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Mycobacterium bovis ESAT-6, CFP-10 and EspC antigens show high conservation among field isolates

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ABSTRACT

ESAT-6, CFP-10 and EspC are virulence factors that have been extensively assayed for bovine and human tuberculosis diagnosis due their potent T-cell inducing activities. While polymorphisms of ESAT-6 and CFP-10 were analyzed, with the description of CFP-10 variants in *M. tuberculosis*, this fact has not been explored in *M. bovis* field isolates. The coding sequences of *esxA* (ESAT-6), *esxB* (CFP-10) and *mb3645c* (EspC) from 58 M. *bovis* strains exhibiting genomic variability (spoligotyping) were analyzed. Two genes –*esxA* and *esxB* – remained invariant while *mb3645c* exhibited one synonymous polymorphism (G to A mutation, position 66bp) in one isolate, compared to *M. bovis* AF2122/97 reference strain. All isolates exhibited a synonymous nucleotide polymorphism simultaneously (G to A mutation, position 255bp), compared to *M. tuberculosis* H37Rv reference strain. This study confirms the high conservation for ESAT-6, CFP-10 and EspC in local *M. bovis* field isolates and reinforce the use of these three antigens in the diagnosis of bovine tuberculosis. Further studies should be performed to globally confirm these findings.

1. Introduction

Bovine tuberculosis (bTB) is a chronic disease caused mainly by *Mycobacterium bovis* (*M. bovis*). This pathogen is genetically related to the main etiological agent of human tuberculosis, *Mycobacterium tuberculosis* (*M. tuberculosis*), both sharing a 99.95% identity at the nucleotide level [1]. Three proteins, ESAT-6 (Mb3905/Rv3875), CFP-10 (Mb3904/Rv3874) and EspC (Mb3645c/Rv3615c) codified by both genomes, have been extensively characterized as recombinant diagnostic reagents due to their role as specific potent T-cell inducers. The use of these three antigens in protein or peptide cocktails has been extensively tested, demonstrating their contribution in the diagnosis of human and bTB [2,3]. The coding sequences of the ESAT-6, CFP-10 and EspC proteins are located in *esxA*, *esxB* and *mb3645c/rv3615c* genes, respectively; sharing *esxA* and *esxB* a 100% and mb3645c/rv3615c a 99.7% similarity in both *M. bovis* AF2122/97 and *M. tuberculosis* H37Rv reference strains according to sequences deposited in the *Bovilist* and *Tuberculist* databases (*Genolist Database*). Nishibe and coworkers compared *M. tuberculosis* H37Rv and several *M. bovis* strains (AF2122/97, AN5, and a field isolate named 04–303). They confirmed the conservation of *esxA* and *esxB* genes, among H37Rv, AF2122/97 and AN5 strains but detected a threonine to alanine substitution (99% identity) among them and *M. bovis* 04-303, a field strain isolate from a wild boar in Argentina characterized as hypervirulent in animal models [4,5].

Considering that ESAT-6 and CFP-10 are relevant diagnostic antigens and also key virulence factors in bovine and human tuberculosis, some investigations focused on the study of polymorphisms in *M. tuberculosis* from humans. Musser and coworkers studied 24 genes coding for targets of the host immune system in 16 M. *tuberculosis* strains from ethnically diverse human patients in Texas, USA, showing that neither ESAT-6 nor CFP-10 exhibited mutations at the nucleotide level [6]. Similarly, Davila and coworkers examined the ESAT-6 coding sequence

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in 88 M. *tuberculosis* clinical isolates obtained from the USA and Turkey and did not find polymorphisms associated with the *esxA* gene [7]. Uplekar and coworkers performed a similar study but including 118 M. *tuberculosis* clinical isolates from different geographical areas such as North Europe, Africa, the Americas and Asia. They found two single nucleotide polymorphisms (SNPs) in the CFP-10 coding sequence encompassing a cellular epitope in a group of 19 isolates. However, the ESAT-6 coding sequence remained invariant in the 118 isolates analyzed, in concordance with previous reports [8].

In spite of *M. tuberculosis* and *M. bovis* being related pathogens, they have evolved to infect different hosts. Antigenic variation in ESAT-6, CFP-10 and EspC has not been explored in *M. bovis* among field isolates. Only one case has been reported, as mentioned above, in a *M. bovis* field isolate (04–303) [4].

Evidence of microevolution events in *M. bovis* were described in herds chronically infected for over 12 months, a relatively short time of infection. This study revealed the emergence of related clonal variants in a strain-dependent manner, including genomic regions with potential functional significance [9]. Previous studies in Argentina showed variability in *M. bovis* molecular genotypes (spoligotypes) among field isolates obtained from bovine granulomatous lesions, some of them associated with hypervirulent phenotypes [10].

Taking into account that *M. bovis* field isolates can experience variation at the nucleotide level and the key role of ESAT-6, CFP-10 and Esp-C for diagnosis and pathogenesis, the aim of this study was to assess polymorphisms associated with the genes codifying ESAT-6, CFP-10 and Esp-C proteins in local *M. bovis* isolates that displayed different spoligotypes.

2. Material and methods

2.1. Features of isolates included in the study

A total of 58 M. *bovis* strains from the main dairy regions of Argentina were analyzed, all of them isolated from lesions in bovines compatible with bTB observed during slaughterhouse inspection. They were sampled as part of a previous study, in which all isolates were confirmed as *M. bovis* by PCR for the insertion sequence (IS) *6110* and spoligotyping characterization [11] in our laboratory [10]. Sampling was performed in different slaughterhouses of Buenos Aires, Santa Fe and Cordoba provinces. To select a variable population strains, *M. bovis* isolates exhibiting different spoligotypes patterns were included as evidence of genomic variability. Furthermore, as ESAT-6, CFP-10 and EspC proteins are virulence factors, additional variation related to virulence phenotypes were weighted through the type and dissemination of macroscopic lesions observed during the slaughterhouse inspection in animals from which the strains were isolated. Details for procedures and protocols regarding spoligotyping and pathology score were previously described [10].

