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ANTIGEN EXPRESSION ENHANCEMENT OF *Mycobacterium bovis* AN5

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Abstract

The revealing value of the purified protein antigens designed for the bovine tuberculosis screening registers considerable variations between the different batches of product, even in the case of the same obtaining protocol and of the same operator.
In order to limit variations in the potency of the purified protein extract and to improve the expression of immunodominant proteins, different culture protocols have been designed, which, under reproducibility conditions, could reduce the variation limits between batches.
The study of the genetic profile identified the AN5 strain used as a virulent Mycobacterium bovis strain belonging to the bovis-caprae lineage. The potency of the purified protein extract was quantified by testing dilutions on guinea pigs. The metabolism modulation of the studied strain, by alternative culture conditions, improved its antigens profile, these being the key compounds for the potency of the tuberculin.

Key words*: AN5 strain, bovine tuberculin, guinea pigs, Mycobacterium bovis, potency.*

INTRODUCTION

Bovine tuberculosis is an infectious disease caused by M*ycobacterium bovis* that affects cattle, other domesticated animals and certain free or captive wildlife species. It is usually characterised by formation of nodular granulomas known as tubercles. Although commonly defined as a chronic debilitating disease, bovine tuberculosis can occasionally assume a more progressive course. Any body tissue can be affected, but lesions are most frequently observed in the lymph nodes (particularly of the head and thorax), lungs, intestines, liver, spleen, pleura, and peritoneum. It should be noted that other members of the *M. tuberculosis* complex, previously considered to be *M. bovis*, have been accepted as new species despite identical 16s RNA sequences and over 99.9% identity of their genome sequences. These include *M. caprae* (Aranaz et al., 2003) (in some countries considered to be a primary pathogen of goats) and *M. pinnipedii* (Cousins et al., 2003), a pathogen of fur seals and sea lions. These two new species are known to be zoonotic. In Central Europe, *M. caprae* has been identified as a common cause of bovine tuberculosis (Prodinger et al., 2005). Disease caused by *M. caprae* is not considered to be substantially different from that caused by *M. bovis* and the same tests can be used for its diagnosis.

The rigorous application of tuberculin testing and culling of reactive cattle has eliminated *M. bovis* infection from farmed bovine populations in some countries, but this strategy has not been universally successful. Extensive investigations of sporadic *M. bovis* reoccurrence have shown that wildlife reservoirs exist in some countries and can act as a source of infection for cattle, deer and other livestock. The risk that these reservoirs of infection constitute for domestic animals and humans is quite variable depending on the specific epidemiological situation for the species and the environment (Corner, 2006; Morris et al., 1994). The detection of infection in a wildlife population requires bacteriological investigation or the use of a valid testing method for the species involved (the tuberculin test is not effective in

all species) together with epidemiological analysis of information. The badger (*Meles meles*) in the United Kingdom (Wilesmith, 1991) and the Republic of Ireland (O'Reilly & Daborn, 1995), wild boar (*Sus scrofa*) in Spain (Naranjo et al., 2008), the brush-tail possum (*Trichosurus vulpecula*) in New Zealand (Animal Health Division, 1986), and several wild living species in Africa have been shown to be capable of maintaining *M. bovis* infection. Other species of *Mycobacteria*, potentially harmful to humans, such as *M. avium*, *M ulcerans*, *M. marinum* and *M. chelonae*, have been isolated from reptiles (Lupescu & Baraitareanu, 2015). Control of transmission from the wildlife population to farmed species is complex and, to date has relied on the reduction or eradication of the infected wildlife population. The use of vaccination to control the disease in some species continues to be investigated.

Although cattle are considered to be the true hosts of *M. bovis*, the disease has been reported in many domesticated and nondomesticated animals. Isolations have been made from buffaloes, bison, sheep, goats, equines, camels, pigs, wild boars, deer, antelopes, dogs, cats, foxes, mink, badgers, ferrets, rats, primates, South American camelids, kudus, elands, tapirs, elks, elephants, sitatungas, oryxes, addaxes, rhinoceroses, possums, ground squirrels, otters, seals, hares, moles, raccoons, coyotes and several predatory felines including lions, tigers, leopards and lynx (De Lisle et al., 2001; O'Reilly & Daborn, 1995).

Mycobacterium bovis has been identified in humans in most countries where isolates of mycobacteria from human patients have been fully characterised.

The official ante-mortem diagnosis relay on the tuberculin's intradermal administration in order to develop cell mediated type IV hypersensitivity, tuberculin most frequently industrially obtained from the AN5 or Vallee *M. bovis* strains.

These products' potency is currently established by statistical analysis using the parallel lines method, based on results of in vivo hypersensitivity tests carried out on guinea pigs and, to the possible extent, on the target species, as compared to the WHO (World Health Organization) reference standard (WHO International Laboratory for Biological Standards). Due to an ongoing concern for the health and welfare of laboratoy animals (Coman et al., 2019), currently, there are attempts to replace the animal tests with in vitro experiments (Spohr et al., 2015).

Tuberculin represents a complex mixture (over 800 proteins) whose production and application did not essentially change since conception (Robert Koch, 1980; Landi, 1982). The concentrate's characterization is challenging, because thermal and chemical treatments applied during production affect the proteins structure and antigenicity (Nagai et al., 1981).

