Rapid Detection of *M. Bovis* and *M. Avium* in Cytological Smears and Tissue Sections by PNA-FISH

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Research article

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Abstract

Background: Bovine Tuberculosis is globally the paramount cause of death from single pathogen in cattle and other species. Rapid and explicit identification of mycobacteria is essential for the control of bovine tuberculosis. We performed a fluorescence Peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) procedure for specific detection of *Mycobacterium bovis* and *Mycobacterium avium* in bovine was optimized on cytological smears and tissue sections of bovines suspected for bovine tuberculosis.

Results: PNA-FISH was performed on lung and lymph node tissues impression smears. The probes were standardized for standard bovine mycobacterial cultures at 50% formamide concentration for *M.bovis* and 30% formamide concentration for *M.avium*. All the cytological smears were positive from *M.bovis* probe (MTBCcy3) which was standardized at hybridization conditions of (55^{o}C and 40% formamide) concentrations. Results revealed 4 out of 25 were positive in tissue sections with a bright red fluorescence with cy3 filter (MTBC probe). No results were seen with (MAV_{TAMRA}) probe for *M.avium* which was standardized at hybridization conditions of (55^{o}C and 30% formamide) respectively. No fluorescence was seen in control tissue sections .In addition, results were compared to other commonly used detection methods like IHC and PCR by targeting *esxA* gene. None of the sample was found positive for *M. avium*.

Conclusion: PNA-FISH can be used in cytological impression smears and tissue sections. It is less time consuming in diagnosis of bTB in post mortem cases than PCR.

1. Background

Infections caused by *Mycobacterium spp*. are often associated with worst clinical outcome including death. Bovine Tuberculosis (bTB) is caused by *Mycobacterium bovis,* a Gram-positive, acid-fast bacterium that belongs to the *Mycobacterium tuberculosis* complex (MTC) which mainly infects cattle [1, 2]. Use of conventional methods for detection of mycobacteria is difficult because of the low numbers of bacilli available, their slow growth, and their fastidious metabolism. The other method of detection of organism can be done by ZN staining as well as Immunohistochemical staining. But these methods suffer from several disadvantages including lower sensitivity. Recently, conventional methods such as acid-fast staining, culture, and phenotypic differentiation have been complemented by nucleic acid probes and amplification-based methods which is rapid and sensitive as well substantially reducing the time to diagnosis [3]. In recent years, molecular methods based on target amplification have become available for identification of *Mycobacterium species* in clinical specimen.Visualization of Mycobacteria by specific techniques, e.g., by fluorescence *in situ* hybridization (FISH), would be a great help in directly identifying bacteria in clinical samples. Peptide nucleic acids (PNAs) are pseudo-peptides with DNA-binding capability. These were the compounds first reported in the early 1990s in connection with a series of attempts to design nucleotide analogues capable of hybridizing, in a sequence-specific fashion, to DNA and RNA [4, 5]. The relative hydrophobic character of PNA as compared to DNA and RNA might be particularly useful in diagnostic applications where access of detection probes to their molecular target will depend upon efficient diffusion of the probe through a hydrophobic environment, such as a cell wall,

under sufficiently mild conditions for the morphology of the cell to be preserved [6]. The *in situ* hybridization procedure is relatively simple, requires only minimal equipment, and permits morphologic evaluation of positive signals. The aim of our study was to detect *M. bovis* and *M. avium* in cytological smears and tissue sections in bovine tuberculosis by PNA-FISH.

2. Results

In the present study, lung and lymph node tissue samples suspected for bovine TB (n=25) were subjected to Ziehl Neelsen staining. 19 out of 25 samples were positive by ZN staining (**Table 1**). The maximum percent positivity of 76% was found in samples with the presence of 15-20 acid fast bacilli per field (Fig.1).

Table 1: Ziehl Neelsen staining of tissue sample

S. No.	No. of tissue samples	ZN staining result	No. of acid fast bacilli	% positivity
1	25	19	15-20	76
Total = 19				

In this study, 4 out of 25 were positive by PNA-FISH. The probes were standardized for standard bovine Mycobacterial cultures at 50% formamide concentration for *M. bovis* and 30% formamide concentration for *M. avium*. All the cytological smears were positive from *M. bovis* probe (MTBCcy3) which was standardized at hybridization conditions of (55^{o}C and 40% formamide) concentrations. All fixed bacteria were stained by the probe and were visible as single cells or clusters (**Fig. 2, 3, 4 5**,). Bright fluorescence was observed with of Cy3-TAMRA filter set. No or weak signals were observed for mycobacterial species with single mismatches in the probe binding region (**Fig. 6**).

Isolation and Immuno-histochemistry

All the four samples were found positive from PNA-FISH technique. Samples were simultaneously ‘processed for Isolation on Lowenstein Jensen media slants. Powdery –buff colonies (colonies exhibited by *M. bovis*) were observed (Fig. 7) on the slants. The colonies of *M. bovis* were positive for PNA-FISH and were further confirmed by IHC and PCR. The central area of caseous necrosis in the granulomas was surrounded by granulomatous reaction comprising of macrophages, epithelioid cells, Langhan’s type giant cells (**Fig. 8, 9A, 9B, 10A, 10B**) and lymphocytes. Four out of 25 positive by (gold standard) isolation were PCR positive by ESAT-6 targeting esx*A* gene with product size of 61 bp (**Fig.11**). The results are given in (**Table 2**) respectively.

Table 2: Results of PNA-FISH in tissue impression smears, of lung and lymph node tissue sections:

Name of Technique	PROBE	Sample screened	POSITIVE	NEGATIVE	ISOLATION (culture)
PNA –FISH (Peptide nucleic acid hybridization)	MTBC-$_{CY3}$ *M. tuberculosis complex (M. bovis)*	25 tissue samples (lung and lymph node sections)	4 (*M. bovis*)	21	4
	MAV $_{TAMRA}$ *M. avium*	25	0	25	0

3. Discussion

The discovery of PNA has raised a number of novel possibilities relating to molecular diagnostics. We have shown the potential of labeled PNA oligomers as a powerful means of identifying *Mycobacteria spp*. directly in cytological tissue and impression smears of lungs and lymph node by PNA-FISH. The method presented here provides a combination of the high specificity offered by molecular techniques and the simplicity of direct microscopy [7]. Although detection of Mycobacteria with oligonucleotide probes is difficult since probe penetration is hampered by mycolic acid in mycobacterial cell walls. The development of PNA probes that enter mycobacteria without further pretreatment was, hence, a breakthrough [8]. Labeling of probes with TAMRA or Cy3 resulted in advanced signal intensity and succeeded in direct FISH detection of mycobacteria in tissue sections and cytological smears. In addition, fluorescent labeled PNA probes represent an economical way to identify mycobacterial cultures isolated from clinical specimens [9]. All four of the isolates obtained in this study were identified unequivocally as *M. bovis*. Assuming a time to result of about 3 hours for a FISH procedure (including fixation, hybridization, and microscopy) and considering its low cost, FISH is a suitable method for fast identification of isolated mycobacterial species. Another advantage of FISH is that no biosafety level 3 laboratory is required. It provides a top to bottom identification of microorganisms at different taxonomical levels without the need to determine traditional phenotypic characteristics, extract and amplify DNA, or to sequence. Laboratory methods used for diagnosis are conventional, mainly based on acid-fast staining, microscopy which is low in sensitivity and identification of mycobacteria causing the disease, so the interpretations of results by conventional methods are highly subjective and prone to errors [10]. Histopathology is considered a reliable tool for diagnosis of bovine TB but cannot differentiate between mycobacterial species, therefore in the present study the histopathological lesions were used for screening the cases for bovine TB and they were later subjected to PNA-FISH to detect *M. bovis* and *M. avium* organisms. Further the samples positive from PNA-FISH were confirmed by IHC and PCR. These tests aided in proving that the results from PNA –FISH were promising and authentic and there were no cross reactions with other mycobacteria. PNA-FISH is less time consuming, a 3-4 hr process time is required to obtain the results whereas other techniques are more laborious. Thus, the results of the present study suggested that IHC and molecular methods like PCR are required for confirmation of *M.*

bovis. The advantage of IHC is that it is robust and can even detect fragmented tubercle bacilli [11]. In our study, PNA FISH procedure was used to identify and visualize mycobacteria in clinical specimens and directly within the tissue sections, and was shown to be a fast and appropriate tool for research and diagnostic purposes. In the present study, acid fast bacilli were observed in nineteen out of twenty five cases, detected by ZN staining technique. The conventional microscopic examination of an Acid fast stain is simple and fast, and a positive AFB stain is the first indication of possible TB. However, staining yields poor positive predictive values for TB in clinical settings in which NTM is frequently isolated, because it does not allow differentiation of MTB from NTM [12]. Hence, there arise chances of more number of false positive results. PNA-FISH was performed on 25 lung and lymph node impression smears. Out of 25 samples subjected to PNA FISH, 4 were found positive for *M. bovis*. No tissue impression smear was positive from *M. avium* probe (MAV_{TAMRA}) which was standardized at hybridization conditions of 55^{o}C and 30% formamide respectively). No results were seen with (MAV_{TAMRA}) probe for *M. avium*.

