities.

(iv) Lack of information on high-risk behaviours, including cultural and social factors, that are associated with risk of transmission of emerging zoonoses in the community;

(v) Inappropriate or inadequate vector control operations.

(vi) Lack or insufficient evidence on some of the public health control measures.

5. Current strategies for control of zoonotic infections

The current strategies for prevention and control of zoonotic diseases remain fragmented with no coherence between the animal and human health sectors. Globally, no strategies exist, either, for prevention and control of emerging zoonotic infections. Due to lack of resources and appropriate policy response, there have not been any focussed efforts in the region to develop any plan for management and control of zoonotic diseases and its public health risks. Despite the public health threats from emerging zoonotic infections, the efforts taken by the Regional Office to advocate for control of zoonotic diseases remain on the margin. Two Regional Committee resolutions-(i) EM/RC54/R.4 Growing threats of viral haemorrhagic fevers in the Eastern Mediterranean Region: a call for action; and (ii) EM/RC58/R.4(D) Dengue: Call for urgent interventions for a rapidly expanding emerging disease have elaborated on the need to control zoonotic infections but efforts are far from adequate.

Given the scale and magnitude of the problem and the evolving public health risks associated with zoonotic infections, now is the time, perhaps, for a paradigm shift and make changes to the way WHO wants to address this imminent threat to global and regional health.

6. Strategic directions for control of zoonotic infections

Considering the growing importance of zoonotic diseases in the region, the most appropriate direction for the Regional Office would be to develop a strategic framework for prevention and control/elimination of zoonotic diseases in the region with a view to minimizing the health, social and economic impact of zoonotic diseases in the countries of the region. The most important and critical technical areas that will need to be considered will include the following strategic approaches:

(i) Building effective collaboration between animal and human health sectors

As it is difficult to predict when or where the next zoonotic disease will emerge, close collaboration between veterinary and public health specialists is important. The goal of this inter-sectoral collaboration would be to enhance inter-personal and inter-organizational communication. An inter-agency taskforce can lead this process of fostering collaboration through regular exchange of scientific information proactively between the two sectors. The task force can also guide joint field investigation and share institutional resources within a coordinated framework of partnerships and agreement for the One Health concept. This would synergize effective prevention and control efforts at the animal–human interface.

(ii) Improving surveillance for early detection of disease threats in humans

As most of the emerging zoonotic infections have reservoirs in animals or/and in arthropods, and the occurrence of such diseases in humans often cannot be precisely predicted, investigation at the first sign of emergence of a new disease in animals that has the potential to jump species barrier is particularly important to early detect any disease threats from zoonoses. The integration of disease surveillance system between the animal and human health sectors is critical for timely gathering and analysis of animal disease data that have the potential to cross species barrier. The use of syndromic surveillance system may also be helpful in detection of any threats in real-time and can accelerate appropriate mitigation and prevention efforts.

(iii) Strengthening laboratory diagnostic capacities for novel pathogens

Laboratory services would be more effective in early detection of any zoonoses when there is a common and agreed communication protocol for sharing laboratory surveillance data between animal and human health sectors in real-time. In addition, a mechanism needs to be put in place for sharing of laboratory investigation data within the health sector, principally between the disease surveillance and the clinical services departments. Establishing laboratory networks both within and outside the countries will enhance rapid transfer and shipment of specimens for timely diagnosis of zoonotic disease threats.

(iv) Improving case management and infection control

To ensure preparedness of health care facilities to the threats of zoonotic infections, an infection prevention and control program should be implemented before the emergence of a disease with the standard precautions as an essential component. Standard precautions should be used in the care and treatment of all patients irrespective of their perceived or confirmed infectious status. If consistently applied, the standard precautions would help prevent most transmission through exposure to blood and body fluids before any zoonotic disease with unknown origin are recognized. As the clinical manifestations of many of the emerging zoonoses are often indistinguishable, leading to confusion and misdiagnosis by health-care workers, the use of a clinical decision algorithm for acute febrile illnesses with a more sensitive case definition may be useful for early detection of any suspected cases. Use of such a decision tree will help in guiding initial therapeutic decisions and trigger the protocol steps for further laboratory diagnosis and follow-up. Implementation of this clinical algorithm needs to be backed up with training of health-care workers on case management and infection control measures as well pre-positioning of strategic supplies.

(v) Integrating vector control management

An integrated vector control management (IVM) approach should be considered for all arthropod-borne viruses as the most rational decision-making process to optimize the use of resources for effective vector control. The IVM strategy is based on evidence and integrated management of mosquito vectors including rodents and promotes the use of a range of interventions, either alone or in combination, which are selected on the basis of local knowledge about the vectors, diseases and disease determinants. IVM would therefore be the most effective strategy for the control of vectors including rodents that are responsible for transmission of a number of arthropod-borne viral haemorrhagic fevers. As a first step, countries need to carry out a detailed mapping of the vectors, and their breeding sites to identify the spatio-temporal distribution of the vector species. Such information may be useful for targeting control measures for breeding sites during the inter-epidemic period. The vector control measures should seek to reduce the potential breeding sites of adult mosquito populations or their interactions with human below that which can sustain an epidemic. Setting up a sentinel site for entomological surveillance in areas of high vector densities and integrating it with that of epidemiological and viral surveillance systems can provide meaningful information through reporting of unusual clusters of acute febrile illnesses, a suddenly rise in vector density or a fortuitous isolation of a novel zoonotic virus. Such information can be helpful to understand the anticipated, prevailing or evolving risk.

(vi) Reducing transmission through social and behavioural interventions

The success or failure of interrupting the transmission chain for most of the emerging zoonoses especially those involving intermediate vertebrate hosts, will rely on the relevance of the behavioural response of the exposed populations. To design appropriate social and behavioural interventions for sich disease threats, the community's risk perception and how this relate to actual or intended behaviour, socio- or psycho-cognitive factors that characterize the exposed population's behaviour and the cultural factors that influence protective factors and sustainability of adherence to such protective behaviour will need to be considered.

(vii) Developing epidemic preparedness and response capacities for emerging zoonoses

The main strategy should begin with developing a national plan involving all important stakeholders. The plan should consider developing a geographic map of the distribution of zoonoses occurring in the countries using geographic information systems and other information technologies, and conducting a detailed risk assessment. Furthermore, areas at risk for expansion of zoonosis should also be identified. Human, animal and vector surveillance should be strengthened and if possible integrated with data and shared, so that vital information on risks are exchanged on a regular basis between the partners through a well-coordinated mechanism. The plan should encompass pre-positioning of strategic supplies (investigation kits, personal protective equipment, etc.), development of appropriate guidelines and standards to measure the effectiveness of response operations and public education programmes aimed at limiting exposure to risk. Setting up a multi-disciplinary coordinating body to foster collaboration and integration between all partners and to guide, lead and provide emergency response operations during an outbreak would be the key for a successful epidemic preparedness and response plan.

Finally, there will be need to develop appropriate monitoring and evaluation tools and indicators to measure the progress of implementation of the strategic framework over time. For ensuring that the programme for prevention and control of emerging zoonotic infections are sustainable, the countries will require to consider the followings while developing their own programme:

(i) Enhancing political commitment, national planning and coordination mechanisms: Policies will be required to be developed for building effective inter-sectoral collaboration between the animal and human health sectors through increased communication between the sectors, joint planning and setting up an effective coordination structure between these two sectors;

(ii) Strengthening preparedness, surveillance and response: Developing a multi-sectoral preparedness and response plans for control of zoonotic diseases through a comprehensive risk assessment, improving laboratory diagnostic capacities, joint surveillance activities at the animal-human interface, etc;

(iii) National capacity building and promoting research: This will include developing plans for national capacity building for prevention and control programme of zoonotic diseases including building a robust scientific evidence-base for new approaches to control zoonotic diseases in the country;

(iv) Enhancing regional and international cooperation and collaboration: Reducing public health risks from zoonotic diseases will require commitment and extensive cooperation and collaboration from all partners towards a common vision, goal and purpose. This will need to be incorporated in the proposed strategic framework;

(v) Health education, risk communication and social mobilization: Policies will need to be established in the countries on the effective use of risk communication and community engagement for addressing the public health risk associated with the emergence of zoonoses;

7. Conclusion and future perspectives

The region is now an emerging focus for global health after the discovery of MERS-CoV. The region has borne the brunt of several emerging zoonotic infections. The lessons of our present time is that emerging zoonotic infections are unexpected and unpredictable events. Another lesson that has been learned is that any disease outbreak anywhere today could be a problem for the world tomorrow. These novel diseases will continue to confront and challenge the national health authorities' resilience and responsiveness to respond in a timely manner. Likewise, the ability of regional and global communities to cooperate to control these diseases that cross national boundaries will be a real test for the global health security.

While the global efforts should continue to fill the current gaps in knowledge associated with the origin and transmission of many zoonotic infections much of which are novel in origin, much greater regional cooperation would be needed to protect the health of the people from all types of zoonotic infections. The current situation in the region with regards to the global and regional response to MERS-CoV should trigger a clear and articulated need for establishing a

sustainable public health programme for detection, prevention and control of emerging zoonoses in the region

The premise for strategic framework for control of zoonotic infections should lie on the concept of “One Health” approach which is a common coordination mechanism, joint planning, joint implementation, community participation, capacity building and joint monitoring and evaluation framework between the animal health and human health sector. The “one Health” approach also identifies five key areas where One Health is likely to make a difference. These are:

(i) sharing health resources between the medical and veterinary sectors;

(ii) controlling zoonotic diseases in animal reservoirs;

(iii) early detection of and response to emerging diseases;

(iv) prevention of epidemics and pandemics; and

(v) generating insights and adding value to health research and development.

The strategy will also require the Member States to initiate and consolidate measures, which integrate technical, social, political, policy and regulatory issues to strengthen their capacities adequately to reduce the public health burden and economic impact imposed on their people and livestock by zoonotic diseases. The implementation of a viable strategy is the way forward for the prevention and control of emerging and re-emerging zoonotic diseases in the Eastern Mediterranean Region of WHO. The Regional Committee is invited to consider and adopt the strategic directions described in this paper. Effective control of emerging zoonoses will present an opportunity for containing health risks of zoonotic infections that are of international concern as has been stipulated in the IHR (2005) and make the world safer from emerging and re-emerging pathogens

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Table-1: selected outbreaks from emerging zoonoses in the Eastern Mediterranean Region of WHO, 2000-March 2013

Disease Country Period Host/reservoir Human health impact

Cases* Fatalities

Rift Valley Fever Yemen 10 September-19 October 2000 Cattle, sheep, goats; Aedes mosquitoes (vector) 653 80

Saudi Arabia 26 August 2000-

22 September 2001

886 123

Sudan 18 October 2007-

15 January 2008 747 230

CCHF Pakistan # 2000-2012 Wild and domestic animals (Cattle, goat and sheep);

Hyalomma tick 585 113

Iran 2000-2011 3235 122

Afghanistan 2007-2012 104 15

Sudan ****

Yellow Fever Sudan September-December 2005 Primates (mainly monkey); Aedes mosquitoes (vector)

605 163

02 Sep-24 Dec 2012 849 171

South Sudan May-June 2003

178 27

Ebola haemorrhagic fever South Sudan

24 May-26 June 2004 Monkeys 17 7

Monkey pox Sudan, Unity state

20 September 2005–31 January 2006 Unknown but rodents, sun squirrels even monkeys are implicated 49 0

Al-khurma haemorrhagic fever ## Saudi Arabia

2001-2009 Camel and sheep;

Mammalian tick

Sand fly fever Lebanon

01 July-18 September 2007 Phlebotomine sandflies 800 -

Avian Influenza** (H5N1) Iraq Jan-March 2006 Poultry, Birds, wild fowl 3 2

Djibouti 23 April 2006 1 -

Pakistan 29 October-21 November 2007 4 2

Egypt Jan 2006-march 2013 172 62

Plague Libya 09-18 June 2009 Rodents; Fleas 5 1

Pandemic influenza All countries

25 May 2009-6 August 2010 Swine 1019

Q Fever ***

Afghanistan 29 May-02 June 2011 Domestic animals (sheep, cattle, goats)/Birds

147

Chikungunya Yemen Oct 2010-Mar 2011

Monkey

Aedes mosquitoes (vector) 1657

West Nile virus fever

Tunisia 14 August-14 November 2012 Birds; mosquitoes (vector) 63 10

Novel coronavirus infection

Saudi Arabia, Qatar , Jordan and UAE 21 March 2012-30 April 2013 Unknown but bats are being suspected 14 9

* Suspected cases including those laboratory-confirmed

** Laboratory-confirmed cases

*** World Health Organization. Eastern Mediterranean Regional Office. Weekly Epidemiological Monitor. Vol-5; Issue-28 & 29, Sunday 17 July 2011.

**** Nosocomial transmission

Cases reported during the outbreak are included

Sporadic cases continue to be reported since 2001

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ANNEXURE - 3

World Organisation for Animal Health

Bovine Tuberculosis

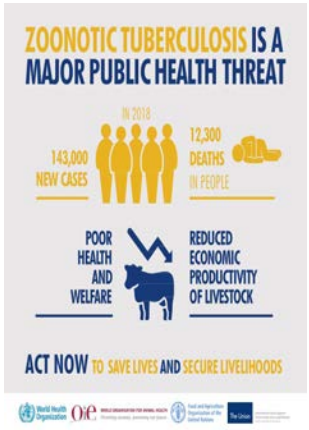
- What is Bovine Tuberculosis? Transmission and spread Public health risk Clinical signs Diagnostic Prevention and control Roadmap for zoonotic tuberculosis Geographical distribution
- **Standards**
  - Terrestrial code
  - Terrestrial Manual

  TB worldwide situation
  - World Animal Health Information Database (WAHIS)

  Ask our experts
  - Reference Laboratories

  Global & Regional activities
  - Roadmap to tackle zoonotic TB
    Asia
  - FAO-APHCA/OIE Regional Technical Workshop on the Prevention and Control of Animal Brucellosis and Tuberculosis in Asia

  Other references
  - The Center for Food Security and Public Health, Iowa State University
  - Merck Veterinary Manual
  - Technical card (CFSPH)



KEY FACTS

- In 1882, Robert Koch announced his discovery of the tubercle bacillus as the cause of human tuberculosis (TB). Theobald Smith subsequently published his findings on human and bovine tubercle bacilli in 1898, where he demonstrated the causative bacteria to be two different organisms that are now known as *Mycobacterium tuberculosis* (*M. tuberculosis*) and *Mycobacterium bovis* (*M. bovis*).
- From January 2017 to June 2018, of the **188 countries and territories** reporting their bovine tuberculosis situation to the OIE, 82 countries (44%) reported the presence of the disease.
- Although the infection in cattle herds has been controlled in most countries, complete elimination of the disease is complicated by persistent infection of wild animals, such as European badgers in the United Kingdom, white tailed deer in parts of the United States of America and brushtail possums in New Zealand.
- Bovine TB remains a serious problem for animal and human health in many developing countries.

What is bovine tuberculosis?

Bovine tuberculosis (bTB) is a chronic bacterial disease of animals caused by members of the *Mycobacterium tuberculosis* complex primarily by *M. bovis*, but also by *M. caprae* and to a lesser extent *M. tuberculosis*. It is a major infectious disease among cattle, and also affects other domesticated animals and certain wildlife populations, causing a general state of illness, pneumonia, weight loss, and eventual death.

The name Tuberculosis comes from the nodules, called 'tubercles', which form in the lymph nodes and other affected tissues of affected animals.

Cattle are considered to be the major reservoir *M. bovis*, and are the main source of infection for humans. Nevertheless, the disease has been reported in many other domesticated and non-domesticated animals.

Mycobacterium bovis has been isolated from numerous wildlife species, including African buffalo, domestic Asian buffalo, bison, sheep, goats, equines, camels, pigs, wild boars, deer, antelopes, dogs, cats, foxes, mink, badgers, ferrets, rats, primates, llamas, kudus, elands, tapirs, elks,

elephants, sitatungas, oryxes, addaxes, rhinoceroses, possums, ground squirrels, otters, seals, hares, moles, raccoons, coyotes and several predatory felines including lions, tigers, leopards and lynx.

Bovine tuberculosis is an OIE-listed disease and must be reported to the OIE as indicated in its *Terrestrial Animal Health Code*.

"Most cases of human TB are caused by the bacterial species, Mycobacterium tuberculosis*. Zoonotic TB is a form of TB in people predominantly caused by a closely related species,* M. bovis*, which belongs to the* M. tuberculosis *complex."*

Geographical distribution

Bovine tuberculosis is found throughout the world, but some countries have never detected TB, and many developed countries have reduced or eliminated bovine TB from their cattle population and kept the disease limited to one or more zones. However, significant pockets of infection remain in wildlife. The highest prevalence of bovine tuberculosis is in Africa and parts of Asia, but the disease is also found in countries in Europe and the Americas.

Transmission and spread

The disease is contagious and can be transmitted directly by contact with infected domestic and wild animals or indirectly by ingestion of contaminated material.

The usual route of infection within cattle herds is by inhalation of infected aerosol, which are expelled from the lungs (by coughing). Calves can be infected by ingesting colostrum or milk from infected cows.

Humans can become infected by ingesting raw milk from infected cows, or through contact with infected tissues at abattoirs or butcheries.

The course of disease is slow and takes months or years to reach the fatal stage. Consequently, an infected animal can shed the bacteria within the herd before the appearance of clinical signs. Therefore, movement of undetected infected domestic animals is a major way of spreading the disease..

Clinical signs

Bovine tuberculosis may be subacute or chronic, with a variable rate of progression. A small number of animals may become severely affected within a few months of infection, while others may take several years to develop clinical signs. The bacteria can also lie dormant in the host without causing disease for a long periods.

The usual clinical signs include:

- weakness,
- loss of appetite and weight,
- fluctuating fever,
- dyspnoea and intermittent hacking cough,
- signs of low-grade pneumonia,
- diarrhoea,
- enlarged, prominent lymph nodes.

Diagnosis

Bovine TB clinical signs are not specifically distinctive and, therefore, do not enable veterinarians to make a definitive diagnosis based on clinical signs alone.

The tuberculin skin test is the standard method of TB diagnosis in live domestic animals. It consists of injecting bovine tuberculin (a purified protein extract derived from M. bovis*) intradermally and then measuring skin thickness at the site of injection 72 hours later to detect any subsequent swelling at the injection site (sign of delayed hypersensitivity associated with infection).*

Blood-based in vitro tests that detect bacteria, antibodies, or cell-mediated immunity are also currently available, or under development. The most widely used blood-based test is a gamma interferon release assay which detects a cell-mediated immune response to infection with *M. bovis*. This test is based on the principle that bovine blood cells that have previously been exposed to *M. bovis* through an infection are known to produce elevated levels of gamma interferon following in vitro incubation with *M. bovis* antigens.

Meanwhile, the definitive diagnosis is confirmed by bacterial culture and identification in the laboratory, a process that can take eight weeks or more.

The recommended diagnostic methods, including the procedures for manufacturing and administering bovine tuberculin, are described in the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*.

Public health risk

The most common form of TB in people is caused by *M. tuberculosis*. However, it is not possible to clinically differentiate infections caused by *M. tuberculosis* from those caused by *M. bovis*, which is estimated to account for up to 10% of human tuberculosis cases in some countries. Diagnosis may be further complicated by the tendency of *M. bovis* infections to be located in tissues other than the lungs (i.e. extrapulmonary infection) and the fact that *M. bovis* is naturally resistant to one of the antimicrobials that is commonly used to treat human tuberculosis, pyrazinamide.

The OIE *Terrestrial Animal Health Code* and OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* provide technical standards and recommendations that are intended to manage the human and animal health risks associated with infection of animals with a member of the Mycobacterium tuberculosis complex, including M. bovis.

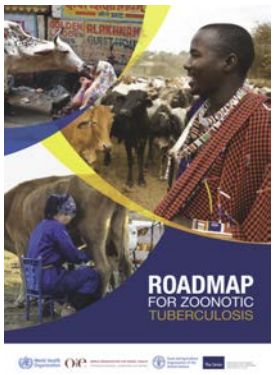
Roadmap for zoonotic tuberculosis

Human tuberculosis is a major cause of illness and mortality worldwide. It is primarily caused by *M. tuberculosis* and is usually transmitted through the respiratory route by close contact and inhalation of infected aerosols. Zoonotic tuberculosis is a less common form of human tuberculosis that is caused by a related member of the *Mycobacterium tuberculosis* complex (*M. bovis*). The zoonotic form is primarily transmitted indirectly, through the consumption of contaminated milk, dairy products, or meat containing infected material. In regions where food hygiene is consistently applied, the risk to the general public has been reduced, however zoonotic tuberculosis infection remains an occupational hazard for farmers, abattoir workers, and butchers.

The OIE, the World Health Organization (WHO), the Food and Agriculture Organization of the UN (FAO) and the International Union Against Tuberculosis and Lung Disease (The Union) jointly launched the first-ever roadmap to tackle zoonotic TB in October 2017. It is based on a One Health approach recognising the interdependence of human and animal health sectors for addressing the major health and economic impacts of this disease.

This roadmap calls for concerted action from government agencies, donors, academia, non-governmental organizations and private stakeholders across political, financial and technical levels. It defines ten priorities for tackling zoonotic TB in people and bovine TB in animals. These fall under three core themes:

- Improve the scientific evidence base
- Reduce transmission at the animal-human interface
- Strengthen intersectoral and collabortive approaches



Zoonotic TB Roadmap

Zoonotic TB factsheet

Zoonotic TB in human beings caused by M. bovis - a call for action (The Lancet)

Prevention and control

National control and eradication programs based on test and slaughter of infected animals have been successfully implemented in many countries, as the preferred approach to managing bovine tuberculosis. However, this approach remains impractical in some heavily infected countries because it could necessitate slaughtering large numbers of cattle, and this may not be feasible, due to human resource or financial limitations within the animal health program, or for cultural reasons. Therefore, countries use varying forms of test and segregation in early stages, and then switch to test-and-slaughter methods in the final stage.

Several disease eradication programmes have been very successful in reducing or eliminating the disease in cattle, by employing a multi-faceted approach that includes:

- post mortem meat inspection (looking for tubercles in the lungs, lymph nodes, intestines, liver, spleen, pleura, and peritoneum), for detection of infected animals and herds,
- intensive surveillance including on-farm visits,
- systematic individual testing of cattle,
- removal of infected and in-contact animals,
- adequate local legislation,
- effective movement controls,
- individual animal identification,
- effective traceability.

Detecting infected animals prevents unsafe meat from entering the food chain and allows Veterinary Services to trace-back to the herd of origin of the infected animal which can then be tested and eliminated if needed.

Pasteurisation or heat treatment of milk from potentially infected animals to a temperature sufficient to kill the bacteria has proven effective for preventing the spread of disease to humans.

Antimicrobial treatment of infected animals is rarely attempted because of the doses and duration of treatment that would be required, high cost of medications, and interference with the primary goal of eliminating the disease, and potential risk of developing resistance.

Vaccination is practiced in human medicine, but it is, so far, not used as a preventive measure in animals, due to the lack of availability of safe and effective vaccines, and potential interference with bovine tuberculosis surveillance and diagnostic tests, due to false positive reactions in vaccinated animals. Researchers are actively investigating potential new or improved bovine tuberculosis vaccines and alternate routes of vaccine delivery for use in domestic animals and wildlife reservoirs, as well as new diagnostic tests to reliably differentiate vaccinated animals from infected animals.

Responsible: Antimicrobial Resistance and Veterinary Products Department

Top

ANNEXURE - 4

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ORIGINAL ARTICLE

WILEY Transboundary and Emerging Diseases

Prevalence of Bovine Tuberculosis in India: A systematic review and meta-analysis

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Abstract

Bovine tuberculosis (bTB) is a chronic disease of cattle that impacts productivity and represents a major public health threat. Despite the considerable economic costs and zoonotic risk consequences associated with the disease, accurate estimates of bTB prevalence are lacking in many countries, including India, where national control programmes are not yet implemented and the disease is considered endemic. To address this critical knowledge gap, we performed a systematic review of the literature and a meta-analysis to estimate bTB prevalence in cattle in India and provide a foundation for the future formulation of rational disease control strategies and the accurate assessment of economic and health impact risks. The literature search was performed in accordance with PRISMA guidelines and identified 285 cross-sectional studies on bTB in cattle in India across four electronic databases and handpicked publications. Of these, 44 articles were included, contributing a total of 82,419 cows and buffaloes across 18 states and one union territory in India. Based on a random-effects (RE) meta-regression model, the analysis revealed a pooled prevalence estimate of 7.3% (95% CI: 5.6, 9.5), indicating that there may be an estimated 21.8 million (95% CI: 16.6, 28.4) infected cattle in India—a population greater than the total number of dairy cows in the United States. The analyses further suggest that production system, species, breed, study location, diagnostic technique, sample size and study period are likely moderators of bTB prevalence in India and need to be considered when developing future disease surveillance and control programmes. Taken together with the projected increase in intensification of dairy production and the subsequent increase in the likelihood of zoonotic transmission, the results of our study suggest that attempts to eliminate tuberculosis from humans will require simultaneous consideration of bTB control in cattle population in countries such as India.

KEYWORDS
bovine tuberculosis, buffaloes, cattle, control program, cows, India, meta-analysis, prevalence, review

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1 | INTRODUCTION

Bovine tuberculosis (bTB) is a chronic granulomatous inflammatory disease that is predominantly caused by *Mycobacterium bovis*. While primarily affecting bovines, the pathogen has a broad host range that includes humans. It has been estimated that *M. bovis* causes ~10% of the total human TB cases in developing countries and subsequently poses a significant threat to global health (Olea-Popelka et al., 2014) (Etchechoury et al., 2010) ("OIE, Bovine Tuberculosis: General Disease Information sheets,"). Prior to mandatory pasteurization of milk in many countries, *M. bovis* accounted for ~25% of all TB cases in children (Roswurm & Ranney, 1973). In addition to being a threat to public health, bTB is also a major economic concern, costing an estimated USD 3 billion worldwide annually due to losses from reduced cattle productivity, culling and movement and trade restrictions (Waters, Palmer, Buddle, & Vordermeier, 2012).

Bovine TB is well controlled in most developed countries where national control programmes have been implemented, although complete eradication and maintenance of bTB-free status are challenging given the potential of spillover from wildlife reservoir hosts. Such control programmes for bTB were successfully adopted over a century ago in many developed countries by applying test and cull strategies, resulting in enormous benefits to human health and more than 10-fold return on investment in animal productivity (Olmstead & Rhode, 2004). In contrast, bTB remains endemic in developing countries like India that lack disease control programmes because of the associated economic costs and social barriers to test and cull strategies. This current level of endemicity is likely to increase in the coming years due to a confluence of factors including the growing intensification of dairy and cattle farming and increased emphasis on improving animal productivity in these countries.

In conjunction with possessing the largest population of cattle in the world (nearly 300 million cows and buffaloes) (Basic Animal Husbandry and Fisheries Statistics, Government of India 2017), India's lack of a control programme poses a potential threat for bTB infection and transmission worldwide. In the absence of a national surveillance programme, accurate prevalence data are lacking and, to our knowledge, there has thus far not been a comprehensive review of the existing literature to determine an estimate of the overall prevalence of bTB in the country. Such an estimate will prove crucial in future efforts to accurately assess risk and inform policy for the development of effective control strategies. In this systematic review and meta-analysis, we sought to address this critical gap and determine the overall prevalence of bTB in the cattle of India. This systematic review conforms to Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (Liberati et al., 2009).

2 | METHODS

2.1 | Literature search strategy

A systematic search for published articles reporting prevalence data for bTB in cows and buffaloes in India was conducted on 11th September 2017. The four databases used in our search (CAB Direct, Web of Science, Web of Science Biological Abstracts and PubMed) were selected in order to comprehensively capture articles published in both international and local journals and minimize journal biases. After examining common MeSH terms for pre-identified and relevant publications, the following search terms were used across all four databases: (("mycobacterium bovis" OR tuberculosis) AND (cows OR cattle OR bovine) AND (epidemiolog* OR prevalen* OR inciden* OR surve*) AND (India)). No restrictions were placed on the date of publication. The citation software program EndNote X8 (Clarivate Analytics, Philadelphia, PA) was used to organize and remove duplicate articles between the databases. Additional articles were also identified manually from the reference lists of articles generated in the database search.

2.2 | Study inclusion criteria

The inclusion/exclusion criteria for data extraction are detailed in Table 1. Included studies reported the prevalence of bTB in cows and/or buffaloes in India based on commonly accepted methods for the diagnosis of bTB. More specifically, studies whose main objectives were not to determine bTB prevalence but required a preliminary prevalence study for determining initial disease status were included as long as data were reported and animals were not preselected for bTB symptoms. Prevalence studies that examined the effects of bTB control strategies were excluded in order to avoid the introduction of potential sampling bias, as the primary aims of these studies were to compare the effectiveness of control strategies. For instance, Dhanda et al. have reported an increase in prevalence in herds at Puri, Orissa, from 9.1% in 1937 to 84.7% in 1942 (Dhanda & Lall, 1959). The cattle populations that were tested were part of farms that did not practice any bTB control strategies. We believe that inclusion of studies conducted on pre-selected herds as

TABLE 1 Study inclusion/exclusion criteria

Inclusion	Exclusion
Cross-sectional prevalence study	Wrong type of study: not a cross-sectional study or animals chosen for bTB symptoms
Study conducted in India	Study conducted elsewhere
Tested for *Mycobacterium bovis* using standard diagnostic tests	Study not addressing bTB
Any breed of cow or buffalo	Study neither performed on cow nor on buffalo
Reported the prevalence of bTB and the number of total animals screened	No statistics reported
In English	Language limitation: Not in English
Full text of publication obtained	Full text unavailable
	Other

Note. Criteria for study inclusion or exclusion to our systematic review on the prevalence of bTB in India.

opposed to randomly sampled prevalence studies would not be truly representative of the prevailing prevalence in the region. Also, all other publications that did not precisely fit the main exclusion categories were excluded within the "Other" category. Finally, all included studies were cross-sectional in nature.

2.3 | Data extraction

Before beginning data extraction, a template was created based on population demographics and other conditions common to bTB prevalence studies. The data set recording general study characteristics included author, publication year, study period, location of study, diagnostic test used, criteria for positivity, sample size, prevalence by different production system, overall prevalence for cow and buffalo combined, overall prevalence for specific cattle breeds, and overall prevalence for male and female animals. Headings for prevalence data broken down by more specific characteristics were production system (organized farm, rural, Gaushala and other), cow breed (exotic, indigenous and cross-bred), sex, age (younger or older than 6 months) and species (cow versus buffalo). Data extracted from studies' individual farm-level data by each of the three of the authors (SS, LE and BR) were assigned to different strata targeted in this study. The determination of bTB infection status was accepted as reported by the studies.

A pilot test on 20 randomly selected papers was performed in order to test the inclusion and exclusion criteria and finalize the data extraction form. For the formal review of all articles generated, an initial screening for inclusion was made based on the titles and abstracts, and publications that were clearly based on different species, countries or diseases were immediately excluded. Otherwise, full texts were read for any prevalence data that could be extracted. Three of the authors (SS, LE and BR) independently reviewed all publications before comparing their respective data forms. When discrepancies were found amongst the forms, the authors (SS, LE and BR) collectively discussed their reasoning before reaching a final consensus. All studies included and excluded are publicly available at https://doi.org/10.18113/d37s9x.

2.4 | Statistical analysis

All quantitative analyses were performed in RStudio (version 1.0.143) ("R core team, R: A language and environment for statistical computing.," R core team 2016) where the "meta" package was used to estimate models (Schwarzer, 2007) (Viechtbauer, 2010). Codes used for the statistical analyses are publicly available at https://doi.org/10.18113/d37s9x. The prevalence estimates from individual studies were logit-transformed, and the pooled prevalence was estimated using meta-analytic models. Cochran's Q statistic (Cochran, 1954) was computed to test for heterogeneity, and Higgin's statistic (Higgins, Thompson, Deeks, & Altman, 2003) ($I^2 > 50\%$ represents at least moderate heterogeneity) helped describe the variability in the pooled prevalence estimate due to heterogeneity between studies.

A univariate screen was used to select a parsimonious set of moderator factors for multivariate analysis. Diagnostic test type was excluded from this selection procedure and forced into the final model in order to adjust for the well-known variability in sensitivity and specificity of diagnostic tests for bTB (Farnham, Norby, Goldsmith, & Wells, 2012). Univariable meta-regression models were estimated using both the random-effects (RE) and fixed-effects (FE) models for each potential moderator variable. Analysis of variance (ANOVA) tests were run on all moderators to assess their significance when compared to the full model with all other variables included. For the purposes of variable selection, given the low power of these tests and precedence set in other systematic reviews and meta-analyses, variables with a p-value < 0.25 were retained for inclusion in the final model (Sibhat et al., 2017) (Asmare et al., 2016) (Dohoo, Martin, & Stryhn, 2009).

The fit of the resulting multivariable meta-regression models and evidence of publication bias was assessed through funnel plots, Egger's asymmetry test (Egger, Davey Smith, Schneider, & Minder, 1997) and Begg's rank correlation (Begg & Mazumdar, 1944) test.

To visualize the prevalence of bTB in the different states of India, we generated a map utilizing an open-source library called D3.js (Data-Drive Documents) (Bostock, Ogievetsky, & Heer, 2011). This allowed us to plot data positions via the centroids of given shapefile locations represented in the map and control graphical elements based on their values (Cleveland & McGill, 1984). We utilized a continuous log scale for circle size to represent bTB prevalence and a power function for circle lightness to represent the confidence in the prevalence estimates of each state, ratifying values to visual variables on a linear scale (Bertin, 1983).

3 | RESULTS

3.1 | Characteristics of included studies

From the 285 publications screened, 44 articles were included in the systematic review (Figure 1). In the instance that a publication reported prevalence data for multiple states, years, cattle breeds, species or production systems, they were considered as separate strata level data. A total of 106 strata level data were extracted from the 44 articles for meta-analysis. For example, as seen in https://doi.org/10.18113/d37s9x, the study by Iyer (1944) has been extracted into three strata level data, the strata being the three locations in which the study was performed. The same was done for other studies that included data on different production system, breed, species, etc. These studies included in the quantitative analyses spanned from 1942 to 2016 and provided bTB prevalence data for a total sample size of 82,419 of which 29,037 were buffaloes and 53,382 were cows (Table 2).

Included studies used common diagnostic techniques for bTB testing including the single intradermal test (SIT), single intradermal comparative tuberculin test (SICT), double intradermal test (DIT), enzyme-linked immunosorbent assay (ELISA), interferon-gamma release assay (IGRA), Ziehl–Neelsen (ZN) staining and detailed postmortem (PM) examinations; some studies performed multiple tests

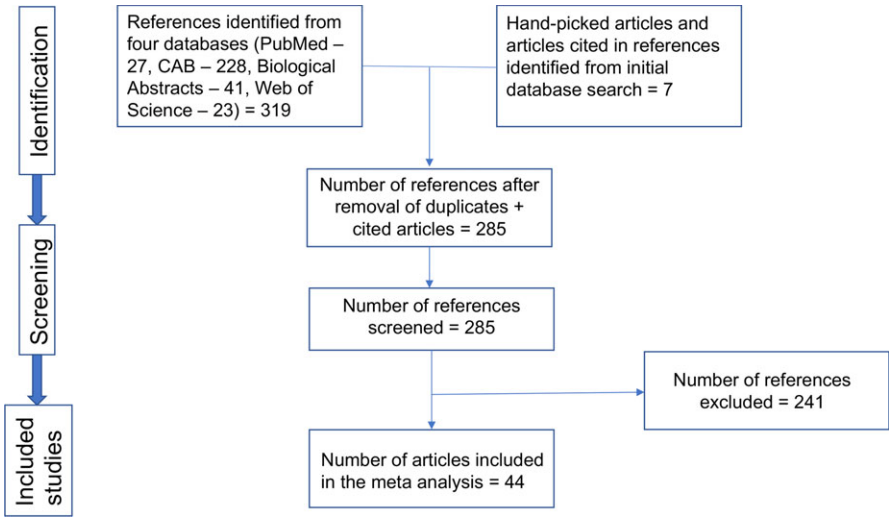

FIGURE 1 Schematic representation of the literature selection procedure for the systematic review of bTB prevalence in India

that included SIT, IGRA and ELISA (SIE). While most studies followed OIE recommended guidelines for skin test positivity at ≥4 mm after 72 hr ("International Office of Epizootics (OIE)," OIE, 2006) (the cut-off for both SIT and SICT tests), some studies defined their cut-off point as ≥5 mm; however, a small number of publications did not report criteria for test positivity (NR). A few studies classified animals as "doubtful" if the increase in skin thickness was between 3 and 4 mm. We did not use any cut-off values on the number of animals for classification of the various production systems. Most included publications explicitly mentioned the type of production system that was used in their studies. In the instance that a study did not specify the production system, we did not include that study under any production system strata. To examine any effect of time on the prevalence of bTB, the study periods were separated into four time intervals: 1941–1960; 1961–1980; 1981–2000; and, 2001–2016.

3.2 | Meta-analysis

To assess for potential publication bias, a funnel plot was constructed of the logit prevalence against standard error (Figure 2). The lack of symmetry in the funnel plot illustrates potential publication bias towards smaller studies with lower prevalence. Egger's asymmetry test was significant and showed presence of bias (p-value < 0.001), while Begg's rank correlation test did not (p-value > 0.05).

