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Genotyping and spoligotyping of *Mycobacterium bovis* isolates from positive tuberculin skin test cattle in Albania

ANITA KONI[1*], SILVA TAFAJ[2], DANIELA LODA[3], M. BEATRICE BONIOTTI[3], M. LUDOVICA PACCIARINI[3], XHELIL KOLECI[1]

[1]Faculty of Veterinary Medicine, Agricultural University of Tirana-Albania

[2] National Reference Laboratory for Tuberculosis- University Clinic ″Shefqet Ndroqi″, Tirana-Albania

[3]Istituto Zooprofilattico Sperimentale della Lombardia e dell´Emilia-Romagna: Centro Nazionale di Referenza per la Tuberculosi Bovina, Brescia- Italy

*Correspondent author: anitakoni@yahoo.de

Abstract

Bovine tuberculosis is a contagious, chronic bacterial disease caused by *Mycobacterium bovis.* Typically, it is a respiratory infectious disease, but any body organ can be affected, especially well-oxygenated tissues. A very large range of domestic and wild animals are susceptible to *M. bovis*. Lack of clinical signs in the infected animals from early stage, presence of reservoirs and limitation on diagnostic methods interfere with successful control and eradication program of the disease. Differentiation of *Mycobacterium tuberculosis complex* by bacteriological and biochemical classic methods are time consuming. The last years, there are available new molecular methods used in diagnose and research and they play a crucial role in control and eradication programs of bovine tuberculosis. The aim of this study was to characterize *M. bovis* isolates by using updated molecular methods. In this study we analyzed four DNA samples extracted from *M. bovis* bacteria isolates. The bacteria culture was identified as *M. bovis* by cultural and staining methods. Molecular identification of isolates was done by a multiplex PCR assay and PCR products were visualized by electrophoresis in 2% agarose gel. The molecular characterization of bacterial DNA was performed by *gyr*B - restriction fragment length polymorphism (*gyr*B-RFLP), variable – number of tandem repeat (VNTR) typing using 12 markers ETR-A-B-C-D-E-QUBS 11a-11b-26-1895-15-3232-MIRU26 and spoligotyping.All extracted DNA samples were identified as *Mycobacterium tuberculosis complex*, species *Mycobacterium bovis*. The *gyr*B-RFLP method identified that isolates belong to *M. bovis*. Genotyping method VNTR showed 7-5-3-3-3-27/28-3-3-4-3-6-4 tandem repeaters for each specific marker in correspondence to the exact same order described previously in material and methods. In addition, results of spoligotyping assay showed that *M. bovis* isolates in Albania are characterized by SB0989 spoligotype described for the first time in Germany-Europe. This study indicates that all mycobacteria isolates belong to *Mycobacterium bovis* subsp. *bovis*. Further molecular study based on genotyping and spoligotyping methods are needed for molecular epidemiological study and to link human cases with source of infection in framework of "One Health" philosophy.

Kew words: Albania, bovine tuberculosis, *Mycobacterium bovis*, spoligotyping,

1. Introduction

Bovine tuberculosis (bTB) is a chronic bacterial disease spread worldwide due by *Mycobacterium bovis* (*M. bovis*) [5, 12]. Cattle are the main host for *M. bovis,* however other mammals, including humans can be affected. Zoonotic tuberculosis is due either by *M. bovis* or *M. caprae,* and only in 2016, both were responsible for at list 147000 new cases and 12500 human deaths [14]. Silent nature of bovine tuberculosis, as other mycobacterial disease, interfere with identifying of infected animals and explain, at list partially, failing of successful control program [2]. In the center of any control program for more than hundred years was and remain tuberculin skin test, which identify the reactors. Alongside delayed hypersensitivity skin test, many attempts are undertaken in order to develop a range of diagnostic tests for detecting the infected animals. Isolation of *M. bovis* is a gold standard test, however it takes 3-8 weeks, does not distinguish *Mycobacteria* spp and it is not appropriate to use