4. Conclusion

It was concluded from the study that among conventional and molecular diagnostic methods, PNA-FISH can be used in cytological impression smears and tissue sections. It is less time consuming in diagnosis of bTB in post mortem cases than PCR.

5. Methods

5.1 Source of animals: Fresh tissue samples (lymph nodes, lungs with tuberculous lesions) were collected from postmortem hall (dairy farm Ludhiana ,Punjab),(n=25) suspected for bovine tuberculosis. Tissue impression smears were made from these tissues fixed with methanol (100%w/v) for further use. The tissue samples were collected in two containers separately, one in 10 % NBF for histopathology and frozen tissues in sterile container for PCR studies.

5.2 Clinical Specimen

A total of 25 tissue samples (lung and lymph node sections) from bovine tuberculosis suspected animals above 2 yrs of age at postmortem were routinely Acid-fast stained, formalin-fixed and paraffin embedded.

5.3 Cytological Smear preparation

Approximately 2 g of tissue from each sample (n= 25) was cut into small pieces and homogenized with 1.0 ml of sterile distilled water using a pestle and a mortar. The tissue homogenates (200 ml each) were decontaminated with 4% NaOH. Inoculated onto two slants of Lowenstein-Jensen (LJ) media with and without glycerol and incubated for 6–8 weeks at 37^{0}C.

5.4 For identification of Culture

Two loops full of tissue homogenate were smeared on glass slides. The smears were dried, heat fixed, stained with Ziehl-Neelsen (ZN) and examined for Acid Fast Bacilli (AFB).

5.5 PNA synthesis and labelling

Samples included in the study were identified by the MTBCcy3 Probe and MAV_{TAMRA} hybridization assay (PNA Bios Probe, USA). Probes MTBCCy3 and MAVTAMRA, were used for specific detection of members of the *M. tuberculosis* complex and *M. avium* respectively, with the 16S rRNA Sequence database and the probe design program [18]. The probe sequences were customized from (PNA Bios USA). *M. bovis* and *M. avium* standard cultures were used for the standardization of PNA-FISH assay. Sequence of the probes is depicted the **Table 3**.

Table 3: PNA probe Sequences used for Assay **[18].**

Probe	Sequence (orientation)	Target species
$MTBC_{Cy3}$	TCC TGG TGC CCT ACG-Cy3 (3–5)	*M. bovis*
	AGG ACC ACG GGA TGC (5-3)	*(M. tuberculosis complex)*
MAV $_{TAMRA}$	CTG GAG TTC TGC GTA-TAMRA (3_5)	*M. avium*
	GAC CTC AAG ACG CAT (5_3)	

5.6. Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) using PNA probes was performed on postmortem samples from animals suspected to have died of tuberculosis, to demonstrate and identify *Mycobacterium bovis* and *Mycobacterium avium*. The procedure for the FISH technique was performed as per [13, 14] with slight modifications. Standard Culture-grown and fixed bacteria (20 µl) were spotted onto three-field microscope slides (Thermo scientific, India) and air dried. Then, the slides were dehydrated in 100% (v/v) methanol for 1 min and 100% (v/v) ethanol for 5 min, air dried again, and preheated to hybridization temperature (~55°C). Slides with impression smears made from organs with lesions and corresponding tissue sections were processed in similar pattern. Impression smears and tissue sections were applied with a 20 µl of Triton X 100 with a cover slip and kept for 20 minutes in dark chamber. The slides were then subjected to hybridization mixture containing 10% (w/v) dextran sulfate (Mp Biomedicals, India), 10 mM NaCl (Mp Biomedicals, India), 30 to 50% (v/v) formamide (Hi media India), 0.1% (w/v) sodium pyrophosphate (Mp Biomedicals, India),0.2% (w/v) polyvinyl pyrrolidone (Mp Biomedicals, India), 0.2% (wt/vol) Ficoll (Mp Biomedical, India), 5 mM disodium EDTA (Mp Biomedicals, India), 0.1% (vol/vol) Triton X-100 (Mp Biomedical, India), 50 mM Tris-HCl (pH 7.5), and a fluorescent probe(s) with a final concentration of 1 to 1.5 mol/liter were applied to each sample. Slides were incubated at a temperature optimized for each PNA probe (Table 3) in a preheated moisture chamber in the dark for 90 min. Coverslips were removed by submerging each slide in approximately 20 ml of prewarmed 5 mM Tris (pH 10), 15 mM NaCl (Mp Biomedical), and 0.1% (vol/vol) Triton X-100 (Mp Biomedical) (FISH wash buffer) in

a water bath at 55^{o}C, following hybridization. The slides were then kept in water bath for 30 minutes. After brief immersion in FISH wash buffer, the slides were washed with double distilled water, air dried and mounted with 1 drop of imaging mounting fluid (Vector Laboratories Inc., Burlingame, Calif.). Microscopic examinations were conducted using a fluorescence microscope (Nikon CFi-l microscope (Nikon Corporation, Japan) and photographs were captured using Nikon DSFi-2 camera.). Nonspecific hybridization of $MTBC_{Cy3}$ to *M. bovis*, was avoided by high-stringency hybridization conditions (55°C, 50% formamide). For impression smears and tissue sections hybridization conditions of formamide, were required at 40% and 50% (**Table 4**). For *M. bovis,* the $MTBC_{Cy3}$ probe sequence, hybridization conditions (55°C, 40% and 50% formamide) were sufficient to prevent unspecific binding. For probe, MAV_{TAMRA} (*M. avium*) unspecific binding was avoided by hybridization at 55°C and formamide concentration of 30%, (impression smears and tissue sections) respectively.

Table 4: PNA probe hybridization conditions

Target species	Hybridization and washing temperature^{o}C	Formamide (%) (vol/vol) Standard culture	Formamide (%) (vol /vol) Impression smears	Formamide (%) (vol/vol) Tissue sections
MTBC Cy^3 *M. tuberculosis complex*	55	50	40	50
MAV_{TAMRA} *M. avium*	55	30	30	40

Immunohistochemical Studies

Detection of antibodies (ESAT-6 monoclonal and polyclonal, CFP-10 polyclonal) in tissues was done by immunohistochemical analysis. All tissue samples were separately collected and fixed in 10% Neutral Buffered Formalin and were further processed as per conventional methods [15]. Thick paraffin tissue sections were spread on Superfrost positively charged microscopic slides (Fisher Scientific, USA). Antigen retrieval was done in EZ antigen retrieval solutions using EZ-Retriever System (Bio Genex Laboratories Inc., California). After endogenous peroxidase and nonspecific protein blocking, the sections were incubated with standardized dilution of (ESAT-6 and CFP-10) antibodies **(Table 5**) in a humidified chamber at 4°C overnight. Secondary antibody conjugated with HRP (Vector Laboratories, USA) was added and incubated for 30 min at room temperature. Visualization of antigen antibody complex was performed using ImmPACT DAB Peroxidase Substrate Kit (Vector Laboratories, USA) followed by counterstaining with hematoxylin. Presence or absence of Mycobacterial antigens was evaluated by

observing the stained cells showing positive reactivity (macrophages, giant cells, epithelioid cells) using light microscopy under oil immersion [16, 17].