The batch of bovine PPDs should be controlled for its diagnostic value, more specifically for the ability of secreted proteins and somatic components - released from autolysis, replication, mechanical and chemical interventions during production - to induce a delayed type IV cell mediated hypersensitivity reaction (Mattow et al., 2003; Sonnenberg et al., 1997). It is a well-known fact that the proteins profile of *in vitro M. bovis* cultures depends on a serie of factors, such as type of media, temperature, time, stirring, etc, which explains the differences between commercial tuberculins obtainned by different producers, or different batches of the same producer. (Sonnenberg et al., 1997; Andersen et al., 1991; Downs et al., 2012).

Also, in field studies in certain epidemiological circumstanses, differences of senzitivity and specificity have been demonstrated between PPDs produced with the same strain by different manufacturers (Rennie et al., 2010). The worldwide use for PPD production of the glycerol adapted AN5 strain, unlike field *M. bovis* strains, has led to the emergence of local variants depending on the cultivation and storage conditions of the producer. Comparative proteomics studies of different AN5 variants (UK, Brazil, Korea, Spain, Italy, Holland), but also of MTC strains (MTC, *Mycobacterium tuberculosis complex*) have proven the existence of a variable number of common proteins to *bovis* (AN5) or *tuberculosis* PPD products, but also particularities regarding the presence or expression level of *M. bovis* specific major antigens, involved in T cells activation, such as MPB70, MPB83 and ESAT-6 like (Roperto et al., 2017; Cho et al., 2015; Borsuk et al., 2009; Prasad et al., 2013; Pelayo

et al., 2009). Comparative genome analysis of the virulent and attenuated, or virulent and AN5 *M. bovis* strains, has demonstrated, on one hand, the lack of deletions or major restructuring, highlighted in the case of the BCG strain, and on the other hand, an acummulation of SNPs (single nucleotide polymorphisms), responsible for the alteration of the global gene expression profile (Pelayo et al., 2009; Canevari et al., 2014; Smith et al., 2003; Smith et al., 2006; Inwald et al., 2003).

MATERIALS AND METHODS

Bacterial strain and cultivation methods. The *M. bovis* AN5 strain was cultivated on modified Dorset-Henley (mDH) medium, with occasional returns to the glycerinated potato. A *M. avium* strain was used as a negative control for the taxonomic classification per genus/species, gene expression and, implicitly, membership of MTC.

For the purpose of the current study, the *Mycobacterium* strains were inoculated on Lowenstein-Jensen agar (LJ, Becton-Dickinson) and glycerinated potato/mDH medium at 37°C for 3 weeks, using 100µl of cellular suspension with the same density as McFarland standard 1. Colonies propagation was performed by 2 successive steps on mDH medium (in house prepared) at 37°C for 3 weeks each: small and medium volumes of 150 ml and respectively 1 liter were inoculated with few mDH adapted colonies (origin: glycerinated potato) and respectively ≈ 1-5 cm^2 of mDH aerobic culture by biofilm transfer.

Isolation and purification of nucleic acids and proteins. The ZR Fungal / Bacterial DNA Kit (ZymoReasearch - miniprep), whose protocol includes a glass beads lyses, was used to extract genomic DNA. For mRNA isolation and purification two different kits were used, both based on the phenol-chloroform and guanidine isothiocyanate extraction method: Ambion ™ TRIzol ™ Plus RNA Purification Kit (Fischer Scientific) and Direct-zol MiniPrep (ZymoResearch). Intracellular (membrane and cytosolubile) proteins were extracted (+4°C) and stored (-85°C) in a cell lyses buffer (total lyses buffer, TLB: 62.5 mM Tris pH 6.8, 2% (v/v) SDS; 10% (v/v) glycerol, 6M urea, 0.01% (w/v) blue bromophenol, 0.01% (w/v) phenol red, 5% (v/v) beta mercaptoethanol) starting from the bacterial sediment (centrifugation: 3500 g, 30 min.) generated by aerobic biofilm or anaerobic cell deposit. Proteins secreted in the culture medium were concentrated (+4°C) and stored (-85°C) in a preservation buffer (4x native buffer, 4x NB: 40% (v/v) glycerol, 0.5M Tris pH 6.8) after precipitation in ammonium sulfate (0.476% w/v) at +4°C overnight and sedimentation by centrifugation at 20000 g for 1 hour at +4°C of the entire proteins' suspension.

Preparation of M. bovis AN5 sensitizing antigen. The bacterial culture (age: 45 days post inoculation, medium: mDH; cultivation: 37°C) was thermally inactivated (1 h, 100°C), aseptically harvested and 3 times washed with a sterile phenol saline solution (5‰ v:v) by centrifugation at 1600 g for 30 min. The sediment was resuspended in a phenol saline solution (10‰ v:v) and mineral oil (Montanide ISA206/Seppic) and homogenized until a white homogenous suspension was obtained. The suspension represents the sensitizing antigen of guinea pigs used in the biological dilutions test of the bovine PPD products (potency test).

Preparation of bovine AN5 PPD. The bovine AN5 PPD was prepared following a protocol previously published (Patrascu et al., 1986), with minor modifications. A fresh inoculums (aerobic biofilm: 1-5 cm^2, age: 2-3 weeks, medium: mDH, temperature: 37°C) was seeded on mDH medium (flask: Roux plate, temperature: 37-39°C, time: ≈3 months).