This evidence of publication bias suggests that RE model will be more appropriate for these data. To explore this further, we estimated both random- and fixed-effects models and constructed funnel plots to compare their fit and look for evidence of systematic bias (Supporting information Figures S1a,b). The RE model demonstrated greater symmetry than the FE model comparatively, suggesting that the RE model is a better fit to the data. Visual inspection of the predicted versus empirical observations (Normal Q-Q plot) also suggests that the RE model is a better fit to the data (Supporting information Figures 2a,b). As a final check, we constructed receiver operating characteristic (ROC) curves for the two models. The ROC analyses found no difference between the two models in terms of their classification ability (AUC ~ 0.74 for both models).

Given the evidence for publication bias and improved qualitative fit of the RE model, we focus on this model, which accounts for heterogeneity between individual studies, to estimate the prevalence of bTB in India from these data. The RE model was estimated from logit-transformed prevalence rates from individual publications, and the pooled prevalence estimate of bTB in India was determined to be 7.3% (95% CI: 5.6, 9.5). Cochran's (Q) value ($Q = 3939.85$, $df = 105$ and $p < 0.0001$) and Higgins statistic ($I^2 = 98.9\%$) were computed to test for heterogeneity. The meta-analysis, and comparison to the RE model, is graphically summarized in a forest plot (Figure 3).

3.3 | Meta-regression

3.3.1 | Univariable meta-regression

Due to the presence of statistical heterogeneity, we conducted univariable meta-regression in order to determine the effect of study-level covariates on the estimates of cumulative prevalence. The moderators considered for the analyses were study period, study location, sample size, production system, species, cattle breed and diagnostic technique used.

As seen in Table 3, the proportion of each predictor variable's effect on heterogeneity (R^2) ranged from 0% to 16.5% in the RE model. Further, under the RE model, the highest value of R^2 was observed for study location while, diagnostic technique, and sample size exhibited no effect on heterogeneity ($R^2 = 0\%$).

3.3.2 | Multivariable meta-regression

All moderators from the univariable meta-regression were subjected to multivariable meta-regression (Table 4), which showed that these moderators accounted for 31.4% of the observed heterogeneity. Hence, the significant variables included in our regression model explain only a fraction of the variability observed.

TABLE 2 Reported bTB prevalence for included studies

Authors	Study location	Dx. Test	Sample size	Reported prevalence‡ (%)
Mallick, Aggarwal, and Dua (1942)	Punjab	DIT	1217	23.2
Iyer (1944)	Uttar Pradesh	PM Exam	250	2.4
Iyer (1944)	Maharashtra	PM Exam	120	13.3
Iyer (1944)	West Bengal	PM Exam	130	2.3
Taneja (1955)	Haryana	DIT	102	26.5
Dhanda and Lall (1959)	Gujarat	SIT	25142	16.7
Lall, Singh, and Sen Gupta (1969)	Uttarakhand	DIT	128	0.0
Lall et al. (1969)	Punjab	DIT	111	13.5
Lall et al. (1969)	Haryana	DIT	1567	2.7
Lall et al. (1969)	Bihar	DIT	169	4.7
Lall et al. (1969)	Uttar Pradesh	DIT	1418	4.9
Lall et al. (1969)	Rajasthan	DIT	727	2.6
Lall et al. (1969)	Telangana	DIT	426	1.9
Lall et al. (1969)	Maharashtra	DIT	194	1.0
Lall et al. (1969)	West Bengal	DIT	65	0.0
Lall et al. (1969)	Himachal Pradesh	DIT	177	0.6
Purohit and Mehrotra (1969)	Rajasthan	SICT	1010	1.8
Rawat and Kataria (1971)	Madhya Pradesh	DIT	1830	2.4
Nagaraja, Krishnaswamy, Adinarayanaiah, Murthy, and Nanjiah (1973)	Karnataka	DIT	3250	5.2
Joshi, Sharma, Dhillon, and Sodhi (1976)	Punjab	DIT	1081	10.5
Bali and Khanna (1979)	Haryana	SIT	663	1.4
Bali and Khanna (1979)	Haryana	SIT	624	4.6
Paily, Georgekutty, and Venugopal (1979)	Kerala	SIT	608	0.8
Appuswamy, Batish, Parkash, and Ranganathan (1980)	Haryana	Culture	308	4.6
Kulshreshtha, Jagjit, and Chandiramani (1980)	Haryana	SIT	13089	2.5
Bali and Singh (1980)	Haryana	SIT	628	2.4
Bala and Sidhu (1981)	West Bengal	NR	475	41.5
Bala and Sidhu (1981)	Haryana	NR	712	1.1
Bala and Sidhu (1981)	Uttar Pradesh	NR	732	13.1
Murti and Hazarika (1982)	Meghalaya	SICT	302	8.9
Sharma et al. (1985)	Uttar Pradesh	PM, ZN staining	1268	13.3
Bapat and Bangi (1985)	Maharashtra	SICT	2043	1.2
Maity, Deb and Pramanik (1992)	West Bengal	PM, ZN staining	1571	0.4
Sharma, Kwatra, Joshi, and Saharma (1994)	Punjab	SIT	2623	4.0
Rakesh Sisodia, Shuykla and Sisodia (1995)	Madhya Pradesh	SIT	465	9.0
Rajaram, Rao and Manickam (1996)	Tamil Nadu	SIT	1339	14.6
Mishra, Panda, and Panda (1997)	Orissa	SIT	670	3.4
Dev, Purohit, and Joshi (1998)	Rajasthan	SICT	75	10.7
Kumar, Sharma, Iyer, and Prasad (1998)	Uttar Pradesh	PM, ZN staining	1435	9.8
Aswathanarayana et al. (1998)	Karnataka	SIT	1189	25.7
Kumar and Parihar (1998)	Uttar Pradesh	PM Exam	2028	0.8
Chowdhury, Sarkar, Pal, Roy, and Chakraborty (2001)	West Bengal	PM, ZN staining	1050	3.9
Mukhopadhyay, Antony, and Pillai (2001)	Pondicherry	SICT	41	51.2
Shringi (2004)	Rajasthan	SIT	353	4.8
Singh, Gumber, Randhawa, Aradhana and Dhand (2004)	Punjab	SIT	627	9.1

(Continues)

TABLE 2 (Continued)

Authors	Study location	Dx. Test	Sample size	Reported prevalence‡ (%)
Dali et al. (2004)	Maharashtra	NR**	340	6.2
Raval, Sunil, Belsare, Kanani and Patel (2006)	Gujarat	SIT	164	1.8
Raval et al. (2006)	Gujarat	SIT	167	0.0
Raval et al. (2006)	Gujarat	SIT	172	0.0
Raval et al. (2006)	Gujarat	SIT	152	3.3
Raval et al. (2006)	Gujarat	SIT	161	1.9
Ganesan (2006)	Tamil Nadu	SIT	63	65.1
Nishath and Ganesan (2006)	Tamil Nadu	SIT	63	49.2
Taggar and Bhadwal (2008)	Jammu and Kashmir	SIT	40	37.5
Phaniraja, Jayaramu, Jagadeesh and Kumar (2010)	Karnataka	SIT	2668	2.4
Aneesh, Mandeep, Katoch, Prasenjit, and Katoch (2010)	Himachal Pradesh	SIT	440	14.3
Trangadia, Rana and Srinivasan (2013)	Gujarat	SIT	2310	2.3
Trangadia et al. (2013)	Uttar Pradesh	SIT	338	0.6
Bhanu Rekha, Gunaseelan, Pawar, and Giri (2014)	Tamil Nadu	ELISA	357	4.5
Neeraja et al. (2014)	Karnataka	SIE	45	26.7
Ashish, Amit, and Deepak (2014)	Uttar Pradesh	SIT	245	14.3
Thakur, Sinha and Singh (2016)	Uttar Pradesh	SIT	442	16.1
Thakur et al. (2016)	Uttarakhand	SIT	99	0.0
Filia, Leishangthem, Mahajan, and Singh (2016)	Punjab	SICT	121	14.0

Notes. The reported bTB prevalence for each included study on a state-by-state basis. Diagnostic techniques (Dx. Tests) used were single intradermal test (SIT), single intradermal comparative tuberculin test (SICT), double intradermal test (DIT), ELISA, interferon-gamma release assay (IGRA), multiple tests that included SIT, IGRA, and ELISA (SIE), Ziehl–Neelsen (ZN) staining and detailed post-mortem examinations (PM). While most studies reported which Dx. test was used, some were not reported (NR) or ‡were unconventional. **Confidence intervals were reported for only a few studies and thus not included in the table above.

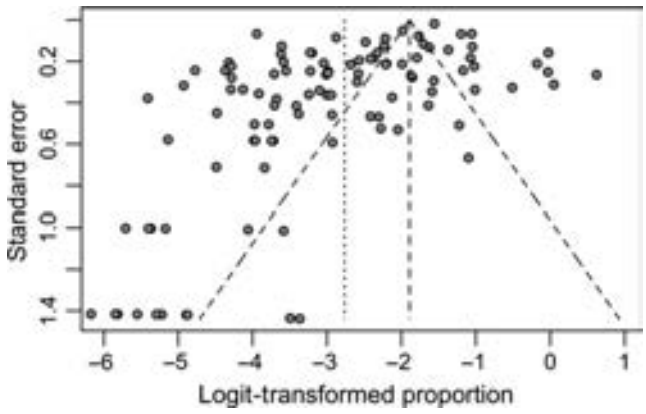

FIGURE 2 Funnel plot of standard error and logit-transformed prevalence demonstrates potential publication bias

Analysis of variance (ANOVA) tests indicated that five (study period, study location, species, diagnostic test and breed) of the seven moderators were significant ($p < 0.25$) when the other variables were included (Table 5).

3.4 | Effect of moderators on prevalence of bTB

Prevalence estimates using both the RE and FE models are reported in Table 6. As noted above, the values reported from RE model are likely more appropriate given the observed heterogeneity in the studies as the FE model is biased by studies with larger sample size. Based on the RE model, the prevalence of bTB in cows, 6.3% (95% CI: 4.9, 8.0), was marginally higher than the prevalence in buffaloes, 4.3% (95% CI: 2.7, 6.7). Amongst cows, prevalence by breed did not vary greatly as cross-bred cows were found to have the highest prevalence with 8.1% (95% CI: 4.6, 13.8), followed by indigenous cows with 7.4% (95% CI: 4.0, 13.1), and exotic cows with 7.0% (95% CI: 3.7, 12.9). Unlike cattle breed, larger differences were seen amongst production systems as cattle housed in Gaushalas (protective shelters for unproductive or destitute cows in India) had a higher prevalence, 19.1% (95% CI: 13.0, 27.1) than those kept in organized farms, 5.1% (95% CI: 3.8, 6.7) and rural conditions, 4.4% (95% CI: 1.0, 16.5). The time period, 1941–1960, was found to have the highest prevalence, 13.8% (95% CI: 10.5, 17.9), while 1961–1980 was found to have the lowest, 3.6% (95% CI: 2.6, 4.9). A total of 28,073 animals had been tested during 1961–1980. The time period between 1981 and 2000 showed a prevalence of 7.0% (95% CI: 4.8, 10.2), and the prevalence of the most recent time period between 2001 and 2016 was determined to be 6.8% (95% CI: 4.3, 10.7) (Table 6).

3.5 | Geographical distribution of included studies in India

Study reports from included publications encompassed 18 states and one union territory in India. No reports were found for Arunachal

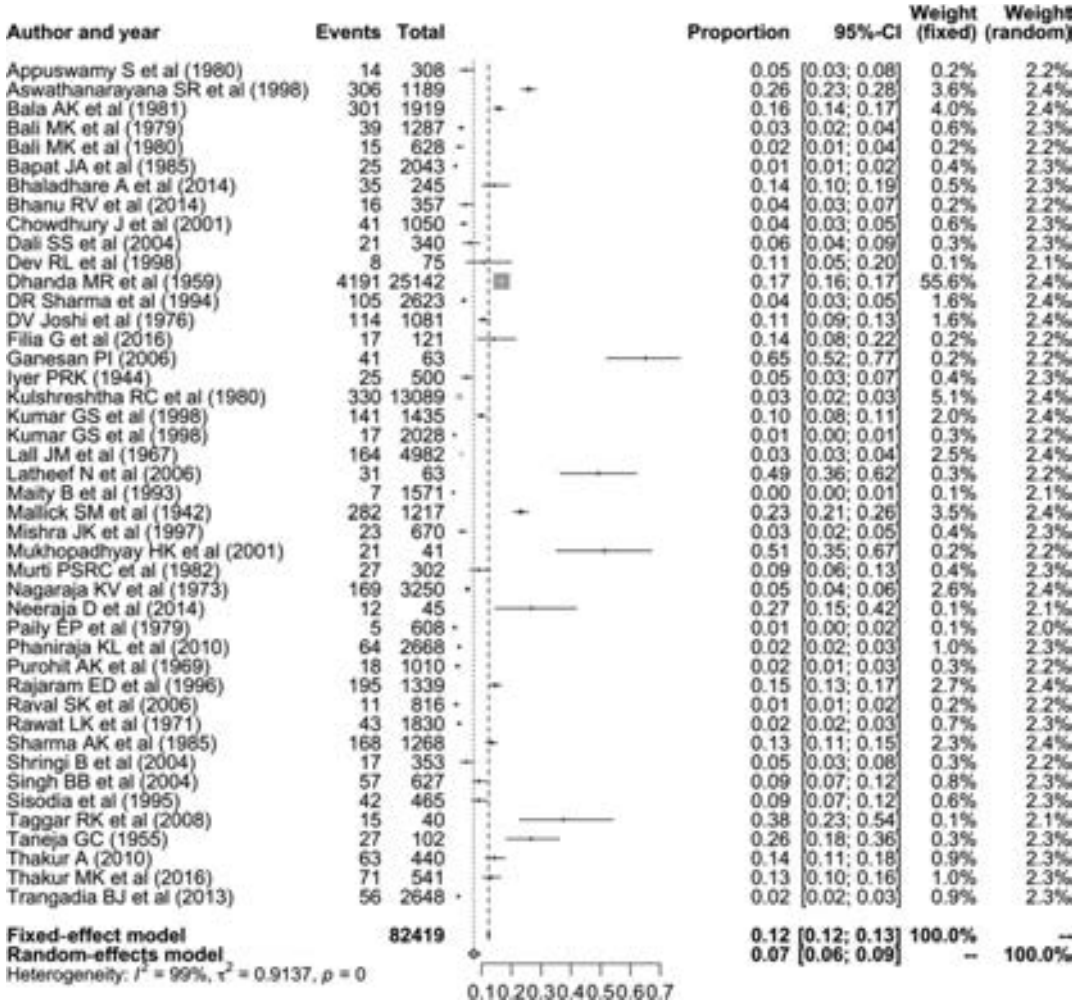

FIGURE 3 Forest plot visualizing the varying bTB prevalence reported for each included publication in the meta-analysis. Weightage given to each included publication by both RE and FE models have been shown for rigorous comparison. "Total" refers to the number of animals in each publication, while "Events" refers to the number of bTB-positive animals. "Proportion" reports the bTB prevalence for each publication

TABLE 3 Univariable meta-regression

Predictors	Proportion (R^2) (%)	p value (RE)
Study period	7.0	0.04
Study location	16.5	0.01
Diagnostic technique	0.0	0.70
Species	0.7	0.22
Breed	0.7	0.40
Production system	2.5	0.16
Sample Size	0.0	0.95

Note. Proportion of effect of predictors on heterogeneity. All variables had a $p < 0.01$ in the FE model.

Pradesh, Assam, Chhattisgarh, Goa, Jharkhand, Manipur, Mizoram, Nagaland, Sikkim, Telangana, Tripura, Andaman and Nicobar Islands, Chandigarh, Dadra and Nagar Haveli, Daman and Diu, Delhi, and Lakshadweep, comprising a total of 11 states and six union territories. It can be observed from the map that the prevalence of bTB varied highly between states (Figure 4) (Table 7).

4 | DISCUSSION

After screening of 285 publications, we extracted data from 44 cross-sectional studies published in peer-reviewed journals that report the prevalence of bTB in India and conducted meta-analysis. The pooled prevalence estimate (RE model) for all of India was found to be 7.3% (95% CI: 5.6, 9.5). Despite being a disease of antiquity with significant animal and public health costs that have been controlled in most developed countries over a half-century ago, bTB has a high and widespread prevalence in India as no national control strategies have been implemented in the country (Figure 4). These data suggest that India, as the world's largest producer of milk (~156 MMT), accounting for ~18.5% of the world's total milk production and the world's largest red meat exporter (~1.9 MMT), has an urgent and as yet unmet need for control of bTB for both economic and public health reasons (DADF, 2015).

Following the White Revolution (Bellur, Singh, Chaganti, & Chaganti, 1990) (Nair, 1985), a rural development programme in India that resulted in making India the largest producer of milk and milk

TABLE 4 Multivariable meta-regression

Predictors	Categories	No. of studies	Odds ratio (95% CI)	*p*-value (RE)
Study period	1941–1960	7	Reference	
	1961–1980	36	0.15 (0.04, 0.65)	0.01
	1981–2000	29	0.21 (0.05, 1.01)	0.05
	2001–2016	34	0.14 (0.03, 0.65)	0.01
Production systems	Gaushala	6	Reference	
	Organized	71	0.34 (0.09, 1.20)	0.09
	Rural	4	0.24 (0.04, 1.52)	0.13
	Semen station	1	1.05 (0.03, 34.89)	0.98
	Slaughterhouse	9	0.57 (0.06, 5.51)	0.61
Species	Buffalo	23	Reference	
	Cow	83	0.60 (0.28, 1.27)	0.16
Study location	Andhra Pradesh	2	Reference	
	Bihar	1	2.57 (0.13, 52.46)	0.54
	Gujarat	10	0.33 (0.03, 3.59)	0.36
	Haryana	15	0.51 (0.06, 4.48)	0.54
	Himachal Pradesh	3	3.88 (0.30, 49.20)	0.29
	Jammu and Kashmir	1	7.74 (0.27, 218.94)	0.22
	Karnataka	7	1.82 (0.19, 17.33)	0.60
	Kerala	2	0.22 (0.01, 5.80)	0.36
	Madhya Pradesh	5	1.56 (0.14, 17.54)	0.72
	Maharashtra	7	0.81 (0.08, 8.70)	0.86
	Meghalaya	2	1.22 (0.06, 24.31)	0.89
	Orissa	2	0.73 (0.03, 17.07)	0.84
	Pondicherry	1	58.57 (2.16, 1595.91)	0.01
	Punjab	12	2.12 (0.26, 17.49)	0.48
	Rajasthan	6	1.89 (0.18, 19.82)	0.58
	Tamil Nadu	5	8.17 (0.55, 121.89)	0.12
	Uttar Pradesh	16	1.32 (0.15, 11.50)	0.80
	Uttarakhand	2	0.13 (0.01, 3.21)	0.21
	West Bengal	7	2.39 (0.23, 24.87)	0.46
Diagnostic test	SIT	46	Reference	
	Culture	2	3.99 (0.53, 30.28)	0.18
	DIT	25	0.69 (0.23, 2.10)	0.52
	ELISA	2	0.71 (0.09, 5.52)	0.75
	PM Exam	6	0.08 (0.01, 0.77)	0.03
	SICT	11	0.69 (0.18, 2.65)	0.59
	SIE	1	0.07 (0.00, 1.03)	0.05
Breed	Cross-bred	19	Reference	
	Exotic	10	1.08 (0.37, 3.18)	0.88
	Indigenous	15	0.97 (0.39, 2.37)	0.94
Sample size			1.00	< 0.0001

Note. Multivariable meta-regression of the selected predictors on the prevalence of bTB in India. (R^2 = 31.4%, n = 106).

products, organized dairy farming has expanded rapidly. These farms have comparatively high (and still increasing) animal densities, paving the way for higher probabilities of disease transmission. In contrast, rural farms are owned by small-holder farmers and have much lower stocking densities, resulting in lower likelihoods of disease transmission. Our results show the prevalence of bTB in animals housed under organized farming systems to be 5.1% (95% CI: 3.8, 6.7) and rural conditions to be 4.4% (95% CI: 1.0, 16.5). The overlap of CI in

TABLE 5 ANOVA results

Predictors	*p*-value (RE)
Study period	0.04*
Study location	0.001*
Production system	0.55
Species	0.16*
Diagnostic test	0.12*
Breed	0.13*
Sample size	0.93

Note. ANOVA results of individual predictors subjected to multivariable meta-regression. All variables had a $p < 0.01$ in the FE model. *represents significance.

prevalence between organized and rural settings as suggested by the RE model is curious given the prevailing dogma that organized farming poses higher risk of disease transmission, suggesting that further investigation is needed for clarification of this issue. Yet, amongst the three different production systems in which the animals were housed, Gaushalas had the highest prevalence of bTB, 19.1% (95% CI: 13.0, 27.1). Gaushalas are protective shelters for destitute or unproductive cows in India. There are over 5,100 of these "old age homes" for cows in India (DADF, G. o. I., 2015), which may account for the higher prevalence observed in this group of animals as bTB is a chronic infection. While noteworthy and in line with expectations of observing greater prevalence in such a high-risk setting, the lower sample size for Gaushalas compared with sample sizes of other production systems must be kept in consideration and warrants further investigation.

Overall, the ordering of prevalence estimates determined using the FE model for different production systems follows the same trend as in the RE model (i.e., prevalence in Gaushala > Organized farms > Rural farms) (Table 6). However, given the observed heterogeneity in the studies, it is difficult to assess the validity of the FE model, and hence, further study is necessary to clarify the exact influence that each production system has on bTB prevalence before definitive conclusions can be made. We note that accurate estimates of prevalence rates for each production system are particularly important in the Indian context where the magnitude of animals housed in Gaushalas and the increasing population of cattle being reared under intensive conditions have the potential to considerably impact overall prevalence and influence assessment of bTB transmission rates and targeted interventions.

Regarding animal species (cow versus buffalo), the meta-analysis (RE model) shows prevalence to be higher in cows [6.3% (95% CI: 4.9, 8.0)] than in buffaloes [4.3% (95% CI: 2.7, 6.7)]. However, we note that the prevalence in buffaloes determined using the FE model was 16.0% (95% CI: 15.5, 16.4) and that in cows was 10.2% (95% CI: 9.8, 10.5). The high prevalence observed in buffaloes using the FE model is most likely driven by a single study that sampled 21,592 buffaloes (of a total buffalo sample size of 29,037 included in this meta-analysis) and recorded a prevalence of 17.4% (Dhanda & Lall, 1959). As per the Government of India's Department of Animal, Dairy and Fisheries (DADF) 2016–2017 Annual report, the share of milk contribution from buffaloes is 49% and that of cows is 48% (DADF, 2016-2017). Assuming a conservative 10% loss in milk productivity due to bTB (Thoen, 2008) and the overall estimated bTB prevalence rates based on the RE model, the annual costs to farmers only from loss in milk production in cows and buffaloes in India are estimated to range from 375 to 544 million USD (Supporting information Table S1). We note that the need for intensification of dairy production to meet increased milk demand and national priorities for nutritional improvement and rural development is likely to significantly increase bTB disease prevalence as the disease is known to more easily spread amongst intensively reared cattle. With the inevitable increase in bTB prevalence, this already large economic cost will only continue to grow if no intervention measures are implemented.

Published studies on the influence of breed on genetic susceptibility to bTB showed that the native breed of cattle is more resistant to the disease than exotic breed (Vordermeier et al., 2012) (Soparkar,

TABLE 6 Pooled prevalence estimates (derived from both RE and FE models) of the various predictors namely, cattle species, breed, production system and study period

	Predictors	Sample size	Prevalence (95% CI) (RE model)	Prevalence (95% CI) (FE model)
Species	Buffalo	29,037	4.3% (2.7, 6.7)	16.0% (15.5, 16.4)
	Cow	53,382	6.3% (4.9, 8.0)	10.2% (9.8, 10.5)
Cattle breed	Exotic	2,011	7.0% (3.7, 12.9)	16% (14.1, 18.2)
	Cross-bred	9,548	8.1% (4.6, 13.8)	13.5% (12.7, 14.5)
	Indigenous	4,169	7.4% (4.0, 13.1)	15.5% (14.0, 17.1)
Production systems	Gaushala	576	19.1% (13.0, 27.1)	18.7% (15.7, 22.3)
	Organized farm	43,847	5.1% (3.8, 6.7)	8.4% (8.1, 8.7)
	Rural farm	1,607	4.4% (1.0, 16.5)	3.3% (2.2, 4.7)
Study period	1941–1960	26,961	13.8% (10.5, 17.9)	17.0% (16.6, 17.5)
	1961–1980	28,073	3.6% (2.6, 4.9)	3.9% (3.6, 4.2)
	1981–2000	16,927	7.0% (4.8, 10.2)	13.9% (13.2, 14.6)
	2001–2016	10,458	6.8% (4.3, 10.7)	9.2% (8.5, 10.0)

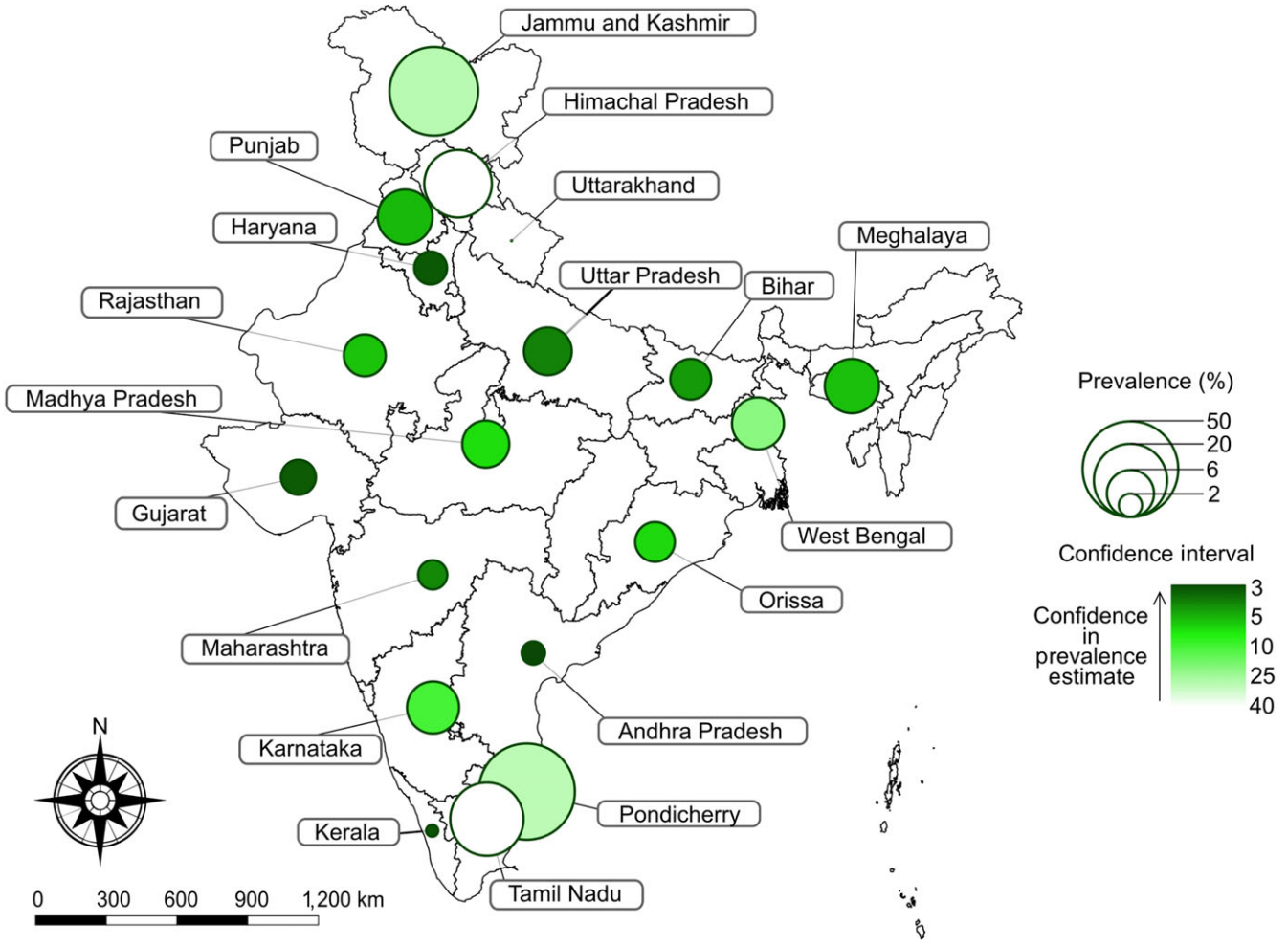

FIGURE 4 Geographical distribution and pooled prevalence estimates (RE model) of bTB in the different states of India. For the confidence intervals, a power scale was used to map colour lightness and represented as $y = mx^k + b$, where $k = 0.5$. Prevalence was mapped to a log-scale where data were uniformly corrected with allow for visual properties. Note that although scales were altered, the original data set is provided in Table 7 to afford accurate measures

1925) (Liston & Soparkar, 1917) (Sharma, Vanamayya, & Parihar, 1985), affirming a generally held and commonly disseminated dogma. In contrast, our results note no significant differences in bTB prevalence between cow breeds in either the RE or the FE models (Table 3). However, given the heterogeneity observed in the studies, rigorous investigations of the true differences in susceptibility amongst different cattle breeds to bTB will be essential for evidence-based formulation of a rational approach to control this disease in India.

India has a cattle population of nearly 300 million, and we attempted a conservative estimation of the number of infected cattle in India. As per DADF, G. o. I., 2015 (Basic Animal Husbandry and Fisheries Statistics, 2015), there were 39.7 m exotic and cross-bred cows, 151.2 m indigenous cows and 108.7 m buffaloes in 2015 (DADF, G. o. I., 2015). Applying bTB prevalence estimates (obtained from our meta-analysis) of 7.3% (95% CI: 5.6, 9.5) across all cattle types, there are likely to be ~21.8 m (95% CI: 16.6, 28.4) bTB-positive cattle in India, suggesting that India likely also has the highest burden of bTB-infected animals in the world, exceeding even at the lower confidence interval the total number of dairy cattle in the United States (USDA, 2016). We note that *M. bovis* may not necessarily be the only causative agent of bTB in all reactor animals as isolation of *M. tuberculosis* from cattle samples has also been reported (Srivastava et al., 2008) (Sweetline Anne, Ronald, Kumar, Kannan, & Thangavelu, 2017).

The multivariable logistic regression model accounted for ~31% of the heterogeneity between studies, suggesting that additional factors not part of our model are also contributors to bTB prevalence. These factors may include animal age, sex and herd size that were not represented with enough frequency in the papers included in the systematic review to be subject to robust and rigorous meta-analysis. Hence, future studies should strive to understand how these factors contribute to overall bTB prevalence.

We note that the findings of this study must be considered in conjunction with the limitations inherent in systematic reviews and meta-analyses. For example, studies were limited to those included in the four databases used and no single study sample can provide a perfect representation of the cattle in a state or across the country. Further, our review consisted of only published studies written in English and did not capture any unpublished data, subjecting our analysis to

TABLE 7 Pooled prevalence estimates (RE model) of bTB prevalence in India by state

STATE	Sample size	Prevalence (95% CI) (RE model)
Andhra Pradesh	426	2% (1.0, 3.9)
Bihar	169	4.7% (2.4, 9.2)
Gujarat	28,268	3.6% (2.2, 5.8)
Haryana	17,693	3.3% (1.9, 5.4)
Himachal Pradesh	617	15.4% (4.2, 43.4)
Jammu and Kashmir	40	37.5% (24.0, 53.2)
Karnataka	7,152	7.9% (3.0, 19.2)
Kerala	608	1.0% (0.3, 3.6)
Madhya Pradesh	2,295	6.3% (2.7, 14.00)
Maharashtra	2,697	2.7% (1.0, 6.9)
Meghalaya	302	8.7% (5.1, 14.3)
Orissa	670	4.5% (1.5, 12.5)
Pondicherry	41	51.2% (36.3, 66.0)
Punjab	5,780	8.9% (5.5, 14.2)
Rajasthan	2,165	5.0% (2.1, 11.5)
Tamil Nadu	1,822	19.6% (6.6, 45.9)
Uttar Pradesh	8,156	6.5% (4.3, 9.8)
Uttarakhand	227	0.4% (0.1, 3.1)
West Bengal	3,291	7.8% (2.1, 25.7)
Grand Total	82,419	

publication bias (Figure 4). This potential publication bias is supported by the significant result of Egger's regression test, the test with greater statistical power (Hayashino, Noguchi, & Fukui, 2005).

In addition, our findings are limited by the variation in experimental design and methodology of each included article. Variation in the reporting details of each article also contributes to variations in study quality. It is important to note that the pooled prevalence estimate of 7.3% (95% CI: 5.6, 9.5) was derived from studies that, despite efforts during the screening process to exclude studies with biased sampling methods, may not have been entirely random surveys and needs to be refined with proper cross-sectional national level surveys using internationally recognized and well-standardized methods. In addition, acceptance of each study's reported number of positive animals as truly positive is also a limiting but unavoidable reality of lacking access to the original data and an inability to know the sensitivity, specificity and other performance characteristics of the tests used. In addition, while most studies' objectives were to determine bTB prevalence in the study population, studies differed in their examination or reporting of specific moderators of interest.

Our analysis also indicated the presence of temporal heterogeneity (R^2 = 7.04%) over the 74-year time frame (1942–2016) represented by the included studies (Table 3). While the specific source(s) of this heterogeneity is unclear, contributors may include differences in environmental conditions over time (Humblet et al., 2010) (Bekara, Azizi, Bénet, & Durand, 2016), the number of studies within each time interval, animals tested, test operators' skills/methods and the diagnostic tests themselves. Recent studies have also shown that the quality, origin and source of tuberculin used are variable within tuberculin-based tests, highlighting a lack of standardization (Bakker et al., 2005). In addition to such variation within individual tests, the performance, sensitivity and specificity vary across tuberculin-based tests making comparisons difficult and imprecise (Hartnack & Torgerson, 2012) (Varello et al., 2008) (Cousins & Florisson, 2005) (Ameni, Miörner, Roger, & Tibbo, 2000). While most tests are tuberculin-based, there are potential causes for heterogeneity that remain to be explored. Thus, combined with the existing limitations of non-standardized and varying performance characteristics of current diagnostic tests, we underscore the need for a national surveillance programme using a single, well-standardized skin test performed by independent, well trained operators using OIE approved protocols and well-standardized tuberculin antigen to enable accurate monitoring of bTB prevalence over time and the impacts of any potential intervention or control programme.

Several previous studies have reported prevalence of bTB in farms that used control strategies for the disease. Although test and slaughter of reactor animals as a control strategy are practically impossible in developing countries like India due to both economic and social considerations, the above-mentioned studies provide preliminary evidence that even test, and segregate approaches have the potential to help reduce the prevalence of bTB in India, at least in intensively farmed animals that are regularly tested using well-standardized tests (Dhanda & Lall, 1959) (Krishnaswamy, Nagaraja, Keshavamurthy, Nanjiah, & Adinarayanaiah, 1973; Mukherjee, 2006).

Taken together, the meta-analysis highlights a critical and hitherto unmet need for the development of a national surveillance programme and the implementation of an effective strategy for control of bTB in India—a need that will only continue to grow in conjunction with India's increasing cattle population and demands on milk production and an inability to cull potentially diseased cows. Given the likely inability of implementing a test and cull programme at any scale due to social and economic considerations, the need for a vaccine that can reduce the burden of infection and transmission is critical. In this context, we note that recent reports suggest that the century-old BCG vaccine may have considerable utility in this regard (Ameni, Vordermeier, Aseffa, Young, & Hewinson, 2010) (Ameni et al., 2017), but requires further study to evaluate its ability in reducing onward transmission. However, if effective, there is also an unmet need for a validated and accepted fit-for-purpose DIVA (Differentiating Infected from Vaccinated Animals) diagnostic test for the detection of bTB-infected cattle that can be used in conjunction with a vaccine programme.

Mycobacterium bovis has also been isolated from milk samples of tuberculous cattle (Aswathanarayana, Rao, Krishnappa, Ramanatha, & Raghavan, 1998) (Veerasami et al., 2012). Given the fact that over 70% of the milk in India is sold unpasteurized (FAO/OIE/WHO, 1993), this raises concerns regarding the potential for zoonotic transmission of bTB and continued spread of human tuberculosis (India has the world's largest burden of human TB) (Thoen, LoBue, & de Kantor, 2006). In May 2014, the World Health Assembly adopted a new strategy to attain an ambitious goal of ending the global TB

epidemic by 2035: the End TB strategy (Uplekar et al., 2015). Given the prevalence of bTB and the potential for zoonotic transmission, particularly to children and others who consume unpasteurized or unprocessed milk from infected cows, there is a critical need for a national bTB control programme in India and other developing countries as attempts to eradicate the disease from humans without eradicating it from cattle are likely to prove futile. Importantly, implementation of a national control programme would not only enable accurate temporal trends and estimates of bTB prevalence, risk and economic costs, but would equally importantly improve the health and productivity of cattle in India.