Table 5: Panel of antibodies used for immunohistochemical characterization of tuberculous lesions

S. No.	Antibody (Catalog No.)	Company	Clone	Dilution Used
1.	ESAT-6 Polyclonal	Thermo Scientific	Polyclonal	1:100
2.	CFP-10 Polyclonal	Thermo Scientific	Polyclonal	1:100

5.7. PCR Primers:

The primer sequences for ESAT-6 were: Forward- 5'-GTACCAGGGTGTCCAGCAA AA-3' and Reverse 5'-CTGCAGCGCGTT GTTCAG-3' [12] giving a product size of 61 bp respectively was used for PCR amplification. All 4 samples found positive by PNA-FISH were further confirmed by **esxA** (ESAT-6) PCR. Tissue sample DNA was amplified by esxA (ESAT-6 PCR, for detection of *M. bovis*. Amplicons of 61 bp were considered positive for ESAT-6 PCR respectively.

Declarations

Ethics Approval and Consent to participate

We are thankful to the ethical committee for approving the use of animals and the approval of IAEC/CPCSEA is obtained vide reference no. IAEC/2015/26/013.The current study was approved by the Institutional Ethics Committee, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana (Approval number -IAEC/2015/26–013). The study was conducted from January 2017 to June 2019.

Consent for publication.

Not applicable

Availability of data and material

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Competing interest

No financial or personal relationships between the authors and other people or organizations have inappropriately influenced this work

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Authors’ contributions

RJ, DN and MC conceived and designed the study. RJ performed the lab work. KG helped in Immunohistochemistry; SD helped in FISH. RJ and DN drafted the manuscript. RJ, DN, KG, MC and SD revised the manuscript. All authors read and approved the final manuscript. DN led the research project.