Well-developed cultures were thermally inactivated (2 h - 100°C) and subjected to a sequential filtration: I. bacterial mass retention, II. medium clarification and III. tangential ultra-filtration, in order to obtain the exoproteins concentrate. Next, the exoproteins concentrate was mixed in a ratio of 1:1 (v:v) with a preservative diluents solution (1.64% (w/v) Na_2HPO_4; 0.36% (w/v) KH_2PO_4; 1% (w/v) NaCl; 20% (v/v) glycerol) and subjected to tyndallization (1 h, 65-70°C). The result represents the exoproteins fraction of the bovine PPD preparation. The filter retained bacterial mass was resuspended in a physiological saline solution (pH 7.2, 5-10% of the initial volume) and 2-3 times washed by tangential ultra-filtration. The bacterial mass was subjected to hydrolysis (2 h/100°C) after

homogenization in a 0.3-0.4% (v/v) HCl solution, using a mass:volume ratio of 1:10. Endoproteins' solubilization was accomplished by alkalization with 33% (w/v) NaOH by stirring till the pH reach 7.5. The liquid phase was filtered, concentrated and tyndallized by the same method as the medium filtrate. The result represents the endoproteins fraction of the bovine PPD preparation. Both fractions were pooled, homogenized and dispensed into flasks. The end product was tyndallized and stored at +4°C, light protected.

Molecular identification and characterization. Amplification of representative DNA sequences for the *M. bovis* species identification (e.g., *mtp*40, *lep*A, *lpq*T), *tuberculosis* complex membership (e.g. IS1081, IS6110, MPB70) and phylogenetic classification to the *bovis-caprae* lineage (e.g. MIRU-VNTR, RD) were performed according to the literature (Supply et al., 2005/2006; Reddington et al., 2011; Warren et al., 2006; Parsons et al., 2002; Mokaddas and Ahmad, 2007; Liebana et al., 1996; Mahmoudi et al., 2013; McNabb et al., 2004; Houben et al., 2009; Greisen et al., 1994) and the dedicated website recommendations (http://www.miru-vntrplus.org/MIRU), using a GeneAmp® PCR 9600 system (Applied Biosystem) and the High Fidelity PCR and Fast Start High Fidelity PCR System kits (Roche) as recommended by the manufacturer. The amplification products were visualized with ethidium bromide on TAE 1x agarose gels (Sigma-Aldrich) using a SubCell system (BioRad) and photographed with a ChemiDoc XRS + cabinet (BioRad).

Gene expression. Analysis of gene expression at mRNA level was performed for the main virulence factors, replication and structural markers according to the literature (Supply et al., 2005/2006; Reddington et al., 2011; Warren et al., 2006; Parsons et al., 2002; Mokaddas and Ahmad, 2007; Liebana et al., 1996; Mahmoudi et al., 2013; McNabb et al., 2004; Houben et al., 2009; Greisen et al., 1994), using a GeneAmp® PCR 9600 system (Applied Biosystem) and 2 Roche kits: Titan One RT-PCR system and Transcript One Step RT-PCR kit, as recommended by the manufacturer. The amplification products were visualized with ethidium bromide on TAE 1x agarose gels (Sigma-Aldrich) using a SubCell system (BioRad) and photographed with a ChemiDoc XRS + cabinet (BioRad).

Western-blot. Total cellular proteins and secreted proteins - after thermal (5min/95°C) and chemical (0.1% (v/V) beta-mercaptoethanol) denaturation - were separated by SDS - PAGE (denaturation gel electrophoresis) in 10% or 12% polyacrylamide and transferred to the nitrocellulose membrane (TransBlot Semi-Dry BioRad system, 18V-30 min) with 1x buffer solution (Towbin w. SDS/Santa Cruz, sc-24954). The major virulence factors highlighting was achieved with polyclonal antibodies (dilution 1:1000) against to MPB83 and ESAT-6 / CFP-10 binary system as follows: I. primary antibodies - rabbit anti CFP10 (abcam, ab45074 - IgG rabbit anti-ESAT-6 (abcam, ab45073 - IgG) and chicken anti MPB83 (LSBio, LS-C130858 - IgY); II. secondary antibodies - goat anti rabbit IgG - HRP (Santa Cruz Biotechnology, sc-2004 - IgG) and goat anti chicken IgY - HRP (antibodies-online, ABIN101022 - IgG).

Biological value control. The potency of the bovine AN5 PPD product was tested through the biological dilution method. The test compares the inflammatory reactions generated by intradermal (id) inoculation of a 0.1 ml/dilution (3 times: 1 to 5) of the AN5 PPD and the International Reference Standard (NIBSC - 58500 IU) at 28-35 days post sensitizing of 400-600 grams guinea pigs with 0.5 ml inactivated *M. bovis* AN5 culture, intramuscular administered (im)(OIE, 2017; European Pharmacopoeia, 04/2007; WHO International Standard Purified Protein Derivative (PPD) of *M. bovis* tuberculin). Dilutions were performed in isotonic phosphate buffer (containing 0.005% (v/v) Tween 80) and administered according to the Latin square principle, at 3 points on each side of the guinea pigs body (see experimental chart, Table 1). The test and control groups consisted of 10 animals for the bovine PPD product and respectively the reference standard, plus 5 uninoculated animals were used as negative control. After 24-48 h post-inoculation, lesion diameters were measured at each inoculation site and the PPD potency was calculated by the of parallel lines statistical method using the CombiStats v.5.0 logic program (CombiStats 5.0).

