

Case Study

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A Case Study of Bovine Tuberculosis in Calf in Albania

Anita Koni¹, Lenida Surraj², Pëllumb Zalla¹, Silva Tafaj² and Xhelil Koleci^{1*}

¹Faculty of Veterinary Medicine, Agricultural University of Tirana, Albania

²University Hospital, Shefqet Ndroqi, Tirana, Albania

*Corresponding author

ABSTRACT

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Outbreak of bovine tuberculosis is rare reported in Albania. In this study we confirmed the bTb diagnosis by using gross pathology examination, microscopic examination of affected tissues, and *Mycobacterium bovis* isolation and identification by culturing, colony morphology and Ziehl–Neelsen stain. Typical mycobacteria were grown in both solid and liquid media, however in liquid media *M.bovis* was grown faster than solid media. In addition, isolates were typed by using spoligotyping method and *M.bovis* was confirmed.

Introduction

Bovine tuberculosis (bTB) is a contagious bacterial infectious disease that affects animals and humans (OIE, 2009). It occurs worldwide and it is a OIE listed disease and interfere seriously with international trade of animals and animal products (Nikki le Roex, *et al.*, 2015). In general, bTB is a typical chronic disease. Humblet, *et al.*, (2009) listed a long list of bTB risk factors and age was classified as an individual factor that affects clinical disease. Despite that animals may be infected at early age of life the clinical disease may appear when animals become older. In addition, older animals are more likely to be exposed to *M.bovis*. The main route of transmission of bTB is by inhalation; however congenital and suckling colostrum and milk from infected animals are described widely (OIE, 2009).

Diagnosis of bTB is an important factor that affects significantly the success of its control. Ante mortem diagnosis of bTB in cattle is based on tuberculin skin test (STs), Interferon Gama Assay (INF- γ), ELISA, Fluorescence Polarization Assay (FPA), etc. Sensitivity of diagnostic methods is low and both false positive and false negative affect their performance. Both STs and INF- γ tests are based on cell mediated immunity, STs have low sensitivity and acceptable specificity, while INF- γ , despite the diagnostic parameters are better than STs, its specificity and sensitivity are still low.

However, using the tests in parallel is useful for detecting more infected animals than each test may be detecting (Gormley, *et al.*, 2006). The serological methods such as

ELISAs and FPA are available. The sensitivity is variable, while specificity of tests is acceptable. Skin tests may detect animals that are infected at list one month before performing the tests as this is the minimal time for late hypersensitivity status to be established (OIE, 2009).

The serological diagnosis based on specific antibodies to *M.bovis* is suitable for detecting the advanced cases of bTB. Young animals react less than adult animals in tests based in late hypersensitivity and serological test. Postmortem diagnosis is based on gross lesion characteristics, microscopic lesions, culturing and ZN staining (Norby, *et al*, 2004, Menin, *et al.*, 2013).

Gross lesions are described as granulomatous inflammation; however, they are not specific for bTB, several other infections may develop the same morphology (Palmer MV *et al*, 2007). The microscopic examination reveals presence of caseous necrosis, epithelioid macrophages, lymphocytes, multinucleated giant cells and clusters of neutrophils. Culturing of *M.bovis* is a gold standard method for bTB diagnosis (Corner, *et al.*, 1994; Ramakrishnan, 2012; Palmer, *et al*, 2007; Kumar, *et al.* 2013). It takes time, is expensive, requires biosafety level three laboratory, trained personnel etc. Characteristics of colonies and ZN staining are used for identification of *M.bovis* (OIE, 2009).

In order to type the isolated strain molecular methods may be used. Molecular typing has a particular importance for tracing back the cases and identifying the source of infection. In this study, we isolated, identified and typed the *M.bovis* from suspected tubercular gross lesions in a calf. The microscopic examination was performed in microscopic smears.

Materials and Methods

We used tissues and organs of abdominal cavity and lungs of a calf that were slaughtered after a severe unspecified gastrointestinal signs.

Gross Lesion Examination

The examination of gross lesions was made by carefully examination of the organs admitted to laboratory of infectious disease at veterinary faculty, Tirana. The visual examination was combined with palpation and incision of the spleen, mesentery, liver, diaphragm and palpation and incision of mesenteric lymph nodes. The mesenteric lymph nodes, spleen and diaphragm were examined in details and were sectioned into 2 cm thick slices to facilitate the detection of visible lesions. The cut surfaces were examined under bright light for the presence of typical tubercular lesions such as: abscess, cheesy mass, and tubercles. The proper samples were selected and in parallel there were used for culturing and for histopathological examination.