5 | CONCLUSION

Overall, the results of our systematic review and meta-analysis conducted on 44 publications indicate high and widespread bTB prevalence in India of 7.3% (95% CI: 5.6, 9.5). Further study is necessary to obtain more robust state-by-state prevalence estimates and explore other moderators of risk (including herd size, animal sex, and age, amongst others) that are likely to impact development and implementation of a rational and effective bTB control strategy. Taken together with the expected dairy farm intensification, growing demands for increased milk production and the zoonotic nature of *M. bovis*, the results of our current studies highlight the importance of developing and implementing a national bTB control programme that will need to include a national surveillance plan using (a) well-standardized method(s) and evidence-based intervention(s) that are likely to work in India and other developing country settings.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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ANNEXURE - 5

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Prevalence of zoonotic tuberculosis and associated risk factors in Central Indian populations

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ABSTRACT

In the present study, we aimed to estimate the occurrence of bovine tuberculosis (TB) and examine the determinants of distribution of the disease in three high-risk populations of Central India. A prospective cohort study was conducted in Central India between March 2014 and June 2015. Based on the requisite inclusion criteria, we recruited a total of 301 participants whose blood samples were subjected to polymerase chain reaction-based detection and differentiation of *Mycobacterium bovis* and *Mycobacterium tuberculosis*. *M. bovis* was detected in 11.4%, 8.9%, and 12.6% of the recruited participants belonging to three distinct population groups (Groups A, B, and C, respectively). The highest proportion of cases infected with *M. bovis* was observed in Group C, who lived in the high TB endemic region. Previous contact with active TB cases (odds ratio = 3.7; 95% confidence interval, 0.9612–14.4533) and raw milk consumption (odds ratio = 5.3472; 95% confidence interval, 1.9590–14.5956) were found to be important determinants of bovine TB in this population. The high incidence rates of bovine TB in the Central Indian populations indicate the substantial consequences of this disease for some population groups and settings. However, more research is necessary to identify the main transmission drivers in these areas.

1. Introduction

Tuberculosis (TB) remains the foremost leading cause of death worldwide, affecting more than 9 million people every year. Although *Mycobacterium tuberculosis* is the most common cause of human TB, unknown proportions of TB cases are considered to be attributable to *Mycobacterium bovis* infection, which is also termed bovine TB [1]. The infection currently poses a major concern in human populations in developing countries, as humans and animals share the same microenvironment. It has been estimated that zoonotic transmission of *M. bovis* is responsible for 10–15% of new human TB cases in developing countries [2]. The disease in humans primarily occurs through close contact with infected cattle or consumption of improperly cooked beef and drinking of unpasteurized milk and milk products [3,4]. People in specific occupations such as veterinarians, farmers, and abattoir workers are considered to be more at risk [5].

The prevalence of bovine TB infection and associated risk factors have been insufficiently characterized in the Indian population that is considerably dependent on agriculture for its livelihood. It has been suggested that poverty, poor dietary habits, close physical contact between humans and animals, and inadequate disease control measures facilitate the transmission of zoonoses [6]; however, there is no substantial evidence in the Indian context to justify the hypothesis.

Currently available tests used for identification of *M. bovis* are based on bacterial isolation and biochemical tests, which are both time-consuming with low diagnostic accuracy [7]. Furthermore, TB caused by *M. tuberculosis* in humans is clinically and radiologically identical to TB caused by *M. bovis* [8]. These problems were overcome by molecular techniques to some extent; however, this technique could not identify the mycobacterial pathogens to the

Abbreviations: TB, Tuberculosis; M. *bovis*, *Mycobacterium bovis*; MTB, Mycobacterium tuberculosis; MTBC, Mycobacterium tuberculosis complex species; RD, Region of difference; PCR, Polymerase chain reaction; AFB, Acid fast bacilli; CIIMS, Central India Institute of Medical Sciences; PBS, Phosphate buffered saline; EDTA, Ethylenediaminetetraacetic acid; CTAB, Cetyl-trimethyl-ammonium bromide; BCG, Bacille Calmette Guerin; ESAT-6, 6 kDa Early Secretory Antigenic Target.

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species level. In the present study, with the purpose of detecting zoonotic transmission of *M. bovis* and simultaneous differentiation of members of *M. tuberculosis* complex species (MTBC), we targeted the regions of difference (RDs) through a duplex polymerase chain reaction (PCR) assay.

As there are limited reports on the occurrence of bovine TB in India, we aimed to estimate its incidence among participants belonging to three distinct population groups and settings based on assessment of risk factors and occupational exposure to animals. The participants for this study were recruited from Achalpur, Amravati, Pilkapar, and Nagpur, located in the Central Indian region of Maharashtra, India. A comparative analytical study was then carried out to evaluate the determining factors that may influence the distribution of the disease in each population.

2. Material and methods

2.1. Ethics statement

The study was approved by the Ethical Committee of Central India Institute of Medical Sciences, Nagpur. All clinical investigations were conducted according to the principles expressed in the declaration of Helsinki 1975, as revised in 1983. Written consent forms were obtained from all recruited participants after they were given a detailed oral explanation of the study.

2.2. Study design and participants

A total of 433 participants were enrolled from three different populations within Central India region through camps organized between March 2014 and June 2015. These population groups were approached with the help of a local health care practitioner. A survey using a standardized questionnaire investigating exposure to TB cases; consumption of meat, unpasteurized milk, and milk products; as well as other sociodemographic parameters such as age, sex, occupation, duration of exposure to animals, and type of animal reared along with clinical history was conducted (Table S1). Active TB was investigated in individuals with respiratory or systemic symptoms by acid fast bacilli (AFB) smear and culture of appropriate sputum samples and chest X-ray. The details of the recruited populations are described in the following subsections.

2.2.1. Group A: Farmers, dairy workers, and livestock keepers

Participants in this group included farmers from Achalpur and Pilkapar villages of the state of Maharashtra in Central India. Most of the population had agriculture as their major occupation. Some of them were also involved in dairy production and livestock keeping. The farmers or members of their family were personally involved in the maintenance of these animals. The animals reared included Indian breed of ox, cows, buffaloes, and calves. Milking and delivery of pregnant animals were also done by the members of the household.

2.2.2. Group B: Zookeepers and animal handlers

This population included zookeepers and veterinarians from a specific locality within the Nagpur district. The participants from this group were involved in activities such as guarding, cleaning, feeding, and maintenance of animals in the zoo. The veterinarians recruited in this group were involved in routine health checkup, semen analysis, treatment, and *post mortem* of deceased animals.

2.2.3. Group C: Residents of high TB endemic area

This population belonged to a specific locality of Nagpur district, in the Vidarbha region of Maharashtra. The majority of the population routinely included meat and other animal products in their diet. The endemic area had high crowding index with an average of six to eight individuals living in small poorly ventilated rooms. The majority of the population had poor socioeconomic status and living conditions characterized by lack of sanitation and poor hygiene. Some households also participated in cattle and goat rearing.

Of the 433 participants enrolled in this study, 84 participants were excluded based on their refusal to give blood. The remaining 349 participants who matched the inclusion criteria were selected for the study. Among these, pregnant women ($n = 8$), children below the age of 10 years ($n = 18$), and individuals with fungal or viral infections ($n = 22$) were also excluded from the study. Fig. 1 represents the inclusion/exclusion criteria adopted for recruitment of the study populations.

2.3. Sample collection

For DNA isolation, 2.5 mL blood was collected in 5-mL sterile syringe BD and dispensed in a sterile vacutainer with coagulant EDTA). Each sample was labeled with a code that corresponded to the study location and identification of the individual.

2.4. DNA isolation and quantification

DNA was extracted from blood samples using the phenol chloroform extraction method described by Deshpande et al. [9], in which 6 mL phosphate buffered saline (PBS) was added to 2 mL blood and mixed thoroughly. Next, 8 mL of blood + PBS was added slowly from the sides of the tube to 4 mL Histopaque and centrifuged at 2000 rpm for 10 min. The buffy coat was transferred to another tube, and an equal volume of PBS was added and the tube was centrifuged again at 2000 rpm for 10 min. The supernatant was discarded and the pellet was suspended in 500 μL PBS, 15 μL 10% SDS, and 3 μL proteinase K (20 mg/ml), then mixed and incubated at 55 °C for 1 and ½ hour. After incubation, 100 μL of 5 M NaCl and 80 μL of high-salt cetyl-trimethyl-ammonium bromide (CTAB) (containing 4 M NaCl, 1.8% CTAB) was added and mixed followed by incubation at 65 °C for 10 min. An approximately equal volume (350 μL) of phenol and of chloroform/isoamyl alcohol (24:1) was added, mixed thoroughly, and centrifuged for 10 min in a microcentrifuge at 12,000 rpm. The aqueous viscous supernatant was carefully decanted and transferred to a new tube. An equal volume of phenol/chloroform-isoamyl alcohol (1:1) was added, followed by a 10-min spin at 12,000 rpm. The aqueous layer was separated and then mixed with 30 μl of 3 M sodium acetate and 0.6 vol of isopropanol to obtain a precipitate. The precipitated nucleic acids were washed with 70% ethanol, dried and resuspended in 30 μL Tris–EDTA (TE) buffer, and then stored at −20 °C prior to use. DNA concentrations for all samples and strains used in this study were determined with the Quant-iT dsDNA HS assay kit using a Qubit fluorometer (Invitrogen).

2.5. Duplex PCR

For determination to the species level of the mycobacterial pathogens—namely, *M. tuberculosis*, *M. bovis*, and *M. bovis* bacille Calmette–Guerin (BCG)—two genetic regions RD4 and RD1 were amplified using a duplex approach. Primers used in this study are shown in Table 1. RD4 is an RD in the bovine lineage. The use of RD4 flanking primers ensured that the PCR products were formed only if the deletion was present [10]. The genes in the RD1 region belong to the *esat6* gene cluster. Early secretory antigenic target-6 (ESAT-6) is a potent stimulator of the immune system, and is an antigen recognized during the early stages of infection. The RD1 region of *M. tuberculosis* is considered to be

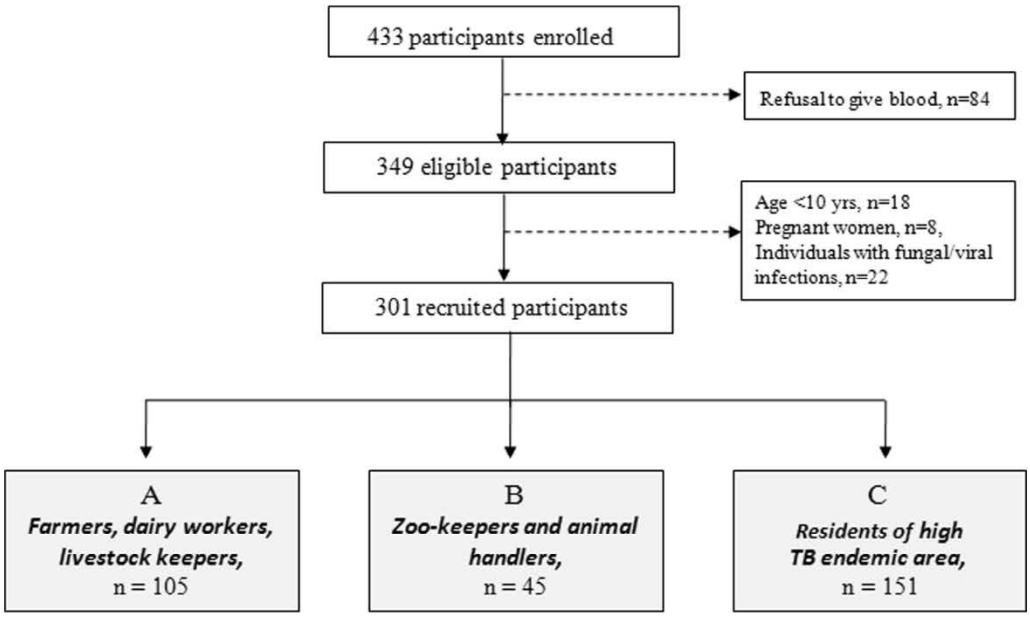

Fig. 1. Inclusion/exclusion criteria adopted for recruitment of the study population. The population was categorized into three groups based on their occupation and origin. Gray boxes indicate the groups included in the final analysis.

Table 1
Primer sequences for RD region analysis.

PCR	Primers	Sequence	Annealing temperature (°C)	Amplicon size (bb)	Reference
RD4	F	5′-AATGGTTTGGTCATGACGCCTTC-3′	58	176	Taylor et al. [10]
	R	5′-CCCGTAGCGTTACTGAGAAATTGC-3′			
RD1	F	5′-CCCTTTCTCGTGTTTATAGTTTGA-3′	60	110	Halse et al. [11]
	R	5′-GCCATATCGTCCGGAGCTT-3′			

PCR = polymerase chain reaction; RD = region of difference.

the primary attenuating deletion in the related vaccine strain *M. bovis* BCG [11].

The duplex PCR reactions were carried out using 10× PCR buffer, 1.5 $MgCl_2$, 0.8 mM dNTP (deoxynucleotide triphosphate), 0.4 μM of RD1 F/R primer, and 0.2 μM of RD4 F/R primer and 1.25 U of *Taq* DNA polymerase. The amplification procedure consisted of initial denaturation at 95 °C for 7 min and 35 cycles each of denaturation at 95 °C for 1 min, annealing at 59 °C for 1 min, and extension at 72 °C for 1 min followed by a final extension step at 72 °C for 10 min. A positive control, DNA extracted from *M. bovis* (ATCC BAA-935) culture, and a negative-no template control were included in each run.

2.6. PCR minimum detection limit

The analytical sensitivity of the duplex PCR assay was determined using 10-fold serial dilutions from 10^7 fg to 10 fg of DNA (equivalent to 10^6–10° genome copies) isolated from *M. tuberculosis* (ATCC 25177), *M. bovis* (ATCC BAA-935), and *M. bovis* BCG Pasteur (ATCC 35734) reference strains. To assess the specificity, the concentration of the DNA templates from each reference strain was adjusted to 10 ng/μL and subjected to PCR.

2.7. Interpretation of the results of duplex PCR

The PCR amplicons were analyzed on a 2% agarose gel and stained with ethidium bromide. The amplified products were then visualized under UV light. Comparative analysis on electrophoresis of the PCR products generated by the two sets of primer pairs showed the ability to distinguish between *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG. The duplex PCR was considered positive for *M. bovis* when bands of both 176 bp and 110 bp were seen; the result was considered positive for *M. tuberculosis* when a band of only 110 bp was seen and positive for *M. bovis* BCG when a band of 176 bp was visualized on the gel.

2.8. DNA sequencing analysis

The PCR products were purified and sequenced using Sanger's dideoxy chain termination method at the SciGenom Labs (Cochin, India). Sequences were verified by Basic Local Alignment Search Tool (BLAST) search using the NCBI National Center for Biotechnology Information (NCBI) website.

2.9. Statistical analysis

The frequencies (percentage) of demographics, clinical factors, and risk factors were measured on a nominal scale. Comparison between groups was performed using the chi-square test in MedCalc statistical software (version 10.1.2.0), and a difference with $p < 0.05$ was considered significant. Odds ratios (ORs) along with 95% confidence intervals (CIs) were obtained for each factor using the bivariate analysis done using the SPSS software (version 22.0).

3. Results

3.1. Baseline characteristics of the populations under study

The baseline characteristics of the populations under study are represented in Table 2. A total of 179 (59.5%) males and 122 (40.5%) females were enrolled in this study. A significantly higher

percentage of the population from Group A (59.1%) and Group C (55%) belonged to the 18–40 years age group. On the contrary, a higher proportion of the population from Group B (75.6%) belonged to the >40 years age group. Reported clinical symptoms at the time of the study were compared in each population. A significantly higher percentage of participants with fever (39.7%), chills (34.4%), cough with expectoration (41.7%), abdominal pain (37.7%), chest pain (20.5%), weight loss (27.1%), night sweating (25.2%), headache (55.6%), and loss of appetite (37.1%) belonged to the population from Group C ($p < 0.0001$). By contrast, a significant number of ($p < 0.0001$) participants with body ache (43.8%) belonged to the population from Group A. Risk factors such contact with active TB case (51%) and living in an endemic area (100%) were more incident in Group C (26.82%) as compared to the other groups ($p < 0.0001$). Exposure to animals and raw milk consumption was more common in Group B (80%) and Group A (48.6%), respectively, and the difference was statistically significant ($p < 0.0001$).

3.2. Detection and differentiation of M. bovis and M. tuberculosis by duplex PCR

A total of 105 samples were collected from Group A, among which 25 samples were positive by the duplex PCR assay. Of these 25 cases, *M. bovis* was detected in 12 (11.4%) and *M. tuberculosis* was detected in 13 (12.4%) cases. Forty-five samples were collected from the participants belonging to Group B. Of these, 11 (24.4%) were found to duplex PCR positive with four (8.9%) cases and seven (15.6%) cases infected with *M. bovis* and *M. tuberculosis* respectively. A total of 151 samples collected from Group C were subjected to duplex PCR assay, of which 60 (39.7%) were found to be positive. *M. bovis* was identified in 19 (12.6%) cases and *M. tuberculosis* was identified in 41 (27.2%) cases. The detection and differentiation *M. tuberculosis* and *M. bovis* in blood samples is represented in Fig. 2. The total positivity observed by the duplex PCR assay is shown in Table 3.

3.3. Population wise distribution of bovine TB

Fig. 3 shows the population-wise distribution of bovine TB. PCR positivity for *M. bovis* induced TB was found to be highest in Group C (12.6%), consisting of residents from the high TB endemic region, followed by Group A with 11.4% positivity. This group consisted of farmers, dairy workers, and livestock keepers. PCR positivity was found to be the lowest (8.9%) in Group B, which consisted of zookeepers and animal handlers.

Table 2
Demographic and clinical characteristics of the study populations ($n = 301$).

Characteristics	A[a] ($n = 105$)	B[b] ($n = 45$)	C[c] ($n = 151$)	Chi-square	*df*	Significance level (p)	Contingency coefficient
Demographic characteristics							
Age (y)							
<18	2 (1.9)	0 (0)	12 (7.9)	5.786	1	0.0162	0.541
18–40	62 (59.1)	11 (24.4)	83 (55)	52.73	2	<0.0001	0.503
>40	41 (39)	34 (75.6)	56 (37.1)	5.786	2	0.0554	0.206
Sex							
Male	72 (68.6)	41 (91.1)	66 (43.7)	9.061	2	0.0108	0.22
Female	33 (31.4)	4 (8.9)	85 (56.3)	82.836	2	<0.0001	0.636
Clinical characteristics							
Fever	33 (31.4)	3 (6.7)	60 (39.7)	50.813	2	<0.0001	0.588
Chills	12 (11.4)	3 (6.7)	52 (34.4)	60.925	2	<0.0001	0.69
Cough with expectoration	41 (39)	8 (17.8)	63 (41.7)	41.054	2	<0.0001	0.518
Abdominal pain	19 (18.1)	1 (2.2)	57 (37.7)	63.688	2	<0.0001	0.673
Chest pain	17 (16.1)	2 (4.4)	31 (20.5)	25.24	2	<0.0001	0.579
Weight loss	15 (14.3)	8 (17.8)	41 (27.1)	28.344	2	<0.0001	0.554
Night sweating	13 (12.4)	4 (8.9)	38 (25.2)	33.855	2	<0.0001	0.617
Headache	33 (31.4)	12 (26.7)	84 (55.6)	63.767	2	<0.0001	0.575
Body ache	46 (43.8)	4 (8.9)	29 (19.2)	33.899	2	<0.0001	0.548
Loss of appetite	27 (25.7)	2 (4.4)	56 (37.1)	51.553	2	<0.0001	0.614
Risk factors							
Previous contact with TB case	15 (14.3)	3 (6.7)	77 (51)	97.562	2	<0.0001	0.71
Living in a high endemic area	20 (19)	2 (4.4)	151 (100)	229.399	2	<0.0001	0.755
Animal contact	82 (78.1)	36 (80)	49 (32.5)	20.204	2	<0.0002	0.329
Raw milk consumption	51 (48.6)	2 (4.4)	38 (25.2)	42.484	2	<0.0001	0.564

df = degree of freedom; TB = tuberculosis.
[a] Farmers, dairy workers and livestock keepers.
[b] Zookeepers and animal handlers.
[c] Residents of high TB endemic area.

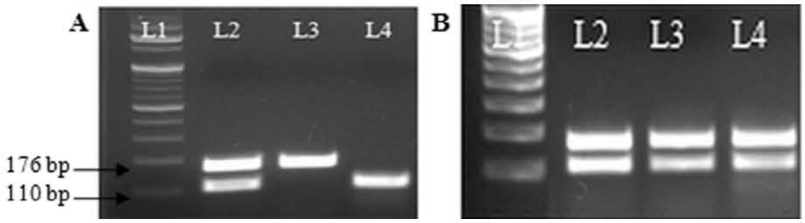

Fig. 2. Duplex polymerase chain reaction (PCR) for detecting and differentiating *Mycobacterium bovis*, *Mycobacterium tuberculosis*, and *M. bovis* BCG. (A) The ethidium bromide-stained amplification products of L2: *M. bovis*, L3: *M. bovis* BCG, and L4: *M. tuberculosis* when electrophoresed on 2% agarose gel. The 176-bp and 110-bp products obtained are indicated. (B) L1: 100 bp molecular ladder; L2: positive control; L3 and L4: samples with *M. bovis* infection. BCG = bacille Calmette–Guerin.

Table 3
Duplex PCR positivity in the three populations under study.

Group	No of samples collected	Duplex PCR positivity No. (%)	*Mycobacterium bovis* No. (%)	*Mycobacterium tuberculosis* No. (%)
A	105	25 (23.8%)	12 (11.4%)	13 (12.4)
B	45	11 (24.4%)	4 (8.9%)	7 (15.6%)
C	151	60 (39.7%)	19 (12.6%)	41 (27.2%)

PCR = polymerase chain reaction.

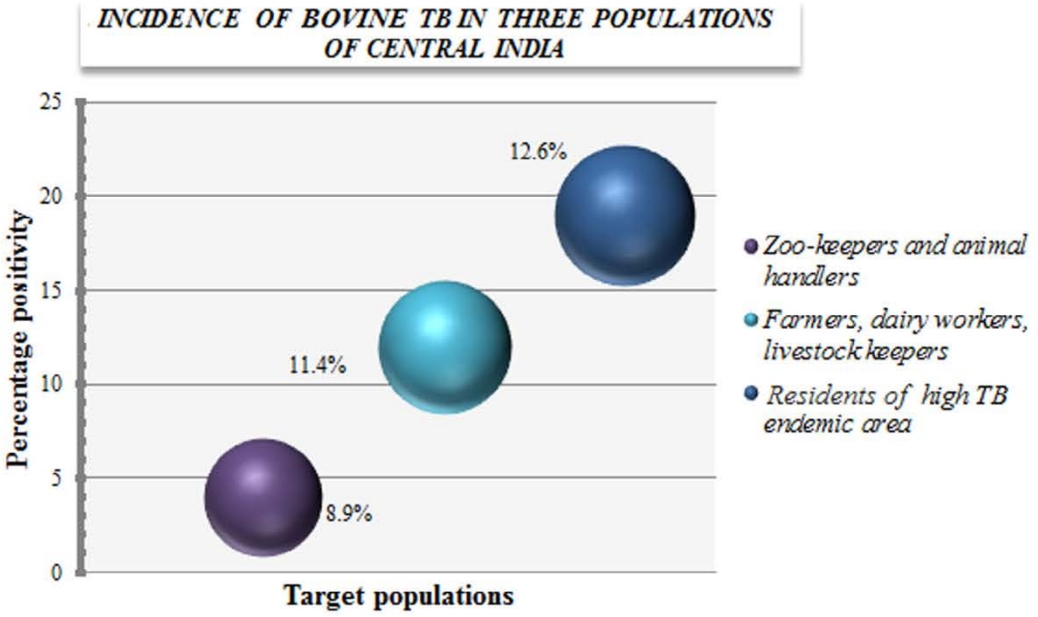

Fig. 3. Population-wise distribution of bovine tuberculosis (TB) in Central India. Positivity (%) of *Mycobacterium bovis*-induced TB infection among three population groups.

3.4. Association between risk factors and bovine TB

Association of risk factors with PCR positivity was studied through bivariate analysis in each of the target population, and results are shown in Table 4. PCR positivity was the outcome, and the risk factors were treated as predictors. In Group A, contact with TB cases (OR = 23.5; 95% CI, 3.06–180.79) and raw milk consumption (OR = 6.34; 95% CI, 1.3161–30.5553) significantly increased the risk of positivity to nearly 20-fold and 6-fold, respectively ($p < 0.05$). A similar trend was also observed in Group C. Contact with active TB cases (OR = 3.7; 95% CI, 0.9612–14.4533) and raw milk consumption (OR = 5.3472; 95% CI, 1.9590–14.5956) significantly increased the likelihood of positivity by 3-fold and 5-fold, respectively. In Group C, exposure to animals (OR = 2.6308; 95% CI, 0.1297–53.3659) doubled the odds in favor of positivity; however, it was not statistically significant.

4. Discussion

In industrialized countries, animal TB control and elimination programs, together with milk pasteurization, have drastically reduced the incidence of disease caused by *M. bovis* in both cattle and humans [12]. In developing countries, however, animal TB is widely distributed, control measures are not applied or are applied sporadically, and pasteurization is rarely practiced [13,14]. In India, very limited data on the zoonotic aspects of *M. bovis* are available. There is a lack of surveys to date to assess the public health concerns posed by bovine TB.

In the present study, we examined the incidence of bovine TB in different populations of Central India and also assessed the risk factors that may influence the occurrence of the disease. In view of the described objective, we primarily recruited participants who were in direct or indirect contact with animals.

The duplex PCR analysis conducted in our study indicated that the residents of the high TB endemic region of Central India had the highest number of *M. bovis*-infected cases (12.6%). This particular population was characterized by individuals living in a community with high crowding index and high prevalence of TB [15]. Majority of the population in this region consumed meat bought from local abattoirs. Some of the respondents during recruitment noted that no proper inspection of meat and meat products was done prior to consumption. Thus, the existing eating culture (eating of raw meat and drinking of raw milk) and the prevailing low standard of hygiene practices may be potential risk factors that favor the spread of bovine TB in this population. A report by Hubbert and Hagstad [16] suggested that ingestion of meat and meat products from slaughtered infected cattle that have not been thoroughly cooked may pose a serious risk of zoonotic infection.

Another important factor that may be contributing to the spread of zoonotic infection in this population was contact with TB index cases. The participants from this group lived in houses that were poorly ventilated and were household contacts of active pulmonary TB cases. In this study, we observed that the highest number of participants with clinical symptoms consistent with active TB such as fever, cough with expectoration, night sweats, chest pain, and abdominal pain, belonged to this group. It has been reported that *M. bovis* causes clinical presentations identical to those of *M. tuberculosis*-induced TB [8,14]. Also, most culture-based systems cannot differentiate between the two pathogens to the species level [17]. It may be likely that some cases that were earlier diagnosed with TB in our previous studies may in fact be overlooked cases of bovine TB.

Table 4
Represents association of PCR positivity (%) with risk factors.

Sr. No.	Risk factors	A			B			C		
		Odds ratio (95% CI)	*p*	*Z* statistic	Odds ratio (95% CI)	*p*	*Z* statistic	Odds ratio (95% CI)	*p*	*Z* statistic
1	Previous contact with TB case									
	No	1	0.0024	3.034	1	0.8997	0.126	1	0.0571	1.903
	Yes	23.5 (3.0568–180.7912)			1.2 (0.0540–27.6783)			3.7 (0.9612–14.4533)		
2	Living in a high endemic area									
	No	1	0.3415	0.951	1	0.2683	1.107	1	0.8914	0.137
	Yes	0.1472 (0.0028–7.6335)			4.28 (0.3296–54.0878)			0.8941 (0.1793–4.4584)		
3	Animal contact									
	No	1	0.0187	2.352	1	0.5288	0.63	1	0.0502	1.959
	Yes	0.2237 (0.0642–0.7790)			2.6308 (0.1297–53.3659)			2.6496 (0.9993–7.0251)		
4	Raw milk consumption									
	No	1	0.0213	2.302	1	0.7294	0.346	1	0.0011	3.273
	Yes	6.3415 (1.3161–30.5553)			1.7556 (0.0723–42.6042)			5.3472 (1.9590–14.5956)		

CI = confidence interval; PCR = polymerase chain reaction.

The present study has significant clinical relevance because *M. bovis* is intrinsically resistant to an important first line anti-TB drug, i.e., pyrazinamide, and the standard regimen including this drug have to be altered [14]. Therefore, such studies would significantly help in patient management programs.

Subsequent high positivity was observed among participants belonging to Group A, consisting of farmers, dairy workers, and livestock keepers from rural settings. These individuals not only had close contact with the animals while feeding and milking, but also lived in close proximity at night, sometimes even under the same roof. A prospective cohort study among farmers in the United Kingdom suggested that agricultural workers may acquire bovine TB by inhaling cough spray from infected cattle and develop typical pulmonary TB [18]. According to one of the respondents, they share their room with the newly born calves at night to protect them from wild animals. Consumption of raw milk was also a common practice in this group. During the interviews, it was recorded that many participants preferred unpasteurized milk over boiled milk because of its richer taste. This could be one of the reasons for transmission of *M. bovis* from cattle to humans [19]. It has also been postulated by Michel et al. [20] that pastoralist and rural communities would be at greatest risk for bovine TB, but the lack of data for these population groups prevents confirmation of this assumption.

The third population recruited for the study consisted of zookeepers and animal handlers. The possible mechanisms of transmission of *M. bovis* in this particular population may include close contact while handling, cleaning the barn, participating in animal necropsies, and living in close proximity to the cages. A report by Michalak et al. [21] described the first case of reverse zoonosis wherein *M. tuberculosis* was transmitted from humans to elephants. Therefore, reliable diagnosis and prevention of TB in all domesticated and exhibited animals is ideal.

To the best of our knowledge, this is the first report on the prevalence of bovine TB in the Central Indian population. A systematic literature search on the occurrence of zoonotic TB by Müller et al. [22] showed that there is lack of data for the World Health Organization region of Southeast Asia, including major cattle producing middle- and low-income countries (e.g., India, Bangladesh, Pakistan, Myanmar, and Indonesia). Recorded incidence rates for zoonotic TB in Europe, the United States, Australia, and New Zealand were consistently below 1/100,000 population/y. The incidence rates were not available for other countries [22].

Individual studies from various regions reported high proportions of zoonotic TB for specific population groups and settings. For example, in the Hispanic community in the United States, zoonotic TB appeared to be a considerable proportion of all TB cases and was associated with the consumption of unpasteurized cheese from Mexico. The highest median proportions for TB caused by *M. bovis* were observed in countries in Africa: Ethiopia, Nigeria, and Tanzania. However, the specific populations affected and risk factors of zoonotic TB in these settings remained largely elusive [14]. In keeping with the earlier reports, our present study also indicates pockets of zoonotic transmission of TB for specific population groups and settings.

In the Indian context, studies by Shah et al. [17] and Prasad et al. [23] have shown high incidence of *M. bovis* and *M. tuberculosis* in extrapulmonary samples of humans and cattle, respectively. A similar study by Mittal et al. [24] has demonstrated the importance of screening and differential diagnosis of MTBC in humans and livestock.

Despite being an insightful study, our work suffers from the limitation of insufficient data on animal health. It was learned that worshipping of cattle has spiritual significance in some regions; therefore, withdrawing blood or performing any tests on their animals was not possible. The present study thus needs further evaluation in animal population to identify the main transmission drivers in these areas.

5. Conclusion

In conclusion, this study has documented the prevalence of neglected bovine TB in human population in Central India. Diagnosis and monitoring of this disease are essential, especially in developing countries such as India, where humans and animals have close association in routine life. This study would thus be valuable in guiding policy makers for further studies in bovine TB epidemiology.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jegh.2017.08.007.

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ANNEXURE - 6

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Isolation and Identification of *M. tuberculosis* from Sheep Tissue Samples and Sero-Diagnosis Study in an Organized Sheep Farm

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A B S T R A C T

A 2 years old female Madras Red sheep with the medical history of reduction in feed intake, poor weight gain and emaciation was found dead in an organised farm. The sheep did not have any obvious respiratory symptoms. Edematous and caseous lesions were observed in mesenteric, bronchial, mediastinal and prescapular lymph nodes of the sheep during post mortem examination. Other internal organs were free of any specific lesions. The lymph node samples were decontaminated and cultured by inoculating into BACTEC Mycobacteria Growth Indicator Tube (MGIT) system and Lowenstein Jensen slants. The cultures turned positive and acid fast staining of the bacterial culturerevealed the presence of Mycobacteria. The bacteria was further confirmed as *Mycobacterium tuberculosis* by multiplex PCR and nucleotide sequencing. A Tuberculosis sero-diagnostic study was conducted for all the animals in the farm using commercially available ELISA kit to know the incidence of tuberculosis in the farm. Three sheep out of the total 205 sheep were positive for tuberculosis by ELISA with the estimated 1.5% positivity. This shows the active circulation of tuberculosis in sheep farm and there may be possibility of human to animal transmission and vice versa. The role of sheep in the epidemiology and transmission of tuberculosis needs further study.

Introduction

Tuberculosis (TB) is a chronic bacterial disease caused by *Mycobacterium tuberculosis* complex (MTC) leading to decreased productivity, economic losses and poses a significant threat to human health. Among MTC organisms, the major agents are *M. tuberculosis* and *M. bovis*. The primary host for *M. bovis* is cattle and *M. tuberculosis* is human. However, occurrence of *M. tuberculosis* in animals and *M. bovis* infection in humans has been reported previously (Ocepek *et al.,* 2005).

In ovine, the occurrence of tuberculosis is very rare although there are few reports indicating the presence of *M. bovis* in sheep and goat (Kassa *et al.,* 2012; Marianelli *et al.,* 2010). This primarily occurs in areas with high intensity sheep population and when there exists close contact between infected cattle and sheep facilitating transmission between these species. India accounts for one fourth of the global TB burden (Central TB division, GOI, 2017).

Tuberculosis in animals is not well studied in India; the lack of nation-wide epidemiological studies makes the disease burden largely unknown (Neeraja *et al.,* 2014a). Few studies have documented the prevalence of TB in animals in India (Parmer *et al.,* 2014; Srivastava, 2008). Tuberculosis causes huge economic loss in farm animals and the production loss in infected animals will be 10 to 20 percent (Verma *et al.,* 2004).

Tuberculosis is often unnoticed in animals and the infected animals continue to spread the disease to other susceptible animals and human by excreting the organisms through milk, faeces and respiratory droplets. Hence to control tuberculosis both animals and human has to be monitored for disease prevalence. In this study Mycobateria was isolated from a TB infected sheep and M. tuberculosis was identified by multiplex PCR and gene sequencing. Then all the sheep in the farm were screened for TB sero-positivity.

Materials and Methods

Sample collection

Post mortem examination was carried out on one Madras red sheep that had died in an organized farm. The mesenteric, pre-scapular, bronchial and mediastinal lymphnodes were edematous and caseous. Samples from these lymphnodes were collected in sterile PBS and transported to laboratory on ice for mycobacterial culture.

Isolation *Mycobacterium* Sp. from tissue samples

The samples were decontaminated and processed following the modified Petroff's method (Kent and Kubica, 1985). A portion of the decontaminated sediments were inoculated into Mycobacterial Growth Indicator Tubes (MGIT)^{TM} from Becton Dickinson (BD) and incubated in BACTEC MGIT 960 instrument for 49 days at 37 °C.

The remaining sediments were inoculated into one tube each of OADC-supplemented Middlebrook 7H10 agar and Lowenstein-Jensen (LJ) medium with sodium pyruvate and glycerol and each tube was incubated for 8 weeks at 37 °C.

Acid fast staining

Heat fixed smears prepared from the sediment and MGIT cultures declared as positive by the BACTEC 960 and typical growths on Middlebrook 7H10 and LJ media were screened for presence of acid fast bacilli. The heat-fixed smears were stained for acid fast bacilli as per the standard protocol.

Polymerase chain reaction confirmation and sequencing

The DNA extraction from MGIT liquid culture and colonies on 7H10 agar/LJ media was performed according to the CTAB –NaCl method. These DNA samples were subjected to conventional polymerase chain reaction (PCR) with specific primers reported by Zumarrga *et al.,* (1999) and Bakshi *et al.,* (2005). Then amplified PCR products were sequenced to confirm the mycobacterium species.

Sero prevalence study using ELISA

Sheep sera samples from study farm were screened for tuberculosis antibodies using the commercial ELISA kit (IDEXX), USA as per manufacturer's instruction.

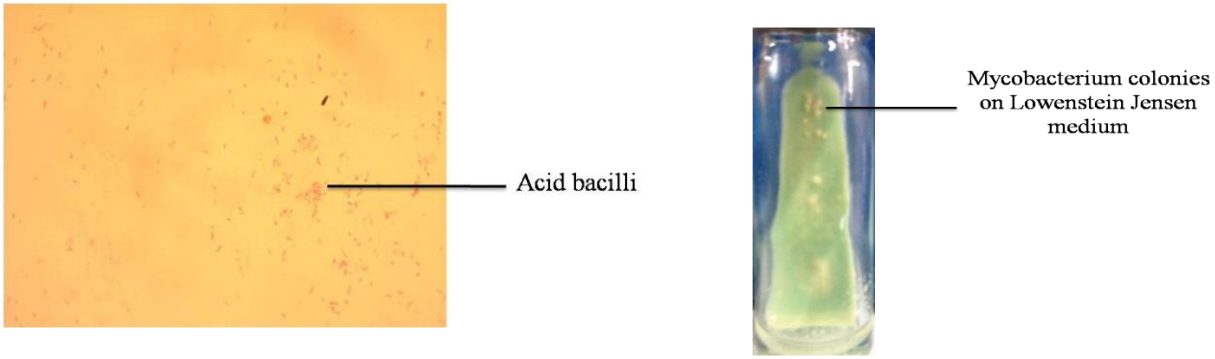
Result and Discussion

The post mortem caseous, edematous lymphnode tissue samples collected from the tuberculosis-suspected sheep were subjected to acid fast staining, bacterial culture and PCR. Staining of tissue smear from sheep lymph node revealed that presence of rod shaped, acid fast bacilli indicating the presence of mycobacterium infection (Figure 1a).