Table 1. Experimental chart of bovine PPD inoculation in guinea-pigs sensitized with inactivated *M. bovis* AN5 antigen. T, PPD test preparation (dilutions: T1 1/20; T2 1/100; T3 1/500); S, standard (dilutions: S1 1/32.5; S2 1/162.5; S3 1/812.5)

Guinea pig no.	LEFT			RIGHT		
	Head	Trunk	Tail	Head	Trunk	Tail
1	T_3	S_3	T_1	T_2	S_2	S_1
2	S_2	T_3	S_1	S_3	T_1	T_2
3	S_3	T_2	S_2	S_1	T_3	T_1
4	T_2	S_1	T_3	T_1	S_3	S_2
5	S_1	T_1	S_3	S_2	T_2	T_3
6	T_1	S_2	T_2	T_3	S_1	S_3
7	T_2	S_3	S_1	S_2	T_1	T_3
8	S_3	T_1	T_3	S_1	S_2	T_2
9	S_1	S_2	T_2	T_3	S_3	T_1

RESULTS AND DISCUSSIONS

Cultivation of bacterial strains and biological sampling. *Mycobacterium* strains originally grown on the solid medium (Lowenstein-Jensen and glycerinate potato) formed rough white-yellowish colonies, confluent after 3 weeks. Propagation on modified Dorset-Henley medium of these colonies allowed the formation of an aerobic biofilm (thickness about 1-2 cm). The aging of culture led to an anaerobic deposit formation and change of the culture medium pH (direct observation of the color change: yellow to brown). The biological sampling is described in Table 2 - initial analisys and Table 3 - readaptation of *M. bovis* AN5 strain from Lowenstein-Jensen onto glycerinated potato/mDH.

Table 2. Biological sampling for the analisys and gene expression experiments and for potency testing of AN5 bovine PPD

Strain	Sample labeling	Growth medium	Pasage	Biological sample
M. bovis AN5	1	Lowenstein Jensen	initial/tube P3	Aerobic biofilm
M. bovis AN5	2	glycerinated potato / mDH	initial /tube P2	Aerobic biofilm
M. avium	3	Lowenstein Jensen	initial /tube P3	Aerobic biofilm
M. avium	4	glycerinated potato / mDH	initial /tube P2	Aerobic biofilm
M. bovis AN5	5	mDH	intermediary/ Roux plate	Aerobic biofilm
M. bovis AN5	6	mDH	intermediary/ Roux plate	Cellular suspension (3ml)
M. avium	7	mDH	intermediary/ Roux plate	Aerobic biofilm
M. avium	8	mDH	intermediary/ Roux plate	Cellular suspension (3ml
M. bovis AN5	9	mDH	final/cultivation flask	Aerobic biofilm
M. bovis AN5	10	mDH	final/cultivation flask	Cellular suspension (3ml
M. avium	11	mDH	final/cultivation flask	Aerobic biofilm
M. avium	12	mDH	final/cultivation flask	Cellular suspension (3ml

Table 3. Readaptation experiment of *Mycobacterium bovis* AN5 strain from L-J medium to glycerinated potato

Pasage	Growth medium	Inoculum origin
(K)2	glycerinated potato (K)	Lowenstein-Jensen pasage 2
(L)3	Lowenstein-Jensen (L)	Lowenstein-Jensen pasage 2
TL(mDH)3	mDH (Erlenmeyer)	L3/K3
(K)4	glycerinated potato (K)	Lowenstein-Jensen pasage 4
(L)5	Lowenstein-Jensen (L)	Lowenstein-Jensen pasage 4
(K)5	glycerinated potato (K)	Lowenstein-Jensen pasage 5
(L)6	Lowenstein-Jensen (L)	Lowenstein-Jensen pasage 5

Molecular identification and characterization. MTC complex. The association of the AN5 strain to the *Mycobacterium* genus and the MTC complex (*M. tuberculosis* complex) has been demonstrated by amplifying the specific genus and complex sequences, such as the following loci: 16-23S ITS region (internal transcribed sequence) – specific to *Mycobacterium* genus (Mokaddas & Ahmad, 2007); IS1081 (IS, insertion sequence), IS6110, MPB70 - specific MTC, and *mtp*40 - specific *M. tuberculosis* (Liébana et al., 1996). Detection of an amplicon (405-408 bp), complementary to the ITS 16-23S region, demonstrated that AN5 strain belongs to the *Mycobacterium genus* (see Figure 1). The *mtp*40 sequence (396bp) was not detected (see Figure 2A - non-specific amplicons) for none of the samples used (*M. bovis* and *M. avium* - different media or passages). However, the target fragment of the MPB70 locus (372 bp) confirmed the *bovis* samples belong to the MTC complex (see Figure 2B - samples 2, 5, 9). In the case of *M. avium* extracts, there was an inconsistent amplification, present at the intermediate passage on the mDH medium, and

absent at the final passage used for the PPD preparation (see Figure 2B - samples 7 and 11). Similar results were generated by the amplification of the IS1081 sequence (see Figure 2C, amplicon 238bp). IS6110 amplification generated a 245bp product for all used DNA extracts (see Figure 2D), except for sample 1 (*M. bovis* AN5, passage on the Lowenstein-Jensen medium).

