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Impact of bovine tuberculosis on public health hazards from frozen bovine meat consumption in world

Abstract

Bovine Tuberculosis or bTB is a chronic zoonotic infection at abattoir in India. Despite its existence in India very little information available about Bovine Tuberculosis extent or nature. Generally, frozen buffalo meat is exporting from India to many other countries. The existence of Mycobacteria spp in bovine meat increases the risk of zoonosis transmits from animal to human. To validate the risk assessment of BTB in frozen buffalo meat and to validate the hypothesis, current study was designed to record the prevalence of Mycobacterium bovis, M. tuberculosis and M. cosmeticum from the frozen buffalo meat and chances of transmission of disease. There are few samples of microscopically infected lesion tested through Zeihl-Neelsen staining technique from infected area of buffalo and found AFB (Acid Fast Bacilli) positive for bTB. Approximately 45 samples of frozen buffalo meat sample processed in Lowenstein Jensen media containing glycerol, pyruvate, egg yolk and found 27 samples positive of Mycobacteria. Among them M. bovis positive in 24 samples, M. tuberculosis positive in one sample and M. cosmeticum positive in 5 samples. Apart from that 25 samples water at abattoir also tested for risk assessment of BTB, but observed as negative. These results indicate that probably all buffalo carcasses inspected in slaughter area in New Delhi, India, are suggestive of Bovine Tuberculosis and consistent serious threat for rest of the world for public health threat. To overcome the overall BTB disease inspection of carcasses during post mortem should be mandatory, diagnostic examination carried out in house laboratory, sanitation and disinfection ratio should maintain at abattoir.

Keywords: frozen bovine meat, ziehl-neelsen microscopy, bovine tuberculosis, mycobacterium, abattoir

Volume 2 Issue 4 - 2016

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Received: July 24, 2016 | **Published:** September 22, 2016

Introduction

Bovin Tuberculosis is chronic infectious zoonotic disease infecting buffalo in India. Bovine tuberculosis (BTB) caused by *Mycobacterium bovis*, a bacterial pathogen that is part of the *Mycobacterium tuberculosis* complex that causes clinical tuberculosis (TB) in humans and other mammals. BTB is transmitted primarily via aerosol pathways, but also through alimentary routes including saliva, intrauterine and milk consumption pathways.[1] *M. bovis* is considered to be the most frequent route of infection in cattle, but infection by ingestion of contaminated material may also occur. TB, described by John Bunyan as 'Captain of these men of death', can sometimes be food borne and is therefore of more concern to us here. It is estimated that *M. bovis*, the etiological organism of TB in bovines is also responsible for 5% of all TB infections in humans.[2,3] Cattle and buffalo both belong to family bovidae and are considered natural host of *M. bovis*.[4] Among the infectious diseases, TB is considered to be the second most common disease around the world and killing almost 2million people annually. According to the report in 1999 around 8million new cases of TB are recorded annually, thereby representing a major economic burden on individuals and countries.[5] According to the report of World Health Organization in 2009 the highest incidence rate of TB was reported in Sub- Sahara Africa while Bangladesh, Pakistan, India, China and Indonesia together account for half of the TB burden round the globe. Pakistan has been reported as one of the twenty two countries accounting for the total TB burden worldwide and is categorized in one of the five countries responsible for the tuberculosis worldwide.[6] The government of veterinary journal in England declared that the cost of dealing with the disease escalated in recent year and financial year 2004-2005 spent £90.5million.[7] Bovine tuberculosis is widely distributed throughout the world and mainly affects animals with occasional human involvement.[8] In Africa, the BTB is widespread and is affecting the animal industries and human health, posing serious public health threats.[2,9,10] In Egypt, the trend in the annual risk of *M. bovis* infection of human has decreased in the last decades.[11] A prevalence of 12.2% of the total number of human tuberculosis cases in 1953, falling to 10 % in 1969, and 5.4% in 1980 has been reported.[2] In the United States of America (USA) and Great Britain, greater than $40 and £100million annually, respectively, have been spent for the eradication of bTB in 2008/09. The USA total includes appropriated and emergency funding.[12,13] Although bovine tuberculosis was once found worldwide, control programs have eliminated or nearly eliminated this disease from domesticated animals in many countries. Nations currently classified as tuberculosis-free include Australia, Iceland, Denmark, Sweden, Norway, Finland, Austria, Switzerland, Luxembourg, Latvia, Slovakia, Lithuania, Estonia, the Czech Republic, Canada, Singapore, Jamaica, Barbados and Israel. Eradication programs are in progress in other European countries, Japan, New Zealand, the United States, Mexico, and some countries of Central and South America. Although bovine tuberculosis has been eradicated from the majority of U.S. states, a few infected herds continue to be reported, and a few states may periodically lose their disease-free status.[14] Epidemiology of *Mycobacterium bovis* in wild life of different countries like UK, New Zealand, Africa have