Histopathological Examination

Histopathologic examination was based on tissue samples fixed in 10% neutral buffered formalin and processed by standard paraffin wax techniques. Samples were cut and stained using the standard hematoxylin-eosin (H&E) method. Slides were evaluated microscopically and classified as positive based on presence of tubercular granuloma displaying central necrosis with or without mineralization surrounded by macrophages, lymphocytes, plasma cells, neutrophils, epithelioid cells, and Langhan's giant cells, and enclosed partly or completely by a thin capsule.

***Mycobacterium bovis* Isolation**

M.bovis was recovered from different tissue lesions by using both solid liquid media. The samples were decontaminated by using the standard protocol described by Stewart L.D. *et al.*, (2013) and classical inoculation procedure was applied for both media.

Briefly, selected samples were homogenized, decontaminated with oxalic acid 10% and Buffer Phosphate Saline as previously described by Markey *et al.*, 2013. At the end the pellet was re-suspend and 0.2 ml were inoculated in both solid and liquid media respectively.

***M.bovis* Isolation in Solid Media**

Although there are several laboratory techniques for diagnosis of *M.bovis*, isolation in solid media Lowenstein-Jensen with pyruvate still remains a very liable and widely method in use. We used Lowenstein-Jensen with sodium pyruvate because it enhances *M.bovis* growth.

Briefly media was prepared by using commercial base of Lowenstein-Jensen, distilled water follow by adding sodium pyruvate. After sterilization, fresh eggs were added to the suspension in slope bottles. The proper slope bottles were used after coagulation was developed by heating at 85°C for 45 minutes. The inoculated media were incubated at 37⁰C and were checked every week.

***M.bovis* Isolation in Liquid Media**

Liquid media was prepared the day of performing the decontamination of the tissues and the samples inoculation. The Bactec MGIT 960 system was used. The media content is as follow: MGIT 960 Growth-Supplement, BBL MGIT PANTA,

BBL MGIT Tube (7 ml). The preparation steps of liquid media are: preparing PANTA solution by adding the Middlebrook OADC growth supplement to the lyophilized PANTA by mixing, in order to be dissolved properly. In every Bactec MGIT 960 tube was added 0.8 ml from PANTA solution and than from every sample is inoculated 0.2 ml of final solution of decontamination samples.

Ziehl-Neelsen (ZN) Staining

As part of identification procedure of *M.bovis*, bacterial isolates in either liquid and/or solid media were stained by Ziehl-Neelsen (ZN) or Acid-Fast method described by Markey *et al* 2013. Briefly, the prepared microscopic smears, were floated into carbol fuchsin and heat for 10 minutes, slides were flooded into alcohol acid decolorizer for 15 minutes with several changes and finally methylene blue was added for 20 seconds.

At the end of every step slides were rinsed with tap water. Stained slides were allowed to air-dry, microscopic examination was done by using LEICA microscope and proper digital picture that show presence of *M.bovis* were saved.

***M.bovis* Typing**

Isolates of *M.bovis* were typed by employing the spacer oligonucleotide typing (spoligotyping).

This is a valuable tools for molecular epidemiology of *M. bovis* infections and can be used to identify distinct groups of strains. The aim of typing was to characterize the strains of *M. bovis* from the calf and to compare it with other isolates either from follow up this case or within districts, country and region.

Results and Discussion

Results of gross lesions are presented in Table 1. Typical lesions were evident in abdominal cavity, organs and lymph nodes.

Isolation of *M.bovis*

M.bovis was recovered from tubercular lesions of diaphragm, spleen and from lymph nodes of abdominal cavity (Table 2).

Bacteria Identification

Based on weekly examination we noticed Mycobacteria spp. growing at four to five weeks in liquid media and six to eight weeks on solid media. Typical colonies were grown in solid media, they are cream colored, central roughness and seems to be fragile. The culture smears prepared and stained by ZN method allowed us to identify presence of acid fast mycobacteria. In addition, based on spacer oligonucleotide typing, *M.bovis* was identified as cause of tubercular lesions detected in five-month tissue calf.