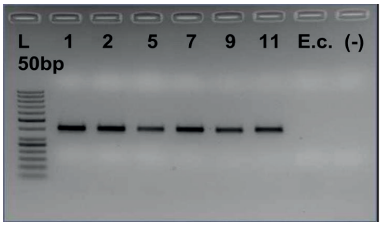

Figure 1. Identification of the *Mycobacterium* genus: 16-23 ITS / 405-408bp. The biological samples used (total genomic DNA) are numerically coded: 1 - initial passage *M. bovis* AN5 /LJ; 2 - initial passage *M. bovis* AN5 /glycerinated potato; 5 - intermediate passage *M. bovis* AN5 /mDH; 7 - intermediate passage *M. avium* /mDH; 9 - final passage *M. bovis* AN5 /mDH; 11 - final passage *M. avium* /mDH. E.C. - *Escherichia coli*; (-), only reagents; L, Ladder 50bp (Thermo Scientific); LJ, Lowenstein-Jensen medium; mDH, modified Dorset Henley medium. 2% agarose gel, 100V-30 min, TAE1x. Representative results for double experiments conducted in triplicates per biological sample

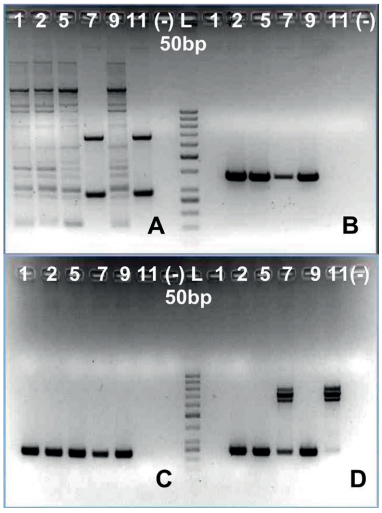

Figure 2. PCR - MTC discrimination: A - mtp40/*M. tuberculosis* (396bp) members; B - MPB70/MTC complex (372 bp); C - IS1081/MTC complex (238 bp); D-IS6110/*Mycobacterium* genus (245 bp). The biological samples used (total genomic DNA) are numerically coded: 1 - initial passage M. bovis AN5 /LJ; 2 - initial passage *M. bovis* AN5 /glycerinated potato; 5 - intermediate passage *M. bovis* AN5 /mDH; 7 - intermediate passage *M. avium* /mDH; 9 - final passage *M. bovis* AN5 /mDH; 11 - final passage *M. avium* /mDH. (-), only reagents; L, Ladder 50bp (Thermo Scientific); LJ, Lowenstein-Jensen medium; mDH, modified Dorset Henley medium. 2% agarose gel, 100V-45 min, TAE1x. Representative results for double experiments conducted in triplicates per biological sample

Bovis-caprae lineage. The differentiation between the zoonotic pathogens of the *bovis-caprae* evolutionary lineage (Reddington et al., 2011) is based on the amplification of the *lep*A (elongation factor/bacterial DNA replication), *lpq*T (essential lipoprotein/in vivo mycobacterium growth) and RD1 (region of difference 1, absence to *M. bovis* BCG strains) (Reddington et al., 2011; Qin et al., 2006; Rezwan et al., 2007; Sassetti & Rubin, 2003; Huard et al., 2006; Behr et al., 1999). The specific primers for *Mycobacterium caprae lep*A sequence determined the amplification of a 155 bp fragment for all *bovis* extracts but not for *avium* ones (negative control/species, see Figure 3A). The *bovis-caprae* lineage was confirmed for the *M. bovis* AN5 strain by usage of *lpqT* specific sequences that generated a 141 bp amplicon (see Figure 3B). The presence of the differentiation region 1 in the *M. bovis* strain genome (see Figure 3C, amplicon 117bp) indicates virulence stability reckless of the number of passages, both on solid media (LJ and glycerinate potato) or liquid media (mDH).

VNTR- RD profile. The degree of phylogenetic relatedness of the AN5 strain with *M. bovis* circulating strains has been established by amplification of 24 MIRU-VNTR regions (mycobacterial interspersed repetitive units - variable number tandem repeats) and 8 RDs (region of difference), an essential character for the diagnostic value of the bovine PPD product (Ghielmetti et al., 2017; Afaghi-Gharamaleki et al., 2017; Supply et al., 2007; Warren et al., 2006; Parsons et al., 2002). The amplification conditions of the MIRU-VNTR and RD sequences are those previously published, using the Roche PCR kits (Warren et al., 2006; Parsons et al., 2002; Supply, 2005). The designation of the MIRU-VNTR alleles took into account the amplicon size and the repeating unit length according to the known data (see Table 4). For MIRU 20 and 24 loci (as well for RD 5 and 11 regions no amplicons were obtained (see Table 4; Afaghi-Gharamaleki et al., 2017; Sun et al., 2012; Jeon et al., 2008; Iwamoto et al., 2007). The AN5 strain membership to the *bovis* virulent type has been determined based on the VNTR-RD profile according to the published algorithm: www.miru-vntrplus.org, as compared to the declared profile of registered *Mycobacterium* strains.