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Figures

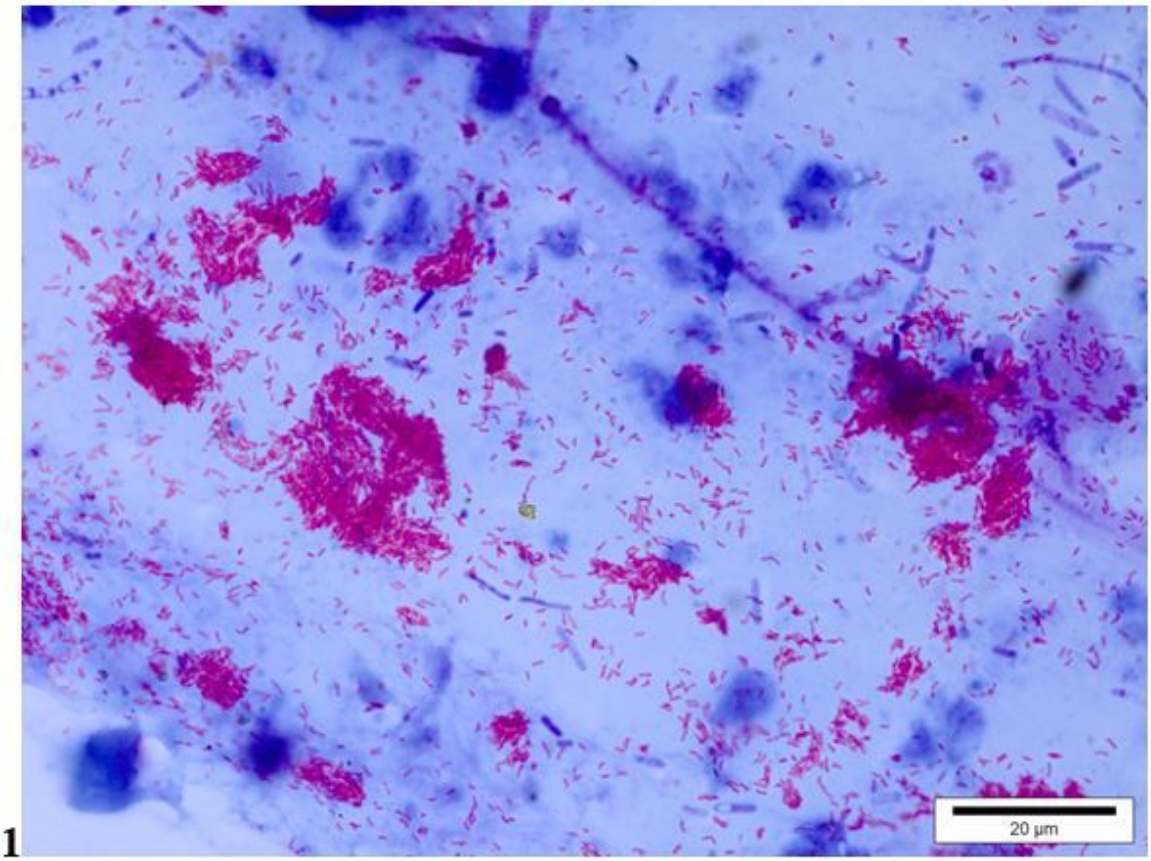

Fig. 1

Figure 1

Acid fast stained bacilli with Ziehl Neelsen Staining, Bar=20 µm

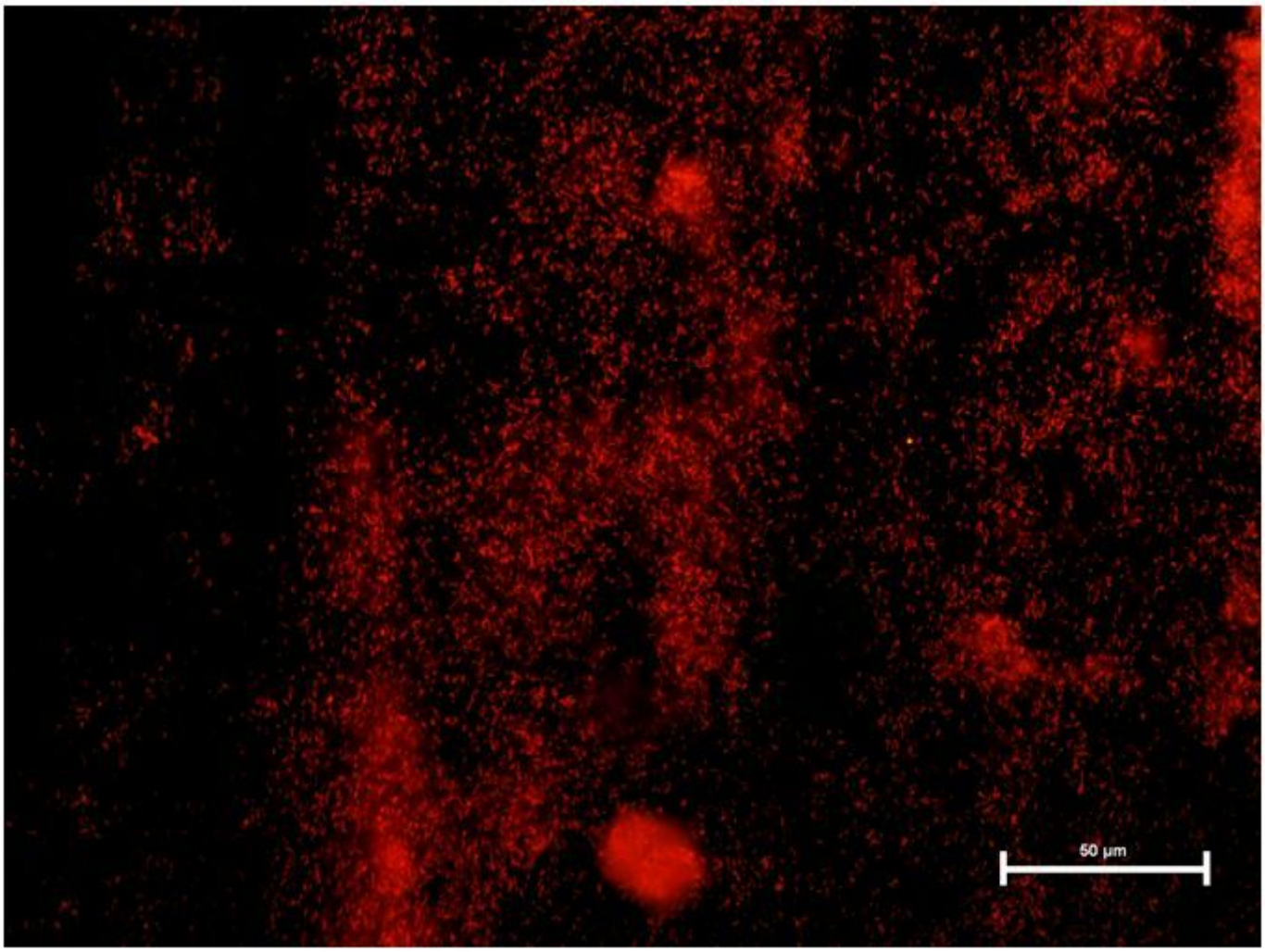

Figure 2

Visualization of M. bovis in a standard culture -PNA-FISH, (MTBC cy3) probe Bar=50 µm

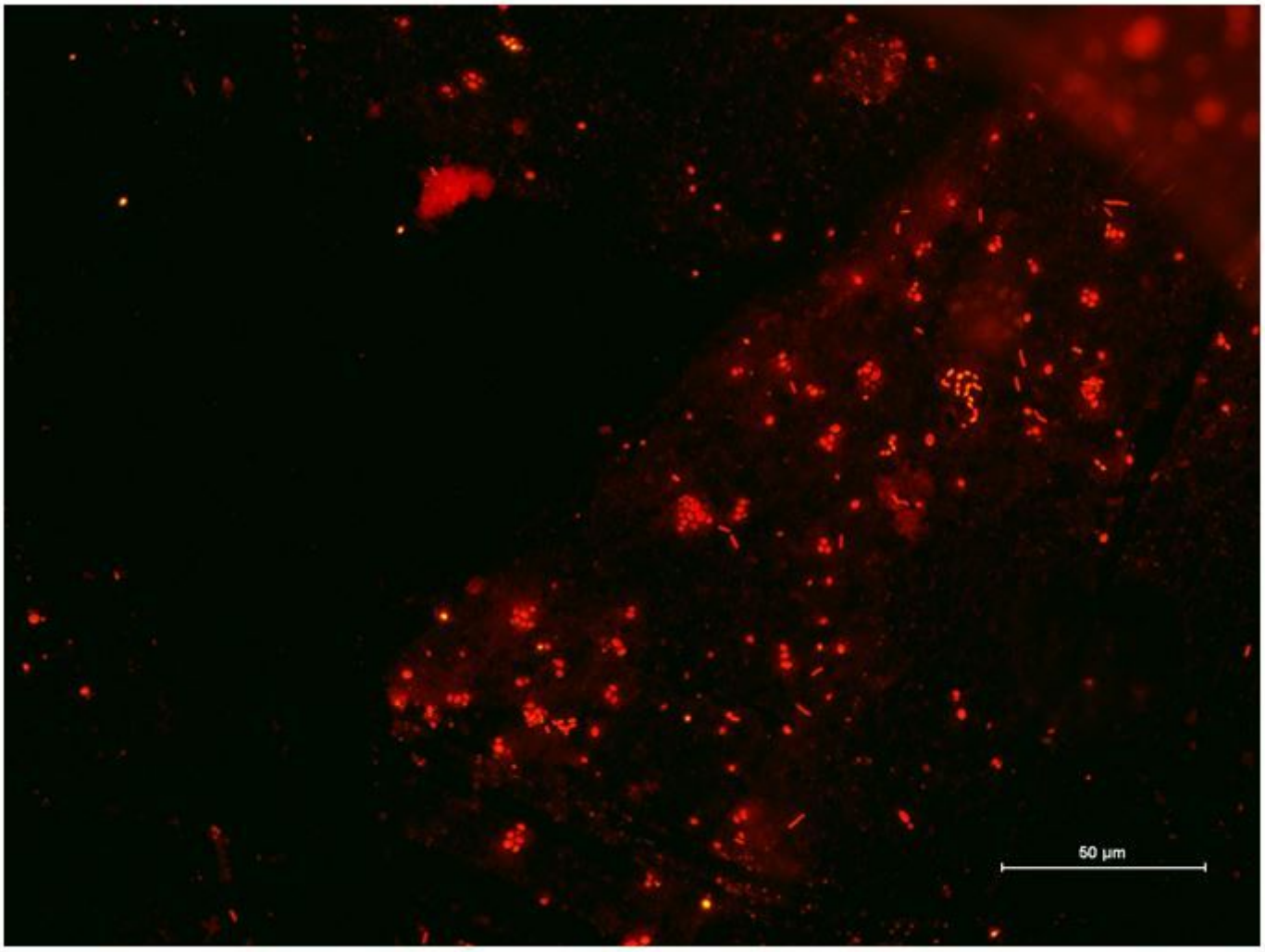

Figure 3

Visualization of M.avium from a Standard culture - PNA FISH, (MAVTAMRA) probe. Bar = 50µm

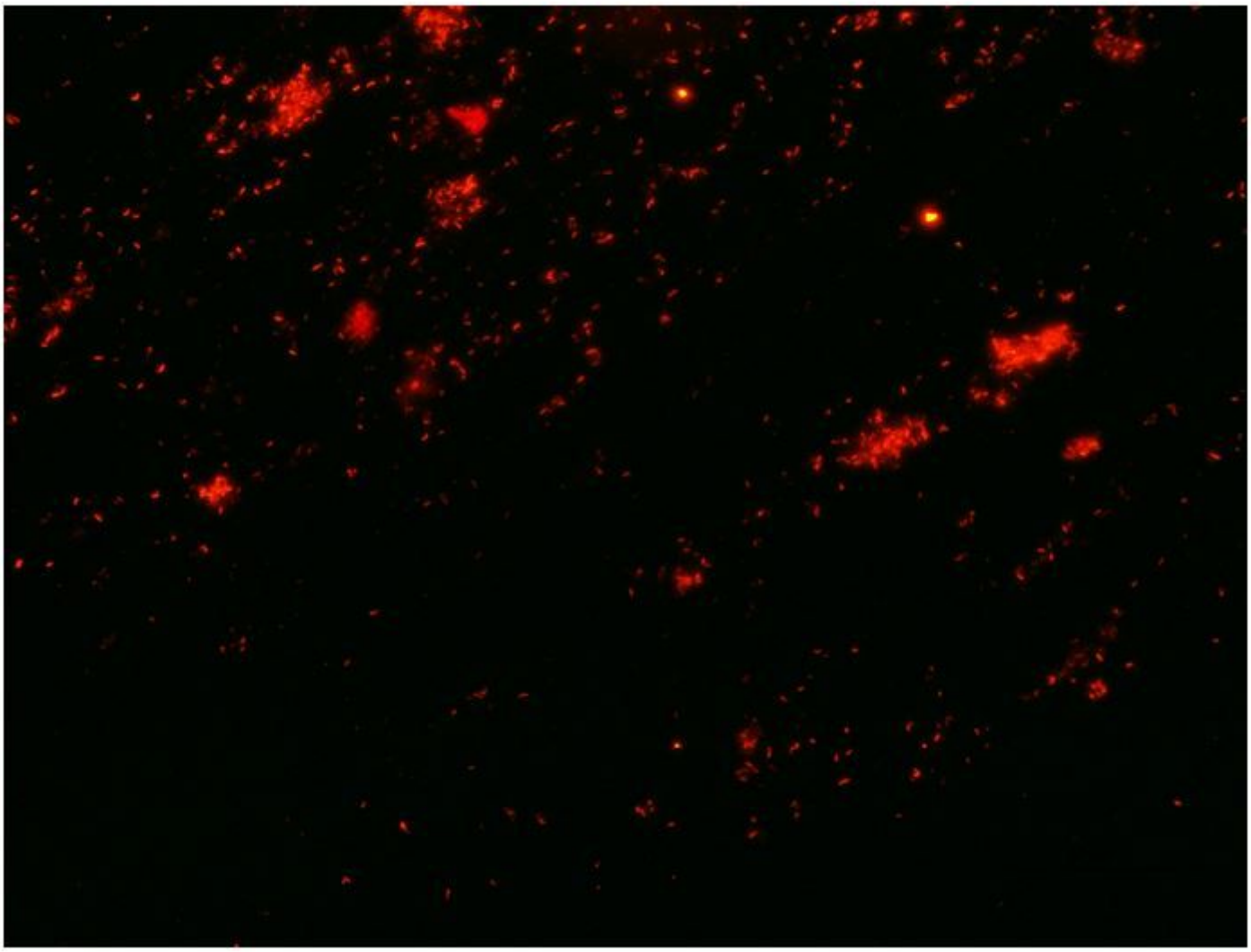

Figure 4

Visualization of M. bovis in a cytological smear of infected bovine lung - PNA FISH (MTBC- cy3) probe