There are few reported cases of bovine tuberculosis in cattle and more rarely in calves in Albania. In addition, there is not running any program for following a typical

protocol for making definitive diagnosis or following up the index case. In general, ante mortem available diagnostic test have low sensitivity. Postmortem diagnosis is based on evidence of gross lesions, histopathological changes, ZN staining (either direct on affected tissue, excretions, secretions and on cultures), bacterial culturing in proper media and detecting the genetic material of *M.bovis*. Based on presence of lesions in different tissues of the calf we identified them as typical granulomas spread in abdominal organs and visceral tissues. Despite that we didn't used any classification score system (Menin *et al* 2013), we conclude that tubercular formations were at different stages of development. Typical yellowish and calcified in consistency granulomas were obviously detected in spleen, which were covered with a fibrous capsule Fig 1 (3, 4) and lymph nodes, Fig.1 (1, 2). Routine examination detected only five granulomas with diameter ranged from 2-5 cm, seen from spleen surfaces. However, detailed examination based on carefully palpation and section every 2 cm detected at list 17 tubercles with different diameters. During dissection of affected tissues there were present a lot of multiple intra-granulomatous areas of caseous necrosis, Fig 1(4).

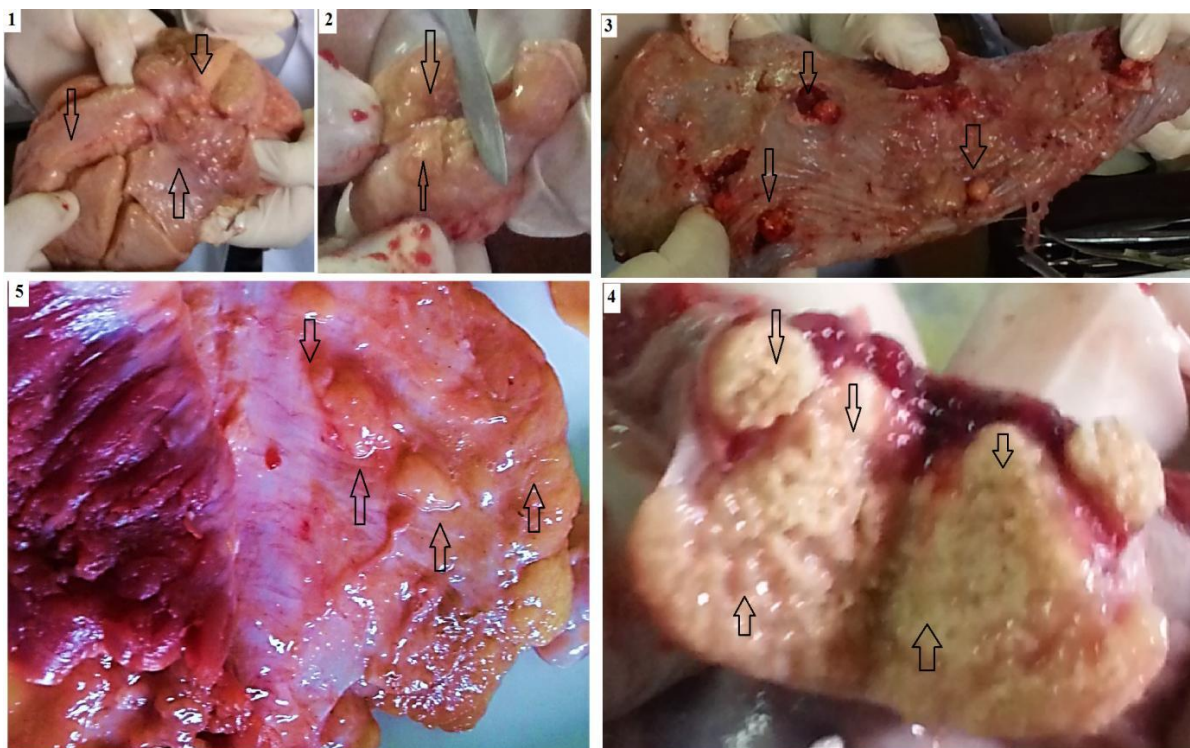
Table.1 Distribution of Tubercular Lesions in Organs and Tissues of a Calf