been reported here.[15] Bovine Tuberculosis is a contagious zoonotic disease of domestic animals and it posses public health threat and economic losses due to abattoir condemnation of infected carcasses during meat inspection of slaughtered animals. Bovine tuberculosis is widespread in Africa including Nigeria affecting both cattle and humans.[16]

In bovine, after infection, nonvascular nodular granulomas known as tubercles may develop. Characteristic tuberculosis lesions occur most frequently in the lungs and the retropharyngeal, bronchial and mediastinal lymph nodes. Lesions can also be found in the mesenteric lymph nodes, liver, spleen, on serous membranes, and in other organs. Bovine Tuberculosis infection in cattle is usually diagnosed in the live animal on the basis of delayed hypersensitivity reactions. Infection is often subclinical; when present, clinical signs are not specifically distinctive and can include weakness, anorexia, emaciation, dyspnoea, enlargement of lymph nodes, and cough, particularly with advanced tuberculosis. After death, infection is diagnosed by necropsy and histopathological and bacteriological techniques. Rapid nucleic acid methodologies, such as the polymerase chain reaction (PCR), may also be used although these are demanding techniques and should only be used when appropriately validated. Traditional mycobacterial culture remains the gold standard method for routine confirmation of infection.

Identification of *M. bovis* by culture and biochemical methods is important for definitive diagnosis.[17] However, because of the technical problems and cost, they have not come into widespread use in veterinary diagnostic laboratories.[18] Most of the abattoirs in India do not have diagnostic facility. The objective of this study was to determine the prevalence of BTB among bovine slaughtered carcasses and frozen buffalo meat product at abattoir in India.

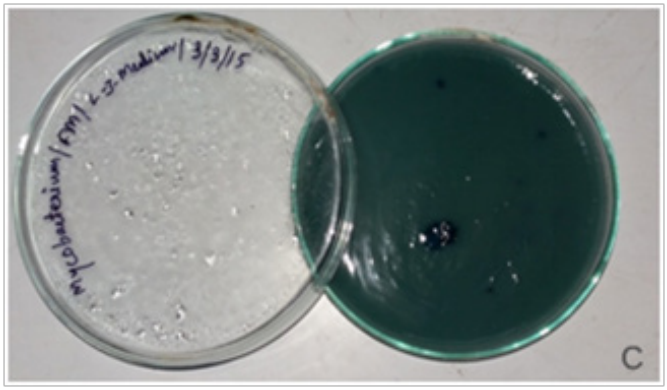
Screening of bovine tuberculosis in Indian continent