Bacterial culture study is the gold standard for laboratory confirmation of TB. Hence the tissue samples were cultured in LJ medium resulting in colonies that were rough, granular and whitish initially and later on the colonies turned yellowish (Figure 1b).

DNA amplification by PCR provides a rapid and sensitive method for the detection of *M. tuberculosis* complex (MTC) from post-mortem samples and cultures (Clarridge *et al.,* 1993). DNA extracted from LJ medium culture were subjected to multiplex PCR method. PCR product was further analyzed by agarose gel electrophoresis. There was no band around 168 bp which is *M. bovis* specific whereas *M. tuberculosis* specific band around 337 bp was visualized (Figure 2).

Fig.1a Acid Fast bacilli in Ziehl-Neelsen staining; **Fig.1b** Characteristic Mycobacterium colonies on Lowenstein Jensen medium



1a 1b

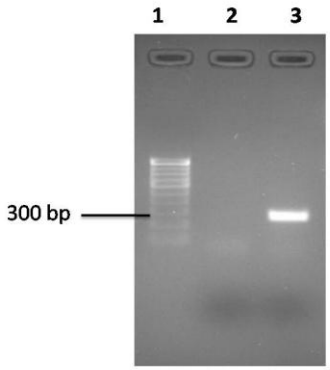


Figure 2 – Multiplex PCR; Lane 1 – 100 bp marker
Lane 2 – Negative control; Lane 3 – Sample

The PCR product was subjected to gene sequencing and confirmed as *M. Tuberculosis*.

Further, the circulation TB in the sheep farm was identified by using ELISA to estimate sero-prevalence. Generally humans are the maintenance hosts for *M. tuberculsois*. The sheep is considered to be the spill-over hosts for *M. bovis*, can maintain the organism only when its population density is high and is generally considered very rare in small ruminants (Tschopp *et al.,* 2011).

However, presence of MTB in sheep indicates a possible transmission of infection from human to animal. In this study out of 205 sheep 3 were sero-positives and indicates 1.5% sero-prevalence of TB was observed in study population. Lack of a robust animal TB surveillance system and vaccine use in animals aids in the transmission of TB between animals and from animals to human or *vice versa*. Thus there is an urgent and unmet need for implementation of animal TB control programs in developing countries through extensive surveillance. The license for the use of BCG vaccine in animals also warrants further studies.

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ANNEXURE - 7

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Review Article

Tuberculosis in Birds: Insights into the *Mycobacterium avium* Infections

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Tuberculosis, a List B disease of World Organization for Animal Health, caused by *M. avium* or *M. genavense* predominantly affects poultry and pet or captive birds. Clinical manifestations in birds include emaciation, depression and diarrhea along with marked atrophy of breast muscle. Unlike tuberculosis in animals and man, lesions in lungs are rare. Tubercular nodules can be seen in liver, spleen, intestine and bone marrow. Granulomatous lesion without calcification is a prominent feature. The disease is a rarity in organized poultry sector due to improved farm practices, but occurs in zoo aviaries. Molecular techniques like polymerase chain reaction combined with restriction fragment length polymorphism and gene probes aid in rapid identification and characterization of mycobacteria subspecies, and overcome disadvantages of conventional methods which are slow, labour intensive and may at times fail to produce precise results. *M. avium subsp. avium* with genotype *IS901*+ and *IS1245*+ causes infections in animals and human beings too. The bacterium causes sensitivity in cattle to the tuberculin test. The paper discusses in brief the *M. avium* infection in birds, its importance in a zoonotic perspective, and outlines conventional and novel strategies for its diagnosis, prevention and eradication in domestic/pet birds and humans alike.

1. Introduction

Avian tuberculosis is one of the most important diseases that affect domestic and pet birds. Several mycobacterial species can be involved in the aetiology of avian tuberculosis. The disease is most often caused by *Mycobacterium avium* belonging to serotypes 1, 2, 3, and 6 (genotype *IS901*+ and *IS1245*+) and *M. genavense* [1–3]. Other species, such as *M. intracellulare, M. scrofulaceum, M. fortuitum, M. tuberculosis,* and *M. bovis* can also cause avian tuberculosis, but the incidences are rare [2, 4–6]. *M. avium* causes avian tuberculosis in probably all avian species, especially in waterfowl, galliformes, columbiformes, passerines, psittacines, raptors, and ratites [1, 7–10]. The disease has a worldwide distribution but is seen most frequently in the North Temperate Zone [11–14]. Susceptibility to disease varies from species to species. Hejlicek and Treml [15] broadly classified bird species into four groups according to their susceptibility to avian tuberculosis as highly susceptible: domestic fowl, sparrows, pheasants, and partridges; less susceptible: guinea fowl and domestic turkeys; moderately resistant: domestic goose and duck, highly resistant: the domestic pigeon. In any avian species, stress factors appear to enhance the development of the disease and this is particularly noteworthy in case of birds living in captivity [4]. Infected birds and contaminated water and soil are the main source of infection as the *Mycobacteria* can survive for several months in the environment [2, 5]. The disease is more prevalent in places with high population density and poor sanitation and hygienic conditions. The practices of allowing birds to roam freely and keeping the

breeders for several years are highly conducive to the spread of tuberculosis [11]. In a flock if once established, TB induces unthriftiness, decreased egg production, and increased mortality, which culminates into severe economical losses.

Mycobacterium avium complex (MAC), comprising *M. avium subsp. avium, M. avium subsp. paratuberculosis, M. avium subsp. Silvaticum,* and *M. intracellulare*, may also infect different animal species like swine, cattle, deer, sheep, goat, horses, cats, dogs, and exotic species besides causing infection in immunocompromised human beings [3, 4, 16, 17]. *M. genavense* has also been reported in a dog and an immunocompromised cat. *M. intracellulare* is a closely related pathogen of birds with a lower prevalence [18]. Although successful experimental infections with *M. a. paratuberculosis* in poultry have been reported [19], however, this subspecies, known to cause Johne's disease (paratuberculosis) in ruminants and other mammals, has not been encountered during any of the cases of avian tuberculosis till date. *M. avium subsp. avium* (MAA) is considered as the most important pathogen causing tuberculosis in domestic birds [2, 20]. On the basis of genetic and phenotypic differences it has been proposed to categorize MAA into two subspecies, namely, *M. a. hominissuis* for human and porcine isolates and *M. a. avium* for bird-type isolates [21]. In humans, *M. avium* is capable of inducing a progressive disease that is refractory to antibiotic treatment and is recognized as localized primary lymphadenitis, pulmonary disease, and a disseminated form of infection [4, 22]. Hence the handling of infected birds in farms or live cultures of *M. avium* in laboratories should be carried out with adequate care.

2. Etiology

M. avium, the causative agent of avian tuberculosis, considered as "atypical mycobacteria", comprises aerobic, nonspore-forming and nonmotile rod shaped bacteria that vary in length from 1–3 μm and cords are not formed, unlike *M. tuberculosis* [2]. They are weakly Gram-positive and stained specifically by acid fast (Ziehl-Neelsen) staining method, due to high levels of lipids in mycobacterial cell wall. *M. avium* is highly resistant to environmental challenges and can survive in soil for up to 4 years, and this makes eradication of the organism difficult [1, 2, 23]. *M. avium* is resistant to high and low temperatures, dryness, pH changes, and many commonly used disinfectants. However, the unprotected organism is killed by direct sunlight. In contrast to *M. tuberculosis* and *M. bovis*, *M. avium* grows at temperatures ranging from 25–45°C, the most favorable range being 29–45°C and for primary isolation, growth can be enhanced with 5–10% CO_2 tension [3, 23]. Strains of *M. avium* can be identified by serological procedures. To date, 28 MAC serotypes have been identified from which the serotypes 1–6, 8–11, and 21 belong to *M. avium* subsp. *avium* (MAA). Serovars 7, 12–20, and 25 have been ascribed to *M. intracellulare*. However, no consensus was achieved on other serovars, and some isolates cannot be typed [3, 24]. Serotypes 1, 2, and 3 are considered virulent for chickens (Table 1) [2, 11, 23]. Serotypes 1 and 2 are most commonly isolated from domestic birds, and serovar 3 is recovered sporadically from wild birds. Serotypes 1 and 2 can affect animals, whereas 4–20 are mainly found in humans. Serovar-1 is the most common organism isolated from birds and from human beings. Distinguishing serovars can help provide a means for studying origin and distribution of specific strains. According to the current taxonomy, *M. avium* contains four subspecies, namely, *M. avium* subsp. *avium*; *M. avium. hominissuis*; *M. avium. Paratuberculosis; M. avium. silvaticum,* which is diagnosed rarely in birds [3, 21]. It is well established that most *M. a. avium* isolates from birds have a repetitive sequence *IS901* in their genome and also produce a characteristic three band pattern in *IS1245* restriction fragment length polymorphism (RFLP) [25]. It has been postulated that the presence of *IS901* correlates with pathogenicity in birds [25–27]. Other than *M. a. silvaticum*, IS901 has only been detected in *M. avium* strains with serotypes 1, 2, and 3 [5].

Table 1: Serotypes of *M. avium* complex (MAC) and their susceptibility to various species of certain birds and mammals [2, 9].

Species	MAC serotypes	Susceptibility
Domestic fowl (*Gallus domesticus*)	1, 2	High
Turkey	1, 2	Moderate
Pheasants	1, 2	High
Wild birds	2, 3	High
Cattle	1, 2	Moderate
Swine	1, 2, 4, 8	High
Rabbit	1, 2	High
Man	1, 4 to 20; 23, 25	Low (in healthy individuals); High (in immunocompromised)

M. avium is the most significant cause of poultry disease. Disease onset in birds is normally more rapid with *M. genavense* than with *M. avium*. In wild birds, though the disease is uncommon, TB may develop when they are in contact with infected chickens. *Mycobacterium avium* complex and *M. intracellulare* can also infect an extensive range of different animal species. *M. tuberculosis* is less commonly the cause of infection in birds, often as a result of transmission from pet bird owners, and also clinical signs differ from those caused by the more commonly occurring species of mycobacteria. In case of psittacine birds, apart from this, tuberculosis due to *M. tuberculosis* or *M. bovis* has also been reported. In canaries, tuberculosis may be caused frequently by *M. tuberculosis* [2].

3. Transmission

The main source of infection is infected birds as they shed large amounts of organism into the environment. The bacilli are exuded from ulcerated lesions of the intestine and are voided in droppings. The most common route of infection for susceptible birds is the alimentary tract [1, 2]. Respiratory tract is also suggested as a potential source of infection. The disease gets transmitted to the susceptible birds by ingestion and inhalation of aerosolized infectious organisms.

Persistence within flocks is associated with keeping older stocks without following adequate cleanliness and hygiene [2]. Further, maintaining birds closely confined under stressful conditions provide favorable ways for the spread of the disease. The ability of the organism to persist in the environment for many years, especially in soil and litter favor the disease transmission to a great extent [5]. Litter, pens, equipment, and pasture contaminated with excreta of infected domestic birds and the hands, feet, and clothing of attendants play an important role in disease transmission. Wild birds, pigs, and some mammals may also act as significant reservoirs of infection [2, 11]. Wild birds, such as sparrows, crows, and pigeons may be infected with *M. avium* and may spread it to poultry flocks [7]. Also, rats and other rodents are known to act as mechanical carriers in transmission of the disease. The agent can also be disseminated by infected carcasses and offals. Occasionally, skin invasion and spread via infected eggs may occur. *M. avium* has been isolated from eggs of naturally infected chickens, but hatched chicks have not developed the disease [2]. The bacilli does not survive in eggs after proper boiling.

4. The Disease and Manifestations

Avian tuberculosis is a contagious disease which occurs in chickens, pheasants, quail, guinea fowl, turkeys, parrots, budgerigars, ducks, goose, doves, partridges, pigeons, and other captive and wild game birds and has also been reported in ostriches, emus, and rheas in many zoological parks. Tuberculosis in birds is most prevalent in chickens and in wild birds raised in captivity. In poultry, the disease follows a slow course through the flocks. The classical presentation is characterised by chronic and progressive wasting and weakness. Avian tuberculosis in domestic birds is primarily an intestinal and hepatic disease with dissemination to other organs including the lungs, air sacs, spleen, bone marrow, and skin [2, 6, 11, 23]. Similarly, avian tuberculosis reported in free living birds including raptors were presented with the disseminated form involving the digestive tract, liver and spleen [8, 28, 29]. The disease has a long incubation period and a protracted course and if appreciable, the symptoms can prolong for weeks or months. Because of the chances to become established through a longer exposure, the disease is less prevalent in young fowls and lesions are less severe in them when compared to adult birds. Usually the losses are experienced more in older stocks of age group 18–20 months. The disease process can be divided into three phases: latency, lesion development, and period of cachexia [2, 5, 11]. During cachexia, massive tubercles with large numbers of bacilli develop. In the classic form of infection the tubercles or granulomas develop in multiple organs; a second form is manifested with lesions in the intestinal tract; a third type of infection often experienced as a nontuberculous one, mainly seen in finches, canaries, and psittacines [2, 5]. Some birds show respiratory signs and sudden death may occur, dyspnoea is less common, and granulomatous ocular lesions [30] and skin lesions have been reported.

Clinical signs are not pathognomonic in avian TB and vary depending on the organs involved. Birds with the intestinal form of tuberculosis often present with chronic wasting disease. In majority of cases of tuberculosis in birds, especially in the initial phase of infection, clinical signs are not grossly observable. However, in advanced cases, birds may develop symptoms like progressive weight loss, depression, white diarrhea with soiled feathers, increased thirst, respiratory distress, fatigue, and decreased egg production [11, 23, 31–33]. Feathers are often dull or ruffled and comb, wattle, and earlobes often appear pale, thinner and dry. Birds eventually become lethargic and emaciated with marked atrophy of breast muscles manifested as "knife edged" keel [2, 5]. In extreme cases, the body fat disappears, and the face of the bird appears smaller than normal. If a jerky hopping gait is observed due to unilateral lameness then it should be assumed that there could be the presence of tubercular lesions in bone marrow of the leg bones or joints. Some birds may adapt a sitting position. Tuberculous arthritis can even lead to paralysis. Fatal results often occur due to massive hemorrhage caused by ruptured liver or spleen. In this case, occasionally birds may die suddenly in good bodily condition and yet show advanced lesions of tuberculosis. The body temperature of the affected bird remains normal, even in severe cases. In most cases, an infected bird without overt clinical signs may serve as carrier that result in the persistence of infection in flocks. In commercial broiler production units, generally avian tuberculosis is uncommon primarily due to the short life span and in layers and breeders, the infection is a matter of much concern. Mortality over a short period may be insignificant, but the intermittent loss of adult birds in valuable breeding stock and decreased egg production in layers are detrimental. Occasionally, heavy losses may occur in pullets on multiage sites where the infection is endemic and the hygienic standards are poor.

After entering the host, *M. avium* prevents the fusion of phagosomes with lysosome and the subsequent bacteremia provides a generalized distribution of lesion. The gross lesions are characterized by the presence of epithelioid cells containing large numbers of organisms that may either diffusely infiltrate the organ or form discrete granulomas [6]. There is presence of tubercular nodules in intestine, liver, spleen, ovaries, testes, and bone marrow but the pulmonary lesions, which are a striking feature of tuberculosis in other species, are rarely observed in birds [2, 5]. Pulmonary avian tuberculosis is only seen occasionally as in case of tuberculosis of pigeons and water fowl [1, 2]. The principal lesions of tuberculosis in birds are seen in intestine, where affection often presents with studded greyish-white to greyish-yellow nodules. Before the intestinal tract is opened, the ulcerated areas appear as tumour-like masses attached to the gut wall, but when the intestine is opened, the true nature of the mass becomes evident. The nodules bulge from the serosal surface of the intestine and can be palpated. Due to this, spleen takes irregular "knobbly" appearance. Lesions evident as deep ulcers filled with caseous material discharges the organism into the intestinal lumen and get excreted via the droppings. Typical caseous lesions, without calcification, are always found in the liver and spleen, with considerable enlargement of the organs [2, 5]. Nodules are firm but can be incised easily since mineralization is rare in avian TB (this is

Figure 1: Spleen of Demoiselle cranes (Anthropoides virgo) showing caseous nodules, measuring 1–5 mm in size, on the cut surfaces of the organ.

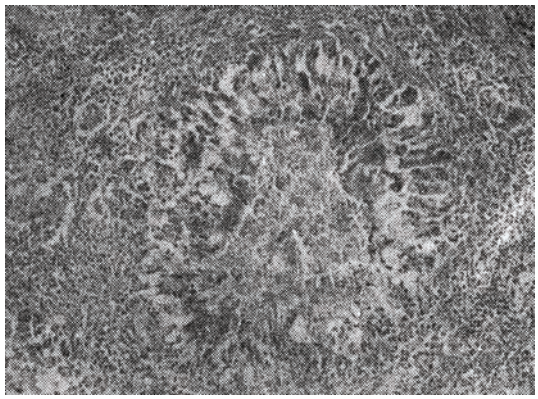

Figure 2: Section of spleen showing a granuloma with associated inflammatory cells, H&E × 120.

in contrast to leucosis, in which lesions cannot be enucleated from the surrounding tissue). The bone marrow of the long bones frequently contains tubercular nodules. Some exotic bird species may have lesions in the liver and spleen without intestinal involvement. Microscopically, lesions consist of granulomas with a central necrosis, either coagulative or caseous, and multinucleate giant cells. Acid fast bacilli are numerous in the central or necrotic zone of the tubercle [2, 11]. Gross and microscopic lesions in spleen of Demoiselle cranes (*Anthropoides virgo*) are depicted in Figures 1 and 2.

The incidence of avian tuberculosis in pet birds kept in captivity appears to exceed the prevalence in poultry [22, 34]. Some of the reasons of the incidence of the infection in pet birds are age of the host, population density, and the ability of organism to survive environmental inclemency [2]. Contact with contaminated water, soil, or feed predisposes to infection [22]. In case of pet birds, the etiology of avian tuberculosis is rarely identified due to the difficulty in isolating some mycobacterial species [23]. Weight loss, diarrhea, dyspnea, lameness, and poor feathering are the usual signs in pet birds. Earlier, most cases of infection were assumed to be caused by *Mycobacterium avium* complex (MAC). However, the use of molecular techniques brought to light the prominent role of fastidious mycobacteria, primarily *M. genavense*, in avian tuberculosis of pet birds [22]. *M genavense* is responsible for the majority of avian mycobacterial infections (up to 80%) in pet birds while the MAC was found responsible for 5% to 10% of mycobacterial infections [22]. In pet birds, *M. genavense* causes a disseminated disease with clinical and histopathological features indistinguishable from infection caused by members of the MAC [34].

Recently, avian tuberculosis in domestic poultry have declined due to changes in poultry husbandry practices, namely, integrated poultry farming, emphasizing all-pullet flocks rather than older hens and maintaining one-age flocks, all in all out farming system, along with better hygiene, disinfection, and biosecurity practices. However, the occurrence of avian TB in birds in zoo aviaries is still an economically important affair since certain species of exotic birds are of high value and most of these birds will be in endangered or near extinction categories. Avian TB is more common in zoological parks, perhaps because of inadequate cleaning and disinfection of pens. Caged birds are reported to soon succumb to avian TB.

M. avium can infect and cause disease in some domesticated mammals but lesions usually are localized and less severe. It multiplies in tissue for a considerable period and induces sensitivity to tuberculin. Swine, rabbit, and mink are readily infected; infection has been reported in cattle and horse; monkey is also susceptible; while goat, guinea pig, rat, and mouse are relatively resistant to infection, cat and dog are highly resistant to *M. avium* infection [16, 17].

5. Infection and Immunity

The cellular arm of the immune system is more important than the humoral arm in preventing and controlling mycobacterial infections [2, 5]. Delayed type of hypersensitivity (DTH), judged by the thickness of wattle, is evident at 2 days after infection and increases as the disease progresses. The organism after entry when phagocytosed by nonactivated macrophages is able to downregulate its killing mechanism by preventing normal fusion of the phagosome with lysosomes. Macrophages that lack microbicidal components are destroyed by the intracellular growth of the organism, and a lesion develops. Also, during infections, thymus is consistently colonized by *M. avium* and as the T-cell differentiation depends on the antigens encountered within the thymus, infection of this organ can alter the immune response to infection [35]. However, if activated, the macrophages can readily destroy and degrade phagocytosed mycobacteria [11, 36]. They have usually good killing potential against the invading mycobacterial species [37, 38]. This is augmented by the release of lymphokines like tumor necrosis factor (TNF) and interleukin-2 (IL-2), which helps in killing *M. avium.* Macrophage activation is also performed by interferon gamma (IFN-γ), which is released by a subset of CD4+ T lymphocytes and natural killer (NK) cells on stimulation by the interleukins released by the macrophage during its encounter with the mycobacteria. The Tlymphocytes also stimulates B cells to produce antibodies against mycobacteria but these antibodies do not appear to have a major protective

effect for the host against infection and high antibody titers can be correlated with serious infections [6, 36, 39]. Recently, it has been identified that lipoarabinomannan, an important outer cell wall component of mycobacteria, are highly potent nonpeptidic molecules which can be used to modulate the host immune response [40].

6. Diagnosis of Infection

The diagnosis of *M. avium* infection is based on clinical signs, postmortem gross lesions, and by demonstrating the acid-fast bacilli in crushed lesions using microscopy, which is sufficient for a positive diagnosis [2–5]. If acid-fast bacilli are not found, but typical signs or lesions are present in the birds, culture of the organism must be attempted. In necropsy, liver or spleen is usually the best organ to use, but if the carcass is decomposed, bone marrow may prove more satisfactory as it could be less contaminated. In live birds, cultural examination using feces or tracheal swabs is necessary to isolate and identify the etiological agent [23, 41]. But usually a definitive diagnosis is performed by culturing the organism in suitable media,namely, Dorset's or Herrold's egg yolk medium, Lowenstein-Jensen medium, Middlebrook 7H10 and 7H11, or Coletsos medium, with 1% sodium pyruvate [3–5, 23]. For growth of *M. avium*, media containing whole egg or egg yolk is desirable and the incubation temperature should be 37–40°C. Growth may be confined to the edge of the water of condensation. Cultures should be incubated for at least 8 weeks. Typically *M. avium* produces "smooth" colonies, within 2–4 weeks; rough variants do occur; smooth transparent colonies are virulent for chickens while variants with smooth domed or rough colonies are avirulent. The colonies, observed only after 10–21 days of incubation, are small, slightly raised, discrete, and grayish white in appearance [2, 11]. Colonies are larger if the medium contains glycerin. Shorter incubation times can be achieved using the liquid culture BACTEC system. Some strains of *M. avium* have been identified to have special requirement of mycobactin as a growth factor.

Recently, comparison of the different methods, namely, the conventional culture method (solid Herrold's and Stonebrink media and liquid Sula medium) and newly developed liquid culture systems, the manual mycobacteria growth indicator tube (M-MGIT), and the fully automated BACTEC MGIT 960 system (A-MGIT), for the detection of *M. avium* subsp. *avium* (MAA) in naturally infected hens revealed overall detection rates to be 60, 70, and 76%, with the mean time of mycobacteria detection being 32.6, 17.6, and 14.6 d, respectively [42].

In live birds, during life time, besides the culture and isolation techniques, immunological tests, namely, tuberculin test; whole blood agglutination test and enzyme-linked immunosorbent assay (ELISA) are also valuable diagnostic tools; and nowadays various molecular tools are also being employed for identification of the causative agent at subspecies level and epidemiological studies (Table 2). Using standard purified protein derivative (PPD) of heat-treated culture of *M. avium*, tuberculin test can be performed in the wattle, which is considered as the test of choice in domestic fowl/poultry. This test is less useful in other species of bird. Birds are tested by intradermal inoculation of 0.05 mL or 0.1 mL tuberculin (2000 IU) and the test is read after 48 hours [2]. A positive reaction is identified as a hot and oedematous swelling at the site or by the presence of a small firm nodule of approximately 5 mm in diameter [5, 11]. It serves as a means of identifying birds infected with or sensitized to the same species of tubercle bacillus. Tuberculin test has 80% accuracy in detecting infective birds relative to gross lesions but in an advanced stage of infection birds may give no reaction. In whole blood agglutination test, a drop of antigen (*M. avium* stained with 1% malachite green) is mixed with a drop of blood and a positive reaction is indicated by agglutination within few minutes [5, 48]. It is a better test, especially for waterfowl. Advantage of this test is that stock has only to be handled once, but false positive reaction is a disadvantage which makes the test a less specific one. The tuberculin test or the haemagglutination (stained antigen) tests are most frequently used for export testing of poultry. However, neither the tuberculin test nor the agglutination test is likely to be of any value in cases of *M. tuberculosis* infection in caged birds. ELISA which is reported to be less specific than tuberculin test can detect specific antibodies and thereby help determine exposure to *M. avium*. However, false positives may be common in ELISA. The identification of immunogenic proteins of *M. avium* may favor the development of more precise serodiagnostic tools [49]. Tuberculin test and serological tests are normally used to determine the prevalence of disease in a flock, or to detect infected birds. When used to detect the presence of tuberculosis in a flock they should be supported by the necropsy of any birds that give positive reactions. IFN-γ assay used to diagnose human tuberculosis may also be useful in diagnosing the infection in birds.

Species and subspecies level typing of mycobacteria requires a specialised laboratory. Conventional biochemical tests for species identification are lengthy and fail to distinguish between *M. avium* and *M. intracellulare*. Classification of MAC organisms into 28 serovars has been made by seroagglutination [3]. The MAC colonies can be identified using high performance liquid chromatography (HPLC) for detecting mycolic acid [4]. HPLC and use of monoclonal antibodies to major serovars in ELISA also facilitates typing of mycobacteria. In the past decade, biotechnological tools, exploiting nucleic acid detection methodology, like DNA probes, polymerase chain reaction (PCR), and PCR-restriction fragment length polymorphism (RFLP) are being widely employed for specific detection of the etiological agent [3, 4, 50, 51]. Commercial nucleic acid hybridisation probes have become a "gold standard" for distinction between *M. avium*, *M. intracellulare,* and *M. genavense* [2, 5, 41, 52]. For intraspecies genotyping, pulsed-field gel electrophoresis of large DNA restriction fragments has proved to be highly sensitive [53]. The PCR approach, using species-specific primers is also capable of specifically detecting DNA fragments of *M. avium* genome, thus acting as a diagnostic alternative to the conventional procedures [41, 54–57]. Also, a multiplex PCR method has been developed for the determination of the subspecies within *M. avium* species,

Table 2: Diagnostic methods and tests used in birds [2, 3, 5, 23, 43–47].

Type of test	Performed in	Time required	Merits	Demerits
Observing gross lesions	Dead birds	1 hour	Easy diagnosis	Only presumptive diagnosis
Acid fast staining	Dead birds	1 hour	Easy definitive diagnosis	Less sensitive, Not able to distinguish amongst species
Isolation/Culture	Dead birds	About 4 weeks	Definitive diagnosis	Time consuming
Tuberculin test	Live birds	48 hours	Easy to perform Definitive diagnosis	Time consuming, Test is not very sensitive, Possibility of false positive and false negative results
Agglutination test	Live birds	Few minutes	Can differentiate serotypes. Useful for screening large flocks for immediate culling	Occasionally false positive reactionsNot reliable in caged birds
ELISA	Live birds	2 hours	Definitive diagnosis Can be used for exotic and pet birds	Less specific than tuberculin test False positives may be there
DNA probes	Bacterial cultures	4–6 hours	Highly sensitive and specific	Probe may react with isolates that genetically or biochemically do not fit within the MAC
PCR	Dead/live birds/cultures	4 hours	Highly sensitive and specific	Requires specialized laboratory and trained personnel
RFLP	Bacterial cultures, clinical samples	1 day	Differentiates mycobacteria to the species level Discriminative for the analysis of strain relatedness	Insufficient quantities of gene makes visualization of digested fragments difficult
Multiplex PCR	Bacterial cultures/clinical samples	5–8 hrs	Rapid and inexpensive technique for subspecies identification and differential diagnosis of the MAC complex	Requires specialized laboratory
Sequencing of the 16S rRNA gene	Bacterial cultures	2 days	Powerful technique for differentiating species	Labor-intensive and difficult to implement in routine diagnosis
HPLC	Bacterial cultures	1 day	Can identify *Mycobacterium*isolates to the species level	Uses costly equipment and requires substantial amounts of the test organism.
Real-Time PCR	Bacterial cultures/clinical samples	4–6 h	Low risk of sample contaminationOffers the possibility to quantify bacterial load	Sensitivity could be affected by the initial volume of DNA present
MIRU-VNTR/MATR-VNTR typing	Bacterial cultures/clinical samples	1 day	Improves RFLP discrimination Useful for determination of genotypic diversity of *M. avium* subspecies	Requires specialized laboratory
Pathogenicity tests	Live young birds	5–6 weeks	Likelihood of the etiological agent can be knownUseful in cases where the typing facilities are not available	Time consuming and concerned to ethical issues

and for differentiating *M. avium* from *M. intracellulare* and *M. tuberculosis* complex [56, 58, 59]. Efficient differentiation of MAC species and subspecies by use of five-target multiplex PCR, designed to amplify a 16S rRNA gene target common to all Mycobacterium species, has been proved to be rapid, reliable, and simple [60]. Lappayawichit et al. [61] reported the differentiation of species of mycobacteria by amplifying 16-23S ribosomal DNA and further digesting with restriction enzyme like *Hae* III, *Msp* I, and *Bst* XI.

Likewise, for differentiation of various mycobacterial species, insertion sequences (IS) in DNA molecule have been identified. *IS 901* and *IS 1245*, which are virtually *M. avium* specific, has been shown to be the most discriminative for the analysis of various strains based on PCR-RFLP [1, 5, 25, 62]. Generally, the PCR-RFLP analysis of suspected tissue samples like liver, spleen, and gonads can be performed targeting 16S-rRNA gene for *Mycobacterium* spp., IS6110 for *M. tuberculosis*, IS1245 for MAC, IS901 for *M. avium* subsp.

Avium, and hsp65 for *M. genavense* [34, 58, 63–66]. Utility of PCR-RFLP of hsp65 has been reported for the identification of *M. avium* [67]. O'Grady et al. [43] performed RFLP investigation using probes derived from IS901, IS1245 and IS1311 to study the molecular epidemiology of *M. avium,* and *M. intracellulare* infection, in particular to gain an understanding of the sources of infection in humans. 16S rRNA and hsp65 sequencing may also be used to differentiate between mycobacterial strains and for distinguishing the *M. avium* subsets [68–72]. The real-time TaqMan PCR assay targeting the hsp65 gene of *M. genavense* and MAC subsp. may provide a useful tool for evaluating clinical samples for DNA from mycobacteria species that most commonly infect birds [44]. Slana et al. [45] has recently developed a real-time quantitative PCR for the identification and quantification of *Mycobacterium avium* subsp. *avium* and *M. a. hominissuis*. Other novel tests like IFN-γ assay, GenoType assay, and DNA microarrays that are used to diagnose human tuberculosis may also be useful in diagnosing the infection in birds [22, 73, 74]. More recently, the use of molecular techniques for species identification brought to light the prominent role of nonculturable mycobacteria, primarily *M. genavense*, in several cases of avian tuberculosis in pet birds [5, 34].

Utilization of new variable-number tandem-repeat markers (VNTRs) of genetic elements called mycobacterial interspersed repetitive units (MIRUs) for typing *Mycobacterium avium* subsp. *paratuberculosis* and *M. avium* strains has been reported to be fast typing method, and which in combination with other methods, might prove to be optimal for PCR-based molecular epidemiological studies [46]. More recently, the usefulness of a MIRU-VNTR typing has been described for determination of genotypic diversity of *M. avium* subspecies (*M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, and *M. avium* subsp. *Silvaticum*) from human and animal origins [47]. Inagaki et al. [75] reported MATR (*Mycobacterium avium* tandem repeat—MATR)-VNTR typing method (MATR-VNTR) to be having excellent discriminatory power compared with MIRU-VNTR and IS1245-RFLP typing; and its concomitant use with IS1245-RFLP typing increases the discriminatory power. MATR-VNTR typing is inexpensive and easy to perform and thus could be very useful for epidemiological studies.

In case the typing facilities are not available, pathogenicity tests are performed for knowing the likelihood of the etiological agent, which should be carried out on the species of bird being investigated, but failing that, domestic fowl or Japanese quail may be used. Young adult birds are best, and when inoculated intravenously with 1 mL of the suspension (culture at 0.1 mg/mL) the bird will die in 5–6 weeks if the organism is virulent, or, by that time, the bird will have extensive lesions filled with acid-fast bacilli [3].

Avian tuberculosis should be differentially diagnosed from those diseases that are known to develop tumorous or granulomatous lesions in gastrointestinal (GI) tract and other visceral organs. Diseases that are to be differentially diagnosed are pseudotuberculosis (common in ducks and turkeys caused by *Yersinia pseudotuberculosis*), Coligranuloma (Hjarre's disease-*Escherichia coli*), neoplasia due to lymphoid leucosis (Retrovirus) or Marek's disease (Herpes virus), fowl cholera (*Pasteurella multocida*), Pullorum disease (*Salmonella* Pullorum), and enterohepatitis (Black head, *Histomonas meleagridis*) [9].

7. Therapy in Avian Tuberculosis

Generally, mycobacterium infections caused by *M. tuberculosis* and *M. bovis* are treated with antibiotics such as isoniazid, ethambutol, rifampicin, and pyrazinamide in human beings [6]. Treatment of infected animals is rarely attempted because of the high cost and prolonged time. Moreover it is considered illegal in some countries. *M. avium*, on the other hand, is resistant to these antituberculosis drugs [2]. Due to this fact and also because of the economical considerations, treatment is not considered a viable option, particularly in poultry sector. However, in case of *M. avium* infection of exotic pet birds or birds maintained in zoo aviaries, treatment against *M. avium* has to be considered and therapy duration can go up to 12–18 months. In avian therapeutics related to mycobacterial infections, the major difficulty is that the pharmacokinetics in birds for most of the antimycobacterial drugs is unknown [6]. Also, the relative hydrophobicity of the mycobacterial cell wall acts as a barrier that restricts the activity of many hydrophilic antibiotics like the aminoglycosides, fluoroquinolones, and macrolides [6]. Besides, the slow growth and intracellular location of mycobacteria necessitate the need for extended periods of therapy.

There are clinical reports documenting the apparent successful treatment of parrots with mycobacterial infections, but no studies to date investigate the treatment of mycobacterial infections in birds [6]. *M. avium* has been reported to respond to trimethoprim-sulfamethoxazole, sulfisoxazole, amikacin, gentamicin, and kanamycin, during *in vitro* studies [76]. *M. avium* infections in pet birds have been treated with isoniazid, rifampin, rifabutin, ethambutol, clofazimine, ciprofloxacin, enrofloxacin, streptomycin, and amikacin and successful therapy of *M. genavense* infections with clarithromycin in humans has been reported [6]. The apparent effectiveness of the newer macrolides like clarithromycin and azithromycin against both *M. avium* and *M. genavense* make them suitable for treating mycobacterial infections in birds. However, the initial therapeutic regimen should include rifabutin and ethambutol, and later azithromycin or clarithromycin can be administered concurrently. Birds that respond poorly to therapy should have either a fluoroquinolone or an aminoglycoside added to the regimen. An alternative or additional drug that may prove useful, especially in birds with a marked inflammatory response, would be clofazamine. All these drugs may be curative at a total daily dose of 85 mg/kg for clarithromycin, 43 mg/kg for azithromycin, 56 mg/kg for rifabutin, 56 to 85 mg/kg for ethambutol, and 6 to 12 mg/kg for clofazamine as per the reports of VanDerHeyden [6]. In another study, to augment the potential of existing drugs, a mycobacteriolytic preparation called "stazyme" has been developed from the *Staphylococcus* strain Clavelis. Stazyme was able to break the permeability barrier of *M. avium* isolates, significantly enhancing the activity of anti-tuberculous drugs like ethambutol, rifampicin, and amikacin [77].