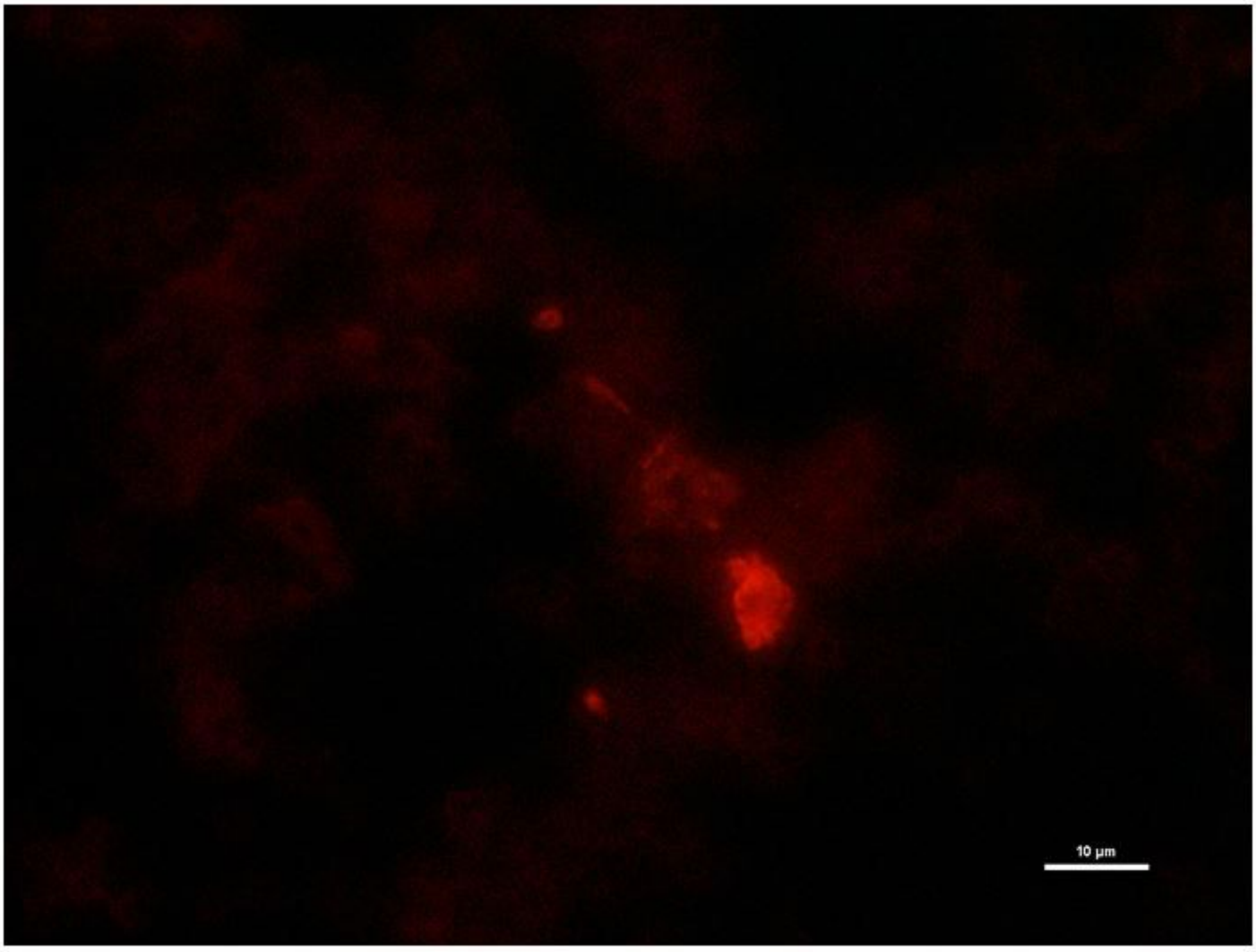

Figure 5

Visualization by FISH of M. bovis (MTBC-Cy3) in (clumps) Lymph node tissue impression smears)