Tuberculosis is one of the top three infectious diseases along with HIV/AIDS and malaria. It infects nearly a third of the world's population and still kills well over two million people a year, with India heading the list. Jawaharlal Nehru's wife Kamala died of it in her early thirties. It is the fastest spreading disease in our country. In large part, because we continue to drink milk and eat meat. Human tuberculosis is from a germ called *Mycobacterium tuberculosis*. A large percentage of cattle in India are infected with a strain of tuberculosis called *Mycobacterium bovis*. One interesting discovery, after unveiling the genomes of both bacterial villains, which share 99.95% identical gene sequences, was that *M. bovis* came from *M. tuberculosis*, and not the other way around. Most cows and buffaloes in India are in filthy dung-filled sheds, standing in their own faces for days. In cattle faces *M bovis* will survive 1-8weeks. Animals are probably more likely to be infected by *M. bovis* when they are poorly nourished or under stress. Tuberculosis lesions can affect any part of the body but generally affect the lungs and lymph nodes and the chest cavity. A soft, chronic cough occurs once or twice at a time. In more advanced cases, there is a marked increase in the depth and rate of respiration as well as shortness of breath. In advanced stages, animals may show weakness, weight loss, and fluctuating fever. *M. bovis* is spread in a number of ways by infectious animals - in their breath, milk, discharging lesions, saliva, urine or droppings. The government has made a The Roadmap to Combat Zoonoses in India - a mission to combat diseases spread from humans to animals. Tuberculosis is No 1 on the list - even above rabies. Even worse, a study done by AIIMS called "Bovine tuberculosis in India: potential basis for zoonosis" did TB tests of cattle and humans, 15.7 % humans had *M. tuberculosis*, 26.8% of cattle had *M. bovis*. BUT 8.7% of humans had *M. Bovis* TB and 35.7% cattle had 'mixed infections" as well. The detection of mixed infection with the mycobacterial pathogenic nature duo in humans and bovines denotes the prospect of potential transmission of these pathogens from humans to cattle (zoonosis) and vice versa (reverse zoonosis). Animals infected with *M. tuberculosis* potentially constitute a grave public health hazard as virulent bacilli can be transmitted to humans". As recently as last year, seeing the increase in TB in Mumbaikars, doctors of the preventive social medicine department of Jalgaon's Ulhas Patil Medical College have asked the civic authorities to look at the cattle sheds as a source of human TB.

Morphology

The name *"Mycobacterium"*, meaning fungus like bacterium is derived from mould-like appearance of *Mycobacterium tuberculosis* when grown on liquid media. Mycobacteriaare complex unicellular organisms with a wide range of antigenic determinants. They are basically bacillary or cocco-bacillary and vary from 0.5 to 10µm in length. *Mycobacterium species* are generally non-fastidious, Gram-positive, strictly acid-fast or called acid-fast bacilli (AFB), nonsporeforming, pleomorphic aerobes 1-4mm in length. *Mycobacterium bovis* is mesophilic and is not heat-resistant, being readily killed by normal milk pasteurization conditions. They are also very resistant to drying and therefore can persist and remain infectious in the environment for long periods. The cell wall is more resistant to degradation by lysosomal enzymes in phagocytes enabling the pathogenic mycobacteria to survive and grow in macrophages. Their cell wall rich in chemically diverged lipid (upto 60% of the cell wall). They are gram positive but many species stain poorly with this stain. The thickness of the cellwall is due to long chain fattyacids (mycolic acids) which form a thick palisade. This was first noted by Ehrlich in 1882 and is detected using the Ziehl-Neelsen staining procedure in which cells are stained with hot carbol fuchsin subsequent decolourization with acid alcohol. *Mycobacterium cosmeticum* first isolated by Dr. Robert Cooksey and Dr. Jacobus H. de Waard from cosmetic patient (Figure 1A & 1B).[19] Many Mycobacteria produce carotenoid pigments under appropriate conditions. Some organisms produce pigments in the dark (scotochromogens) and others only on exposure of light (photochromogens). Thus pigment production can be a useful aid of identification. *Mycobacterium tuberculosis* colonies on Lowenstein Jensen Medium Base (L. J. Agar) appear as dry, rough, raised, irregular with wrinkled surface, creamy-white initially becoming yellowish or buff colored on further incubation. They also appear as brown, granule like colonies. They incubate sufficient length of time, usually fourweeks, due to slow doubling time of *M. tuberculosis* than other bacteria. It is an aerobe and can grow only at 37°C. *Mycobacterium bovis* appear on L. J. Agar as flat, smooth, moist, white or non- pigmented and break up easily (Figure 2C & 2D). Growth is enhanced by sodium pyruvate in culture media, giving eugenic colony rather dysgonic (small, smooth colonies) colonies appearance on glycerol egg. It is Microaerophilic and incubate at 37°C for 4weeks. In L. J. Agar *Mycobacterium cosmeticum* colonies appear as moist, smooth surfaces and edges, are domed and are scotochromogenic, visible in 3days incubation in aerobic atmosphere at 28°C or 35°C. L. J. Agar supplemented with glycerol and pyruvate egg yolk.[20,21]

Figure 1A & 1B Indiacate *Mycobacterium cosmeticum* on L. J. Agar.