8. Preventive Measures

The eradication of M. avium infection is difficult due to the chronic carrier state and intermittent shedding of organisms by the infected birds. Measures to eliminate disease and establishing/maintaining TB-free flock should be followed. Gill and Blandy [78] and Dhama et al. [11, 79] described measures like sacrificing the affected flocks, abandoning the equipments and housing materials, removal of litter and contaminated soil, elimination of older flocks, following of strict biosecurity procedures besides regular monitoring with tuberculin and agglutination tests. Stress is a key factor as it causes an increase in the rate of shedding to precipitate outbreaks. The best way to control this disease is to remove infected ones and carriers and also to reduce stress factors by improving the environmental parameters [2, 11]. Prevention is best done by minimizing overcrowding, providing proper ventilation and supplementing adequate amounts of vitamins and minerals in diet. In case of avian TB in a farm, birds in other flocks in the same farm should be quarantined and tested at 6–12 week intervals. Neither the tuberculin nor the agglutination tests can be depended upon for the detection of every infected bird, therefore, as long as one infected bird remains in a flock, dissemination of disease is possible. So entire flock needs to be depopulated and repopulation on noninfected soil and fresh litter may be the best approach. Frequent removal of fecal material is the single most important factor in preventing transmission. Disinfection of the poultry houses should be done frequently. The practice of managing poultry in free-range system should not be followed. Provide proper biosecurity measures to prevent unrestricted movement of chickens, thus preventing exposure from previously infected premises or from wild birds, pigs, and other mammals. For exotic birds prevent contact with infected birds or the premises and housing previously used by them. Monitoring for infection and disposing of positive reactors should be followed along with practicing all reasonable hygienic precautions to prevent entry of the infection. During the import of exotic or domestic birds, tuberculin testing must be mandatory in order to identify the presence of M. avium and the newly introduced birds should be quarantined for a minimum of 2 months [2, 5]. The difficulty of tuberculin testing of all chickens or even a majority of flocks makes it impossible to obtain exact data on the incidence of *M. avium* infections of chickens. No vaccines are available for use in birds. Experimental vaccines with killed and/or live mycobacteria for protecting chickens against TB have been evaluated. Satisfactory protection was obtained when M. avium serovar 6 was given orally [5, 9, 11]. Combination of inactivated and live M. avium serovars 7 and 19 can also give protection to a limited extent. Nucleic acid-based vaccines may also be experimentally tried using M. avium genes that can generate proteins to elicit cell mediated immunity in birds [80, 81]. Simple, whole cell or lysate vaccines and combinations of vaccine preparations were identified that led to high levels of protection [82].

9. *Mycobacterium avium*: The Zoonotic Implications

Mycobacterium avium subsp. *avium* (MAA) represent veterinary and economic risks in birds (mainly poultry) as well as mammals (pigs, etc.). Infected animals and their products (mainly eggs) often come from small household production and pose a risk for human health [83]. Exposure of humans to infected birds with the MAA microorganism may cause a zoonotic infection, particularly in those with immunocompromised diseases such as HIV/AIDS [84]. In addition, the situation worsens due to the spread of HIV infection in developing countries [85]. Unlike *M. tuberculosis*, human beings are generally resistant to *M. avium* infection but occasionally they can get infected. Human pulmonary tuberculosis due to avian tubercle bacilli has been reported during the early 1940's [86]. High incidence of sensitivity to avian tuberculin in man has also been identified [87, 88]. It is essential to bear in mind that *M. avium*, *M. intracellulare,* and *M. genavense* are of public health concern mostly in immunocompromised hosts. Infections of humans and animals caused by this *M. intracellulare* agent are expected to rise. The *M. avium* infection, seen in many AIDS patients, is a progressive disease that is refractory to treatment [65, 89–91]. This is especially true in cases of exposure to large numbers of organisms [6]. In humans, *M. avium* is capable of inducing localized primary lymphadenitis, pulmonary disease, and a disseminated form of infection particularly in case of immunosuppressed individuals or patients under transplant therapy [4, 22, 92, 93]. *M. avium* also causes local wound infections with swelling of regional lymph nodes. In adults, the organism frequently affects the lungs, producing respiratory signs and in children, the cervical lymph nodes are often involved. Eccles and Ptak [93] reported that *M. avium* causes a serious disseminated bacterial infection in up to 40% of patients with advanced HIV infection. In AIDS patients, the main route for *M. avium* infection is the gastrointestinal tract and *M. avium* is naturally tolerant to the low pH that exists in stomach [94]. The transmission occurring via aerosols results in pulmonary infections as the organism frequently affects the lungs with endobronchial lesions [95]. During the infection, *M. avium* can be demonstrated *in vivo* in lymph nodes, bone marrow, urine, and sputum [87, 91, 95, 96]. Primarily, the serotype-1 of *M. avium* subsp. *avium* has been isolated from such individuals, clearly pointing role of birds in acquiring infection [4]. It should also noteworthy that *M. avium* is a pathogen that infects several hosts including birds, humans, cattle, and pigs [45, 97, 98]. They are also encountered in environmental sources like soil and water, having considerable ability to overcome adverse and competitive conditions thanks to a major preprotein translocase subunit that is coded by *secA* gene of the species [99].

During a study from 1953 to 1968, in cattle and swine of Great Britain, 13% of the total tubercle bacilli were typed as *M. avium* and in pig population it was an astonishing 81%. This should be correlated with the ever increasing number of recorded cases of tuberculosis in man caused by *M. avium* [64, 100]. Contaminated food originating from pig or other

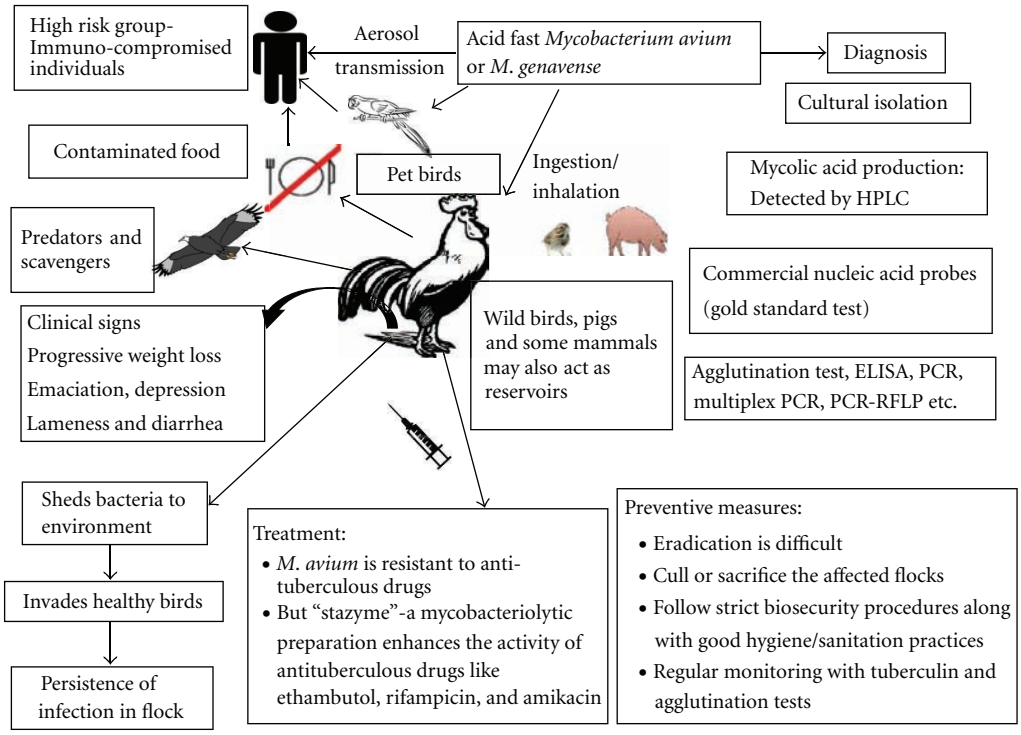

Figure 3: Salient features of avian tuberculosis.

livestock is identified as potential source of human infection. *M. avium* can infect and cause disease in some domestic mammals but lesions usually are localized and less severe. When domestic farm animals are infected, particularly cattle and pigs, tuberculous lesions are commonly found localized to the head and intestinal lymph nodes [1, 101]. As reported by some workers, *M. avium* isolates from swine represent the major threat to human beings. The similarity of the IS1245 RFLP patterns of the human and porcine isolates indicates close genetic relatedness, suggesting that *M. avium* is transmitted between pigs and humans [64, 102].

Regarding the therapeutics, *M. avium* is of special concern because drug regimens commonly used for treating tuberculosis in humans are not effective [22]. *M. avium* strains are notorious in being resistant to isoniazid, the most popular anti-TB drug [103, 104]. However the infection was found subsided when treated with azithromycin or clarithromycin together with ethambutol [6, 105]. Further, the *M. avium* isolates have been demonstrated to get inhibited by sufficient concentrations of sulfamethoxazole in serum [106]. Rifabutin prophylaxis may also help in controlling the disseminated infection [93]. Dunne et al. [107] suggested the use of azithromycin as a safe, effective, and convenient option during disseminated infection in HIV-infected patients. As per the findings of Horgen et al. [108], rifampin-clarithromycin and rifampin-amikacin are the most potent two-drug combinations, while rifampin-amikacin-clarithromycin has been identified as a potent three-drug combination. Likewise, Saito et al. [109] suggested the use of benzoxazinorifamycin in combination with clofazimine to be highly efficacious in the therapy of *M. avium* infections. In case of infection with *M. genavense*, clarithromycin is the better choice when compared to azithromycin [6].

Besides therapeutic interventions, there have been numerous attempts to check the *M. avium* isolates in both environment and in host. Lin et al. [110] suggested that copper-silver ionization of drinking water is a better option for the effective control of *M. avium*. David et al. [111] proposed the use of synthetic macrocyclic compounds as a new family of compounds that are capable of acting against *M. avium* infections. The use of recombinant cytokine molecules for the effective killing of *M. avium* especially interleukin-4 (IL-4) has been well studied [112–114]. The use of adjunctive immunomodulatory therapy by using recombinant granulocyte-macrophage colony-stimulating factor has also been reported [115]. Salem et al. [116] reported that by encapsulating antibiotics like amikacin, streptomycin, ciprofloxacin, sparfloxacin, and clarithromycin, their effect against *M. avium* can be enhanced. Iron accumulation has been suggested to contribute to an increase of the susceptibility to mycobacterial infections. Iron deprivation, by the use of iron chelators, restricts *M. avium* growth and this offers a novel approach in controlling infections in man [117].

So it is considered prudent to keep infected birds away from humans, particularly the elderly, and individuals with poor immune status. Hence the handling of infected birds in farms should be carried out with adequate care, and manipulation of material from infected birds or open live cultures of *M. avium* in laboratories must be performed with appropriate biohazard containment [3]. Healthy individuals with normally functioning immune system have a high

resistance to this infection. However, it is recommended to take proper precautions and avoid contact or exposure to infected birds or their carcasses.

Salient features of avian tuberculosis are presented in Figure 3.

10. Conclusion and Future Perspectives

Members of the *Mycobacterium avium* complex (MAC) are ubiquitous bacteria that can be found in water, food, and other environmental samples and are considered opportunistic pathogens for numerous animal species, mainly birds and pigs, as well as for humans. Infections caused by the MAC are on the rise in both human and veterinary medicine. Avian tuberculosis is an important disease which affects companion, captive exotic, wild, and domestic bird, and has public health significance too. The most significant cause of poultry disease is *M. avium*. In recent years, the incidence of avian tuberculosis in domestic poultry have declined due to introduction of novel poultry husbandry practices, namely, maintaining one-age flocks, all in-all outfarming system; provision of better hygiene and sanitation; stringent implementation of biosecurity practices. But the inevitable occasional stress and production demands in the poultry sector could create dynamics similar to those that occur in immune-compromised individuals. Also, *M. avium* pose a significant threat in layer and breeder farms, where high age groups are maintained. Unless eliminated from the domestic birds, tuberculosis will remain an economic burden on the swine industry too and the role of pigs in transmitting the disease to humans has been well documented. Disseminated form of infections with *M. avium* is seen in increasing numbers in immunocompromised individuals. *M. avium* subsp. *avium* may have wild birds as major reservoirs that are responsible for its shedding into environment and facilitating its spread for years. The diversity of strain types indicates that infections are acquired not from a single reservoir alone. This is in contrary to the belief that existent infected birds are the primary sources of infection through fecal contamination of the environment. Under these perspectives more studies should be performed on identifying avian reservoirs and environmental sources of *M. avium*. RFLP analysis and multiplex PCR methods can further discriminate between different isolates, which is particularly useful for epidemiological studies. Identification of the MAC members based on culture examination followed by biochemical testing, can take up to several weeks, as opposed to molecular biology methods that provide fast and accurate identification to the species level, which is important in diagnosis and treatment of avian tuberculosis. A means of effectively discriminating among closely related yet pathogenically diverse members of the MAC would enable better diagnosis and treatment as well as further our understanding of the epidemiology of these pathogens. Moreover, viewing the importance, the advanced diagnostic tools and novel prevention strategies that are employed against *M. tuberculosis* and *M. avium* in man needs to be standardized for *M. avium* infections in birds and animals as well.

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ANNEXURE - 8

PERSPECTIVE STUDY ON TUBERCULOSIS IN PIGS – POSTMORTEM FINDINGS

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PERSPECTIVE STUDY ON TUBERCULOSIS IN PIGS - POSTMORTEM FINDINGS

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ABSTRACT

The prevalence of tuberculosis among pigs caused by *Mycobacterium spp* is described in this paper. The period between May 2008 to February 2010, a total of 108 pigs/piglets were subjected to postmortem examination showed gross pathological lesions indicative of tuberculosis in nine pigs. Amongst this, 3(2.8%) pigs belonging to three way crosses and 6(5.6%) were 75% crossbred pigs were found positive for Acid Fast Staining (AFS) technique based on the retention of primary stain by the bacilli. The infected pigs with primary tuberculous lesions in the parenchymatous organs were demonstrated with typical reproducible AF bacilli. The sources of infection might have been either concentrate feed to the pigs or cow's milk that was provided as supplement.

Key words : Pig tuberculosis, Acid fast bacilli, Epidemiology, Zoonosis.

INTRODUCTION

Tuberculosis is an infectious disease occurring in several animal species including domestic and wild animals, as well as humans. Pigs usually acquire infection by consumption of unpasteurised milk, milk products from infected cows and/or unsterilised by byproducts from slaughter houses. *M.avium complex* (MAC) and /or opportunistic *Mycobacteria* were obtained on necropsy of pigs infected with *tuberculosis* (Pavlik *et al.*, 2005 and Cvetnic *et al.*, 2006). Sadana (1975) and Padmanaban and Rai (1975) also reported that pigs from Delhi, Haryana, Punjab and Tamilnadu had 0.5% caseous and 13% of calcified foci in the parenchymatous organs. In Asia, nearly 94% of cattle and 99% of buffalos are either partly controlled for bovine tuberculosis or not at all controlled (Cosivi *et al.*, 1998). *M.bovis* and *M. tuberculosis* have been isolated from human and animals, but pigs and other wild animals are not at all screened against tuberculosis in India (Verma and Srivastava, 2001). Tuberculosis in pigs remains to be the highly infectious and the most important bacterial disease resulting in colossal economic losses, causing higher morbidity and mortality amongst pigs as well as major global public health problem (WHO, 1982). However, the origin and transmission of infections between human and animals has not been investigated. The present study is reported on occurrence of tuberculosis lesion in pigs during postmortem examination.

MATERIALS AND METHODS

The period from May 2008 to February 2010, a total of 108 postmortem of pigs/piglets were

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conducted at Livestock Research Station, Kattupakkam, TamilNadu. Amongst nine pigs, three animals of three way cross (Large White Yorkshire + Landrace + Durac) and six animals of 75% cross (LargeWhiteYorkshire+ Desi pigs) were detected to have mild to moderate caseous and calcified tuberculous lesions in the parenchymatous organs on necropsy. Liver, lungs, mescentric lymph node and intestine were removed and examined for calcified (White cheese material) nodules, typical (characteristic) microorganism of Acid Fast bacilli. Smears from nodules of parenchymatous organs were subjected to Ziehl-Neelsen staining technique. After the complete necropsy, tissue specimens were collected and fixed in 10% formalin for the routine histopathological studies. The bacteriological culture of the *Mycobacterium* was not conducted in these specimens. The source of concentrate feed for these animals was received regularly from Poultry Research Station (PRS), Nandanam and hotel swill feed was used particularly in 75% of crossbred pigs. Weak piglets were provided with cow's milk as supplement. There is no major disease outbreak reported during this period in this farm.

RESULTS AND DISCUSSION

The incidence of typical tuberculous lesion of 2.8% in three way crossbred and 5.6% in 75% of crossbred pigs recorded in this study was in agreement with the findings of Sadana (1975) and Padmanaban and Rai (1975) they also reported 0.5% and 13% of caseous and calcified foci in the parenchymatous organs of slaughtered pigs. However, in this report caseous and calcified foci in the parenchymatous organs (Figures 1, 2 and 3) had the typical (characteristic) tuberculous lesions invariably in all the nine animals. Four out of nine pig's tissue smears revealed that 3.7% of cases had typical AF bacilli by microscopic examination based on the retention of primary stain by the bacilli. However, caseous and calcified foci from the lungs, mescentric lymph node smear yielded *Mycobacteria* organisms other than microorganism such as *Nocardia, Staphylococcus, Actinobacillus* and

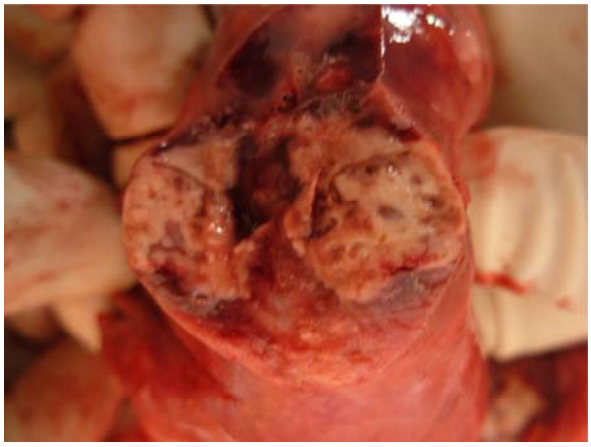

Fig 1 : Tuberculous lesion in lungs of pig.

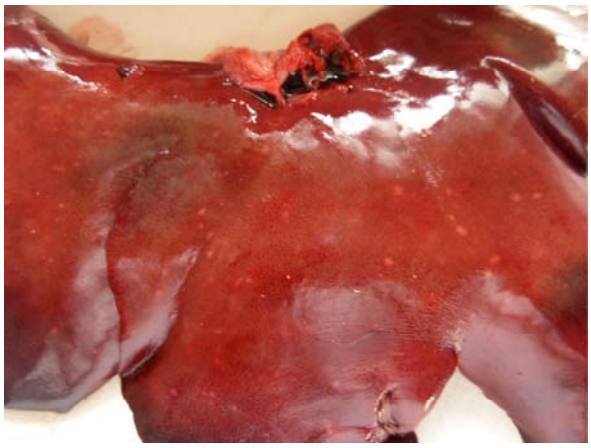

Fig 2 : Millet shaped tuberculous lesion in liver-spread over its surface of parenchyma in pig.

Actinomyces spp (Padmanaban and Rai, 1975 and Cosivi *et al.*, 1998). Concurrent with Sadana (1975) and Pavlik *et al.* (2005), the present study revealed some pigs were revealing progressive weight loss, emaciation and debility (Fig. 1). Lesions included caseous and calcified foci found in all the parenchymatous organs with typical cheesy white nodules of varying sizes (0.5mm to 1cm) diffusely spread over its surface. Lungs appeared congested and consolidated in most cases with typical calcified nodules (3-7cm in size).

The most important observation in this study was the prevalence of *Mycobacterium* in pigs and other animal species is to a considerable extent, the sources of infection might have been either

concentrate ration to the pigs or unpasteurized cow's milk that was provided as supplement was in agreement with Sharma *et al*. (1985) and Verma *et al*. (1987). The reasons for the occurrence of *tuberculosis* in pigs due to different levels of infection in poultry in individual countries make themselves felt in differing contribution of *Mycobacterium* species which causes tuberculous lesions in pigs and animals.

In addition to the characteristic mortality amongst grower pigs in this investigation, one of the features of *Mycobacterium* spp and/or opportunistic *Mycobacteria* was that it persists for long periods in pigs without clinical disease. This can lead to excretion of organisms via faeces and perpetuation of infection in the shed which can result in faecal contamination of feed and water to other susceptible piglets in the pens (Cosivi *et al*., 1998 and Matlova *et al*., 2005).

The observation of gross pathological lesions and reproduced the same morphological appearance of AF bacilli from the parenchymatous organs (Figure 4) in this study was well corroborated with the results of Padmanaban and Rai (1975); Pavlas *et al*. (1985) and Verma and Srivastava (2001). The present study, therefore, recommends a wide spread screening of pigs to assess the carrier status that should enable to institute effective control measure in view of global prevalence of *tuberculosis* and zoonotic importance of *Mycobacterium* spp. There is an urgent need to evolve technique that not only identify and characterize tubercle bacilli, but also facilitate epidemiological studies in order to back trace of infection thereby facilitating formulation of effective control strategies for both animals as well as humans.

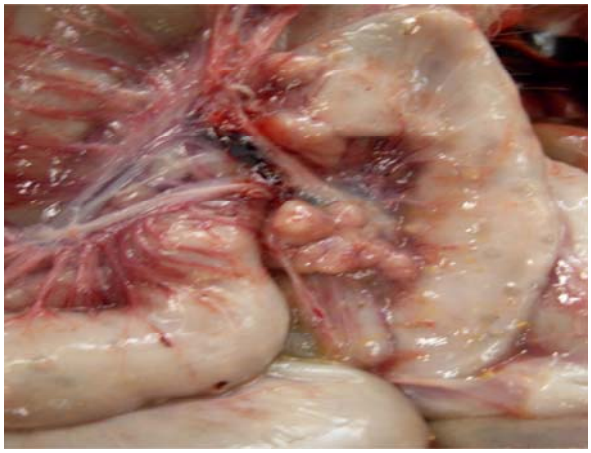

Fig 3 : Tuberculous lesion in mescentric lymph node of pig.

Fig 4 : Impression smears from lungs of pig-revealed typical Acid Fast bacilli (pink rod shaped) x100.

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ANNEXURE - 9

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MANNUTHY - 680 651, THRISSUR, KERALA, INDIA
Ph: 0487 2370451, 2370344 (O), Telefax: 0487-2370388
E-mail: deanvetmnty@kvasu.ac.in

DR. C. LATHA
DEAN

No: PA/Vet/29/2018 Dated 31.03.2018

To

Dr.Manilal Valliyat.
76, Ground Floor,
Defence Enclave, Vikas Marg,
New Delhi 110 092.

Sir,

Sub: CVAS, Mannuthy - - Results of screening of elephants for tuberculosis results in original forwarding of reg.

Ref: 1. Request from Animal Welfare Board of India dated 17.3.2018
2. Letter No. C3/0051/2016 dated 19.3.2018 of the Dean, CVAS, Mannuthy

As per the reference 1st cited, the screening test of the samples for Tuberculosis have been completed and the results in original is forwarded herewith in sealed envelope for favour of further necessary action.

Yours faithfully,

31/3/18

DEAN

Encl: as above.

COLLEGE OF VETERINARY AND ANIMAL SCIENCES MANNUTHY

DEAN
College of Veterinary & Animal Sciences
Kerala Veterinary & Animal Sciences University
Mannuthy, Thrissur-680 651

Results of Screening of elephants for tuberculosis using Chembio Kit

Sl No	Elephant ID No	Date of blood collection	Date of test	Diluent serial No.	RT Sl No.	Result
1	R87	17/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
2	R51	17/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
3	R16	17/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
4	R62	17/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
5	R63	17/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
6	R52	17/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
7	R53	17/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
8	R2	17/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
9	R34	17/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
10	R39	17/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
11	R30	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
12	R117	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
13	R43	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
14	R41	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
15	R55	18/01/2018	19/03/2018	B27083017	VTB0630171	**Reactive**
16	R104	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
17	R72	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
18	R71	18/01/2018	19/03/2018	B27083017	VTB0630171	**Reactive**
19	R1	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
20	R130	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
21	R58	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
22	R131	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
23	R125	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
24	R92	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
25	R27	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
26	R91	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
27	R20	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
28	R133	18/01/2018	19/03/2018	B27083017	VTB0630171	**Reactive**
29	R14	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
30	R98	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
31	R107	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
32	R105	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
33	R56	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
34	R41	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
35	R116	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
36	R113	18/01/2018	19/03/2018	B27083017	VTB0630171	**Reactive**
37	R81	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
38	R76	18/01/2018	19/03/2018	B27083017	VTB0630171	**Reactive**
39	R126	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
40	R99	18/01/2018	19/03/2018	B27083017	VTB0630171	**Reactive**
41	R102	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
42	R68	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
43	R100	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
44	R120	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
45	R110	18/01/2018	19/03/2018	B27083017	VTB0630171	**Reactive**
46	R59	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
47	R74	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
48	R75	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
49	R17	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive
50	R57	18/01/2018	19/03/2018	B27083017	VTB0630171	Non reactive

Dr. BINU. K. MANI

Dr. Deepa. PMV

51	R96	18/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
52	R109	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
53	R82	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
54	R83	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
55	R31	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
56	R23	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
57	R64	19/01/2018	19/03/2018	B27083017	VTB063017/1	**Reactive**
58	R49	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
59	R90	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
60	R18	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
61	R73	19/01/2018	19/03/2018	B27083017	VTB063017/1	**Reactive**
62	R75	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
63	R93	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
64	R33	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
65	R15	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
66	R115	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
67	R123	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
68	R128	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
69	R44	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
70	R7	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
71	R43	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
72	R122	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
73	R89	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
74	R9	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
75	R21	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
76	R117	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
77	R97	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
78	R80	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
79	R74	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
80	R95	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
81	R85	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
82	R111	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
83	R127	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
84	R134	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
85	R48	19/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
86	R77	20/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
87	R65	20/01/2018	19/03/2018	B27083017	VTB063017/1	**Reactive**
88	R94	20/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
89	Sonu	20/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
90	R25	20/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive
91	R79	20/01/2018	19/03/2018	B27083017	VTB063017/1	Non reactive

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ANNEXURE - 10

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PLOS ONE

Serodiagnosis of Tuberculosis in Asian Elephants (*Elephas maximus*) in Southern India: A Latent Class Analysis

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Abstract

Background: *Mycobacterium tuberculosis*, a causative agent of chronic tuberculosis disease, is widespread among some animal species too. There is paucity of information on the distribution, prevalence and true disease status of tuberculosis in Asian elephants (*Elephas maximus*). The aim of this study was to estimate the sensitivity and specificity of serological tests to diagnose *M. tuberculosis* infection in captive elephants in southern India while simultaneously estimating sero-prevalence.

Methodology/Principal Findings: Health assessment of 600 elephants was carried out and their sera screened with a commercially available rapid serum test. Trunk wash culture of select rapid serum test positive animals yielded no animal positive for *M. tuberculosis* isolation. Under Indian field conditions where the true disease status is unknown, we used a latent class model to estimate the diagnostic characteristics of an existing (rapid serum test) and new (four in-house ELISA) tests. One hundred and seventy nine sera were randomly selected for screening in the five tests. Diagnostic sensitivities of the four ELISAs were 91.3–97.6% (95% Credible Interval (CI): 74.8–99.9) and diagnostic specificity were 89.6–98.5% (95% CI: 79.4–99.9) based on the model we assumed. We estimate that 53.6% (95% CI: 44.6–62.8) of the samples tested were free from infection with *M. tuberculosis* and 15.9% (97.5% CI: 9.8 - to 24.0) tested positive on all five tests.

Conclusions/Significance: Our results provide evidence for high prevalence of asymptomatic *M. tuberculosis* infection in Asian elephants in a captive Indian setting. Further validation of these tests would be important in formulating area-specific effective surveillance and control measures.

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Competing Interests: Jacob Varghese Cheeran is employed by Cheerans Lab (P) Limited. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials. Cheerans Lab (P) Limited had no role in developing the serological tests described in the current investigation.

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☯ These authors contributed equally to this work.

Introduction

Conservation medicine enables us to rethink the linkages between human, animal, and environmental health [1], [2]. As wildlife populations become more fragmented and less genetically diverse stochastic events leading to disease outbreaks could become more common. A case in hand is that of the Asian elephant (*Elephas maximus*) an ''Endangered'' flagship species and featuring on the '2010 IUCN Red List of Threatened Species' [3]. About 39,463-47,427 wild elephants are found in their 13 range countries in Asia [4]. Estimates of Asian elephant numbers in the wild in India are 26,000–28,000 of which 14,000 are found in southern India [5]. About 3467–3667 elephants are also held in captivity in India (http://envfor.nic.in/pe/PE%20Note.pdf) at forest camps, temples, zoological gardens and circuses, thus constituting a substantial population living in close proximity to humans.

Both humans and elephants are susceptible to infection primarily with *M. tuberculosis*. Importantly, TB is a serious zoonotic disease in elephants [6], [7], [8], [9], [10] and infects about 11-25% of tested captive elephant populations in USA, India and Nepal [11]. The transmission, pathobiology and immune correlates of TB are poorly understood in Asian elephants. There is paucity of information on the time intervals between exposure, seroconversion, and shedding of the bacilli as also latent versus active disease status. Equivalent to the culture of human sputum [12], [13], [14] trunk wash culture for isolation of *M. tuberculosis* remains the 'gold standard' of ante-mortem TB diagnostics in elephants [15]. In its absence, ante-mortem TB diagnosis presents a conundrum [16]. Clinical signs such as chronic weight loss, weakness, anorexia, exercise intolerance and abnormal discharge

from the trunk [17] are frequently absent or seen at the terminal stages. Intradermal tuberculin test [17], [18] and radiographic thoracic evaluation [17] are unsuccessful in elephants. The GenProbe Amplified *Mycobacterium tuberculosis* Direct Test (MTD; Gen-Probe, San Diego, CA, USA) has found limited use [19], while ELISA [17], [20], restriction fragment length polymorphism (RFLP) [6], [17], [18] and serological tests such as the dual path platform (VetTB test), multi-antigen print immunoassay (MAPIA) and the rapid serum test (RT, ElephantTB STAT-PAK) from Chembio Diagnostics (Medford, USA) have also been evaluated in elephants [21], [22], [23].

In this study, we report screening of elephants with RT and four in-house ELISAs using *M. tuberculosis* H37Rv antigens *EsxA*-6 kDa early secretory antigenic target (ESAT-6) (Rv3875); *EsxB*-10 kDa culture filtrate antigen (CFP10) (Rv3874); PE_PGRS17 (Rv0978c) and PE_PGRS11 (Rv0754). The ESAT-6 and CFP10 proteins function in inducing interferon gamma (IFNγ) from memory effector cells upon infection with pathogenic mycobacteria [24], [25]. The proline glutamic acid (PE) and proline-proline-glutamic acid (PPE) families of acidic, glycine-rich proteins are unique to the Mycobacteria [26] and many function as cell surface antigens. The PE_PGRS11 (Rv0754) is a hypoxia responsive gene that encodes a functional phosphoglycerate mutase [27] and PE_PGRS17 and PE_PGRS11 antigens induce maturation and activation of human dendritic cells [28].

Performance characteristics of a diagnostic test should ideally enable us to distinguish between infected and non-infected animals. Notably, estimation of DSe (the proportion of infected animals correctly identified by the diagnostic test) and DSp (the proportion of non-infected animals accurately identified by the diagnostic test) of any index test is generally derived by comparing it to a standardized and validated 'gold standard' with the assumed sensitivity and specificity of 100% [29], [30], [31]. However, the gold standard could suffer from serious deficiencies. For example, the ante-mortem trunk wash culture for *M. tuberculosis*/*M. bovis* in Asian elephants is reported to suffer from poor sensitivity [17], [22], logistical issues in sample collection and processing and slow turnaround time. In view of these observations, we have utilized Latent Class Analysis (LCA) of five imperfect serological tests to estimate and derive the probability of *M. tuberculosis* infection in elephants.

Results

Sampling in Elephants

About 600 captive Asian elephants were visited for health assessment in the three southern Indian states of Kerala, Karnataka and Tamil Nadu. The sampling included healthy individuals as well as animals with alternative diagnosis such as chronic arthritis, impaction, and other non-specific symptoms such as anemia and emaciation. Sera from 179 animals were randomly selected for this study. Trunk-wash culture for isolation of *M. tuberculosis* in select RT positive elephants was carried out with no elephant testing positive. Post-mortem examination of one RT positive elephant revealed lung nodules from which *M. tuberculosis* was cultured. Ante-mortem serum from this animal was used as positive control in standardizing the ELISAs and immunoblot assay.

Evaluation of Humoral Immunoreactivity

One hundred and seventy nine elephants were screened for differential B-cell responses using RT and recombinant *M. tuberculosis* H37Rv antigens in ELISA format. Only selected reference samples were tested by immunoblot analysis using the four recombinant antigens and the results were not included in the LCA model. The immunoblots (Figure 1) were not quantitative in nature. The RT readout gave either a positive or negative test result (binary outcome), while the ELISA results were continuous numerical outcome values (continuous outcome). We dichotomized each continuous test result using a Weibull mixture model that assumed the elephants were a mixture of two latent groups – those with the antigen and those without. The cut-off value was the point of intersection of the two Weibull distributions. The cut-off values were 0.2 for ESAT-6, 0.337 for CFP10 and 0.22 for both PE_PGRS11 and PE_PGRS17. Test value greater than each cut-off was deemed to be a positive test. Of the 179 elephants 33 tested positive in RT, 37 in ESAT-6 ELISA, 41 in CFP10 ELISA, 64 in PE_PGRS11 ELISA and 78 in PE_PGRS17 ELISA (Figure 1).

Analyzing Diagnostic Test Results using LCA

The LCA assumed in Figure 2 has sixteen latent classes. The sero-prevalence associated with each latent class is listed in Table 1. In the absence of any prior information, we chose to use a 'non-informative' prior distribution for the prevalence of *M. tuberculosis* infection, allowing for equal weight of all values from 0% to 100%. Using the posterior distribution, we report that 53.6% (97.5% CI: 44.6% to 62.8%) of the sera samples we tested did not carry any of the *M. tuberculosis* antibodies measured by the 5 tests. This estimate is higher than the 15% sero-prevalence reported by Abraham *et al.* (2008, Report submitted to Project Elephant, Ministry of Environment and Forests, Government of India) using RT in the same population of captive elephants. We report that the percentage of *M. tuberculosis* infected animals testing positive in all five tests is 15.9% (97.5% CI: 9.8%−24.0%). The DSe and DSp of each test with respect to the target antibody that it is designed to detect is listed in Table 2. Thus the PE_PGRS11 ELISA had the highest DSe of 97.6% (97.5% CI: 88.6%−99.8%) and DSp of 98.5% (97.5% CI: 93.6%−99.9%). Table 2 also lists the DSe and DSp of each test in detecting the presence of at least one antibody (Table 1 lists 15 latent classes that are positive for at least one antibody) as well as the latent antibodies it is not designed to detect. The main difference between our model and other latent class models is that we recognize that the different tests are measuring different latent variables. Thus, for example, we are able to comment not only on how well RT measures the antigens it is supposed to detect but also on how well it measures *M. tuberculosis* infection that is picked up by other antigens. Finally, the observed and predicted numbers of elephants for each combination of test results (Table 3) agree quite well suggesting that the model fits the data adequately.

Discussion

Latent class analysis is used in a scenario wherein the gold standard assessment of disease is unavailable and the true infection status unknown but the results of multiple imperfect tests are known [32], [33], [34]. This analysis is attractive because it does not arbitrarily treat one of the tests as a perfect gold-standard with 100% sensitivity and specificity. It allows intuitive construing of data for input and helps in understanding the uncertainties associated with the predicted prevalence estimates. A large number of reports using LCA in veterinary diagnostic tests have been published [35], [36], [37], [38], [39], [40]. This study uses LCA to estimate the diagnostic test characteristics of five serological tests and is the first report of this analysis used to study TB infection in elephants. This model recognizes that each

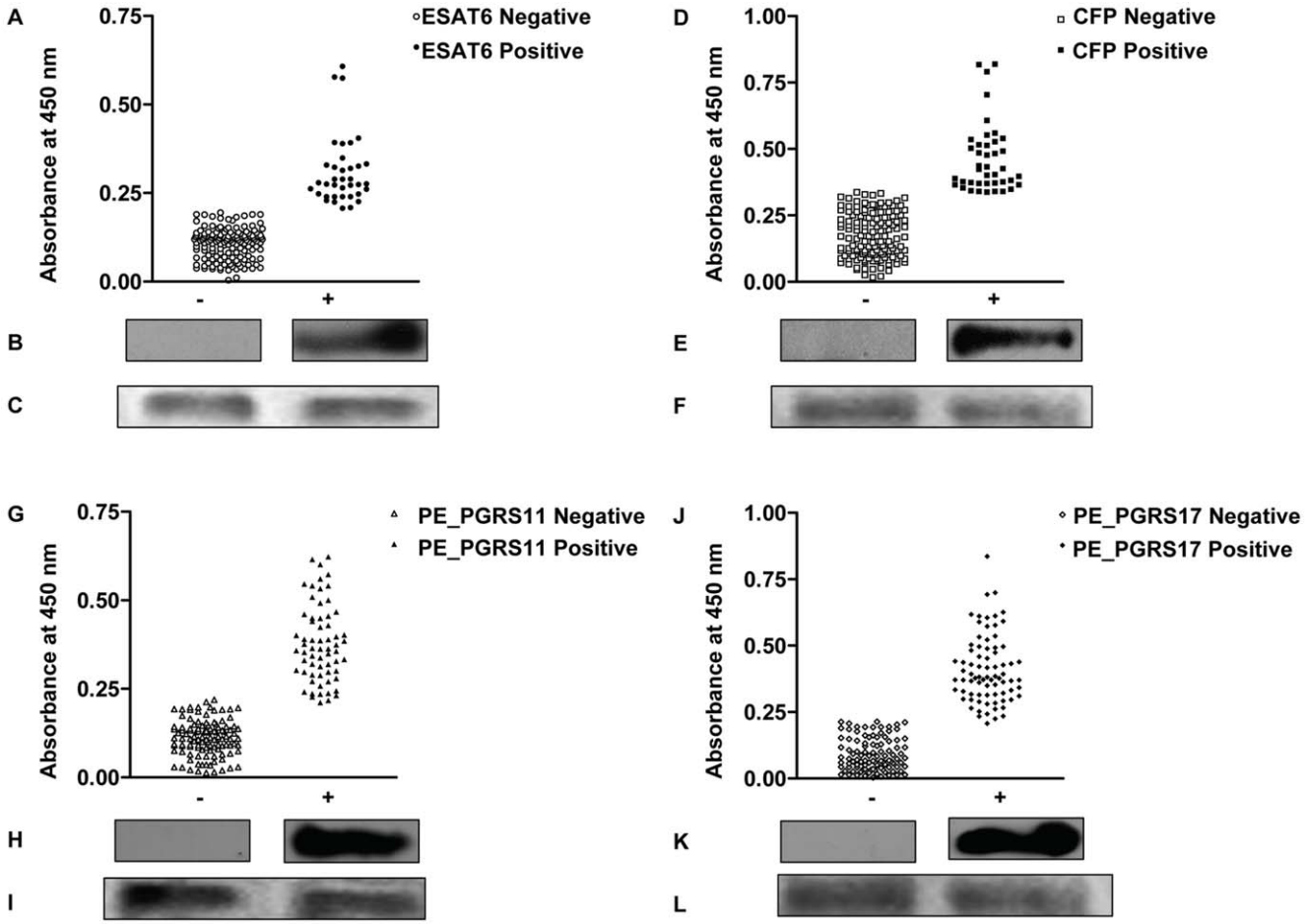

Figure 1. Differential humoral reactivity of four mycobacterial antigens with Asian elephant sera. For ELISA, elephant sera (1:200) was allowed to react with ESAT-6 (1 μg/ml) (A), CFP10 (0.5 μg/ml) (D), PE_PGRS11 (0.25 μg/ml) (G) and PE_PGRS17 (0.25 μg/ml) (J). Scatter plots show the total number of animals testing seronegative and seropositive for each antigen. For immunoblotting, 10 μg of each transferred protein ESAT-6 (C), CFP10 (F), PE_PGRS11 (I) and PE_PGRS17 (L) was first stained with Ponceau to check for loading control. Next, individual lanes were cut out of the blot and probed with sera from reference negative and positive animals. B, E, H, K represent immunoblots for one representative negative and positive animal each. The westerns were not quantitative in nature.
doi:10.1371/journal.pone.0049548.g001

test is measuring a different target latent variable, which is in turn associated with the presence of *M. tuberculosis* infection.