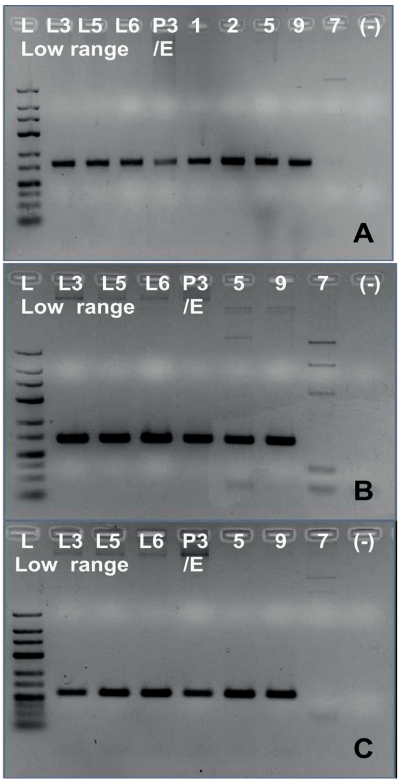

Figure 3. PCR - *Bovis-caprae* lineage: A - *lep*A/ *M. caprae* (*155bp*); B - *lpq*T/*M. bovis*, *M. bovis BCG* and *M. caprae* (*141bp*); C - RD1/complex MTC (*117bp*). The used biological samples (total genomic DNA) are encoded in letters and numbers: L3 - *M. bovis* AN5 strain/passage 3/LJ; L5 - *M. bovis* AN5 strain/passage 5/LJ ; L6 - *M. bovis* AN5 strain/passage 6/LJ; P3/E - *M. bovis* AN5 strain /passage 3/mDH; 1 - *M. bovis* AN5 primary passage/LJ; 2 - *M. bovis* AN5 primary passage/glycerinate potato; 5 - intermediary passage *M. bovis* AN5/mDH; 7 - intermediary passage *M. avium*/mDH; 9 - final passage *M. bovis* AN5 /mDH. (-), only reagents; L, ladder Low range (Thermo Scientific); LJ, Lowenstein-Jensen medium; mDH, modified Dorset Henley medium. 2.5%, agarose gel, 100V-45 min, TAE1x. Representative results for double experiments conducted in triplicates per biological sample

Table 4: MIRU-VNTRs and RDs profile of *M. bovis* AN5 strain (n.a., not applicable/not amplified)

No.	Locus	Repetitive unit (bp)	Amplicon (bp)	Allele
1	MIRU 2	53	380	2
2	Mtub 04 / 42	51	600	1
3	ETR C / 43	58	440	5
4	MIRU 4 / ETR D	77	404	3
5	MIRU 40	54	462	2
6	MIRU 10	53	550	1
7	MIRU 16	53	670	2
8	Mtub 21 / 1955	57	270	3
9	MIRU 20	77	n.a.	
10	QUB-11b	69	620	1
11	ETR A	75	630	6
12	Mtub 29 / 46	57	500	3
13	Mtub 30 / 47	58	480	4
14	ETR B / 48	57	620	5
15	MIRU 23	53	350	4
16	MIRU 24	54	n.a.	
17	MIRU 26	51	480	4
18	MIRU-27 / QUB-5	53	700	4
19	Mtub 34 / 49	54	450	2
20	MIRU 31 / ETR E	53	620	3
21	Mtub 39 / 52	58	380	2
22	QUB-26	111	200	2
23	QUB-4156 / 53	59	500	2
24	MIRU 39	53	650	2
25	RD1	n.a.	117	present
26	RD3	n.a.	500	present
27	RD4	n.a.	172	absent
28	RD5	n.a.	n.a.	absent
29	RD9	n.a.	108/206	absent
30	RD10	n.a.	202	absent
31	RD11	n.a.	n.a.	absent
32	RD12	n.a.	306	absent

Gene expression. RNA samples extracted from the aerobic biofilm or anaerobic deposit were subjected to reverse transcription in order to study the gene expression of the major structural proteins and replication markers as well as of some virulence factors. The gene expression level was normalised by amplification of a short 16S*r*DNA fragment (120bp), according to literature data (see Figure 4A) (Greisen et al., 1994). Starting from the premise of stable expression along the passages on solid or liquid media, we chose the *hsp*65 gene (heat shock protein 65 kDa) as representtative marker of mycobacteria (McNabb et al., 2004). The result of the experiment (amplicon of 441 bp), developed under the published conditions, reinforced the information obtained previously by the 16S*r*RNA fragment revertranscription (see Figure 4B). In the case of the *lepA* elongation factor (see Figure 4C) and lipoprotein *lpqT* (see Figure 4D) - essential elements in the multiplication of mycobacteria in vitro and in vivo – could be seen an accumulation of the mRNA molecules along the passages, especially on the mDH liquid medium (see sample 5 - passage 3 / mDH) (Qin et al., 2006; Rezwan et al., 2007; Sassetti & Rubin, 2003).

In the case of virulence factors, we analyzed few elements involved into pathogen dissemination (MPB70/Figure 5A), required for inhibition of phagolysosomal fusion (pknG/Figure 5B) and bacteria release from phagosome to the cytoplasm at the late stages of infection (ESAT-6/Figure 5C; CFP10 /Figure 5D).