Figure 6

Negative control M. bovis (MTBC Cy3) in lung tissue impression smears

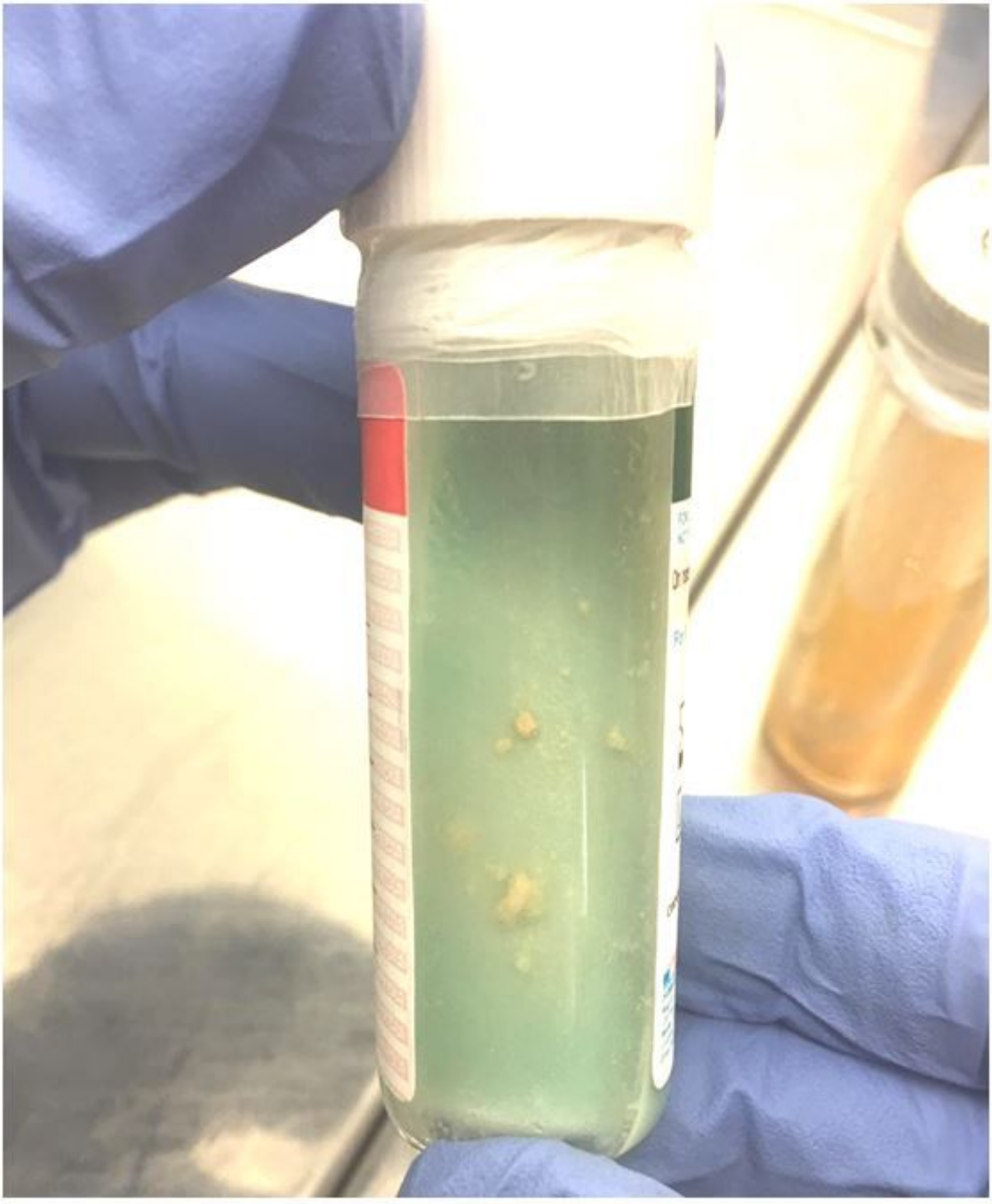

Figure 7

M. bovis (culture growth-powdery buff colonies) isolated from Lowenstein Jensen Media slant

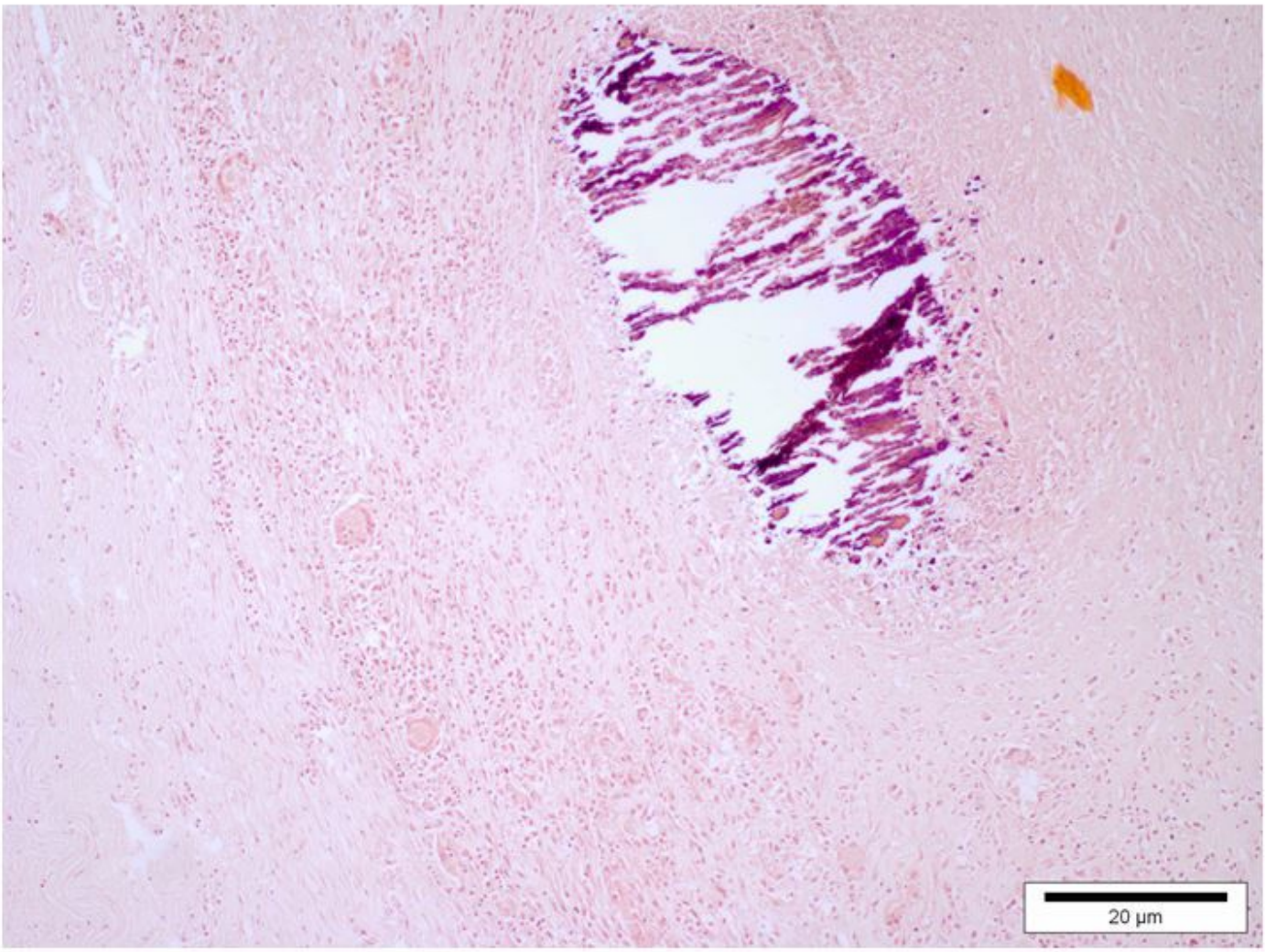

Figure 8

Section of Lung infected with M. bovis caseo calcifying granuloma .H&E Staining, Bar=20µm

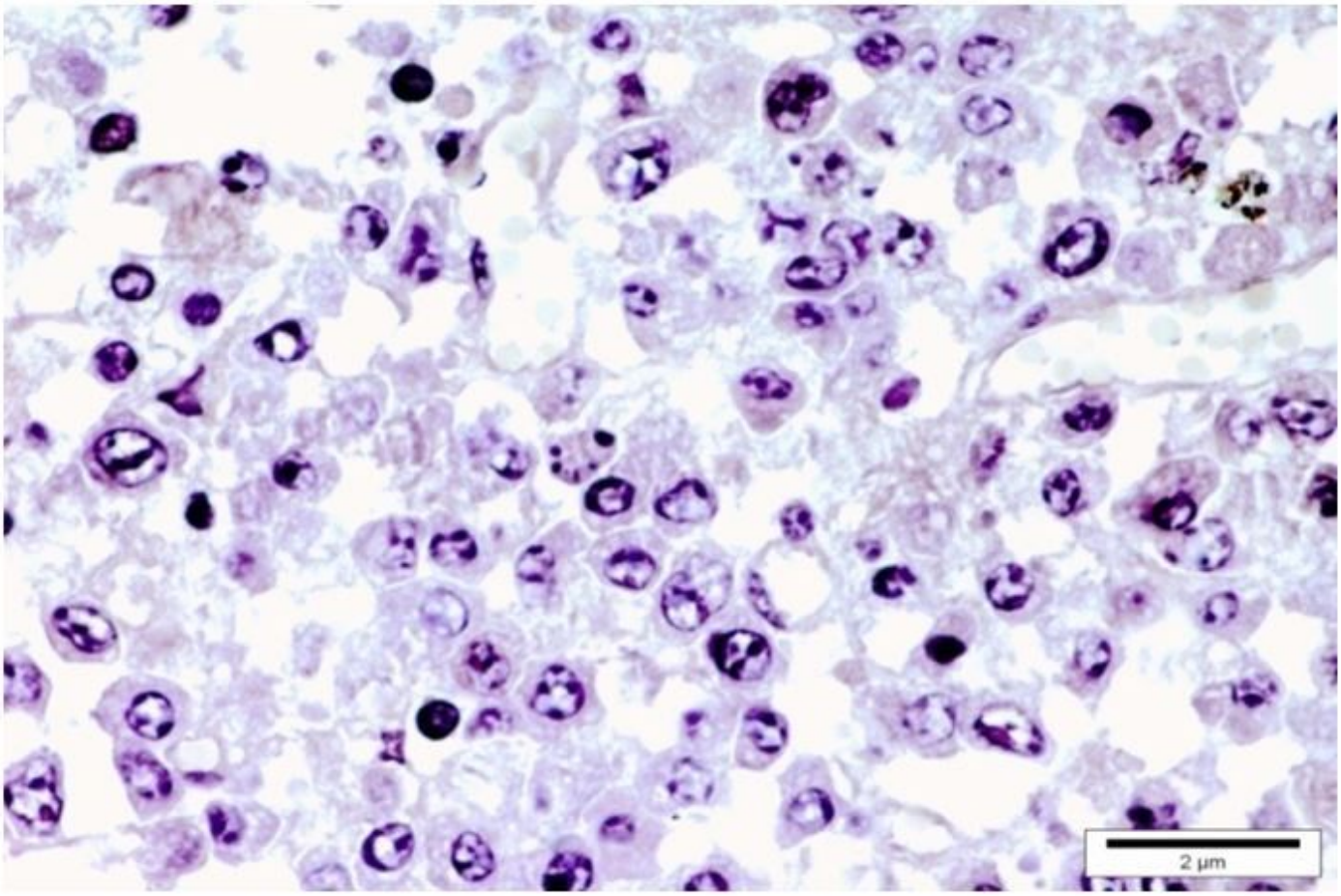

Figure 9

Immuno-histochemical negative control of ESAT-6(Polyclonal antibody) in a section of bovine lung. Bar= 20 µm