Distributions of granulomas	Major organs/tissue	The calf 5 months' old
Thorax	Lung	No
Abdominal	Liver	++
	Spleen	+++
	Diaphragm (abdominal surface)	+++
	Mesentery	++
	Lymph nodes	
Thorax	Mediastinal	No
Abdominal	Hepatic	+
	Mesenteric	+++

Table.2 Culturing Of *M.bovis* from Organs and Lymph Nodes of the Five-Month Calf

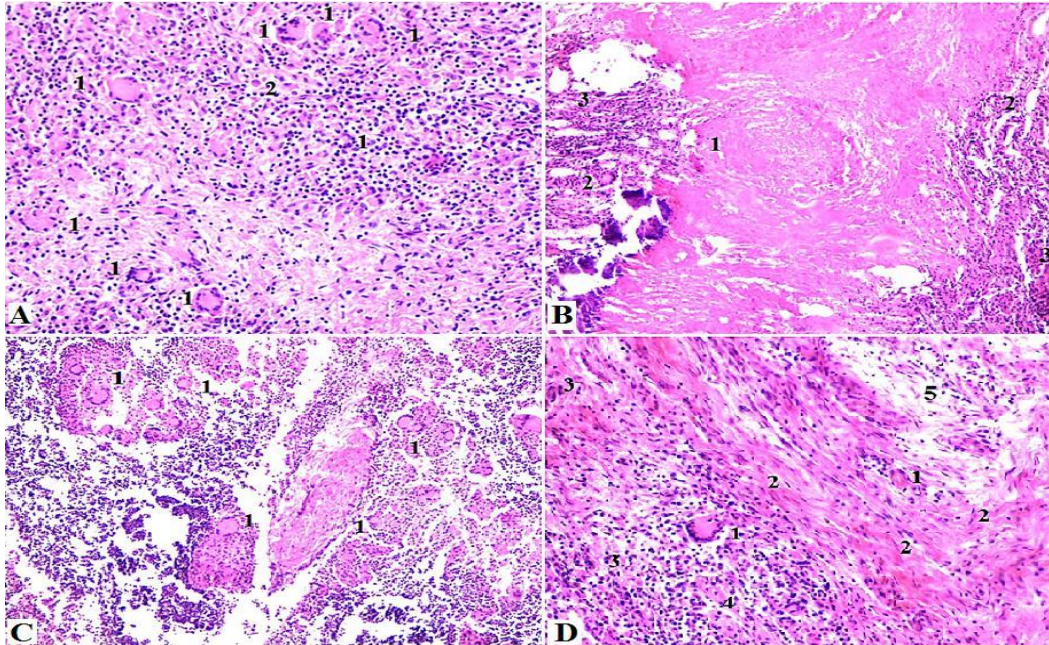
Organs and lymph node	The time in weeks that grown <i>M.bovis</i> was detected according media	
	LJ Media	BACTEC-960 system
Lung	NA	NA
Tubercular lesions of diaphragm	8	4.5
Spleen	7	5
Lymph nodes		
Hepatic	6	5
Mesenteric	6	4

Fig.1 Distribution of Tubercular Lesions (Indicated By Arrow) in a Five-Month Calf



Legend. 1. Mesenteric lymph nodes. Enlargement and tubercular lesions in surface and inside lymph-nodes. 2. A lymph node dissection and typical dense tubercular lesions. 3. Encapsulated granuloma on calf spleen. 4. Dissection of a typical tubercle in the spleen encapsulated. 5. Tubercular lesions in abdominal surface and peritoneum parietal.

Fig.2 Histo-Pathological Analyses in Tubercular Lesions (Indicated By Arrow) in a Five-Month Calf Infected with *M.bovis* (40 X Magnification).