Test validation in the absence of suitable reference samples is extremely challenging [30], [31]. Culture for clinical isolation of *M. tuberculosis* remains the 'gold standard' of diagnostics; however a positive culture result is more likely in animals with advanced stage of disease. Validation of DSe based on positive culture results may result in its overestimation under field conditions [38], [40] and DSe may also vary with severity of disease. Validation of DSp entails testing of individuals and herds free from infection (a condition unlikely in TB endemic countries) [41] and may not be constant across different populations [38], [40]. Variables such as methods and their operational characteristics, expertise of the diagnostician, variations in host-pathogen interactions and difference in true disease prevalence rates may contribute to changes in performance of tests. Thus, test performance characteristics would need careful re-evaluation when used in different settings.

In order to decide the cut-off for our new in-house ELISAs, we had the choice of using either the RT as an imperfect gold standard or a mixture model. Even though the RT and ELISA work on the same biological principle, we did not use the former approach as the RT does not contain any PE_PGRS antigens. The mixture model for continuous data has been used for tuberculin skin test induration data among other applications [42]. The LCA is based on the premise that the true disease status is a common latent (or unobserved) variable associated with several imperfect tests that measure the same disease [43]. For the LCA, we delineated 16 latent classes which are the different combinations of the antibodies detected in the study. It would be interesting to link the latent classes to other disease parameters such as mortality and clinical signs. The disadvantage of using the LCA model is that it dichotomizes test results and thus does not use all the information derived from continuous test results. The choice of model used is dictated by the type of data and whether the model assumptions are satisfied. The model we propose is complex and subject to our interpretation. However, it incorporates results from five serological assays independent of culture results, estimates the true seroprevalence within the sampled population and the true disease status of each animal sampled therein.

Only about 10% of the *M. tuberculosis* proteome generates human antibody responses, and this immunoproteome contains predominantly membrane-associated and secreted proteins [44]. Differences in antibody profiles seen in TB patients need to factor in host characteristics, bacillary burden and metabolic state and

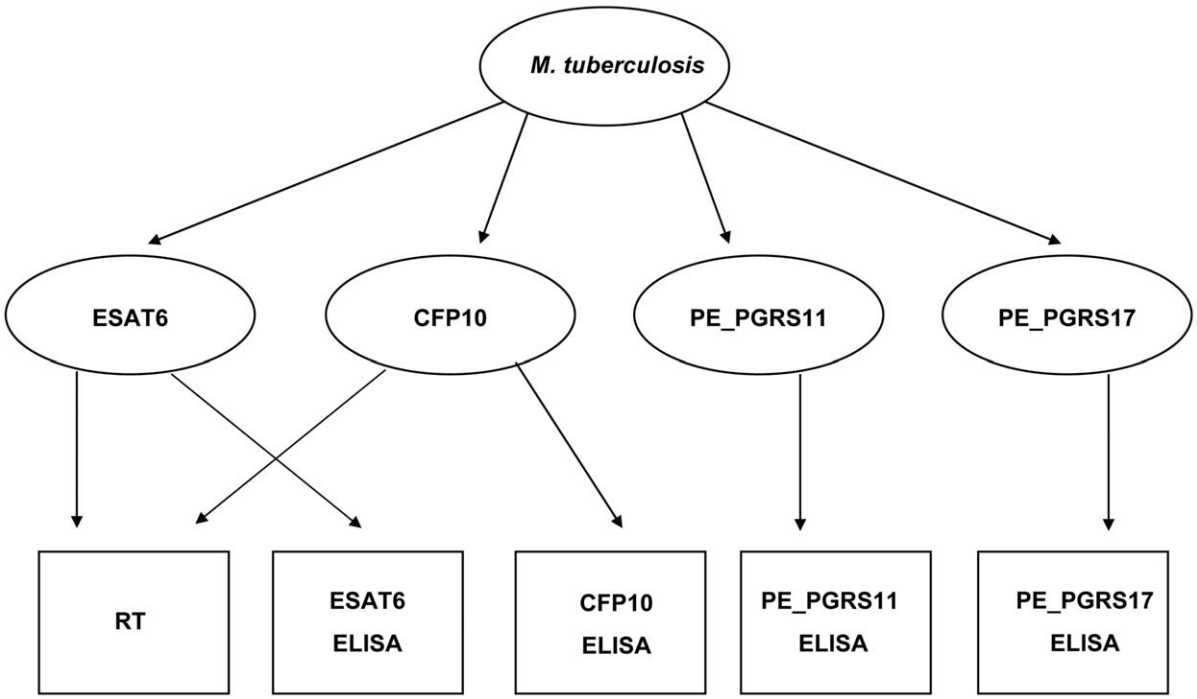

Figure 2. Schematic for the multiple latent variable model used in this study. The parameters to be estimated are depicted in oval shapes and the observed diagnostic test results are represented in rectangular shapes. While RT contains both the ESAT-6 and CFP10 antigens, the ELISAs are specific for one antigen each.
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protein expression by the infecting strain of *M. tuberculosis* [44], [45]. Additional complexity is introduced by the multiple clinical manifestations of disease in humans [46]. This host and pathogen derived heterogeneity has led to several reviews highlighting the shortcomings of TB immunodiagnostics [47], [48], [49], [50].

Circulating antibodies have been evaluated as biomarkers for TB since 1898 [51]. As compared to other members of the PE family (including PG_PGRS) or mycobacterial antigens, PE_PGR11 and PE_PGR17 have been shown to elicit stronger and differential antibody response in humans. Our laboratory has previously reported that PE_PGR11 and PE_PGR17 elicited antibodies in adult humans with active pulmonary infection, and in child patients with pulmonary or extrapulmonary TB [27], [28], [52]. Serology studies have demonstrated that antibodies reactive with a recombinant carboxyterminal fragment of the PE_PGRS protein from Rv1759c [53] or with the PGRS domain of Rv3367 [54] are present in human sera of patients infected with TB. The ESX protein family (for example, Rv3881c and Rv3784) are preferentially recognized antibody targets in active TB in humans [44]. The proteins CFP10 (Rv3784) and ESAT6 (Rv3785) have been evaluated in a number of veterinary serological assays [21], [22], [55], [56]. This approach is now giving way to whole proteome screening to identify TB associated proteins and the dynamics of antibody response they elicit during disease.

The past decade has seen decline of Asian elephant populations in most range countries with the exception of India and Sri Lanka [4]. Population depletion reduces the risk of host-specific infectious diseases except when the pathogen resides in reservoir hosts or when captivity results in increased infectious disease transmission [57]. Thus, conservation strategies that increase population density or cross-species contact such as in zoos, reserves and other captive conditions need careful evaluation in light of the risks of such infection. Cases of cross-species TB outbreaks in zoos are well documented [58]. The management of TB in Asian elephants in India remains inadequate owing to a number of issues including historical and cultural context of captivity of the species, legality of ownership (government owned vs. privately- owned) or the costs associated with the treatment. Albeit the United States Department of Agriculture has drafted clear guidelines [15] current need involves a creation of guidelines in regard to regulation of TB infections in elephants in India. Thus, our current study clearly proposes periodic verification/testing for TB in captive elephants as well as in-contact personnel.

Factors governing the initiation and expansion of ensuing immunity to wide ranging infections in elephants are not well understood. Further, detailed analysis of various effector roles played by the key components of immune systems such as T cells, B cells, macrophages or dendritic cells, complement and cytokines requires extensive investigation. For example, presence of five subclasses of IgG has been demonstrated in African elephant (*Loxodonta africana*) [59], [60]. Further, the genomic organization of the IgH, Igκ, and Igλ loci of the African elephant has been identified [61], [62]. Immunoreactivity analysis demonstrated the role for complement and antibodies during infections with African horse sickness [63], [64] Bluetongue [63] and Mycoplasmosis [65]. Interestingly, Alpha Napthyl Acetate Esterase activity was utilized as a T cell marker to demonstrate T lymphocyte distribution in peripheral blood [66] and presence of functional CD genes in the African elephant [67]. Not much tuberculosis disease stage-specific information is available in Asian elephants and vaccination with *M. bovis* BCG has not been evaluated in elephants. Lyaschenko *et al.* [21] reported that Multiantigen print immunoassay (MAPIA) and RT could pick up serum IgG to ESAT-6 and other proteins up to 3.5 years and 4.0 years respectively prior to culture of *M.*

Table 1. Defining the sixteen latent classes and calculating their sero-prevalence rates.

Class No.	Latent class definition	Seroprevalence	
		Median (95% CI)*	Mean
Class 1	Mtb**+, All antibodies+	15.9 (9.8, 24.0)	16.2
Class 2	Mtb+, ESAT6+, CFP10+, PE-PGRS11+	1.3 (0.1, 4.3)	1.5
Class 3	Mtb+, ESAT6+, CFP10+, PE-PGRS17+	0.4 (0.0, 2.1)	0.6
Class 4	Mtb+, ESAT6+, PE-PGRS11+, PE-PGRS17+	0.8 (0.0, 4.4)	1.2
Class 5	Mtb+, CFP10+, PE-PGRS11+, PE-PGRS17+	3.1 (0.2, 10.3)	3.7
Class 6	Mtb+, ESAT6+, CFP10+	0.8 (0.0, 3.2)	1.0
Class 7	Mtb+, ESAT6+, PE-PGRS11+	0.6 (0.0, 3.2)	0.9
Class 8	Mtb+, ESAT6+, PE-PGRS17+	0.5 (0.0, 2.3)	0.7
Class 9	Mtb+, CFP10+, PE-PGRS11+	0.5 (0.0, 2.6)	0.7
Class 10	Mtb+, CFP10+, PE-PGRS17+	0.4 (0.0, 2.3)	0.6
Class 11	Mtb+, PE-PGRS11+, PE-PGRS17+	11.9 (4.2, 18.5)	11.7
Class 12	Mtb+, ESAT6+	0.7 (0.0, 3.5)	1.0
Class 13	Mtb+, CFP10+	0.5 (0.0, 2.6)	0.7
Class 14	Mtb+, PE-PGRS11+	0.7 (0.0, 3.1)	0.9
Class 15	Mtb+, PE-PGRS17+	4.8 (0.2, 11.8)	5.1
Class 16	Mtb-, All antibodies-	53.6 (44.6, 62.8)	53.6

Latent class definition and the seroprevalence rate for each class thus defined in the LCA model for studying TB in Asian elephants in southern India. The latent classes are different combinations of the latent variables (i.e. true presence of the antibodies) which are present if *M. tuberculosis* infection is present. *Median estimate is at the 50% quantile while the 2.5% and 97.5% quantiles define a 95% credible interval (CI). ***M. tuberculosis* infection.
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tuberculosis from trunk washes. They reported that ESAT6 and CFP10 were the immunodominant antigens elicited upon infection of elephants with *M. tuberculosis*/*M. bovis* [21], [23]. Significantly, in addition to ESAT-6 and CFP-10, we report high DSe and DSp for the two PE_PGRS ELISAs based on a latent class model that recognizes that the different tests are designed to measure different antigens. The DSe and DSp for the ELISAs we developed for ESAT6 and CFP10 were comparable to the commercially available RT test. Importantly, we have attempted to address immunoreactive potential as well as serodiagnostic utility of selected antigens of *M. tuberculosis* H37Rv which would help in our understanding of the pathophysiological attributes of TB infection in elephants.

Serology remains an attractive first step in TB diagnosis in wildlife. It is simple, quick, affordable and does not require repeated handling of animals. Once elicited, the antibody response is sustained while the trunk wash culture may yield intermittent results in elephants. Identifying serological correlates of active TB in elephants and their use in antitubercular treatment monitoring [21], [23], [68] could be potentially useful tools in situations where it is important to keep costs of diagnosis low. We are currently evaluating cell immunity based assays for TB diagnosis in elephants. Further studies into TB transmission and surveillance using accurate, low cost and high throughput assays are also warranted. Such active disease surveillance in elephant range countries would help us to study the dynamic relationship between TB and elephant conservation.

Table 2. Sensitivity (DSe) and specificity (DSp) of the five serological tests used in the study.

Test	w.r.t.**	DSe	DSp
		Median (95% CI)*	Median (95% CI)*
RT	At least 1 antibody	48.6 (37.2, 61.0)	99.3 (96.7, 99.9)
	ESAT6	44.6 (33.9, 56.4)	95.2 (90.4, 98.2)
	CFP10	46.5 (36.0, 58.1)	93.0 (87.8, 96.6)
	PE_PGRS11	79.8 (66.7, 90.8)	98.5 (93.6, 99.9)
	PE_PGRS17	84.5 (75.2, 91.3)	89.6 (79.4, 98.4)
ESAT6	At least 1 antibody	88.8 (73.5, 97.5)	95.1 (90.5, 98.2)
	ESAT6	91.3 (74.8, 99.5)	95.2 (90.4, 98.2)
	CFP10	77.0 (61.9, 88.5)	88.9 (83.2, 93.6)
	PE_PGRS11	85.0 (71.7, 94.0)	75.9 (68.2, 82.3)
	PE_PGRS17	80.3 (66.7, 90.2)	65.7 (57.8, 73.1)
CFP10	At least 1 antibody	88.8 (73.5, 97.5)	95.3 (90.8, 98.2)
	ESAT6	75.2 (60.0, 87.1)	91.2 (85.7, 95.2)
	CFP10	92.3 (76.9, 99.5)	93.0 (87.8, 96.6)
	PE_PGRS11	85.2 (71.5, 94.0)	76.0 (68.5, 82.6)
	PE_PGRS17	81.5 (67.9, 91.4)	66.2 (58.3, 73.5)
PE_PGRS11	At least 1 antibody	50.1 (38.2, 62.1)	93.9 (88.8, 97.2)
	ESAT6	47.1 (35.8, 58.7)	91.5 (85.9, 95.4)
	CFP10	49.1 (38.0, 60.2)	89.4 (83.5, 93.7)
	PE_PGRS11	97.6 (88.6, 99.8)	98.5 (93.6, 99.9)
	PE_PGRS17	87.1 (77.6, 93.6)	81.4 (73.4, 88.1)
PE_PGRS17	At least 1 antibody	44.4 (32.5, 57.6)	91.6 (85.5, 96.0)
	ESAT6	42.2 (30.9, 54.8)	89.6 (83.0, 94.1)
	CFP10	44.8 (33.6, 57.1)	87.8 (81.5, 92.7)
	PE_PGRS11	82.7 (68.2, 94.3)	91.9 (85.3, 96.5)
	PE_PGRS17	97.2 (88.5, 99.9)	89.6 (79.4, 98.7)

The LCA model was used to calculate the DSe and DSp of each test w.r.t. the antibody it is designed to detect as also presence of at least one antibody. *The Median estimate refers to the 50% quantile while the 2.5% and 97.5% quantiles define a 95% credible interval (CI). ** With respect to.
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Materials and Methods

Study Population

In the three southern Indian states of Kerala, Karnataka and Tamil Nadu, there are an estimated 1,000 Asian elephants in captivity. These animals, mostly caught from the wild but also born in captivity, are maintained under different ownership and management regimens. A project for captive elephant health assessment was undertaken by Asian Nature Conservation Foundation (Permit No.8-1/2002-PE, Project Elephant, Ministry of Environment and Forests, Government of India). Apart from photographic documentation of body condition index, wounds and injuries with special reference to eyes and feet, routine haematology, serum biochemistry, urinalysis and dung analysis were performed for individual elephants. In an attempt to provide better healthcare to these elephants, the results of each elephant's health evaluation was then handed over to the veterinarian in-charge of the elephant. Following a convenience/opportunity sampling method, nearly 600 elephants were visited over a period of one year. A random sample of 179 serum samples collected from this heath survey was selected for this study.

Table 3. Number of elephants (observed and predicted according to the latent class model) with each test result.

Binary Input	2.50%	50%	97.50%	Mean	Observed
00000	69	82	92	81	84
00001	9	16	26	17	16
00010	0	3	8	3	1
00011	18	24	29	24	26
00100	1	6	13	6	8
00101	0	1	4	1	1
00110	0	0	2	0	1
00111	0	2	6	2	0
01000	0	4	11	4	4
01001	0	1	3	1	1
01010	0	0	2	0	0
01011	0	1	5	2	2
01100	0	0	2	0	0
01101	0	0	1	0	0
01110	0	0	2	0	0
01111	0	2	8	3	2
10000	0	0	4	1	0
10001	0	0	1	0	0
10010	0	0	1	0	0
10011	0	1	3	1	1
10100	0	0	2	0	0
10101	0	0	2	0	0
10110	0	0	2	1	0
10111	1	4	8	4	4
11000	0	0	2	0	0
11001	0	0	2	0	0
11010	0	1	3	1	1
11011	0	3	8	3	2
11100	0	1	2	1	1
11101	0	0	3	1	0
11110	0	2	5	2	2
11111	12	19	25	19	22

Evaluating the fit of the model by comparing the observed and expected number of elephants with different combinations of tests to see if the assumptions of the substantive model in Figure 2 were satisfied. 50% refers to the median estimate while the 2.5% and 97.5% quantiles define a 95% credible interval (CI).

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Materials

Unless otherwise specified all materials used in this study were purchased from Sigma-Aldrich, St. Louis, USA. Vacutainer needles (No. 301747), Vacutainer tubes (No. 367820), PANTA^{TM} Supplement, BACTEC^{TM} 12B Mycobacteria Culture Vials and BBL^{TM} Mycobactose Lowenstein-Jensen Medium were procured from BD (Franklin Lakes, NJ, USA). GammaBind G, Type 2 affinity matrix was bought from GE Healthcare Bio-Sciences (Uppsala, Sweden), Ni-nitrilotriacetic acid (Ni-NTA) columns from Qiagen (Valencia, CA, USA), Nunc-Immuno Plates (No 44204) from NUNC A/S (Roskilde, Denmark) and polyvinylidene difluoride membranes (PVDF) from Millipore (Bedford, MA, USA). ECL detection system was bought from Perkin-Elmer (MA, USA) and 3,3′,5,5′-tetramethylbenzidine (TMB) and horse radish peroxidase (HRP)-labeling kit from Bangalore Genei (Bangalore, Karnataka, India).

Collection of Serum and Screening with RT

Blood was collected by venipuncture of the middle auricular vein, allowed to clot at room temperature and serum separated within three hours of collection by centrifugation at 500 *g* for 10 minutes. Each serum was screened with RT and stored at −70°C until further testing. The RT (http://www.chembio.com/animaltest4.html) is a point-of-care lateral flow serological test licensed by the USDA in 2007. Greenwald *et al.* [22] reported a DSp of 95.2% (95% CI, 90.1 to 97.9) and DSe of 100% (95% CI, 84.0%−100%) for the RT.

Trunk-wash Culture for Isolation of *M. tuberculosis*

The procedure for trunk wash collection as described in the Guidelines for the Control of Tuberculosis in Elephants, 2008 [15] was modified [69]. Trunk wash specimens from select RT positive elephants were tested for *M. tuberculosis* culture [15]. Briefly, 0.5 ml of sample supplemented with Erythromysin (32 µg/ml) and PANTA^{TM} Supplement was inoculated into BACTEC^{TM} 12B vials and BBL^{TM} Mycobactose Lowenstein-Jensen Medium and grown at 37°C and 10% CO_2 for 8 weeks; this was followed by a niacin-nitrate reduction test for confirming *M. tuberculosis*. Ante-mortem serum collected from one elephant, which showed nodules in the lung tissue during post-mortem examination and from which *M. tuberculosis* was cultured on Lowenstein-Jensen medium, was used as positive control in the serological assays.

Expression and Purification of Recombinant ESAT6, CFP10, PE-PGRS17 AND PE-PGRS11 Proteins

M. tuberculosis H37Rv antigens *EsxA*-6 kDa early secretory antigenic target (ESAT-6) (Rv3875); *EsxB*-10 kDa culture filtrate antigen (CFP10) (Rv3874); PE_PGRS17 (Rv0978c) and PE_PGRS11 (Rv0754) were used as the major antigenic determinants in this study. Recombinant expression vectors for Rv3875, Rv3874, Rv0978c and Rv0754 antigens were obtained from Colorado State University, TB Vaccine Testing and Research Materials Contract (http://www.cvmbs.colostate.edu/mip/tb/recombinant.htm) and expressed as described previously [70]. Briefly, for the expression of His-tagged recombinant antigens, *Escherichia coli* BL21(DE3) cells carrying recombinant plasmids were induced with isopropyl-b-D-thiogalactopyranoside, and the proteins purified under native conditions (ESAT6 and CFP10) and denaturing conditions (PE_PGRS17 and PE_PGRS11) using Ni-NTA columns. In-gel digestions of proteins for matrix-assisted laser desorption/ionization mass spectrometry was carried out for identification.

Raising Rabbit Anti-Asian Elephant IgG-horse Radish Peroxidase (HRP)

Asian elephant IgG was separated from sera [71] and rabbit anti-Asian elephant IgG raised as per [59] and [60] with modifications. Briefly, New Zealand white rabbits were injected subcutaneously at multiple sites with 1 mg of purified Asian elephant IgG emulsified in equal volume of Freund's complete adjuvant followed by a second dose of 500 µg Asian elephant IgG emulsified in Freund's incomplete adjuvant. Antibody titres in sera were determined two weeks post final immunization by ELISA. The rabbit anti-Asian elephant IgG was coupled to HRP and its reactivity to elephant IgG was checked. All animal experiments were approved by the Institutional Ethics Committee for Animal

Experimentation and Institutional Biosafety Committee, Indian Institute of Science, Bangalore.

ELISA

Careful checker board titration for optimum protein concentration, elephant sera dilution, rabbit anti-Asian elephant IgG-HRP was carried out for each individual ELISA. All protein dilutions were made in 1X PBS (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na2HPO4; 1.47 mM KH2PO4, pH 7.4). 1X PBST (1X PBS with 0.05% tween-20) was used as wash buffer and 3% BSA in PBST as blocking buffer. Elephant sera, rabbit anti-Asian elephant IgG-HRP were each diluted in blocking buffer and 100 µl added per well. ESAT-6 (1 µg/ml), CFP10 (0.5 µg/ml), PE_PGRS17 and PE_PGRS11 (0.25 µg/ml each) were coated overnight (o/n) at 4°C into ELISA plates and then washed thrice. Blocking for 1 hour was followed by addition of elephant sera (1:200) and incubation o/n at 4°C. After washing, rabbit anti-Asian elephant IgG-HRP (1:3000) was added and the plate incubated o/n at 4°C. Tetramethylbenzidine was used as chromogenic substrate and the absorbance was read at 450 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Immunoblot Analysis

Ten µg of purified protein was subjected to 12% SDS-PAGE (Laemmeli) or 10% Tricine SDS-PAGE; transferred to PVDF and stained with Ponceau to check for loading control. The PVDF was cut into strips, blocked with 5% nonfat dried milk and each strip probed with individual elephant sera overnight at 4°C, probed with rabbit anti-Asian elephant IgG-HRP and the blot visualized with the ECL detection system.

Statistical Analysis

To adjust for the imperfect nature of the gold-standard reference, our test validation entailed i) determining an optimal cut-off for each ELISA using a mixture model for continuous data ii) using LCA to estimate the DSe and DSp of the five dichotomous tests. The first step was carried out using the *mixdist* library in the R software package [72] assuming that the observed continuous data on each test arises from a mixture of two Weibull distributions among the antibody positive and antibody negative elephants. The point of intersection of the two density functions was chosen as the optimal cut-off.

The LCA was carried out using the *lcmr* library in R software package [43]. This package uses a Bayesian approach to estimate the parameters of interest. We used non-informative prior distributions over all parameters so as to let the data dominate the analysis. The LCA model (Figure 2) assumed that each of the ELISA tests was measuring a different latent variable (i.e. true presence of the antibody) which was present if *M. tuberculosis* infection was present. Thus, this model adjusts for the correlation that may arise between tests within the groups of elephants that are *M. tuberculosis* infection positive or negative. The RT test was assumed to measure the presence of both ESAT-6 and CFP10 antibodies while each of the four ELISA was assumed to measure the presence of the corresponding antibody. The resulting model had 16 latent classes corresponding to different combinations of the antibodies (see supplementary document File S1). The fit of the model was evaluated by comparing the observed and expected number of elephants with different combinations of tests and a posterior predictive check for conditional dependence (Table 3). The predictive values of each test combination were examined to see if the assumptions of the substantive model in Figure 2 were satisfied.

Supporting Information

File S1 Algorithm for LCA model.
(DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: SVK DA KNB RS. Performed the experiments: SVK DA. Analyzed the data: ND SVK. Contributed reagents/materials/analysis tools: RS JVC ND KNB. Wrote the paper: SVK ND DA RS KNB.

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ANNEXURE - 11

October 2013, Vol 144, No. 4_MeetingAbstracts
Chest Infections | October 2013

Cross-Species Tuberculosis Transmission: Two Probable Cases in Mahouts and Captive Elephants

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Chest. 2013;144(4_MeetingAbstracts):215A. doi:10.1378/chest.1704012

Abstract

SESSION TITLE: Infectious Disease Global Case Reports

SESSION TYPE: Global Case Report

PRESENTED ON: Tuesday, October 29, 2013 at 01:30 PM - 02:30 PM

INTRODUCTION: In elephants, tuberculosis is a spillover disease resulting mostly from human cohabitation and most cases are caused by the human strains of Mycobacterium tuberculosis. Captive elephants in southern India are kept in hands-on contact with mahouts (elephant keepers) as well as public. In the field settings in southern India where there is a significant prevalence of tuberculosis in humans, human-to-elephant and elephant-to-human transmission of tuberculosis presents disease risks to both the in-contact species. A collaborative research involving medical physicians, veterinarians and molecular biologists is studying the transmission dynamics of tuberculosis infection among captive elephants and mahouts. Systematic tuberculosis screening of the nearly 1000 captive elephants and 5000 mahouts in southern India is underway. Screening of mahouts is done by clinical examination and tuberculin skin testing, followed by chest X-ray and sputum collection as required. Screening of elephants is done with the USDA licensed rapid serum tests Elephant TB STAT-PAK® and DPP Vet Assay® (Chembio Diagnostics Inc. Medford NY), followed by trunk wash culture for isolation and identification of mycobacteria.

CASE PRESENTATION: We identified two probable cases of cross-species transmission of M. tuberculosis between mahouts and captive elephants. First is case of human-to-elephant and second is a case of elephant-to-human transmission of M. tuberculosis. Case#1: A female elephant tested positive on Elephant TB STAT-PAK® and showed clinical symptoms of tuberculosis. There has been hand-on interaction between this elephant and its mahout for the past 25 years. The mahout was found to be suffering from active tuberculosis and tested positive on tuberculin skin test and showed shadows in his chest X-ray. M. tuberculosis culture isolate

was obtained from one of the three sputum samples collected from the mahout and was sensitive to first line anti-tuberculous drugs. Case#2: Another male elephant tested positive on Elephant TB STAT-PAK® and also showed symptoms of tuberculosis. The mahout associated with this elephant had a cutaneous nodule on the palm of right hand. This nodule, which was later operated and removed, was confirmed by histopathology as a case of tuberculous granuloma. Further inquiry revealed that this mahout previously suffered injuries to his hand while trying to control the elephant and the wound was contaminated with discharges from the elephant's trunk. The mahout tested positive on tuberculin skin test, but chest X-ray did not reveal any lesion suggestive of tuberculosis. Trunk wash samples from both elephants are collected for the isolation and identification of mycobacteria.

DISCUSSION: During the life span of nearly 50 years a captive elephant lives in close contact with humans. Also as a wild animal in captivity surviving in unnatural surroundings that add stress, the captive elephants present a unique animal model for an observational study to assess the disease risks of interspecies transmission of tuberculosis. This study intends to identify modifiable risk factors, if any, for effective policy intervention for prevention and control.

CONCLUSIONS: M. tuberculosis infection spillover and the risk of zoonosis from infected elephants is a well researched subject in the United States. In developing countries, mainly due to ineffective surveillance, such cases are rarely reported and documented. This poster presents a pictorial depiction of the different aspects of these two probable cases of cross-species transmission of M. tuberculosis.

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DISCLOSURE: The following authors have nothing to disclose: Venugopal Kummannoor Parameswaran Pillai, David Abraham

No Product/Research Disclosure Information

ANNEXURE - 12

Type: Poster Presentation

Final Abstract Number: 43.244
Session: Poster Session III
Date: Saturday, March 5, 2016
Time: 12:45-14:15
Room: Hall 3 (Posters & Exhibition)

Viral burden in acute respiratory tract infections in hospitalized children in the wet and dry zones of Sri Lanka

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Background: Acute respiratory tract infection (ARTI) is one of the most common acute illnesses of childhood. Mostly encountered viral etiology of ARTI in children under 5 years are respiratory syncytial virus (RSV), parainfluenza types 1, 2 and 3 (PIV), adenovirus (AV), influenza virus types A and B, coronavirus (CoV), human Boca virus (hBoV) and human metapeumo virus. (hMPV) This study was conducted to identify the viral burden in hospitalized children with ARTIs to map the occurrence of these viruses with local seasonality.

Methods & Materials: Nasopharyngeal aspirates (NPA) of inward patients (1 month - 5 years) with ARTI were collected in Teaching Hospital, Gampola (THG) and Teaching Hospital, Anuradhapura (THA) from March 2013 - August 2014. Following screening of NPA with indirect immunofluorescence assay (IFA) specific viral aetiology was detected by a direct immunofluorescence assay (DFA). IFA negative hundred NPA were tested for hMPV, hBoV and CoV. Viral seasonality and the overall viral burden were evaluated and the descriptive statistics was expressed using measures of central tendency.

Results: Out of 443 and 418 NPAs tested, RSV was detected 94 children (59.96%) in THG and 85 children (51.51%) in THA. In both cohorts RSV was detected throughout the year. In the dry zone, the peak viral incidence was noted from May-July in 2013 and 2014. In the wet zone two peaks were observed: December-January in 2013 (major peak) and in April in 2013 and 2014 (minor peak). Period prevalence of RSV ARTI in THG was 4.7% and in THA was 4.25%. The RSV incidence at THG and THA was 31.3 and 28 /100000 person years. The hMPV distribution was similar to that of RSV.

Conclusion: Knowledge of seasonality of the occurrence of viral aetiologies in children with ARTI is important to implement early preventive measures, such as vaccination for influenza A, use of respiratory precautions and health education. Identifying the viral aetiology by proper virological diagnosis will reduce the empirical use of antibiotics and thus will contribute to reduce the cost and to prevent the emergence of anti-microbial resistance.

http://dx.doi.org/10.1016/j.ijid.2016.02.980

Type: Poster Presentation

Final Abstract Number: 43.245
Session: Poster Session III
Date: Saturday, March 5, 2016
Time: 12:45-14:15
Room: Hall 3 (Posters & Exhibition)

Cross-species transmission of mycobacterium tuberculosis in mahouts and captive elephants: Implications to health policy

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Background: There are nearly a thousand captive Asian elephants and not less than 3,000 mahouts in southern India. In the hands-on and open systems of captive elephant management, diseased mahouts and captive elephants could present the risk of cross-species tuberculosis transmission. With the help of evidence based results, we intend to formulate specific policy guidelines, which can suggest locally relevant preventive and control measures to help mitigate the risk of cross-species infection.

Methods & Materials: Over a period of three years, one time screening of nearly 800 elephants and their mahouts was achieved. Tuberculosis screening of mahouts was done by clinical examination, chest X-ray evaluation, sputum culture and tuberculin skin testing, as required. Screening of elephants was done using the USDA licensed serological test, DPP Vet Assay® (Chembio Diagnostics Inc., Medford, New York) and trunk wash culture, as required. Detailed contact investigation of traceable human and animal contacts of the identified diseased mahouts and elephants were done. We examined three different contexts of tuberculosis transmission among captive elephants and mahouts. First scenario is the risk of infection from an infected mahout to an elephant. Second is the risk of infection from an infected elephant to a mahout and third is the risk of infection from an infected elephant to another elephant.

Results: There is evidence to suggest cross-species tuberculosis transmission. However, under the tropical climatic conditions in southern India, the risk of infection to a captive elephant from a diseased mahout seems to far outweigh the risks of infection to a mahout or another elephant, from a diseased elephant. There are political as well as ethical consequences to the outcomes in each of the three scenarios and they are both varied and complex.

Conclusion: Mahouts and captive elephants in southern India are highly migrant and locating the subjects for contact tracing and follow-up testing is difficult. Hence, systematic and regular tuberculosis screening of mahouts and captive elephants is a challenge. Formulating as well as implementing policy guidelines for prevention and control of cross-species tuberculosis transmission, in the existing cultural and religious contexts of captive elephant managements in southern India, appears to be an even bigger challenge.

http://dx.doi.org/10.1016/j.ijid.2016.02.981

ANNEXURE - 13

World Health Organization | OIE World Organisation for Animal Health | Food and Agriculture Organization of the United Nations | The Union International Union Against Tuberculosis and Lung Disease *Health solutions for the poor*

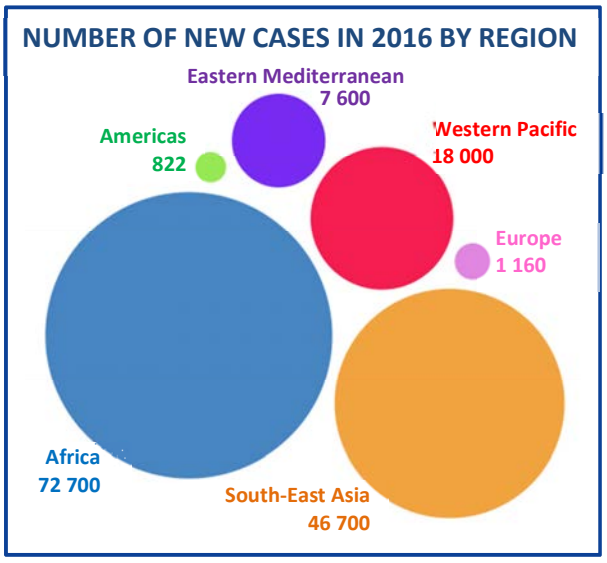
ZOONOTIC TUBERCULOSIS

WHAT IS ZOONOTIC TB?

- Zoonotic tuberculosis (TB) is a form of tuberculosis in people caused by *Mycobacterium bovis*, which belongs to the *M. tuberculosis* complex.
- It often affects sites other than the lungs (extra-pulmonary), but in many cases is clinically indistinguishable from TB caused by *M. tuberculosis.*
- Within animal populations, *M. bovis* is the causative agent of bovine TB. It mainly affects cattle, which are the most important animal reservoir, and can become established in wildlife. The disease results in important economic losses and trade barriers with a major impact on the livelihoods of poor and marginalized communities.

BURDEN

- In 2016, there were an estimated 147 000 new cases of zoonotic TB in people globally, and 12 500 deaths due to the disease. The African region carries the heaviest burden, followed by the South-East Asian region.
- The true burden of zoonotic TB is likely to be underestimated due to a lack of routine surveillance data from most countries.
- Bovine TB is endemic in animal populations in many parts of the world.