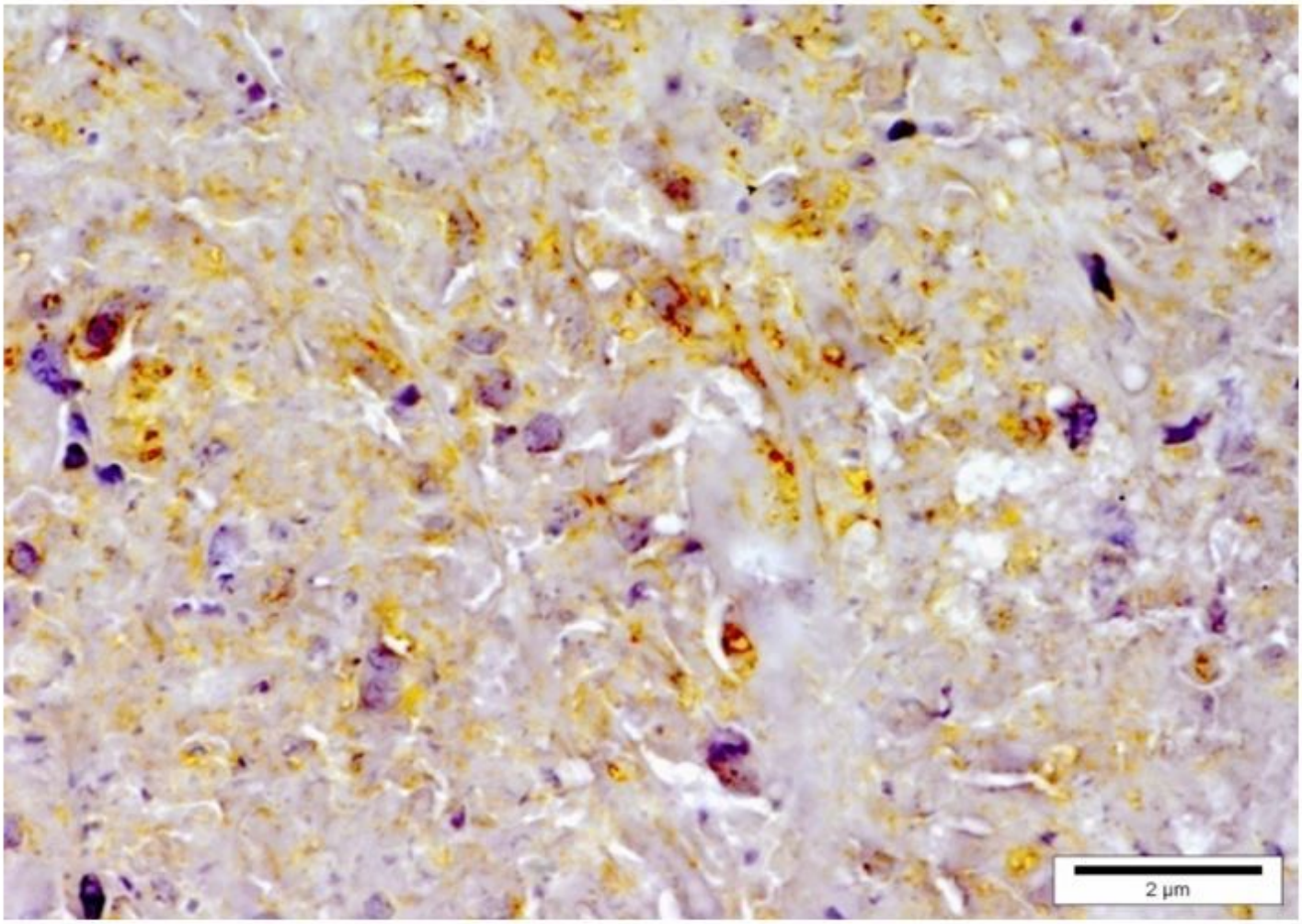

Figure 10

Immuno-histochemical localization of M. bovis in necrotic area, macrophages and the giant cells using ESAT-6 (polyclonal antibody) with Polymer HRP staining. Bar=20 µm

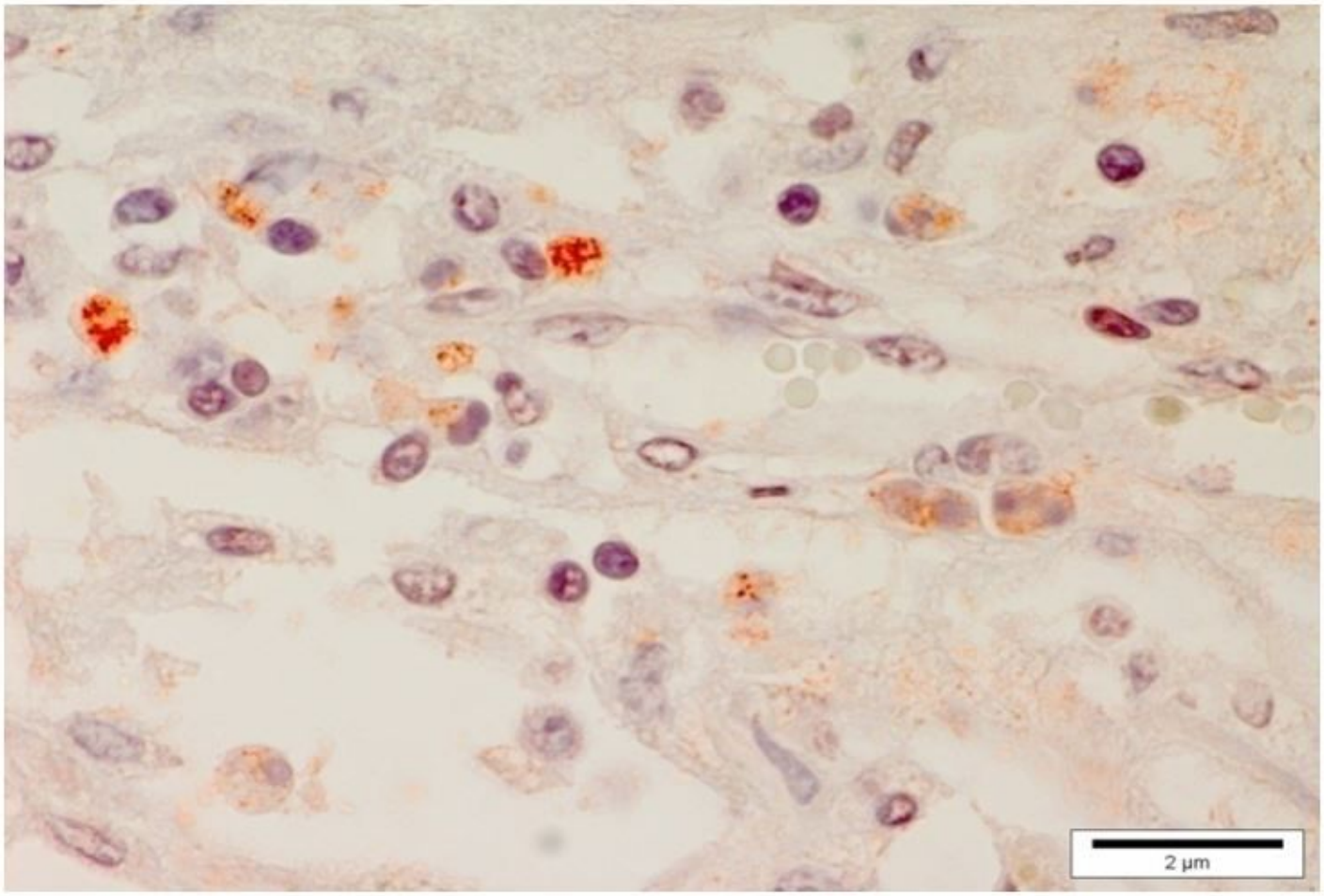

Figure 11

Immuno-histochemical localization of M. bovis in macrophages and the giant cells using CFP-10 in lungs with AEC staining. Bar=20 µm