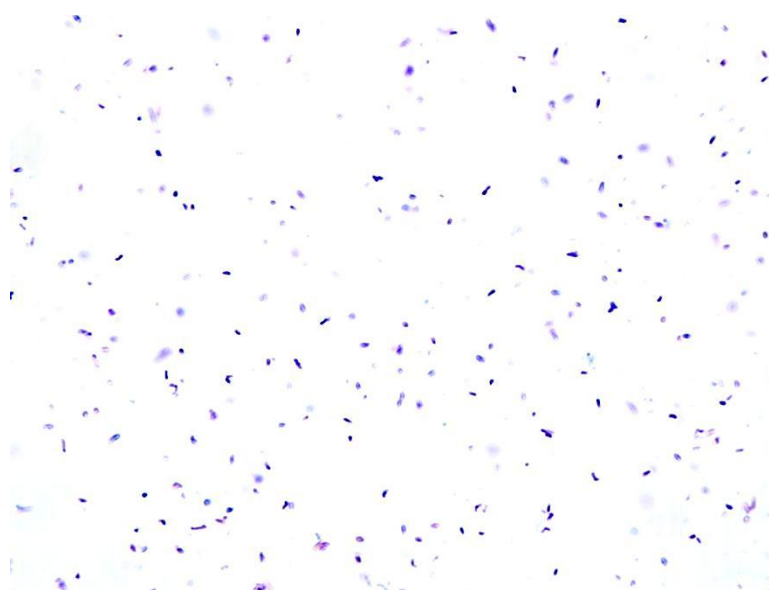


- A. Microscopic slides of mesenteric lymph nodes. 1. abundant typical giant cells (fused macrophages) and 2. macrophages
- B. Microscopic slides of mesenteric lymph nodes H&E stain: 1. single granuloma with central caseous necrosis; 2 giant cell; 3. epithelioid cells
- C. Microscopic slides of spleen H&E stain. 1. giant (Langhans) cells; 2. Fibroblasts; 3. epithelioid cells; 4. macrophages and 5. Lymphocytes
- D. Microscopic slides of mesenteric lymph nodes. 1. Giant cell; 2. fibroblasts; 3. epithelioid cells; 4. macrophages; and 5. lymphocytes

Fig.3 *M.bovis* Isolation in Lj Solid Media (Left) and Liquid Media, Bactec System (Right)



Fig.4 *M.bovis* Visualization Using the Ziehl–Neelsen Stain.



The granulomas were evident in abdominal surface of diaphragm and other abdominal visceral layers. Interestingly, there was not detected any tubercular lesion in lungs and related lymph nodes of head and respiratory system. As previously was described, the granulomas are typical lesion for bovine tuberculosis, however other pathogen may induce this condition, such as other mycobacteria and fungi, *Actinomyces* spp, neoplasma formations etc. All detected granulomas have a tendency to be encapsulated and appeared significant necrotic center. Norby *et al.*, (2004) reported that sensitivity of gross necropsy method has 86.05% sensitivity. The histological examinations show involvement of typical cells and other tissue changes. In spleen and lymph nodes were identified many giant cells, macrophages, epitheloid cells, thin capsule and typical caseous necrosis. At this stage, all detected lesions were defined as highly suspected tubercular granulomas, and most likely were due by *M.bovis*. Isolation of *M.bovis* from suspected tubercular lesions of spleen, diaphragm and mesenteric and hepatic lymph nodes confirmed bovine tuberculosis diagnosis. The typical

mycobacteria colonies were grown in solid and liquid media. Mycobacteria were grown one to 3.5 weeks faster in BACTEC system compares to classical solid LJ media (Fig 3). The ZN stain reveals presence of acid fast bacteria in microscopic slides (Figure 4). ZN staining method may be used direct in suspected tubercular tissue lesions, however its sensitivity is highly affected by number of mycobacteria present. In cattle, the *M.bovis* titer is low, however in captive and wild animals ZN staining is suitable for animals screening. Genotyping analyses identify all four isolates as *M. bovis*. Recently, molecular analyses become more popular and affordable for bovine tuberculosis diagnosis. It is important to emphasize that strain typing is highly recommended for following up the source of infections either in animals and or humans.

In conclusion, a five months' calf suffered from severe bovine tuberculosis. Typical tubercular gross lesions were widespread in all abdominal organs. The granulomatous formations were identified in histopathological examination on microscopically section stained by H&E. In

addition, typical microscopic view and related cells were observed in tissue sections of spleen and lymph nodes. *M.bovis* was recovered from affected tissues by using proper media; BACTEC system was superior compare to solid media; ZN stain revealed acid fast bacteria and isolates were typed as *M.bovis*. Based on our results and epidemiological data it is most likely that source of infection is calf's mother. Further work must be done in order to identify possible infected cattle. Screening of calf's origin farm and entirely epidemiological unit and confirmation of infected animals must be the target future work.

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