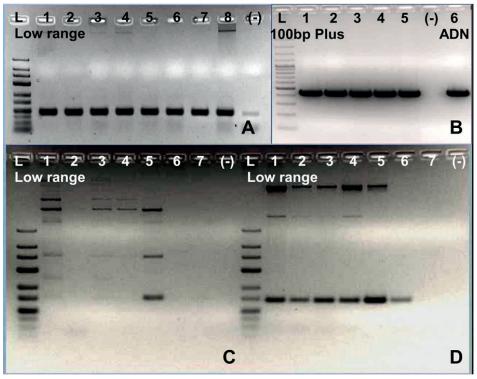

Figure 4. RT-PCR structural marker/*Mycobacterium* replication (genetic material: total RNA). A. 16S*r*RNA/eucariote bacteria (*120bp*): 1- inoculums; 2 - aerobic biofilm (passage 1/mDH);3 - anaerobic deposit (passage 1/mDH); 4 - aerobic biofilm (passage 1/glycerinated potato); 5 - aerobic biofilm (passage 2/glycerinated potato); 6 - aerobic biofilm (passage 4/ glycerinated potato; 7 - aerobic biofilm (passage 5/glycerinated potato); 8 - aerobic biofilm (passage 3/ mDH); B. *hsp65*/universal *Mycobacteria* (*441bp*): 1- inoculum; 2 - aerobic biofilm (passage 1/mDH); 3 – anaerobic deposit (passage 1/mDH); 4 - aerobic biofilm (passage 1/ glycerinated potato); 5 - aerobic biofilm (passage 3/mDH); 6 - DNA / aerobic biofilm (passage 1/LJ); C: *lep*A/specific M. caprae (*155bp*) si D: l*pq*T/ specific *M. bovis, M. bovis* BCG and *M. caprae* (*141bp*): 1- inoculum; 2 - aerobic biofilm (passage 1/mDH); 3 - anaerobic deposit (passage 1/mDH); 4 - aerobic biofilm (passage 1/glycerinated potato); 5 - aerobic biofilm (passage 3/mDH); 6 - aerobic biofilm (passage 4/ glycerinated potato); 7 - aerobic biofilm (passage 5/glycerinated potato). Inoculums - cells separated by centrifugation after washing with diluent/protocol Becton Dickinson. L, ladder Low range and 100bp Plus (Thermo Scientific); (-), only reagents; mDH, modified Dorset Henley medium; LJ, Lowenstein-Jensen medium. 2.5% agarose gel, 100V-45 min. Representative results for double experiments conducted in triplicates per biological sample.

Amplification of the messenger RNA (mRNA) corresponding to MPB70 (372bp) and *pkn*G (321bp) proteins, developed according to the previously published conditions, demonstrated a high transcription capacity of the AN5 strain regardless of passages number (1 to 3, see Figure 5A), nature of the culture medium (liquid/mDH or solid/ glycerinated potato, see Figure 5A) or the growing period (4-12 weeks, see Figure 5B), revealing its virulent feature (Liébana et al., 1996; Houben et al., 2009; Wiker 2009; Charlet et al., 2005). The mRNA sample extracted from the 4 weeks culture did not generated a *pkn*G amplicon. Reverstranscription of ESAT-6/CFP-10 heterocomplex members generated an amplicon only for the CFP-10 sequence (*300 bp*; see Figure 5D) (Mahmoudi et al., 2013). Taking into consideration that both open reading frame (ORF) are under the control of the same promoter, probably we are dealing with a false negative result (see Figure 5C) due to mutations accumulated in the primer binding sequence, data supported by western-blot heterocomplex detection.

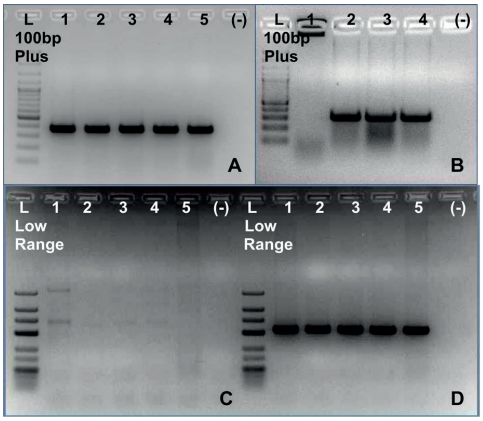

Figure 5. RT-PCR Mycobacterium virulence factors (genetic material: total RNA). A: MPB70/*Mycobacterium bovis* 70 kDa specific *M. bovis* protein (*372bp*); B: *pkn*G/serine-threonine kinase G (*321bp*); C: ESAT-6/early secretory antigenic target 6kDa (*340bp*); D: CFP-10/culture filtrate protein 10kDa (*300bp*). A, C, D: 1- inoculums; 2 - aerobic biofilm (passage 1/mDH); 3 - anaerobic deposit (passage 1/mDH); 4 - aerobic biofilm (passage 1/glycerinated potato); 5 - aerobic biofilm (passage 3/mDH); B: aerobic biofilm/mDH - 1/4 weeks culture; 2/6 weeks culture; 3/8 weeks culture; 4/12 weeks culture. L, Ladder 100bp Plus and Low range (Thermo Scientific); (-), only reagents; mDH, modified Dorset Henley medium. 2.5% agarose gel, 100V-45 min, TAE1x. Representative results for double experiments conducted in triplicates per biological sample

<u>*Translational capacity.*</u> For the correct evaluation of the protein synthesis process of the main virulence factors - especially MPB83 (M70 high structural homology) and ESAT-6/CFP-10 (heterodimer) binary system - intracellular protein extracts (concentrated in total lysis buffer/TLB) and extracellular or secreted proteins (precipitated with ammonium sulfate/$(NH_4)_2SO_4$ - data not shown) were electrophoretically separated into the denaturing polyacrylamide gel and hybridized with polyclonal antibodies specific for M. bovis immunodominante antigens (see Fig.6). Western blot experiments revealed the presence of MPB83 exclusive in the M. bovis samples (see Figure 7A) and the ESAT-6/CFP-10 complex in all Mycobacterium extracts (see Figure 6B/ESAT- 6 and Figure 6C/CFP-10).