in live animals [10, 11, 12]. Despite that, there are developed several diagnostic tests, only few of them are efficient and recommended by OIE for bTB diagnosis. The main laboratory diagnostic methods are IDEXX *M. bovis* Ab Test (an ELISA test that identify positive animals based on humoral immunity) and Gamma interferon test (an ELISA assay wich measure the cellular mediated immunity response) [12]. Aiming to increase the overall tests sensitivity it is recommended to use blood - based *in vitro* tests in certain time after tuberculin skin test and results must be interpret as parallel tests [5]. In addition, both conventional and Real Time PCR are recommended as diagnostic tolls. Neither above mention methods do not distinguish *M. bovis* either form *M. caprae* or *M. tuberculosis.* In addition, those methods are not sufficient for epidemiological surveillance, identifying the source of infection and route of transmission. Recently, molecular methods become more common and advanced molecular methods are not only play important role in providing rapid diagnosis, but for supporting eradication programs [1, 6]. Molecular methods such as multiplex PCRs are employed for identification of isolates and discrimination of mycobacterium species [9]. The more advanced methods, those based on *gyr*B sequence polymorphism are superior and more powerful for differentiation of *Mycobacteria* spp. [4, 7]. In addition, the method based on polymorphism of direct repeaters (DR) loci, such as Spoligotyping is more sensitive compare to others and is designed exclusively for both differentiation and typing of *Mycobacterium tuberculosis complex* group [6]. This is a first study performed in the *M. bovis* bacteria isolated from cattle and aims identifying and typing of *Mycobacterium bovis* isolates based on comprehensive molecular methods.

2. Material and Methods

In this study, we used four isolates of *Mycobacterium,* identified as *M. bovis* based on cultural growth, colonies characteristics and ZN staining. The mycobacteria were isolated Lowenstein Jensen (LJ) media from tissue of positive cattle in single comparative skin test (SCST). *M. bovis* was isolated from retropharyngeal lymph node (sample 1, RF-LN), mediastinal lymph node (sample 2, Med-LN), mammary lymph node (sample 3, Mam-LN) and pulmonary lesion (sample 4, PL).

2.1. DNA extraction.

Initially, 1-2 colonies from each M. bovis isolate were transferred in eppendorfs within 500 µl TE buffer and was homogenized. Heat lyses were performed by using thermoblock PBI TD in 95°C for 15 minutes. DNA extracted was stored in freezer until molecular identification was performed (-80°C).

2.2. Molecular identification

Molecular identification was made by employing multiplex PCR method. The DNA samples were allowed to reach room temperature before they were used. The PCR was performed in a 50µl volume reaction mixture containing dNTPs 200µM, primer reverse (R) and foreword (F) 1pmole/ml, Taq DNA pol 0,05U/µl, 5µl DNA, Buffer 5X and final volume of 50µl was reached with water. There were used three pair primers, respectively: MYCGEN-F, R for *Mycobacterium* spp, TBCX-F, R for *M. tuberculosis complex* and MYGEN-F and MYCAV-R for *M. avium*. The PCR reaction was set as follow: initial cycle at 95°C for 15 minutes, DNA amplification for 40 cycles, each of them of constitute by denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, and an extension step for 2 minutes at 72°C. The amplified PCR products were run in agarose gel electrophoresis. We used agarose gel at 2% concentration by adding 1X TBE buffer (100mM Tris-base, 100mM boric acid, 2mM EDTA {pH 8.3}). The MTBC positive samples based on identifying presence of the IS6110 gene, were analyzed by PCR-RFLP of *gyr*B-gene for further distinguishing.