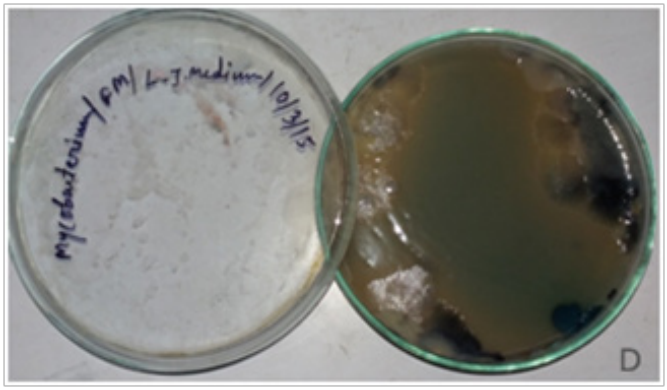


Figure 2C & 2D Indicate white color colonies Mycobacterium bovis on L. J Agar.

Materials and methods

Collection of samples

100gm frozen buffalo meat samples collected and transported in a insulated shipping container enough gel-type should be to maintain refrigerant at 6°C or below. Upon receipt in the laboratory, store the samples at 4°C and analyze as soon as possible. If analysis cannot be started within 4days after collection, freeze samples promptly and store at -20°C until examined. Thaw at room temperature and proceed with analysis as usual. Among the 100gm of sample, 25gm of frozen buffalo meat sample are weighed and add 225mL of 0.1 % peptone water, homogenized the mixture in a blender and used as pre-enrichment solution.[22]

Methods

Pour plate onemL of the pre-enrichment was poured into plate with Lowenstein Jensen Agar (L. J. Agar) supplemented with pyruvate, glycerol and egg yolk. As *Mycobacterium spp.* are microaerophilic in condition it's need anaerobic environment to grow. Hence, L. J. Agar plates are kept wrapped in aluminium foil and incubate or plates may placed into anaerobic jar and incubate. Incubate on can carry out at 37°C or 35°C for 42days or 4weeks or 6weeks or 8weeks.[23]

Identification

The colonies of *M. tuberculosis* appeared as dry, rough, raised, irregular with wrinkled surface, creamy-white initially becoming yellowish or buff coloured on further incubation (Figure 3E). The colonies of *M. bovis* appear as flat, smooth, moist, white or non-pigmented and break up easily. The colonies of *M. cosmeticum* appear as moist, non-pigmented or whitish, smooth surfaces and edges, are domed and are scotochromogenic, it appears on L. J. Agar within 3days of incubation in aerobic environment.

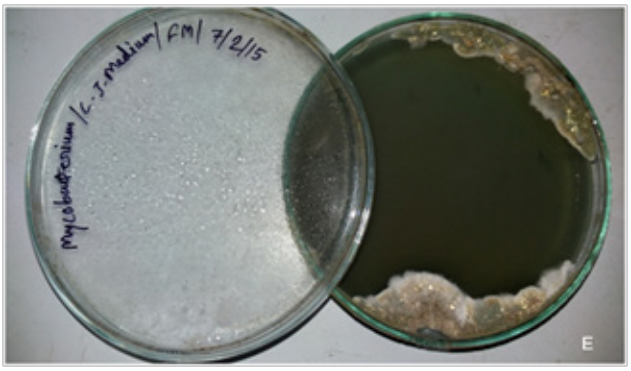


Figure 3E Indicate yellow color colonies Mycobacterium tuberculosis on L. J Agar.