RISK FACTORS

- While the most common route of transmission of *M. bovis* to humans is through contaminated food (mainly untreated dairy products or, less commonly, untreated meat products), airborne transmission also poses an occupational risk to people in contact with infected animals or animal products, including farmers, veterinarians, slaughterhouse workers and butchers.

KEY CHALLENGES

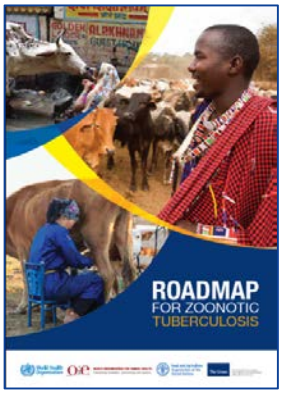
- The laboratory procedures most commonly used to diagnose TB do not differentiate *M. tuberculosis* from *M. bovis*. This leads to under-diagnosis of zoonotic TB.
- Zoonotic TB poses challenges for patient treatment and recovery. *M. bovis* is naturally resistant to pyrazinamide, one of the four medications used in the standard first-line anti-TB treatment regimen. As most healthcare providers initiate treatment without drug susceptibility testing, patients with zoonotic TB may receive inadequate treatment.
- Zoonotic TB in humans is often initially extrapulmonary and may be misdiagnosed, and therefore initiation of treatment can be delayed.

TIMPIYAN LESENI
Zoonotic TB Survivor
Maasai community, Kenya

"I suffered from abdominal TB as a consequence of cultural traditions of drinking unpasteurized milk. I am now working to educate my community - the Maasai people -on how to fight zoonotic TB, through my civil society organization, Talaku."

10 PRIORITIES FOR ZOONOTIC TB

The World Health Organization (WHO), the World Organisation for Animal Health (OIE), the Food and Agriculture Organization of the UN (FAO) and the International Union Against Tuberculosis and Lung Disease (The Union) launched the first-ever roadmap for tackling zoonotic TB in October 2017. The roadmap is centered on a *One Health* approach, recognising the interdependence of human and animal health sectors to address the major health and economic impact of this disease. It articulates clear immediate actions that all stakeholders can take to address this issue across different sectors and disciplines, and defines milestones for the short- and medium-term. The roadmap calls for concerted action from government agencies, donors, academia, non-governmental organizations and private stakeholders across political, financial and technical levels. Ten priorities for action are defined, which will also bring substantial benefits for the control of other zoonotic and foodborne diseases:



IMPROVE THE SCIENTIFIC EVIDENCE BASE

1. Systematically survey, collect, analyse and report better quality data on the incidence of zoonotic TB in people, and improve surveillance and reporting of bovine TB in livestock and wildlife.
2. Expand the availability of appropriate diagnostic tools and capacity for testing to identify and characterize zoonotic TB in people.
3. Identify and address research gaps in zoonotic and bovine TB, including epidemiology, diagnostic tools, vaccines, effective patient treatment regimens, health systems and interventions coordinated with veterinary services.

REDUCE TRANSMISSION AT THE ANIMAL-HUMAN INTERFACE

4. Develop strategies to improve food safety.
5. Develop capacity of the animal health sector to reduce the prevalence of TB in livestock.
6. Identify key populations and risk pathways for transmission of zoonotic TB.

STRENGTHEN INTERSECTORAL AND COLLABORATIVE APPROACHES

7. Increase awareness of zoonotic TB, engage key public and private stakeholders and establish effective intersectoral collaboration.
8. Develop and implement policies and guidelines for the prevention, surveillance, diagnosis, and treatment of zoonotic TB, in line with intergovernmental standards where relevant.
9. Identify opportunities for community-tailored interventions that jointly address human and animal health.
10. Develop an investment case to advocate for political commitment and funding to address zoonotic TB across sectors at the global, regional and national levels.

ZOONOTIC TB MUST BE PRIORITIZED IN THE GLOBAL HEALTH AGENDA

The UN Sustainable Development Goals (SDGs) emphasise the importance of multidisciplinary approaches to improving health. In the context of the SDGs, WHO's End TB strategy calls for diagnosis and treatment of every TB case. This must include people affected by zoonotic TB. Zoonotic TB in people cannot be fully addressed without controlling bovine TB in animals and improving food safety. Through a **One Health** approach, together we can save lives and secure livehoods.

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For more information, please access http://www.who.int/tb/areas-of-work/zoonotic-tb/en

ANNEXURE - 14

रजिस्ट्री सं० डी० एल०—(एन)04/0007/2003—09 REGISTERED NO. DL—(N)04/0007/2003—09

भारत का राजपत्र
The Gazette of India

असाधारण
EXTRAORDINARY
भाग II — खण्ड 1
PART II — Section 1
प्राधिकार से प्रकाशित
PUBLISHED BY AUTHORITY

सं० 29] नई दिल्ली, शुक्रवार, मार्च 20, 2009 /29 फाल्गुन, 1930
No. 29] NEW DELHI, FRIDAY, MARCH 20, 2009 / 29 Phalguna, 1930

इस भाग में भिन्न पृष्ठ संख्या दी जाती है जिससे कि यह अलग संकलन के रूप में रखा जा सके।
Separate paging is given to this Part in order that it may be filed as a separate compilation.

MINISTRY OF LAW AND JUSTICE
(Legislative Department)

New Delhi, the 20th March, 2009/Phalguna 29, 1930 (Saka)

The following Act of Parliament received the assent of the President on the 20th March, 2009, and is hereby published for general information:—

THE PREVENTION AND CONTROL OF INFECTIOUS AND CONTAGIOUS DISEASES IN ANIMALS ACT, 2009

No. 27 OF 2009

[*20th March*, 2009]

An Act to provide for the prevention, control and eradication of infectious and contagious diseases affecting animals, for prevention of outbreak or spreading of such diseases from one State to another, and to meet the international obligations of India for facilitating import and export of animals and animal products and for matters connected therewith or incidental thereto.

WHEREAS economic losses due to infectious and contagious diseases of animals are enormous in the country with some of these diseases constituting a serious threat to the public;

AND WHEREAS many of such animal diseases can be largely prevented by judicious implementation of vaccination programmes or by taking other appropriate and timely measures on scientific lines;

AND WHEREAS such measures are necessary to facilitate the import and export of animals and animal products and to keep in tune with international practices;

AND WHEREAS it has been realised that the prevention, control and eradication of infectious and contagious diseases of animals from India has to be tackled on a national basis so as to avoid adverse impact of such diseases on the economy of the country and for this purpose harmonise the control procedures and to prevent inter-State transmission of animal diseases;

AND WHEREAS the national level handling has to be done with the active involvement of the State Governments, particularly in regard to the precautionary measures required to

be taken within their jurisdiction in respect of certain infectious and contagious diseases and the regulation of movement of animals outside their respective areas by timely adoption of appropriate measures;

AND WHEREAS India is a Member Country of the *Office International Des Epizooties, Paris* and it is necessary to implement the general obligations, decisions and recommendations of the said Organisation and abide by the International Animal Health Code stipulated by the said Organisation;

BE it enacted by Parliament in the Sixtieth Year of the Republic of India as follows:—

CHAPTER I

PRELIMINARY

Short title, extent and commencement.

1. (*1*) This Act may be called the Prevention and Control of Infectious and Contagious Diseases in Animals Bill, 2009.

(*2*) It shall come into force on such date as the Central Government may, by notification, appoint; and different dates may be appointed for different States or for different areas therein as well as for different provisions of this Act, and any reference in any such provision of this Act to the commencement of this Act shall be construed in relation to any State or area or provision as a reference to the coming into force of this Act or, as the case may be, of that provision, in such State or area.

Definitions.

2. In this Act, unless the context otherwise requires,—

(*a*) "animal" means,–

(*i*) cattle, buffalo, sheep, goat, yak, mithun;

(*ii*) dog, cat, pig, horse, camel, ass, mule, poultry, bees; and

(*iii*) any other animal or bird as the Central Government may, by notification, specify;

(*b*) "Check Post" means any place established as such by the Director to carry out checking of animals for the purpose of this Act;

(*c*) "Competent Officer" means any person or officer of the Government notified as a Competent Officer under section 17;

(*d*) "compulsory vaccination" means vaccination of any animal against any scheduled disease in respect of which vaccination is made mandatory under the provisions of this Act;

(*e*) "controlled area" means any local area which has been declared as such by the State Government under sub-section (*1*) of section 6;

(*f*) "defective vaccine" means any vaccine which is expired, breach in seal, contaminated, improperly stored, unlabelled or with mutilated label;

(*g*) "Director", in relation to a State, means any officer in charge of the Department of Animal Husbandry or Veterinary Services, or both, notified by the State Government as such for the purpose of this Act;

(*h*) "free area" means any controlled area which has been declared as such under sub-section (*5*) of section 6;

(*i*) "infected animal" means an animal which is infected with any scheduled disease;

(*j*) "infected area" means an area declared as such under section 20;

(*k*) "notification" means notification published in the Official Gazette;

(*l*) "prescribed" means prescribed by rules made under this Act;

(*m*) "publication" includes propagation of information through the media or newspaper or any other mass media and the means of local communication such as declaration in loud voice and by beating drums in the area;

(*n*) "Quarantine Camp" means any place declared to carry out quarantine of animals and birds for the purpose of this Act;

(*o*) "scheduled disease" means any disease included in the Schedule;

(*p*) "Veterinarian" means a person having a recognised veterinary qualification who, under the law for the time being in force, is allowed to treat animal diseases;

(*q*) "Veterinary Officer" means any officer, appointed as such by the State Government under clause (*b*) of section 3;

(*r*) "Village Officer", in relation to a village, means any person who is authorised or designated as such in accordance with the qualifications prescribed by the State Government.

CHAPTER II

CONTROL OF SCHEDULED DISEASES

Appointment of Veterinary Officers

3. The State Government may, by notification, appoint—

(*a*) such number of persons, as it deems proper, to be Veterinarians to undertake inspection and specifying the local limits of their respective jurisdiction; and

(*b*) such number of Veterinarians, as it deems proper, to be Veterinary Officers, who shall exercise their powers and discharge their duties within the local limits of their jurisdiction as may be specified in the said notification.

Reporting scheduled diseases obligatory.

4. (*1*) Every owner, or any other person, non-governmental organisation, public bodies or the village panchayat, in charge of any animal which he or it has reason to believe to be infective of a scheduled disease shall report the fact to the Village Officer or village panchayat in-charge, who may report the same in writing to the nearest available Veterinarian.

(*2*) The Village Officer shall visit the area falling within his jurisdiction for reporting any outbreak of the disease.

(*3*) Every Veterinarian shall, on receipt of a report under sub-section (*1*), or otherwise, if he has reason to believe that any animal is infected with a scheduled disease, report the matter to the Veterinary Officer.

(*4*) Where in any State there is any occurrence of scheduled disease in relation to any animal, the Director shall send an intimation to the Directors of the States which are in the immediate neighbourhood of the place where there is such occurrence, for taking appropriate preventive measures against the spread of the disease.

Duty to segregate infected animals.

5. (*1*) Every owner or person in charge of an animal, which he has reason to believe is infective of a scheduled disease, shall segregate such animal and have it kept in a place away from all other animals which are healthy, and take all possible steps to prevent the infected animal from coming in contact with any other animal.

(*2*) The owner or other person in charge of, or having control over, the animal referred to in sub-section (*1*) shall confine that animal and prevent it from grazing in a common place or to drink water from any common source including a vessel, pond, lake or river.

(*3*) All other infected animals shall be segregated by the Municipality, Panchayat or other local administration.

Notification of controlled areas and free areas.

6. (*1*) The State Government may, with the object of preventing, controlling or eradicating any scheduled disease, by notification, declare any area to be a controlled area in respect of any scheduled disease affecting any species of animal and any other species that may be susceptible to the disease specified in the said notification.

(*2*) The State Government shall also cause the substance of the notification issued under sub-section (*1*) to be published in a local newspaper in the vernacular language and by declaration in loud voice and by beating drums in the area.

(*3*) Where a notification has been issued under sub-section (*1*), all animals of the species in the controlled area shall be subjected to compulsory vaccination against that disease, and be subjected to such other measures against the disease, in such manner and within such time as the State Government, may, by public notice, direct.

(*4*) The State Government shall make available necessary vaccine and it shall be obligatory on the part of every owner, or the person in charge of an animal which is required to be vaccinated under sub-section (*3*), to get the animal compulsorily vaccinated.

(*5*) Where the State Government is satisfied, on a report received from the Director or otherwise, that, in any controlled area, any of the scheduled diseases affecting any species of animal is no longer prevalent, it may, by notification, declare the area to be a free area in respect of that disease in relation to the particular species of animal.

(*6*) Where a notification has been issued under sub-section (*5*), no animal of the species or of any other susceptible species with regard to which it is a free area shall be allowed to enter the free area unless duly immunized by vaccination against that particular disease.

Prohibition of movement of animals from controlled area.

7. (*1*) Where a notification has been issued under sub-section (*1*) of section 6 declaring any area as a controlled area in relation to any disease affecting any species of animals, no animal belonging to that species shall be moved from the place where it is kept.

(*2*) The Director may, for the purpose of control, prevention or eradication of any scheduled disease, in respect of any area, by order published in the Official Gazette, prohibit the movement of all animals belonging to any species specified therein, from the place where it is kept, to any other place.

(3) Nothing contained in sub-sections (1) and (2) shall be deemed to prohibit—

(a) the movement of any animal referred to therein, from the place where it is kept, to the nearest place where it can be got vaccinated, so long as the animal is being moved for the purpose of its immunization by vaccination; or

(b) the movement of any such animal, so long as it is accompanied by a valid certificate of vaccination to indicate that the animal is duly immunized against the particular disease and it bears proper mark of such vaccination.

Vaccination, marking and issue of vaccination certificate

8. (1) The vaccine to an animal may be administered by any person competent under the law for the time being in force to administer it, and issue a certificate of administration of vaccination.

(2) Where any animal has been vaccinated for any scheduled disease in compliance with the provisions of sub-section (1), the person vaccinating the animal shall cause to put a mark by branding, tattooing or ear tagging, or in such other manner as the Director may, by general or special order, direct and the same shall, unless otherwise specified by the Director, shall not be removed.

(3) The authority issuing a certificate of vaccination shall specify the date of vaccination, dates of manufacture and expiry of the vaccine and the date up to which the vaccination of the animal with the particular vaccine shall be valid.

Contents of vaccination certificate

9. Every vaccination certificate issued under this Act shall be in such form and shall contain such particulars as may be prescribed by the Central Government.

Entry and exit of animals into controlled area and free area

10. (1) Where any area has been declared as a controlled area under sub-section (1) of section 6 in respect of any disease affecting any species of animals, no animal belonging to that species shall be taken out of, or brought into that area save as provided in section 16.

(2) The Director may, by notice duly published in the Official Gazette and at least in one daily local newspaper in vernacular language, extend the prohibition contained in sub-section (1) to any other species of animals, if animals belonging to that species are also likely to be infected with that disease.

(3) No carrier of goods or animal shall carry any animal from or out of a controlled area, free area or infected area by land, sea or air unless he complies with the provisions of section 16.

(4) Nothing contained in sub-sections (1) to (3) shall apply to the carriage by railway of any animal referred to in those sub-sections through any area which, for the time being, is declared as a controlled area or infected area so long as the animal is not unloaded (for whatsoever purpose or duration) in any place within that area:

Provided that the State Government may, by notification, declare that any species of animal so carried through any local area within the State shall be duly immunized against such scheduled disease, in such manner and within such time as may be specified in that notification and a certificate of vaccination shall be a pre-requisite for the transportation of the animals by the railways through that area:

Provided further that, where any notification as referred to in the first proviso has been issued, it shall be incumbent on the State Government to intimate that fact to the concerned railway authorities so as to enable them to satisfy themselves about the immunization of the animal before transporting it through the local area of the State.

Precautionary measures in relation to controlled areas

11. No person shall take out of the controlled area—

(a) any animal, alive or dead, which is infected with, or reasonably suspected to have been infected with, any scheduled disease notified under sub-section (1) of section 6,

(b) any kind of fodder, bedding or other material which has come into contact with any animal infected with such disease or could, in any manner, carry the infection of the notified disease, or

(c) the carcass, skin or any other part or product of such animal.

Prohibition of markets, fairs, exhibition, etc., in the controlled areas

12. No person, organisation or institution shall hold any animal market, animal fair, animal exhibition and carry on any other activity which involves grouping or gathering of any species of animals within a controlled area:

Provided that the Competent Officer may, *suo motu* or on application made to him in this behalf, relax the prohibition in relation to any species of animals, in a case where animals belonging to that species are not susceptible to the scheduled disease and are incapable of carrying it, if he is satisfied that in the public interest it is necessary to accord such relaxation.

Prohibition of bringing of infected animals into market and other places

13. No person shall bring or attempt to bring into market, fair, exhibition or other congregation of animals or to any public place, any animal which is known to be infected with a scheduled disease.

Check Posts and Quarantine Camps

14. (*1*) The Director may establish as many Quarantine Camps and Check Posts within the State as may be required—

(*a*) for the detention of animals suffering from any scheduled disease or of animals which have come into contact with or have been kept in the proximity of any such infected animal;

(*b*) for ensuring the prevention of entry into or exit from any controlled area or infected area or free area, of any animal belonging to the species of animals in respect of which a notification, issued under sub-section (*1*) of section 6, or an order issued under sub-section (*2*) of section 7, is in force.

(*2*) Any animal which is required to be detained, inspected, vaccinated, or marked, may be kept in the Quarantine Camp for such period as the Competent Officer may direct.

(*3*) Every animal detained at a Quarantine Camp shall be under the custody of the person in charge of the camp, and shall be vaccinated and marked.

(*4*) The officer in charge of the Quarantine Camp shall, at the time of release of an animal from the station, grant a permit, in such form as may be prescribed by the State Government, to the person taking charge of the animal, and every such person shall be bound to produce the permit whenever required to do so by any Competent Officer.

Inspection and detention of animals at Check Ppsts and Quarantine Camps

15. (*1*) Every person in charge of any Check Post or Quarantine Camp shall inspect any animal stopped at the Check Post, or detained therein or at the Quarantine Camp.

(*2*) The manner of inspection and the period of detention of the animal at the Check Post or at the Quarantine Camp for the purpose of inspection or for the administration of compulsory vaccination, the marking of animals and the form and manner in which permit for entry in respect of any animal may be issued, shall be such as may be prescribed by the State Government.

Entry and exit of vaccinated animals into controlled and free areas

16. Notwithstanding anything contained in section 10, an animal belonging to the species of animals in respect of which an area has been declared as a controlled or free area in relation to any scheduled disease, which has been duly vaccinated against that disease, shall be allowed to enter into or be taken out of the controlled area or free area, or to be taken out of any other place on the production of a certificate to the effect that vaccine against that disease has been administered and a period of not less than twenty-one days has elapsed thereafter.

Appointment of Competent Officers

17. The State Government may, for the proper implementation of the provisions of this Act, by notification, authorise any person to exercise any power or discharge any duty as a Competent Officer, under this Act, who shall exercise such powers and such duties within the local limits of his jurisdiction as may be specified in the notification.

Cleaning and disinfection of carriers.

18. (*1*) Every common carrier whether a vessel or vehicle shall be cleaned and disinfected immediately before and after the transportation of any animal in that vessel or vehicle, and so also any other place where the animal has been kept in transit.

(*2*) Where any area has been declared as a controlled area or free area in respect of any scheduled disease affecting any species of animal, the Director may, by an order duly published in the Official Gazette and in a local newspaper in the vernacular language, direct the owner of every vehicle in which any animal belonging to that species is carried, to have the vehicle properly cleaned and disinfected.

Powers of entry and inspection.

19. Any Veterinary Officer or other Competent Officer may enter upon and inspect any land or building or place, vessel or vehicle, for the purpose of ensuring compliance of the provisions of this Act or the rules or orders made thereunder, by the persons responsible for such compliance.

CHAPTER III

INFECTED AREAS

Declaration of infected areas.

20. If the Veterinary Officer, upon receipt of a report from a Veterinarian or otherwise, is satisfied that, in any place or premises falling within his jurisdiction, an animal has been infected with any scheduled disease, or that an animal, which he has reason to believe has been so infected, is kept, may, by notification and publication in at least one local newspaper in the vernacular language and by declaration in loud voice and by beating drums, declare such area as he may deem fit (including the place or premises aforesaid) to be an infected area.

Effect of declaration of infected areas.

21. (*1*) Where an area has been declared as an infected area under section 20, all provisions of this Act which are applicable in relation to a controlled area shall *mutatis mutandis* apply thereto as if for the words "controlled area", the words "infected area" have been substituted.

(*2*) Without prejudice to the generality of the provisions contained in sub-section (*1*), the following further provisions shall apply in relation to an infected area, namely:—

(*a*) in respect of every animal in that area which is infected or reasonably believed to be infected, with any scheduled disease, the owner or other person in charge of the animal, shall forthwith get it treated by a Veterinarian;

(*b*) all articles, which are likely to have come into contact with any animal referred to in clause (*a*), shall be treated or disposed off in such a manner as the Veterinarian may direct;

(*c*) every Veterinarian shall, for the purpose of inspection, have the power to enter any place or premises where any animal is kept or is likely to be kept;

(*d*) the owner or any other person in charge of the animal referred to in clause (*a*) shall keep the animal in isolation forthwith, and also take such other measures as may be necessary for the prevention, treatment and control of the disease as the Veterinarian may direct.

Denotification of infected area.

22. If the Veterinary Officer, after such enquiry as he may deem fit, is satisfied that there is no longer the threat or danger of any animal being infected with the scheduled disease in any infected area, by notification and publication in a local newspaper in vernacular language, declare that the area is no longer an infected area as aforesaid, whereupon all the restrictions referred to in section 21 shall cease to apply.

CHAPTER IV

INFECTED ANIMALS

Segregation, examination and treatment of infected animals.

23. (*1*) Where the Veterinarian has, on receipt of a report or otherwise, reason to believe that any animal is infected with a scheduled disease, he may, by order in writing, direct the owner or any other person in charge of such animal—

(*a*) to keep it segregated from other apparently healthy animals; or

(*b*) to subject it to such treatment as may be required under the circumstances.

(*2*) Where any action has been taken in pursuance of sub-section (*1*), the Veterinarian shall forthwith give a detailed report of the incidence of the disease to the Veterinary Officer.

(*3*) On receipt of a report from the Veterinarian, the Veterinary Officer shall, as soon as possible, examine that animal as well as any other animal which could have come in contact with it, and for that purpose, submit the animal to such test and medical examination as may be required under the circumstances.

(*4*) If, after such test and examination, the Veterinary Officer is of the opinion that an animal is not infected with any of the scheduled diseases, he shall issue a certificate in writing that the animal is not infected with any such disease.

Drawing samples from animals.

24. (*1*) Where the Veterinary Officer considers it necessary for the purpose of ascertaining whether the animal which is suspected to have been infected with any scheduled disease or susceptible to such infection is actually infected, or for the purpose of ascertaining the nature of the scheduled disease with which an animal is infected, he may draw such samples, as may be required, from the animal for the purpose of carrying out such investigations as he may deem necessary under the circumstances.

(*2*) The Veterinary Officer or any other Competent Officer shall draw samples from any animal for the purposes of ascertaining whether the animal has been vaccinated against any disease, or whether the vaccination of the animal has been effective in conferring it immunity and have the samples examined, in such manner as he may deem necessary.

Resort to euthanasia for infected animals.

25. If the Veterinary Officer deems it necessary that an animal, which is infected with a scheduled disease, euthanasia has to be resorted to, for preventing the spread of the disease to other animals in the area or to protect public health if the disease is of zoonotic importance, he may, notwithstanding anything contained in any other law for the time being in force, by an order in writing, direct euthanasia of the animal and the carcass disposed of immediately to his satisfaction.

Disposal of carcass.

26. Every person in possession of carcass (or any part thereof) of any animal, which, at the time of its death, was infected with any scheduled disease or was suspected to have been infected, shall dispose it of in such manner as may be prescribed.

Powers of Veterinary Officer and Veterinarian to hold post-mortem examination.

27. (*1*) Where the Veterinary Officer or any Veterinarian has reason to believe that the death of an animal has been caused by an infection of any scheduled disease, he may make or cause to be made a post-mortem examination of the animal and for that purpose he may cause the carcass of any such animal to be exhumed where required followed by proper disposal after necessary examination and post-mortem.

(*2*) Every examination and post-mortem referred to in sub-section (*1*) shall be conducted in such manner, and the report of post-mortem shall be in such form, as may be prescribed.

Seizure and removal of certain animals.

28. Where any animal which is infected or suspected to have been infected is found without any person claiming to be its owner, or where a valid order or direction given in relation to any such animal is not promptly complied with by the owner or other person in control of the animal, it shall be open to the Veterinary Officer or any other Competent Officer, to seize the animal and remove it to a place of isolation or segregation, as he may deem proper.

CHAPTER V

ENFORCEMENT AND PENALTIES

Enforcement of orders and recovery of expenses.

29. (*1*) Where by any rule, notification, notice, requisition, order or direction made under this Act, any person is required to take any measure or to do anything—

(*a*) in respect of any animal, carcass of any animal or other thing in his custody or charge, the same shall be promptly complied with by that person;

(*b*) in case of any stray or ownerless animal, carcass of such animal or parts thereof, the same shall be promptly complied with by the municipality or Panchayat, as the case may be, at its cost.

(*2*) If the measures as referred to in sub-section (*1*) are not taken within such time as may be allowed for the purpose, the authority issuing the notice, requisition, order or direction, may cause the measures to be taken at the cost of the person or municipality or Panchayat, as the case may be, who or which was required to take the measures.

(*3*) The costs of any measures taken under sub-section (*2*), shall be recoverable from the person or the municipality or Panchayat, as the case may be, concerned in the manner provided by the Code of Criminal Procedure, 1973, for the recovery of fines imposed by a Court, as if such costs were a fine imposed by a Court.

Village Officers, etc., to assist

30. All Municipal, Panchayat or Village Officers and all officers of the rural and dairy development, revenue, agriculture, animal husbandry and veterinary departments of the State Government, shall be bound—

(*a*) to give immediate information to the Veterinary Officer and to the Veterinarian having jurisdiction in the area regarding the prevalence of a scheduled disease amongst any animal or species of animals, in the area;

(*b*) to take all necessary measures to prevent the outbreak or spread of any scheduled disease; and

(*c*) to assist the Veterinary Officer and the Veterinarian in the discharge of their duties or in the exercise of their powers under this Act.

Penalty for issuing vaccination certificate without authority or administering defective vaccine.

31. If any person issues a vaccination certificate,—

(*a*) without authority or competence in that behalf, or

(*b*) after administering the vaccine which is known to be defective in any manner,

he shall be guilty of an offence punishable with a fine of five thousand rupees or in case of non-payment of fine with imprisonment which may extend to one month, and in the case of any subsequent offence, with fine of ten thousand rupees or with imprisonment which may extend to three months.

Penalties

32. Any person who contravenes the provisions of this Act or obstructs the Competent Officer in performing his duties shall be guilty of an offence punishable with fine which may extend to one thousand rupees, and in case of failure to pay the penalty with imprisonment for a term which may extend to one month; and in the case of any subsequent offence (whether under the same provision or any other provision of this Act except in case of sections 31 and 33) with a fine of two thousand rupees, or with imprisonment for a term which may extend to two months in case of non-payment of the penalty.

Penalty for placing infected animal or carcass in river, etc.

33. Whoever places or causes or permits to be placed in any river, lake, canal or any other water body, the carcass or any part of the carcass of any animal which at the time of its death was known to be infected, shall be guilty of an offence and, on conviction, be punished, in the case of a first offence with fine of two thousand rupees or with imprisonment of one month in case of non-payment of fine and in the case of subsequent conviction with a fine of five thousand rupees or imprisonment for a term which may extend to three months or with both.

Offences by companies

34. (*1*) Where an offence under this Act has been committed by a company, every person who at the time the offence was committed was in charge of, and was responsible to, the company for the conduct of the business of the company, as well as the company, shall be deemed to be guilty of the offence and shall be liable to be proceeded and punished accordingly:

Provided that nothing contained in this sub-section shall render such person liable to any punishment provided in this Act, if he proves that the offence was committed without his knowledge or that he had exercised all due diligence to prevent the commission of such offence.

(2) Notwithstanding anything contained in sub-section (1), where an offence under this Act has been committed by a company and it is proved that the offence has been committed with the consent or connivance of, or is attributable to any neglect on the part of, any director, manager, secretary or other officer of the company, such director, manager, secretary or other officer shall also be deemed to be guilty of that offence and shall be liable to be proceeded against and punished accordingly.

Explanation.—For the purposes of this section,—

(*a*) "company" means any body corporate and includes a co-operative society registered or deemed to be registered under any law for the time being in force, a firm or other association of individuals; and

(*b*) "director", in relation to a firm, means a partner in the firm.

CHAPTER VI

PRECAUTIONARY MEASURES ON CAUSATIVE ORGANISM, ETC.

Prevention of escape of causative organism

35. (1) In every institution, laboratory or clinic, engaged in the manufacture, testing or research, related to vaccines, sera, diagnostics or chemotherapeutic drugs and aimed at the prevention or treatment of any scheduled disease, adequate precautionary measures shall be taken—

(*a*) to ensure that the causative organism of any scheduled disease does not escape or otherwise get released;

(*b*) to guard against any such escape or release; and

(*c*) to warn and to protect everyone concerned in the event of any escape.

(2) Notwithstanding anything contained in any other law for the time being in force, every animal—

(*a*) used for the manufacture, testing or research as referred to sub-section (1), or

(*b*) which is likely to carry or transmit any scheduled disease,

shall be promptly administered euthanasia and disposed of by the person in charge of or having control of the institution, laboratory or clinic, as the case may be, referred to in that sub-section.

(3) Every person who is in charge of or having control of an institution, laboratory or clinic referred to in sub-section (1) comply with the provisions of sub-section (1) and sub-section (2); and in the event of non-compliance he shall be guilty of an offence punishable with fine which may extend to twenty thousand rupees or imprisonment for a term which may extend to six months or with both, and in case the establishment is in commercial manufacturing of vaccines or medicine, a temporary suspension of licence up to a period of one year may also be imposed.

CHAPTER VII

MISCELLANEOUS

Power to delegate

36. The State Government may, by notification, delegate to any officer or authority subordinate to it, all or any of the powers conferred on it by or under this Act, except the powers to make rules under sub-section (2) of section 42.

Officers and authorities to function subject to Government control

37. All officers and authorities under this Act shall exercise their powers and discharge their duties conferred or imposed on them by or under this Act, in accordance with such orders, not inconsistent with the provisions of this Act, as the Central Government or the State Government may, from time to time, make.

Power to amend the Schedule

38. (1) The Central Government may, by notification, add to, or omit from the Schedule any animal disease and the said disease shall, as from the date of the notification, be deemed to have been added to, or omitted from, the Schedule.

(2) Every notification issued under sub-section (1) shall, as soon as may be after it is issued, be laid before each House of Parliament.

Power to issue directions.

39. The Central Government may, with the object of prevention, control and eradication of any infectious or contagious disease of animals, issue such directions to the State Government or other authorities under this Act, from time to time, including directions for furnishing such returns and statistics on scheduled diseases, and vaccination, as it may deem fit and every such direction shall be complied with.

Certain persons to be public servants.

40. Every Competent Officer, Director and Veterinary Officer, while exercising any power or performing any duty under this Act, shall be deemed to be a public servant within the meaning of section 21 of the Indian Penal Code.

Power to remove difficulties.

41. (*1*) If any difficulty arises in giving effect to the provisions of this Act, the Central Government may, by order published in the Official Gazette, make such provisions, not inconsistent with the provisions of this Act, as appear to it to be necessary or expedient for removing the difficulty:

Provided that no such order shall be made after the expiry of a period of two years from the date of commencement of this Act.

(*2*) Every order made under this section shall, as soon as may be after it is made, be laid before each House of Parliament.

Power of Central Government to make rules.

42. (*1*) The Central Government may, subject to the condition of previous publication, by notification, make rules for carrying out the provisions of this Act.

(*2*) In particular and without prejudice to the generality of the foregoing power, such rules may provide for all or any of the following matters, namely:—

(*a*) the form of vaccination certificate and the particulars which such certificate shall contain, under section 9;

(*b*) the manner of disposal of carcass, under section 26;

(*c*) the manner of conducting examination and post-mortem under sub-section (*1*) and the form of report of post-mortem under sub-section (*2*) of section 27;

(*d*) any other matter which may be prescribed or in respect of which rules are required to be made by the Central Government.

Power of State Government to make rules.

43. (*1*) The State Government may, by notification and with the prior approval of the Central Government, make rules for carrying out the purposes of this Act.

(*2*) In particular and without prejudice to the generality of the foregoing power, such rules may provide for all or any of the following matters, namely:—

(*a*) the form of permit to be granted by the officer in charge of a Quarantine Camp, under sub-section (*4*) of section 14;

(*b*) the manner of inspection and the period of detention of an animal at a Check Post or at a Quarantine Camp for the administration of compulsory vaccination and marking of animals and the form and manner of issue of entry permit, under sub-section (*2*) of section 15;

(*c*) any other matter in respect of which rule is to be or may be made by the State Government.

Laying of rules.

44. (*1*) Every rule made by the Central Government under this Act shall be laid, as soon as may be after it is made, before each House of Parliament, while it is in session, for a total period of thirty days which may be comprised in one session or in two or more successive sessions, and if, before the expiry of the session immediately following the session or the successive sessions aforesaid, both Houses agree in making any modification in the rule or both Houses agree that the rule should not be made, the rule shall thereafter have effect only in such modified form or be of no effect, as the case may be; so, however, that any such modification or annulment shall be without prejudice to the validity of anything previously done under that rule.

(*2*) Every rule made by the State Government under this Act shall be laid, as soon as may be after it is made, before the State Legislature.

Repeal and savings

45. On the commencement of this Act—

13 of 1899 (*i*) The Glanders and Farcy Act, 1899;

5 of 1910 (*ii*) The Dourine Act, 1910; and

(*iii*) any other corresponding law of any State, so far as it is inconsistent with the provisions of this Act,

shall stand repealed:

Provided that nothing contained in this section shall—

(*a*) affect the previous operation of any such provision of law or anything duly done or suffered thereunder;

(*b*) affect any right, privilege, obligation or liability acquired, accrued or incurred under any such provision of law;

(*c*) affect any penalty, forfeiture or punishment incurred in respect of any offence committed against any such provision of law; or

(*d*) affect any investigation, legal proceeding or remedy in respect of any such right, privilege, obligation, liability, penalty, forfeiture or punishment as aforesaid; and every such investigation, legal proceeding or remedy may be continued, instituted or enforced, and any such penalty, forfeiture and punishment may be imposed, as if the aforesaid provisions of law had continued:

Provided further that, anything done or any action taken under any such provision of law, including any notification, order, notice or receipt issued or declaration made, shall in so far as it is not inconsistent with the provisions of this Act, be deemed to have been done, taken, issued or made under the corresponding provisions of this Act, and shall continue in force accordingly, unless and until superseded by anything done or any action taken under this Act.

THE SCHEDULE

[*See* sections 2 (o) and 38]

(*a*) Multiple species diseases

1. Anthrax.
2. Aujeszky's disease.
3. Bluetongue.
4. Brucellosis.
5. Crimean Congo haemorrhagic fever.
6. Echinococcosis/hydatidosis.
7. Foot and mouth disesase.
8. Heartwater.
9. Japanese encephalitis.
10. Leptospirosis.
11. New world screwworm *(Cochliomyia hominivorax)*.
12. Old world screwworm *(Chrysomya bezziana)*.
13. Paratuberculosis.
14. Q fever.
15. Rabies.
16. Rift Valley fever.
17. Rinderpest.
18. Trichinellosis.
19. Tularemia.
20. Vesicular stomatitis.
21. West Nile fever.

(*b*) Cattle diseases

1. Bovine anaplasmosis.
2. Bovine babesiosis.
3. Bovine genital campylobacteriosis.
4. Bovine spongiform encephalopathy.
5. Bovine tuberculosis.
6. Bovine viral diarrhoea.
7. Contagious bovine pleuropneumonia.
8. Enzootic bovine leucosis.
9. Haemorrhagic septicaemia.
10. Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis.
11. Lumpy skin disease.
12. Malignant catarrhal fever.
13. Theileriosis.
14. Trichomonosis.
15. Trypanosomosis.

(*c*) Sheep and goat diseases

1. Caprine arthritis/encephalitis.
2. Contagious agalactia.
3. Contagious caprine pleuropneumonia.
4. Enzootic abortion of ewes (ovine chlamydiosis).
5. Maedi-visna.
6. Nairobi sheep disease.
7. Ovine epididymitis (*Brucella ovis*).
8. Peste des petits ruminants.
9. Salmonellosis (S. abortusovis).
10. Scrapie.
11. Sheep pox and goat pox.

(*d*) Equine diseases

1. African horse sickness.
2. Contagious equine metritis.
3. Dourine.
4. Equine encephalomyelitis (Eastern).
5. Equine encephalomyelitis (Western).
6. Equine infectious anaemia.
7. Equine Influenza.
8. Equine piroplasmosis.
9. Equine rhinopneumonitis.
10. Equine viral arteritis.
11. Glanders.
12. Surra (*Trypanosoma evansi*).
13. Venezuelan equine encephalomyelitis.

(*e*) Swine diseases

1. African swine fever.
2. Classical swine fever.
3. Nipah virus encephalitis.
4. Porcine cysticercosis.
5. Porcine reproductive and respiratory syndrome.
6. Swine vesicular disease.
7. Transmissible gastroenteritis.