2.3. Molecular characterization

a. PCR restriction fragment length polymorphism of *gyr*B: Molecular characterization of bacterial DNA was further analyzed with *gyr*B- restriction fragment length polymorphism (*gyr*B-RFLP). PCR was performed in a 50µl volume reaction mixture containing 5µl DNA sample, Buffer KAPA 2G (GC buffer) 5X, dNTPs 2.5mM, primers *gyr*B R, F 10µM and for reaching final volume was added water. The reaction was set up as follow: first cycle at 94°C for 10 minutes, amplification step was done by running 40 cycles (each of them contains following steps: denaturation at 96°C for 60 seconds, annealing at 60°C for 60 seconds and elongation at

72°C for 70 seconds) and an extension cycle at 72°C for 5 minutes. The PCR product (20µl) for each sample was detect by running in 2% solution agarose gel and same amount, 20µl, of PCR gene *gyr*B products was digested with 10µl RsaI restriction enzyme. This compound was incubated for 2 hours at 37°C and was a gel electrophoresis reaction was run in 2% agarose prepared by using 1xTBE buffer.

b. Variable number of tandem repeater (VNTR) typing. For this objective, PCR reaction was performed in a 25µl volume reaction mixture containing Buffer KAPA 2G (GC buffer) 5x, dNTPs 200µM, primers 10µM for each, Taq DNA pol 5U/µl, 5µl DNA sample and for reaching final volume was added water. PCRs reactions were set in different thermal cycles depending the markers. For QUB15-3232: 1 cycle at 95°C per 15 minutes, 40 cycle, where each of them constitutes from denaturation at 94°C for 30 seconds, annealing at 55°C for 1minute, elongation step at 72°C for 2 minutes and an extension cycle at 72°C for 7 minutes. For QUB11a-QUB11b-QUB26-QUB1895: initial cycle at 95°C per 15 minutes, followed by 40 cycle, where each of them constitutes from denaturation at 94°C for 30 seconds, annealing at 60°C for 1minute, elongation step at 72°C for 2 minutes and an extension cycle at 72°C for 7 minutes. For ETR A-B-C-D-E and MIRU26: the first cycle at 95°C per 15 minutes, 40 cycle where each of them constitutes from denaturation at 94°C for 30 seconds, annealing at 60°C for 1minute, elongation step at 72°C for 2 minutes and at the end of the amplification one cycle at 72°C for 7 minutes. All PCR products were detected by 2% agarose gel electrophoresis and 1xTBE buffer. The calculation of the exact number of complete repeaters per each locus per PCR product is made using a derived allele-naming table.

c. Spoligotyping: For performing spoligotyping method a spoligo membrane is used initiated by stripping step. PCR assay was performed in a 25µl volume reaction mixture containing Buffer 1X resnova, dNTPs 200µM, primer DRa biotinilated and DRb 5pmoli/µl 0.4µM, Taq Hot Start resnova 0.05U/µl, 5µl DNA reached to final volume with water. PCR machine was set up as follow: beginning cycle at 95°C for 7 minutes, followed by 40 cycles, where each of them constitutes from denaturation at 95°C for 20 seconds, annealing at 55°C for 30 seconds, elongation step at 72°C for 30 seconds and ending by an extension cycle at 72°C for 7 minutes. Before hybridization step, 20µl of PCR product was diluted in 150µl 2 x SSPE, 0,1% SDS, incubated at 99°C for 2 minutes. Before use, spoligo membrane is washed in 2 x SSPE, 0.1% SDS at 60°C for 5 minutes and it was transferred carefully in a miniblotter system, well fixed in a strictly horizontal surface. The amplified diluted products were hybridized in a set of 43 oligonucleotides, each corresponding to a unique spacer DNA sequences within the DR locus. Hybridization assay was run at 60°C for one hour and the product was removed by aspiration, membrane was washed twice with 250ml 2x SSPE, 0,5% SDS at 60°C for 10 minutes. Membrane was incubated with 10ml solution containing 2xSSPE, 0.5% SDS, 2.5µl streptavidine-peroxidase conjugate for 90 minutes in 42°C. After incubation the membrane was washed three times; two step wash with 250 ml 2x SSPE, 0,5% SDS at 42°C for 10 minutes and a last wash with 2x SSPE for 5 minutes at room temperature by shaking. In order to detect and visualiase the hybridization, 20 ml liquid detection of chemiluminescent ECL was added for one minute and documented on film.

3. Results and Discussion

Results are presented in Table 1 and figure 1-9.