Biochemical examinations

Mycobacteria are gram-positive but few species does not take the stain. They are strictly acid-fast organism. *M. tuberculosis* glycerol enhanced positive, pyruvate enhanced positive, niacin production positive, pyrazinamide sensitivity is sensitive and aerobic. *M. bovis* glycerol enhanced negative, pyruvate enhanced positive, niacin production negative, pyrazinamide sensitivity is Resistant and microaerophilic. *M. cosmeticum* in niacin test negative, 3-days arylsulfatase activity negative, 14-days arylsulfatase activity positive, nitrate reduction positive, iron uptake positive.

Acid-fast staining of mycobacteria

The acid-fast stain is differential stain. It was developed by Paul Ehrlich in 1882 which was later on modified by Ziehl-Neelsen. Bacteria are classified as acid-fast if they retain primary stain (Carbol fuchsin) after washing with strong acid and appear red or non-acid-fast if they lose their color on washing with acid or counter stained by the methylene blue. The property of acid-fastness appears to be due to the presence of high contents of a lipid called mycolic acid in the cellwall, that makes penetration by stain extremely difficult. In this staining procedure Mycobacterial smear prepare and heat fix.

Citation: Sen U, Garode AM. Impact of bovine tuberculosis on public health hazards from frozen bovine meat consumption in world. *J Bacteriol Mycol Open Access.* 2016;2(4):102–107. DOI: 10.15406/jbmoa.2016.02.00033

Flood with carbol fuchsin, heat the slide to steam for 3-5minutes. Add more stain to become it dry. Cool the slide and wash with distilled water. Decolorize the smear with acid-alcohol for 10-30seconds or until the smear is faint pink color. Washed with distilled water and counterstained the smear with methylene blue for 1-2minutes. Blot dry with blotting paper. Mycobacteria cells appeared as red coloured with slender, straight or curved rods and show a typical acid-fast reaction.[24]

Niacin test

In this research article niacin test is a major test to distinguished between three organisms like *M. tuberculosis, M. bovis*&*M. cosmeticum*as identified here. Niacin test was carried out for Mycobacteria by two methods, i.e modified Runyon Method and Paper Strip Test (Difco laboratories). The paper strip method as found easy to perform, safe and showed good correlation (97%) with the routine test.

Modified runyon method: 0.25ml of autoclaved extract was taken in a test tube and an equal amount of 4% aniline in cthanol and 10% aqueous cyanogens bromide were added to it. Positive results were indicated by the appearance of yellow colour and negative test by no colour.[25,26]

Paper strip method (Difco laboratories): The Bacto-TB Niacin Test strips are prepared with potassium thiocyanate, chloramine T, citric acid and sodium aminosalicylate. The test control is prepared with nicotinamide. The "disk", when used according to the directions, yields a yellow solution, equivalent to approximately 5 meg niacin. A 0.6mL of the autoclaved extract from the test strain is put in a special test tube with a stopper: the negative control is 0.6mL, of distilled water and the positive control is the provided test control. The test strip is dropped in each tube with arrow downwards and stoppered immediately. The tubes are shaken gently but not tilted. After 12-15minutes, but not later than 30minutes, the colour of the extract is compared with the controls. A positive test is indicated by the appearance of yellow colour in the extract tube and positive control and no colour in the negative control.[26,27]

Tuberculin test: At abattoir infected carcasses found with tuberculin lesions undergo tuberculin tests. It is basically a biological antigenic test which used at abattoir sudden detection of tuberculin carcasses and reject them if found positive. The TST represents the OIE prescribed test for interna- tional trade and constitutes a delayed type hypersensitivity test.[28] It measures dermal swelling primarily because of a cell-mediated immune response (CMI) threedays after intradermal injection of purified protein derivative (PPD) in the skin of the caudal fold (CFT) or neck (CIT), respectively. The skin of the neck is regarded to be more sensitive to atuberculin-related hypersensitivity reaction than the skin of the caudal fold. Historically, the TST has been the primary antemortem test available to support bTB eradication campaigns. Advantages of the TST and reasons for its wide use are low costs, high availability, and long history of use and, for a long time, the lack of alternative methods to detect BTB. Importantly a recent comparison of commercially available tuberculins has shown that if applied in a dose of 1mg/ml, the majority of these tuberculins would not meet the required minimum dose of 2000 International Units.[29] In this study, potencies largely varied among the bovine tuberculins and, to a lesser degree, among the avian tuberculins. Marked variations were also found with regard to the relative potencies of tuberculin pairs (PPD-B and PPD-A) from the different manufacturers. As these tuberculins are used in current control and eradication programs, the marked variability of potencies may have direct implications for the diagnosis of bTB cases. Abattoir surveillance with lesion detection during commercial slaughter is used as cost-efficient method for passive surveillance of BTB countries, in the latter to supplement live cattle testing. The finding of a tuberculosis animal at slaughter initiates an investigation through TST of the herd of origin and any other potentially exposed animals.[30,31]