(*f*) Avian diseases

1. Avian chlamydiosis.
2. Avian infectious bronchitis.
3. Avian infectious laryngotracheitis.
4. Avian mycoplasmosis (*M. gallisepticum*).
5. Avian mycoplasmosis (*M. synoviae*).
6. Duck virus hepatitis.
7. Fowl cholera.

8. Fowl typhoid.
9. Highly pathogenic avian influenza and low pathogenic avian influenza in poultry.
10. Infectious bursal disease (Gumboro disease).
11. Marek's disease.
12. Newcastle disease.
13. Pullorum disease.
14. Turkey rhinotracheitis.

(*g*) Lagomorph diseases

1. Myxomatosis.
2. Rabbit haemorrhagic disease.

(*h*) Bee diseases

1. Acarapisosis of honey bees.
2. American foulbrood of honey bees.
3. European foulbrood of honey bees.
4. Small hive bettle infestation (*Aethina tumida*).
5. *Tropilaelaps* infestation of honey bees.
6. Varroosis of honey bees.

(*i*) Fish diseases

1. Epizootic haematopoietic necrosis.
2. Infectious haematopoietic necrosis.
3. Spring viraemia of carp.
4. Viral haemorrhagic septicaemia.
5. Infectious pancreatic necrosis.
6. Infectious salmon anaemia.
7. Epizootic ulcerative syndrome.
8. Bacterial kidney disease (*Renibacterium salmoninarum*).
9. Gyrodactylosis (*Gyrodactylus salaris*).
10. Red sea bream iridoviral disease.

(*j*) Mollusc diseases

1. Infection with *Bonamia ostreae*.
2. Infection with *Bonamia exitiosa*.
3. Infection with *Marteilia refringens*.
4. Infection with *Mikrocytos mackini*.
5. Infection with *Perkinsus marinus*.
6. Infection with *Perkinsus olseni*.
7. Infection with *Xenohaliotis californiensis*.

(*k*) Crustacean diseases

1. Taura syndrome.
2. White spot disease.
3. Yellowhead disease.
4. Tetrahedral baculovirosis (*Baculovirus penaei*).

5. Spherical baculovirosis (*Penaeus monodon*-type baculovirus).

6. Infectious hypodermal and haematopoietic necrosis.

7. Crayfish plague (*Aphanomyces astaci*).

(*l*) Other diseases

1. Camelpox.

2. Leishmaniosis.

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ANNEXURE - 15

Transboundary and Emerging Diseases

RAPID COMMUNICATION

Evidence of Presence of *Mycobacterium tuberculosis* in Bovine Tissue Samples by Multiplex PCR: Possible Relevance to Reverse Zoonosis

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Keywords:
bovine tuberculosis; *Mycobacterium bovis*; *Mycobacterium tuberculosis*; *mce-3* operon; multiplex PCR; reverse zoonosis

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Summary

Bovine tuberculosis, caused by *Mycobacterium bovis*, remains one of the most important zoonotic health concerns worldwide. The transmission of *Mycobacterium tuberculosis* from humans to animals also occurs especially in countries where there is close interaction of humans with the animals. In the present study, thirty bovine lung tissue autopsy samples from an organized dairy farm located in North India were screened for the presence of Mycobacterium tuberculosis complex by smear microscopy, histopathological findings and PCR. Differential diagnosis of *M. tuberculosis* and *M. bovis* was made based on the deletion of *mce-3* operon in *M. bovis*. The present study found eight of these samples positive for *M. tuberculosis* by multiplex PCR. Sequencing was performed on two PCR-positive representative samples and on annotation, and BLAST analysis confirmed the presence of gene fragment specific to *Mycobacterium tuberculosis*. The presence of *M. tuberculosis* in all the positive samples raises the possibility of human-to-cattle transmission and possible adaptation of this organism in bovine tissues. This study accentuates the importance of screening and differential diagnosis of Mycobacterium tuberculosis complex in humans and livestock for adopting effective TB control and eradication programmes.

Introduction

Bovine tuberculosis (BTB) is a chronic bacterial infectious disease of cattle caused by *Mycobacterium bovis* (*M. bovis*), which occasionally gets transmitted to humans and constitutes a major public health concern worldwide. Office International des Epizooties (OIE) classifies BTB as a List B disease, a disease considered to be of socioeconomic and of public health importance within countries and constitutes significant embargo to the international trade of animals and animal products (Cosivi et al., 1998; OIE, 2012). In developed countries, BTB control and eradication programmes, together with culling of affected animals and milk pasteurization, have significantly reduced the incidence of the disease. But in developing countries like India, socioeconomic conditions where human and livestock reside in close interaction and lack of stringency on implementation of preventive and control measures have resulted in the disease becoming endemic. Besides *M. bovis*, transmission of *M. tuberculosis* from infected humans to animals and *vice versa* has been reported and is popularly known as reverse zoonosis (Fritsche et al., 2004). Among domestic animals, infection with *M. tuberculosis* has been most frequently identified in cattle. The disease caused by *M. bovis* is indistinguishable to that caused by *M. tuberculosis*. The bacteriological, biochemical and genetic

similarities of these two species have made it difficult to differentially identify them in clinical samples/cultivated isolates (Niemann et al., 2000).

The diagnosis of BTB mainly depends on the examination of post-mortem lesions in suspected clinical specimens from the dead animals followed by smear microscopy (Isenberg, 1998). Although culture of the organism remains the gold standard test for the diagnosis of tuberculosis, it is tedious and time-consuming (OIE, 2012). Therefore, *in vitro* nucleic acid amplification tests offer a rapid and sensitive alternative to traditional methods for the detection of BTB infection (Vincent et al., 2009). The high degree of variability among the conventional tests warrants the development of molecular biological tools for the identification of *M. tuberculosis* and *M. bovis*. In this regard, multiple gene targets have been used to date to detect and differentiate genetically identical mycobacterial species. The gene targets include *pncA* (Barouni et al., 2004), *gyrB* (Chimara et al., 2004), *oxyR* (Sreevatsan et al., 1996) and *katG* (Haas et al., 1997). Brosch et al. (2002) have extensively studied the region of differentiation to differentiate between the two mycobacterium species. Huard et al. (2003), Richter et al. (2004) and Bakshi et al. (2005) have targeted multiple loci and deletions within the genome to differentiate *M. tuberculosis* from *M. bovis*.

Sequence analysis of the *M. tuberculosis* and *M. bovis* genomes has shown that *M. bovis* lacks a 12.7-kb fragment present in the genome of *M. tuberculosis* (Zumarraga et al., 1999). Further analysis of the 12.7-kb fragment suggested that it represents a deletion in *M. bovis* rather than an insertion in *M. tuberculosis*. This deletion removes most of the *mce-3* operon. All the *M. tuberculosis* isolates show the presence of the 12.7-kb fragment, while all the *M. bovis* strains lacked this fragment (Zumarraga et al., 1999). Therefore, the 12.7-kb fragment may be a useful marker to differentiate *M. bovis* from *M. tuberculosis*. Bakshi et al. (2005) and Kumar et al. (2009) have used this particular deletion in the *mce-3* operon to develop multiplex PCR and molecular beacons to differentiate between *M. tuberculosis* and *M. bovis*, respectively.

The present study has been designed to rapidly differentiate between *M. tuberculosis* and *M. bovis* using multiplex PCR in clinical specimens. The ability to monitor the transmission of the *M. tuberculosis* and *M. bovis* in cattle could provide epidemiological information about human TB infections guiding clinicians and researchers to intervene and adopt proper control measures.

Materials and Methods

Clinical specimens

The present study was undertaken in an organized dairy farm located in Uttar Pradesh, a state in North India. Three breeds of animals were maintained in the farm, namely Frieswal (Frieswal breed has around 57.5–67.5% Holstein Friesian and 32.5–42.5% Sahiwal inheritance), crossbred (cows with less or more than the range of 57.5–67.5% of Friesian inheritance are considered to be crossbred) and pure Sahiwal (*Bos indicus*). Some of the animals in the dairy farm showed symptoms of progressive emaciation, dyspnoea, cough and acute respiratory distress in the terminal stages of the disease. Bovine lung tissue samples were collected from thirty animals which died of suspected tuberculosis infection. Post-mortem lesions in many of the lung specimens showed tuberculous granuloma with a yellowish appearance. Some of the granulomas were mucopurulent, and some had calciferous growth suggestive of bovine tuberculosis. The tissue samples were transported to the laboratory on ice for further characterization and processing as per the OIE guidelines.

Acid-Fast Bacilli (AFB) examination

Acid-fast bacilli examination was carried out on the direct smear films. Processing of the samples was performed as per standard procedures (Kent and Kubica, 1985; Isenberg, 1998; Vincent et al., 2009). Direct smear films were prepared either from tissue exudates or indirectly after homogenization. Aliquot from each sample was digested and decontaminated with N-acetyl-L-cysteine and 2% NaOH method and concentrated by centrifugation at 3000 g for 20 min. Collected specimens were examined for the presence of AFB using Ziehl–Neelsen staining technique.

Histopathological examination

Processing of the lung samples and staining of tissue sections for histopathological examination with haematoxylin and eosin staining was performed as per standard procedure (Fischer et al., 2008).

Nucleic acid isolation from tissue samples

DNA was extracted from lung tissue samples by using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was estimated for quality and quantity using NanoDrop Spectrophotometer (ND-1000, Thermo Fisher Scientific, Wilmington, USA) and stored at −20°C until further use.

Multiplex PCR for differentiation of *Mycobacterium tuberculosis* and *Mycobacterium bovis*

Primer design

Primers specific to *M. tuberculosis* and *M. bovis* were designed based on the deletion of the 12.7-kb fragment of

the *mce-3* operon in the *M. bovis* genome. The primers were designed according to Kumar et al. (2009) with some slight modifications (molecular beacons were not used in our study in contrast to Kumar et al. (2009)). The schematic representation of the primers used in the present study is depicted in Fig. 1. The common forward primer sequence for both the species was FP-5′-ATGACGCCTT CCTAACCAGAA-3′ and the reverse primer of *M. tuberculosis* was RPT-5′-GCCTGACATCTTATTCCGATGCAT-3′ and for *M. bovis* was RPC-5′-TTGACCAGCTAAGATATC CGGT-3′. The primers were custom-synthesized from Bio-Serve Biotechnologies (India) Pvt Ltd, Hyderabad, India.

Standard mycobacterium strains

Standard mycobacterium strains for both *M. tuberculosis* and *M. bovis*, which were used as a positive control in this study, were generously provided by Professor H.K. Prasad of Department of Biotechnology, All India Institute of Medical Sciences, New Delhi, India. The standard *M. tuberculosis* strain was H37Rv, which was maintained at National JALMA Institute for Leprosy and other Mycobacterial Diseases, ICMR, Agra, India. The standard *M. bovis* strain was BCG vaccine strain Danish 1.331, which was maintained at Department of Biochemistry, South Campus, University of Delhi, South Campus, Delhi.

PCR amplification

PCR amplification was performed in thin-walled 0.2-ml PCR tubes containing approximately 100 ng of bacterial genomic DNA, 5 μl of 10X PCR buffer, 2 mM of $MgSO_4$, 1 μl of 10 mM dNTPs, 10 μM of common forward (FP) and two sets of reverse primers (RPT and RPC), 2.5 U of Pfu DNA Polymerase (Fermentas, Lithuania), and the reaction volume was made up to 50 μl with nuclease-free water (NFW). The PCR amplification was performed in a thermocycler (Master Cycler®, Eppendorf, Hamburg, Germany) and consisted of initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 70°C for 1 min, followed by a final extension at 70°C for 10 min. A non-template control was run in all the PCR experiments to rule out the possibility of contamination in the samples. The PCR-amplified products were resolved on 2% agarose gel in Tris–acetate–EDTA (TAE) buffer (1X). The agarose gel was stained with ethidium bromide and visualized under UV light in a gel documentation system (Alpha Imager® EP, Alpha Innotech, San Leandro, CA, USA).

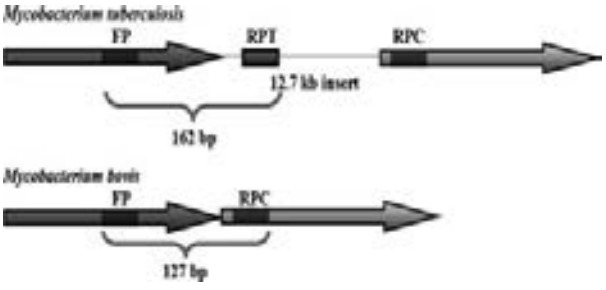

Fig. 1. Schematic diagram showing the position of the primers in the *mce-3* operon used for the differentiation and identification of *M. tuberculosis* and *M. bovis*. A 12.7-kb insert in the *M. tuberculosis* genome prevents Taq polymerase-mediated amplification with FP and RPC primers, but amplification occurs in *M. bovis* with the same set of primers. An amplified product of 162 bp with FP and RPT primers is observed in *M. tuberculosis*.

Sequencing of the PCR products and Bioinformatics analysis

The PCR products were excised from the gel and extracted using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The purified products were assessed for quality and quantity. The sequencing was carried out using the Big dye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc, CA, USA) following the manufacturer's instructions on an automated DNA sequencer (Applied Biosystems 3130 Genetic Analyzer, Applied Biosystems, CA, USA) at Bio-Serve Biotechnologies (India) Pvt Ltd, Hyderabad, India.

The sequence chromatogram was visualized in BioEdit Sequence Alignment Editor software version 7.0.5 (Isis Therapeutics, Carlsbad, CA, USA). Mega Blast was performed with the deduced sequence within the non-redundant nucleotide database (http://www.ncbi.nlm.nih.gov/Blast) to confirm the presence of either *M. tuberculosis* or *M. bovis* strains. Phylogenetic comparison was made using MEGA5.10 software (Tamura et al., 2011). The evolutionary history was inferred using neighbour-joining (N-J) method (Saitou and Nei, 1987). The evolutionary distances were computed using Kimura-2 parameter method (Kimura, 1980) and expressed in the units of the number of nucleotide substitutions per site. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the strains analysed.

Results and Discussion

The post-mortem examination of the thirty clinically BTB-suspected animals revealed the presence of the mucopurulent and calcified granulomas in the lung tissues of ten animals only (Fig. 2a). Direct smear examination of the lung tissue of these ten animals showed the presence of AFB of mycobacterium morphology with Ziehl–Neelsen staining technique in a total of six animals only (Fig. 2b). Histopathological examination of eight lung tissue samples revealed tuberculous granulomatous lesions in eight

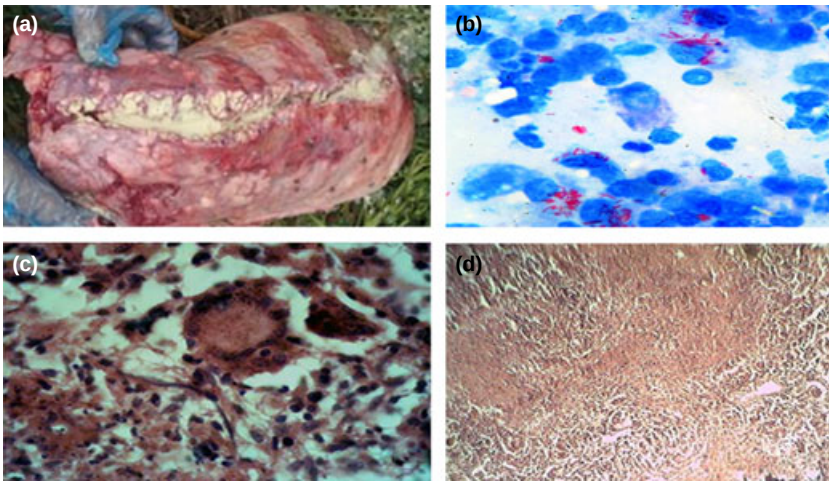

Fig. 2. (a) Gross pathological lesions in the lungs from the post-mortem samples showing mucopurulent and calciferous granulomas. (b) Acid-fast bacilli seen on Ziehl–Neelsen staining of tissue sections (at 1000× magnification). (c) Langhans type multinucleated giant cell by haematoxylin–eosin staining (at 1000× magnification). (d) Multiple granulomatous lesions with diffuse caseous necrosis along with fibrocellular reaction and infiltration of epithelioid and mononuclear cells throughout parenchyma by haematoxylin–eosin staining (at 1000× magnification).

animals. Microscopically, multiple granulomatous lesions comprising central area of necrosis surrounded by epithelioid and mononuclear cells in pulmonary parenchyma, fibrocellular reaction and presence of Langhans type multinucleated giant cells were evident (Fig. 2c and d).

The same eight tissue samples were also positive in PCR amplification. All the eight samples showed an amplified product of 162 bp, suggestive of the presence of *M. tuberculosis* in the clinical specimens. The positive controls, *M. tuberculosis* (H37Rv) and *M. bovis* (BCG vaccine strain Danish 1.331) showed an amplified product of 162 bp and 127 bp, respectively, and the non-template control wells showed no amplification (Fig. 3). To confirm the presence of *M. tuberculosis*, two representative samples 77-Silco and 770-Bamini were excised from the gel and sequenced in an Automated DNA sequencer. Sequence chromatogram was visualized with BioEdit Sequence Alignment Editor software version 7.0.5 and on BLAST analysis matched with *M. tuberculosis* strains in the NCBI database. A sequence homology of 98% at the nucleotide level of the present isolates was observed with other *M. tuberculosis* strains in the NCBI database. Phylogenetic analysis of the isolates revealed that the isolates clustered with other *M. tuberculosis* strains forming a separate clade. *M. bovis* strains formed a separate clade (Fig. 4). Sequence analysis confirmed that all the present isolates belong to *M. tuberculosis*.

Interestingly, of the eight samples positive in histopathological examination and PCR, six were of Frieswal breed and two were of crossbreed animals. None of the animal from Sahiwal breed was found positive in PCR and histopathological studies among the suspected animals for

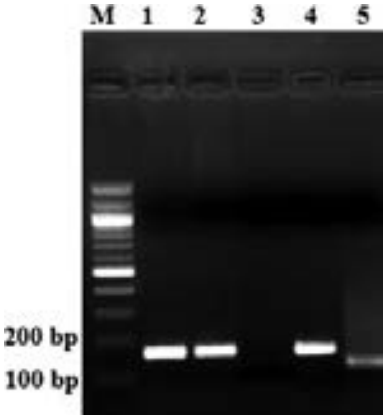

Fig. 3. Agarose gel (2%) showing PCR-amplified products from clinical and positive samples. Lane M: 100-bp DNA ladder (MBI Fermentas), Lane 1: 77-Silco clinical isolate, Lane 2: 770-Bamini clinical isolate, Lane 3: negative control, Lane 4: positive control for *M. tuberculosis* (H37Rv); Lane 5: positive control for *M. bovis* (BCG vaccine strain Danish 1.331).

tuberculosis infection. Similar results were obtained by Vordermeier et al. (2012) in Ethiopia where exotic Holstein Friesian cattle were more susceptible to TB infection than local zebu cattle.

Bovine tuberculosis remains a major infectious disease affecting the livestock sector especially in developing countries like India. The zoonotic potential of the Mycobacterium tuberculosis complex from cattle remains a constant threat to the majority of the Indian population who lives in close association with these animals. India alone accounts

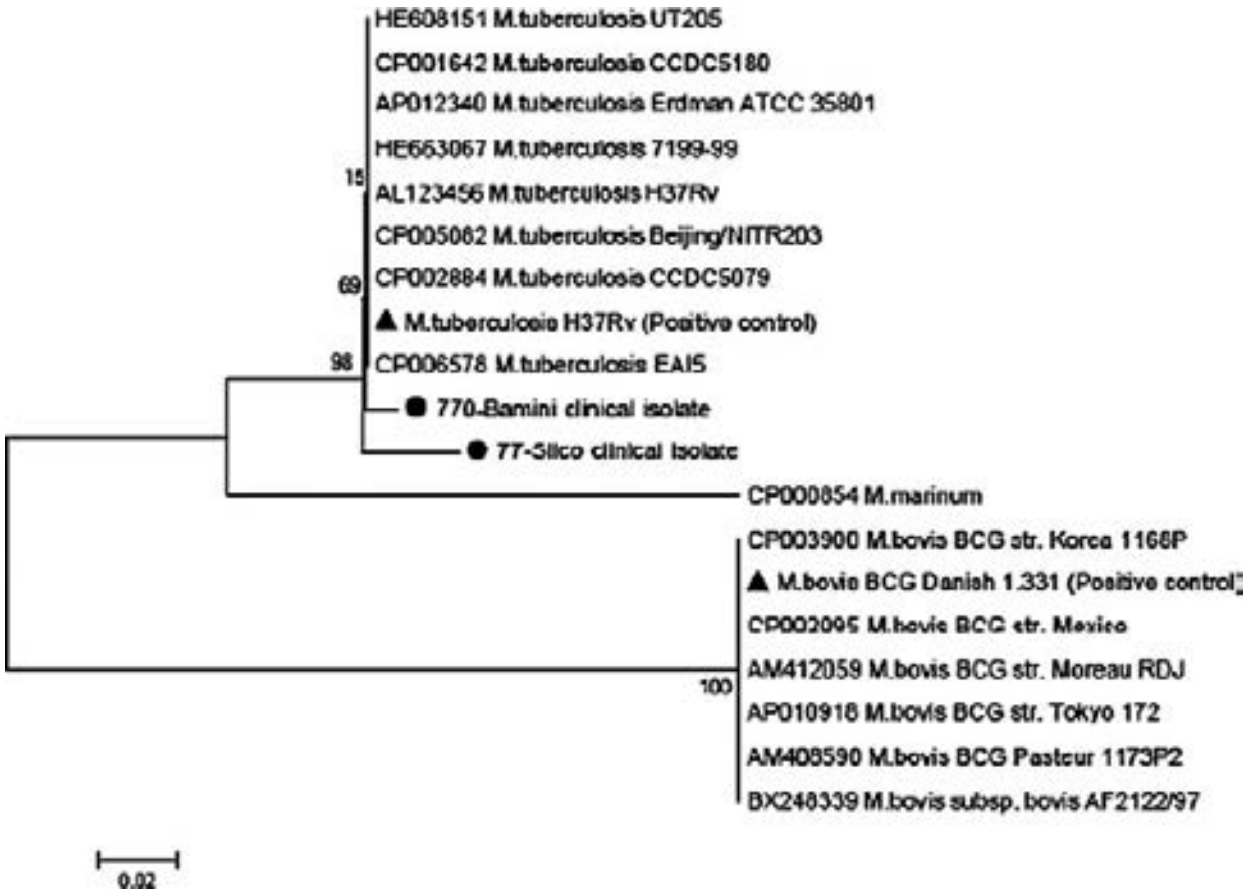

Fig. 4. Phylogenetic relationship of *M. tuberculosis* isolates (77-Silco and 770-Bamini). Isolates sequenced in this study are shown with solid circle (●), and the positive controls (*M. tuberculosis* – H37Rv and *M. bovis* – BCG vaccine strain Danish 1.331) are shown with solid triangle (▲). Bootstrap values (out of 1000 replicates) of relevant nodes are shown near the nodes. Scale indicates nucleotide substitution per site.

for more than 20% of the world human TB cases according to the WHO reports (http://www.who.int/tb/-publications/global_report/en/index.html).

Despite extensive knowledge and research on tuberculosis over the years, diagnosis and identification of the causal organism of tuberculosis still remains a challenge. Species identification of mycobacterial strains is indispensable for knowing the epidemiology of the animal–human transmission and adopting surveillance and control measures. The conventional tools based on smear microscopy and growth of the organism in specific media are time-consuming and suffer from reliability and accuracy. Hence, molecular tools such as nucleic acid amplification techniques over the years have gained popularity due to its sensitivity and specificity of the results in differential diagnosis of tuberculosis (Schiller et al., 2010).

The present study has focused on the diagnosis of *M. bovis* and *M. tuberculosis* from lung tissue specimens from the dead animals. The most interesting and peculiar finding from the present study is that all the samples were positive for *M. tuberculosis*. Sequencing followed by phylogenetic tree construction was employed in two of the randomized selected samples to confirm the presence of *M. tuberculosis*. The two samples clustered with other *M. tuberculosis* strains in the phylogenetic tree.

In domestic livestock, infection with human tuberculosis has been documented ever since the last century (Kraus, 1942). There has been a tremendous increase in the cattle and buffalo population in India owing to the huge demand of milk and milk products; the chances of these animals getting infected with human tuberculin bacilli are on the rise. There has been an increase in the number of *M. tuberculosis* cases reported from cattle from both India and other countries. The reports are especially from settings in South-East Asian and African countries where human and cattle population lives in close interactions. Prasad et al. (2005) developed a specific nested PCR based on the *hupB* gene to differentiate *M. tuberculosis* and *M. bovis*. They found mixed infection of both the organisms in both human (8.7%) and cattle (35.7%). Cadmus et al. (2006)

investigated the presence of human and bovine tubercle from both human and cattle in Nigeria by spoligotyping and VNTR analysis. Srivastava et al. (2008) reported 14 isolates of *M. tuberculosis* and 40 isolates of *M. bovis* by different biochemical tests from a total of 768 specimens in India. A similar study carried out in Ethiopia by Berg et al. (2009) found eight cases of *M. tuberculosis* infection. Chen et al. (2009) in China carried out multiplex PCR and identified six cases of *M. tuberculosis* infection in bovines. Regassa et al. (2008) in Ethiopia carried out a systemic study involving the farmers having active tuberculosis with the transmission of the disease to the cattle population. They found out that there is a strong positive correlation of the presence of *M. tuberculosis* in bovines originating from humans in their study. Ameni et al. (2011) found that 27% of the grazing cattle in Ethiopia had *M. tuberculosis* infection where mouth-to-mouth contact has been practised where chewed tobacco is discharged into the animal to prevent parasitic infection. They have postulated that this may increase the risk of the cattle getting infected with *M. tuberculosis* from farmers having TB infection. Thakur et al. (2012) employed PCR-RLFP of *hsp65* gene to differentiate *M. tuberculosis* and *M. bovis*. Two animals had *M. tuberculosis* and one had mixed infection out of the four positive cases. Some European countries like Slovenia (Ocepek et al., 2005) and Spain (Romero et al., 2011) reported isolated cases of *M. tuberculosis* tracing humans as the source of the infection by molecular typing of both human and bovine isolates.

Most of the previously reported incidences have been traced to South-East Asian and sub-Saharan African countries where animals and humans share close interactions. These regions are the pockets in the world where incidences of high human TB cases have been reported. The results from this study also corroborate with previous findings that incidences of *M. tuberculosis* in bovine TB have traced humans as a source of the infection. In contrast to all previous findings where both *M. tuberculosis* and *M. bovis* have been reported in each of the BTB study, the most peculiar and interesting results in the present study show that all the BTB cases were caused by *M. tuberculosis* only and not by *M. bovis*, the most natural mycobacterial species causing BTB. The literature from the previous studies (Lesslie, 1960; Steele, 1980; Erler et al., 2004) shows that *M. tuberculosis* infection in cattle produces a quickly vanishing infection rather than a progressive disease. The infected cattle show short-term sensitization to tuberculin (Erler et al., 2004). The pathological changes in cattle do not appear to support disease transmission, because *M. tuberculosis* infection usually does not progress beyond the development of small granulomas in several different lymph nodes (Cousins et al., 2004). In contrast to all the previous studies, pathological lesions showing mucopurulent and calciferous granulomas were observed in most of the lung tissues on post-mortem examination. To the best of our knowledge, we report for the first time the presence of *M. tuberculosis* in all the clinical specimens analysed for BTB, which was confirmed by multiplex PCR based on the deletion of the *mce-3* operon in *M. bovis* and further sequence analysis. However, mere presence of *M. tuberculosis* in bovine tissue will not confirm human as the source of infection, unless mycobacterial strains from the animal attendants suffering from suspected tuberculosis infection will be typed. The typing based on molecular methods will confirm the epidemiological transmission from humans to cattle.

In conclusion, the presence of *M. tuberculosis* in cattle raises the pertinent question of possible adaptation of *M. tuberculosis* strains in cattle and subsequent cattle–cattle or cattle–human transmission. Further studies on the host–pathogen interactions and virulence mechanism for the survival of *M. tuberculosis* in cattle tissues need to be studied in greater details.

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ANNEXURE - 16

Mycobacterium tuberculosis in Wild Asian Elephants, Southern India

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Mycobacterium tuberculosis in Wild Asian Elephants, Southern India

Arun Zachariah, Jeganathan Pandiyan, G.K. Madhavilatha, Sathish Mundayoor, Bathrachalam Chandramohan, P.K. Sajesh, Sam Santhosh, Susan K. Mikota

We tested wild Asian elephants (*Elephas maximus*) in southern India and confirmed infection in 3 animals with *Mycobacterium tuberculosis,* an obligate human pathogen, by PCR and genetic sequencing. Our results indicate that tuberculosis may be spilling over from humans (reverse zoonosis) and emerging in wild elephants.

Infection with *Mycobacterium tuberculosis* in domestic and wild animals of various species living in close contact with humans has been reported (*1*). Elephants in captivity are known to be susceptible to infection with *M. tuberculosis*, and there is a potential for transmission of *M. tuberculosis* between humans and elephants (*2–4*). In 2013, a case of tuberculosis (TB) in a wild elephant in Africa, which had been under human care, was reported (*5*), after which another case in a wild Asian elephant in Sri Lanka was reported (*6*). Habitat encroachment and competition for resources brings wild elephants into closer contact with humans, providing opportunities for zoonoses and reverse zoonoses to occur and for a previously unknown pathogen to emerge in captive free-ranging and wild elephant populations.

The Study

In March 2007, an emaciated wild bull elephant, estimated to be 20 years of age, died shortly after it was found recumbent in the Muthanga range of the Wayanad Wildlife Sanctuary in southern India (case 1). Postmortem examination revealed purulent exudates throughout the lungs, an enlarged liver, enlarged mesenteric lymph nodes, and surface nodules containing caseated yellowish-white material (Figure 1). We found serosanguinous fluid in the pericardial sac and slightly hypertrophied heart ventricles. We saw focal areas of necrosis in the renal cortices but noted no other gross lesions. Ziehl-Neelsen staining of lung, liver, kidney, and mesenteric lymph node impression smears revealed numerous acid-fast bacilli. We confirmed the presence of *M. tuberculosis* by using PCR amplification of the targeted bacterial genome, gel documentation of the amplified products, and sequencing.

Subsequently, a surveillance program was initiated (until March 2014), and all fresh elephant carcasses in the study area were examined for evidence of TB (n = 88). In May 2010, a bull elephant, ≈30 years of age, was found dead in the Kurichiyat range (case 2). Postmortem examination revealed extensive caseated lesions in the lungs (Figure 2) and mild mesenteric lymph node hypertrophy. In May 2013, TB infection was diagnosed in a bull ≈40 years of age that was found in the same forest range and had extensive caseated lung lesions (case 3). Both bulls were emaciated.

We fixed samples for histopathological studies in 10% formol saline and embedded them in paraffin. We found numerous acid-fast organisms in lung impression smears and tissue sections. Granulomatous lesions encapsulated by connective tissue with aggregated macrophages and central areas of necrosis were seen during histopathologic examination of the lungs for all 3 cases and of the kidney and liver in case 1. Langerhans-type giant cells were observed in cases 2 and 3 but not in case 1.

Tissues for molecular studies were collected in absolute alcohol. We extracted total DNA from tissues by using DNeasy Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. DNA was subjected to a tetraplex PCR to differentiate between *M. tuberculosis* complex and nontuberculous mycobacteria. DNA was subjected to amplification and sequencing of the 3 target regions separately, 16S–23S internal transcribed spacer region, hsp65, and rpoB separately (*7*). *M. tuberculosis* H37Rv and *M. bovis* bacilli Calmette-Guérin genomic DNA was used as control DNA for the PCR studies.

We observed the expected 4-band pattern after tetraplex PCR. As the MTP40 fragment was amplified, *M. bovis* was ruled out because the *plcA* gene (mtp40), one of the members of the *plc* family of genes that code for the phospholipase C enzyme, is deleted in the *M. bovis* and *M. bovis* bacilli Calmette-Guérin RD5 region (*8*). Sequences that were generated were assembled and

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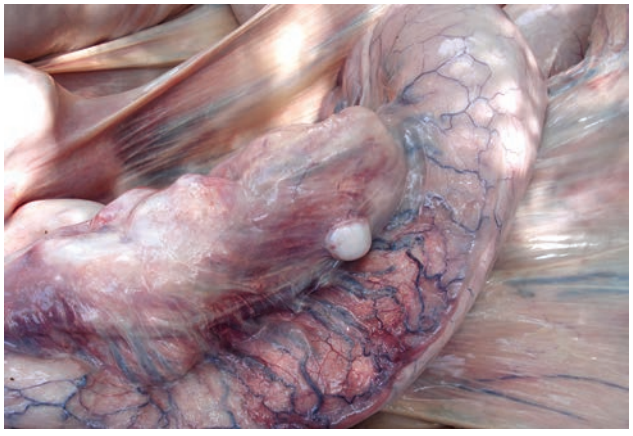

Figure 1. Intestine from a wild bull elephant, estimated at 20 years of age, Wayanad Wildlife Sanctuary, India, 2007. Multiple white-to-tan discrete nodules (granulomas) are protruding from the serosal surface, and less well-defined areas of pale discoloration are visible within the intestinal wall. Serosal blood vessels are markedly dilated, tortuous, and congested

edited by using the alignment software Seqscape (http://www.seqscape.software.informer.com). BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) analysis of the edited sequences revealed that the elephant sequences showed 100% similarity with the *M. tuberculosis* genome fragment. We also used DNA for large sequence polymorphism analysis to determine the lineage of *M. tuberculosis* using RD239 and RD750 primers (*9*,*10*). The genomic deletion analysis revealed a deletion in RD239, which is characteristic of the Indo-Oceanic lineage (*10*), also referred to as the East African–Indian lineage (*11*).

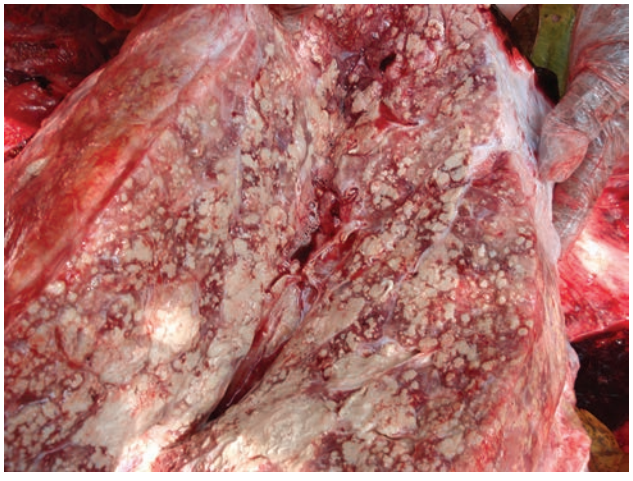

Figure 2. Lung from a bull elephant, estimated at 30 years of age, Kurichiyat Range, India, 2010. Note the multifocal to coalescing pale tan-to-white firm nodules (granulomas) effacing much of the lung parenchyma. Some areas of white chalky mineralization are also present.

Conclusions

There are reports of mycobacterial infections in captive elephants in India from as early as 1925 (*12*). We report *M. tuberculosis* infection in wild elephants in India. In this study, 3 (3.4%) of 88 elephants undergoing postmortem examination were confirmed to be infected with *M. tuberculosis*. All 3 animals were emaciated, and we considered TB to be the cause of death.

The close interaction between humans and captive elephants is presumed to be a key risk factor for the interspecies transmission of TB. The epidemiology of TB among wild elephants, now documented in 3 countries, has yet to be elucidated. In our study, there were no known captive elephant releases or reintroductions into the study area, and the interaction between captive and wild elephants is considered negligible. However, native tribes do live within the park; many tribal members are employed by the forest department for protection and ecotourism activities. Tourists may visit specified areas only under supervision; there are no overnight facilities. Human–elephant conflict is a problem; most conflicts are caused by resident bulls. All 3 TB cases reported here were in bulls. Exposure of bulls to humans infected with TB during conflict activities is a possible explanation.

More than 3,000 native cattle reside within the sanctuary, cared for by the Animal Husbandry Department, Kerala State. No cases of TB among cattle have been reported. Cattle would be more likely to be infected with *M. bovis* than with *M. tuberculosis*, but comprehensive testing would be informative. Cattle living in close proximity to TB-infected humans can become infected with *M. tuberculosis* (*13*). Whether such infected cattle could then transmit *M. tuberculosis* to elephants through contamination of shared grazing lands is yet another research question.

The *M. tuberculosis* complex is thought to have emerged as a human pathogen in Africa rather than arising from an animal source (*14*). Although the epidemiology has not been defined, our study and previous reports indicate that *M. tuberculosis* appears to be spilling over into elephants (reverse zoonosis) and emerging among wild elephant populations. Although these cases may have resulted from individual introductions, if *M. tuberculosis* becomes established, wild elephants and other susceptible species will be at risk.

Ecologic, environmental, or demographic factors that place animals or humans at increased contact can contribute to disease emergence. Certainly, the increased human–elephant conflict in India and other Asian elephant range countries attests to the narrowing interface between humans and elephants. This study suggests that *M. tuberculosis* is emerging in the largest single population of Asian elephants in India. Continued surveillance in India and other Asian elephant range countries is warranted.

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