Table 1: Summary table of genotyping and spoligotyping results

Sample	ETR					QUB						MIRU 26	Spoligo specif	Nomenc-lature
	A	B	C	D	E	11a	11b	26	1895	15	3232			
RF-LN	7	5	3	3	3	27/28	3	3	4	3	6	4	13,23	SB0989
Med-LN	7	5	3	3	3	27/28	3	3	4	3	6	4	13,23	SB0989
Mam-LN	7	5	3	3	3	27/28	3	3	4	3	6	4	13,23	SB0989
PL	7	5	3	3	3	27/28	3	3	4	3	6	4	13,23	SB0989

3.1. Molecular identification

Based on multiplex PCR results, all tested samples were identified as Mycobacterium bovis. Furthermore, they have a specific molecular weight (MW), 746 base pairs, as there are demonstrated in Figure 1. This MW is typical for M.bovis

Figure 1: Electrophoresis result of PCR products from *M. bovis* isolates: **1**- RF-LN **2**-Med-LN, **3**- Mam-LN **4**- PL runed in 2% agarose gel.

3.2 Molecular characterization:

a. PCR restriction fragment length polymorphism of *gyr*B showed presence of 765 bp fragment (Fig.2) and after digestion of amplified product the fragment of 480bp was detected (Fig.3). Both fragments are typically for *M. bovis,* consequently, the samples most likely belong to *M. bovis* sub. *bovis*.

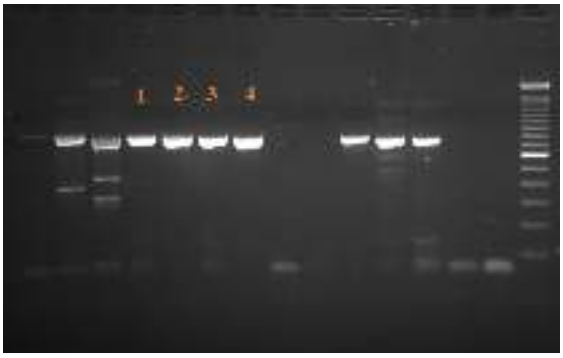

Figure 2. Results of PCR products of M.bovis gyrB runinng in 2% agarose gel electrophoresis for samples: 1- RF-LN, 2-Med-LN, 3- Mam-LN, 4- PL

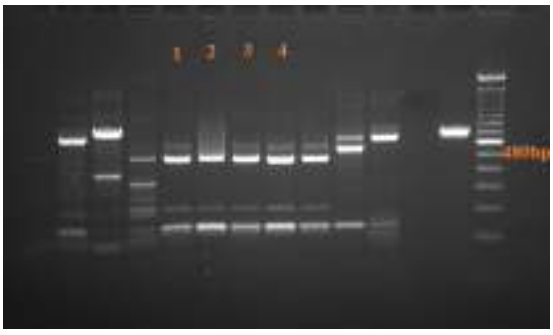

Figure 3. PCR results for evaluation of *M.bovis gyr*B digested by RsaI for samples **1-** RF-LN **2**-Med-LN, **3**- Mam-LN, **4**- PL

b. Variable number of tandem repeater (VNTR) typing: Each DNA sample was amplified with specific primers for each locus in this exact order: ETR A- B- C- D- E, QUB11a, QUB11b, QUB26, QUB1895, QUB15, QUB3232, MIRU 26. Detection of every PCR product was made by electrophoresis in 2% agarose gel

and visualized on UV transiluminator per sample (Fig 4-8). According to molecular weight of each band detected it is established the variable number of tandem repeater.