The standard method for detection of bovine tuberculosis is the tuberculin test, which involves the intradermal injection of bovine tuberculin purified protein derivative (PPD) and the subsequent detection of swelling (delayed hypersensitivity) at the site of injection 72hours later. This may be performed using bovine tuberculin alone or as a comparative test using avian and bovine tuberculins. The tuberculin test is usually performed on the mid-neck, but the test can also be performed in the caudal fold of the tail. The skin of the neck is more sensitive to tuberculin than the skin of the caudal fold. To compensate for this difference, higher doses of tuberculin may be used in the caudal fold. Delayed hypersensitivity may not develop for a period of 3-6weeks following infection. Thus, if a herd/animal is suspected to have been in contact very recently with infected animals, delaying testing should be considered in order to reduce the probability of false-negatives. As the sensitivity of the test is less than 100%, it is unlikely that eradication of tuberculosis from a herd will be achieved with only a single tuberculin test. It should be recognized that when used in chronically infected animals with severe pathology, the tuberculin test may be unresponsive. The tuberculin test has not been well validated in most non-bovid and non-cervid species.[32]

Mycobacteria in abattoir water

Water one of the sources to transmit or contaminate bovine meat abattoir. 25 samples have been tested and none find out that Mycobacteria present. It is observed that they are totally absent. It is published in a paper that Mycobacterium avium subsp. Para tuberculosis can contaminate the drinking water and form chronic infection while human consumption.[33] Water used at abattoir at every stage like washing of floors, carcasses washing, washing of equipments etc. Water contamination one of the greatest risk factor transmitting BTB in meat to human.

Control of BTB

BTB or bovine tuberculosis can be controlled proper sanitation of liarage and vaccination of slaughter bovine. Inspection during postmortem of infected carcasses with Bovine Tuberculosis lesions in its organs can be rejected but in India such rejection rare meet in action. The food handlers should undergo medical examination whether having tuberculosis or not. They have to restrict to enter the abattoir. The frozen buffalo meat should pass through ɣ-irradiation to kill and form safe production of buffalo meat at abattoir. Sanitation for processing hall by fumigation and floor with equipment by chemical reagents also very essential to control BTB. *M. bovis* is relatively resistant to disinfectants and requires long contact times for inactivation. Effective disinfectants include 5% phenol, iodine solutions with a high concentration of available iodine, glutaraldehyde and formaldehyde. In environments with low concentrations of organic material, 1% sodium hypochlorite with a long contact time is also effective. *M. bovis* is also susceptible to moist heat of 121°C

(250°F) for a minimum of 15minutes. Rodent control may also be advisable on affected farms; meadow voles and house mice can be infected experimentally, and voles shed *M. bovis* in feces.[14]

Results

Result obtained from frozen buffalo meat examination on L. J. Agar, it is clear that out of 45 samples, 27 samples observed as positive and 18 samples observed as negative. Tuberculosis like lesions were generalized TB and localized TB not only restricted to lymph nodes and parenchymatous organs, but can infect other organs of buffalo. Carcasses are examined to detect tuberculosis lesions in head, lungs, liver, intestine etc in Indian abattoir. But, main objective given whether *Mycobacterium bovis, Mycobacterium tuberculosis* can kill during the processing of buffalo meat at abattoir at very low temperature. *M. bovis* observed on around 24 of frozen buffalo meat samples. *Mycobacterium bovis* found to be comes under extreme phychrophilic organisms proved by above result. *Mycobacterium cosmeticum* found 5 sample and *Mycobacterium tuberculosis* found in 1 sample which passes through the extremely low temperature freezing condition.