The MPB83 lipoprotein was major identified as the intact 26 kDa form and minor as a 23 kDa N-terminal truncate product (see Figure 6A) for all tested passages (intracellular extract/ TLB, sample 9) (Wiker et al., 1991; Harboe et al., 1998). The partners of the ESAT-6/CFP-10 heteroduplex can be detected intracellular, especially in the multimeric active form at approximately 60 kDa (see Figure 6B and Figure 6C, respectively). Consistent with information gained from gene expression experiment, ESAT-6 has not been identified as a monomer or glycosylated form (see Figure 6B) and instead CFP-10 has been highlighted in all maturation stages: about 25 kDa and 30 kDa with intracellular accumulation to the high passages (see Figure 6C, samples 7 and 9). In contrast to MPB83, members of the RD1 differentiation region are detectable independently of the analyzed species (*bovis* or *avium*). The proteins expression of *M. bovis* AN5 strain at passage 1 on the Lowenstein-Jensen medium is much diminished to undetectable in contrast to the productivity of the same strains on the glycerol potato medium.

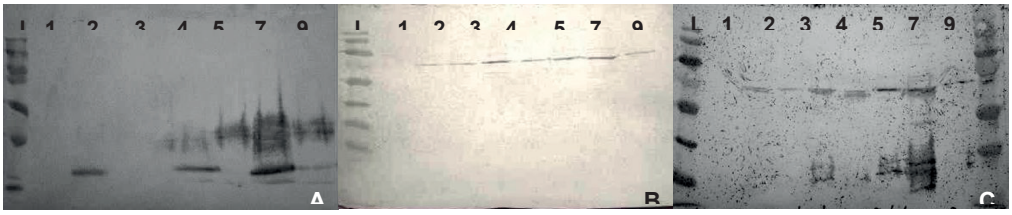

Figure 6. Western Blot of virulence factors. A. Anti-MPB83 (1:1000 dilution) - *26kDa* (intact form) and *23kDa* (N terminal truncated fom); B. Anti-ESAT-6 (dilution 1:1000) - 6kDa/monomer (undetectable) and approximately *60kDa* ESAT-6/CFP-10 hetero-complex; C. Anti-CFP-10 (1:1000 dilution) - *10kDa*/monomer (undetectable), *25kDa* and *30kDa* / probable glycosylated forms and approximately *60kDa* ESAT-6/CFP-10 hetero-complex. A, B, C: 1 - initial passage *M. bovis* AN5 /LJ; 2 - initial passage *M. bovis* AN5/glycerinated potato; 3 - initial passage *M. avium* /LJ; 4 - initial passage *M. avium* /glycerinated potato; 5 - intermediate passage *M. bovis* AN5/mDH; 7 - intermediate passage *M.avium* /mDH; 9 - final passage *M. bovis* AN5 /mDH; 11 - final passage *M. avium* /mDH. L, PageRuler Prestain Protein Ladder/Thermo Scientific; LJ, Lowenstein-Jensen medium; mDH, modified Dorset Henley medium; 10% SDS-PAGE, 120V, 90 min., Tris-glycine-SDS 1x migration buffer. Representative results for double experiments conducted in triplicates per biological sample

<u>*Biological value - PPD M. bovis AN5.*</u> The relative potency of the PPD is determined by analogy with the declared potency of the international standard (NIBSC: 58500 IU). According to the European Pharmacopoeia, this value should be between 66% and 150% when testing on guinea pigs. Following in vivo testing of the AN5 protein concentrate (origin: passage 3/mDH; filter: 0.45 µm; dilution - 1:1 with saline phosphate buffer plus 0.005% Tween 80), the diameters of the inflammatory reactions were measured at 24 hours post inoculation and a relative potency value of 72% was obtained by statistical calculation (soft: CombiStats v. 5.0, model: parallel lines, design: Latin square).

The *in vivo* testing of the bovine PPD preparation, obtained by concentration of intra- and extra-cellular proteins after sequential propagation of the AN5 strain, showed an estimated potency value of 72% by comparing to the international NIBSC standard (58500 I.U.), hovering in interval of 66-150% stipulated by the European Pharmacopoeia.

In the present study we have demonstrated that, despite the presence of major antigens as MPB70/MPB83, stably expressed, the biological value of a bovine PPD, "tuberculin B" reagent, involves the synthesis of several other virulence factors, as ESAT-6/CFP-10, influenced by some cultivation factors like culture medium type, passages number, cell density etc.

CONCLUSIONS

The genetic profile of an *M. bovis* AN5 strain based on 24 repetitive sequences and 8 differentiation regions, classifying and confirming it as virulent *M. bovis*, has been achieved for the first time, by our knowledge, in this work.

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