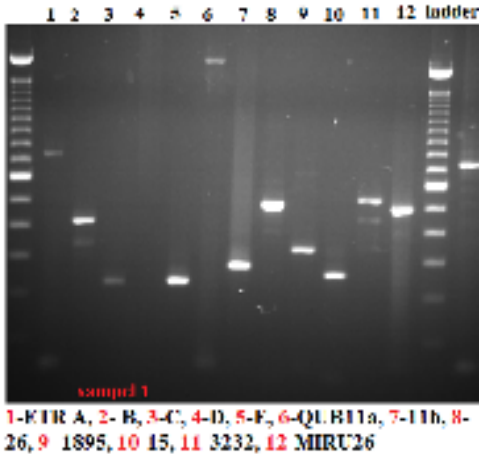

Figure 4. PCR results of VNTR of *M. bovis* isolated from RF-LN

Some of the locus were repeated because it was not clear at first their molecular weight, as it is showed in the picture above.

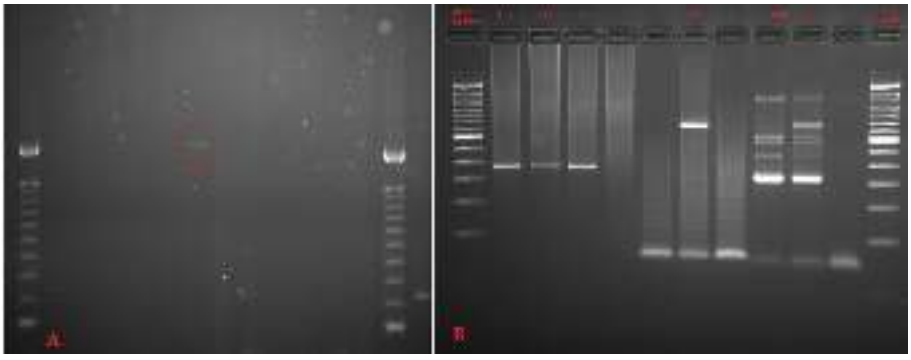

Figure 5. PCR results of VNTR of *M. bovis*: A isolated from RF-LN, locus QUB11a; **B.** Locus ETR-D of sample 1 and 3 (marked in red in top as 1D, 3D respectively). In addition in figure **B** are presented VNTR for locus QUB11a of sample 3 and 1895 of sample 4 [marked in red as 3 11a and 4(1895)]. For each tested locus are used positive and negative control, DNA Ladder marked in red on top of the figure.

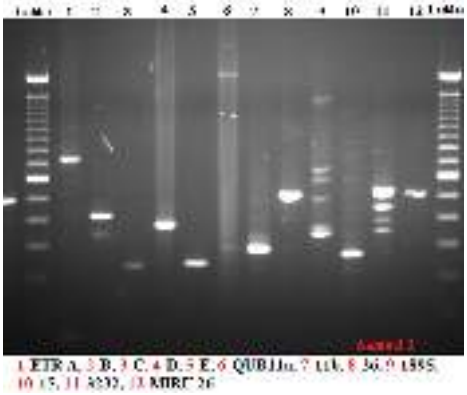

Figure 6. PCR results of VNTR of M. bovis isolated from Med-LN

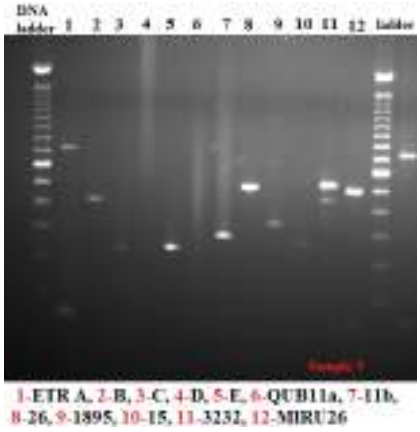

Figure 7. PCR results of VNTR of *M. bovis* isolated from Mam-LN

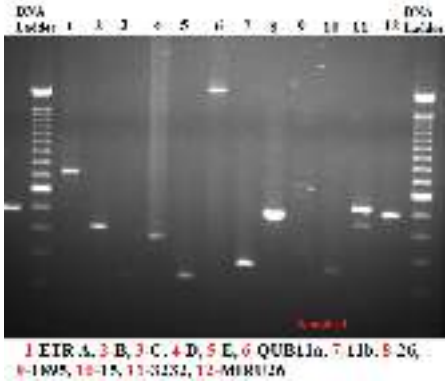

Figure 8. PCR results of VNTR of *M. bovis* isolated from PL

As a result, refering to the interpratation of all views obtained from electroforesis on 2% agarose gel (Fig.4-8), all four genomic DNA analyzed with VNTR typing, as a very sensitive molecular method, reveal the exed genotyping: 7-5-3-3-3-27/28-3-3-4-3-6-4.