Discussion

The public health risk analysis for BTB at denotes that this study is very chronic and infective for cattle and human. Despite of serious public health concern associated with BTB infection littile resources have been commited to screen and control this disease. In current study bovine tissue sample have been screened Mycobacterial infection from the site in Indian abattoir using Ziehl-Neelsen microscopy for direct examination over microscope which found as positive. Here basically direct microscopic examination and post culture of frozen meat sample as per above method have been conducted. At abattoir meat inspection largely depend on time, work load, diligence on the part of the meat inspector.[17,34]

In India buffalo slaughtered abattoirs passes the BTB infected carcasses and processed in Very low temperature at-38°C to -42°C. Those frozen samples collected and post cultured on L. J. Agar. The colony morphology and biochemical detection results as three species of Mycobacteria isolated and identified from the present study. Biochemically Niacin test is very important to distinguish between the three organisms. Tuberculin test of the animals also a major diagnostic test to identify the infected carcasses.

Many stuff, workers and butchers not used protective clothing despite the protection it gives against zoonotic transmission, clearly indicating their high risk of contracting BTB by occupation. This study showed significant association between awareness of the respondents (abattoir staff) of BTB and their occupational status, age, and duration of exposure to cattle carcasses. However, most of the abattoir staff believed in the importance of use of protective clothing while working, but very few of them did not know the importance. Likewise, on its zoonotic nature, most of them knew that bTB can be contracted from cattle. BCG vaccines should received by workers. Public health risk association and risk of BTB found in this study reported economic loss with public health implication due to tuberculosis.[35,16]

Conclusion

The above test methods and observations confirm that investigation of bTB with more sensitive test methods are highly recommended. Future studies also directed to the research result of Mycobacteria in environmental sample and their influence on manifestation of *M. bovis* infection. The tissue samples were examined by culturing and microscopic examination to demonstrate and identify different *Mycobacterima species*. The prevalence of *Mycobacteria spp.* in frozen buffalo meat showed that meat is contaminated with Mycobacteria can transmit bacteria from one animal to another and to humans. So for the better health preventive measures should be used. The detection of bTB among the slaughtered animals indicates the presence of bTB in the Indian animal husbandry with relevance to human zoonoses. Therefore, proper implementation of meat inspection procedures during slaughtering with public awareness are important to control bTB in India. A large-scale surveillance is needed to estimate the apparent and true prevalence of bovine tuberculosis in India. In this research project samples are withdrawn for microscopic examination directly from infected carcasses lesions at abattoir and frozen samples withdrawn from abattoir any part of bovine with even veals or buffalo culves from abattoir. The positive samples of Mycobacteria *like M. tuberculosis, M. bovis and M. cosmeticum* identified here. In Ethiopia *M. bovis* have been reported in abattoir meat found to be the cause of cervical lymphadenitis in human indicating the significance of bovine tuberculosis in human and cattle and prevailing low standard of hygienic status in production farms are potential risk factors that favors the spreading of infection.[36] bTB is still prevalent in cattle slaughtered in Cameroon, with a prevalence of bovine tuberculosis of 0.81% and 1.3% in abattoirs of Yaoundé in Center region and Douala Littoral region of Cameroon respectively.[37] *M. bovis* also detected in slaughtered cattle in west Africa.[38] A paper reveals that bovine tuberculosis spread in abattoir workers and butchers subjected by drawing their blood samples for tuberculin infection test at abattoir through nested PCR examination detected *M. bovis* prevalence in factory workers.[39]

Acknowledgements

The authors are indebted to the slaughter houses managements and other abattoir staff for cooperation and assistance during the research field work in New Delhi, also grateful to the Chancellor of Kalinga University and Principal & Ph.D Supervisor Dr. Anil Mahadeo Garode of Shivaji Science College, Chickli, Maharastra for pursuing the work on BTB. I was unable to carry out the said research without their support.

Conflict of interest

The author declares no conflict of interest.

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