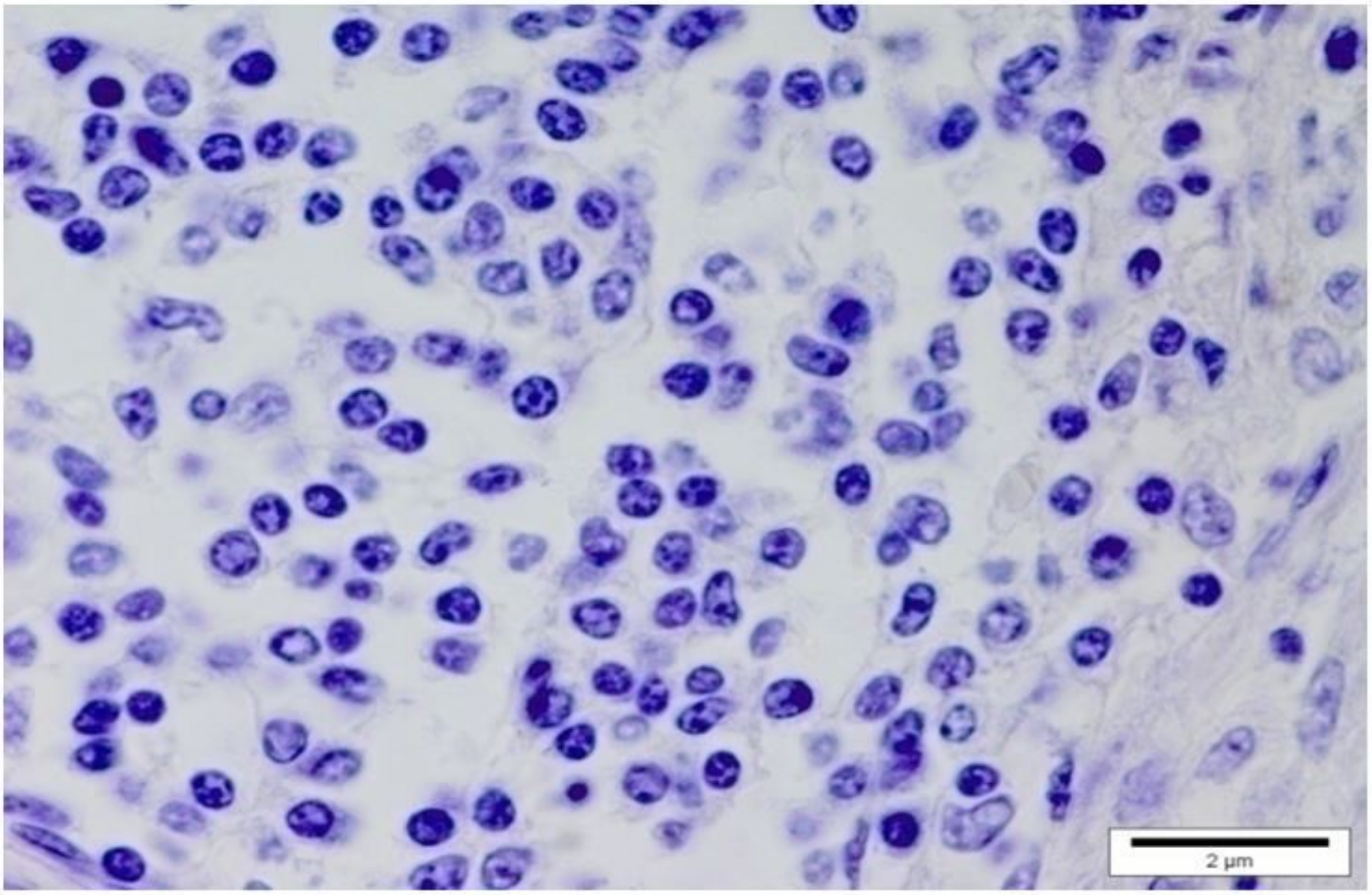

Figure 12

Immuno-histochemical negative control using CFP-10 in a section of bovine lung. Bar= 20 µm

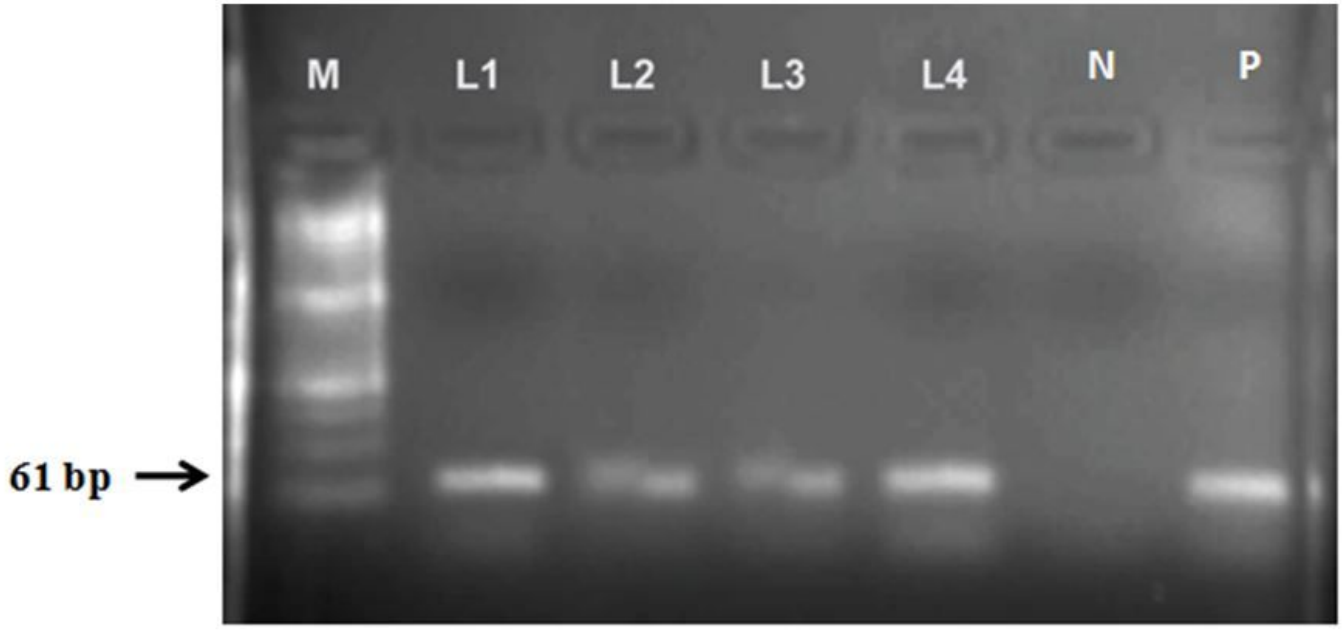

Figure 13

Gel electrophoresis of amplified PCR products of 61bp (ESAT-6 primers) in DNA of tissue samples for bovine tuberculosis (cropped image) • Lane M: 50 bp plus molecular weight marker, • Lane N: Negative control, • Lane P: Positive control, • (Lane 1 to 4: Tissue samples)