c. Spoligotyping: Variability of spacers used for spoligotyping method showed Spoligo+ with specific spacers for this cluster at 3, 9, 12, 16, 23, 39-43 (Fig.9). By putting BIN code obtained in *Mycobacterium bovis* Spoligotype Database, it comes up that cluster analyzed is SB 0989, a spoligotype isolated for the first time in Germany in 2004.

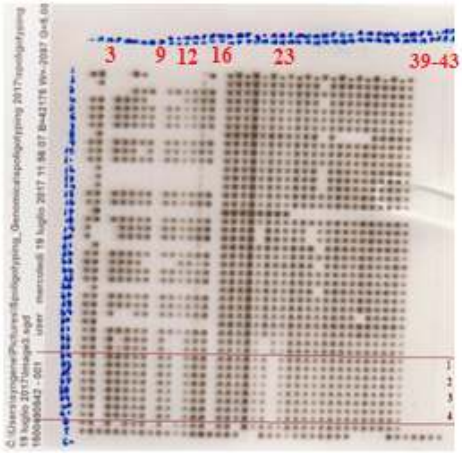

Figure 9. Spoligotyping of *M.bovis* DNAs isolates (marker in red from 1-4), represented isolates from RF-LN, Med-LN, Mam-LN, PL, documented in spoligotyping membrane. Each sample was run in double, and their pattern indicate that all samples posses specific spacer 3, 9, 12, 16, 23, 39-43 and confirm that *M. bovis* belong to SB0989 spoligotype.

Bovine tuberculosis is a zoonotic bacterial disease spread worldwide, particularly in developed countries. It is a re-emergent infectious disease in Albania and an important disease which interfere with animal and human health [8, 13]. In recent years a range of modern molecular techniques are introduced in diagnosing and research work for mycobacterial disease, including bovine tuberculosis [6]. Multiplex PCR assay play an important role for diagnosing of bTB by identifying the specific conservative genes in bacterial culture, nasal swabs, milk and other target tissues. In addition, it could discriminate *Mycobacterim* spp at species level within 24 to 36 hours. However, different PCRs assay have some limitations, particularly sensitivity and specificity when they use in tissue samples, either live animals or tissue from necropsy. To overcome the limitations of PCRs, especially for epidemiological study for following up source of infection and study the relationship between strains more advanced molecular study are required. The *gyr*B-RFLP method has improved specificity of detecting and characterizing the *Mycobacterium tuberculosis complex* strains [3]. Our study results demonstrate that isolates from tissues of tuberculin test positive animals posses specific genes for *M. bovis.* In addition, genotyping analyses show that current isolates are positive for 27/28 tandem repeaters in locus QUB11a, which represent the only identified genotype circulate in Albania. Results of spoligotyping assay indicate that analyzed *M. bovis* isolates were identified as SB0989 spoligotype described for the first time in Germany-Europe in 2004. This data shown circulating a certain genotypes of *M. bovis* subsp *bovis*, however as there are a very limited number of samples, even originate from one epidemiological unit, could not make a general conclusion for the country.

4. Conclusion

This study proved that positive animals identified by singe comparative skin test belong *Mycobacterium bovis* subsp. *bovis*. Furthermore, the certain genotype and spoligotype was identified that circulate in particular cattle subpopulation in Albania. This is the first study that empoyed advanced molecular methods and identify specific genes and genotypes of *M. bovis* in Albania, further larger and detailed study must be carried out in the future. Molecular methods must be employed as ancillary diagnostic methods and as main assay for depper molecular studies. The successfull bTB control program must integrate more collaboration between veterinary and public health as neccesary aproach in framework of "One Health".

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