2.2. PCR amplification of M. bovis esxA, esxB and mb3645c genes.

Template lysates for PCR reactions were prepared by picking a colony former unit (CFU) in 200 µL of free DNase water and boiled at 95 °C for 30 min. Lysates were clarified by centrifugation at 12,000 rpm for 10 min and the supernatants were used as template. In all cases, a high-fidelity DNA polymerase (EasyPfu® DNA polymerase, AP-Biotech) was used. Primers were designed in order to amplify the complete coding sequence for *esxA*, *esxB* and *mb3645c* genes. Primers used were as follows: for the *esxA* gene (encoding the ESAT-6 protein), *esxA*fLM/*esx*ArLM (AGAGATCTCATGACAGAGCAGCAGTGGAATTTC/GTTGGATCCTGCGAACATCCCAGTGACG); for the *esxB* gene (encoding Cfp-10 protein) *esxBf/esxBr* (TGACAACAGACTTCCCGG/CGATACCCGCGAAATTC); and for the *mb3645c* gene (encoding EspC protein) *mb3645cf/mb3645cr* (GGATCCATGACGGAAAACTTGAC/AAGCTTTCAGGTAAACAACCCGT). The PCR reaction mixture was carried out in a final volume of 50 µL under the following conditions: 35 µL of DNAase free sterile water, 5 µL of 10X EasyPfu buffer, 1 µL of each deoxyribonucleoside triphosphate (10 mM), 1.5 µL of each primer (10 µM), 1 µL of EasyPfu® DNA polymerase (AP-Biotech) and 5 µL of template (lysate). The PCR reactions were conducted as follows: one cycle at 94 °C for 2 min; 30 cycles at 94 °C for 30 s (60 °C for *esxA*, 55 °C for *esxB* and 54 °C for *mb3645c*) for 30 s, 72 °C for 1 min; with a final extension of 72 °C for 8 min. PCR products (288bp for *esxA*, 450bp for *esxB* and 312bp for *mb3645c*) were visualized by electrophoresis in a 1.2% agarose gel in 1 X Tris-borate-EDTA buffer (45 min at 100 V). PCR products were purified and then the DNA was quantified by a micro spectrophotometer (NanoDrop).

2.3. DNA sequencing, sequence edition and polymorphism analysis

The PCR products were sequenced to assess the presence of insertions/deletions or SNPs in the coding sequence of *esxA*, *esxB* and *mb3645c* genes of the *M. bovis* isolates included in the study. The sequencing reaction was performed by the fluorescent terminator method in capillary electrophoresis, using Big Dye Terminates v3.1 chemistry and automatic capillary sequencers Genetic Analyzer 3500XL (Applied Biosystems), encompassing double reading frame. The sequences were optimized using the program *Sequencher 4.8* (Gene Codes Co, USA) with the *M. bovis* AF2122/97 strain as reference sequence (GenBank accession no. LT708304). Alignment was performed with the program *Bioedit Sequence alignment Editor Version*7.0.5.3. Sequences generated were deposited in the Genebank database with the following accession no: **MG655378-MG655387**, **MG655389-MG655393**, **MG655395-MG655401**, **MG655403-MG655417**, **MG655419-MG655422**, **MG655425-MG655427**, **MG655429**, **MG655430**, **MG655432**, **MG655433**, **MG655436**, **MG655437**, **MG655439-MG655444**, **MG655446** and **MG655447** for *esxA* coding sequences; **MG655518-MG655531**, **MG655533**, **MG655536**, **MG655538-MG655543**, **MG655545-MG655558**, **MG655560**, **MG655562-MG655565**, **MG655567**, **MG655568**, **MG655570-MG655585** for *esxB* coding sequences; and **MG655448-MG655455**, **MG655457-MG655468**, **MG655470-MG655480**, **MG655483**, **MG655484**, **MG655487-MG655492**, **MG655494**, **MG655496-MG655509**, **MG655511**, **MG655512** and **MG655514-MG655516** for *mb3645c* coding sequences.

3. Results

3.1. Genomic variability of M. bovis isolates

A total of 58 M. *bovis* isolates were selected with different spoligotypes. Therefore, out of the 58 isolates included in the present study, 57 had been classified by 12 different spoligotypes [10], with the most frequent spoligotype being SB0140 (41.4%), followed by SB0145 (24.1%). The remaining ones were detected in three isolates (SB0120, SB0484 and SB0130), in two isolates (SB0131, SB0273, and SB0820) and in one isolate (SB0153, SB0269, SB1033 and SB1055). One isolate remained unclassified.

3.2. Polymorphisms analysis of M. bovis isolates in the coding sequence of esxA, esxB and mb3645c genes

A total of 58 M. *bovis* isolates were included in the present study to investigate the presence of insertions/deletions or SNPs in the *esxA*, *esxB* and *mb3645c* genes encoding ESAT-6, CFP-10 and EspC proteins, respectively.

The nucleotide composition of the codifying sequences showed that *esxA* and *esxB* remained invariant when compared to the *M. bovis* AN5 strain (which is used to produce the protein purified derivative for the intradermal tuberculin skin test), *M. bovis* AF2122/97 and *M. tuberculosis* H37Rv.

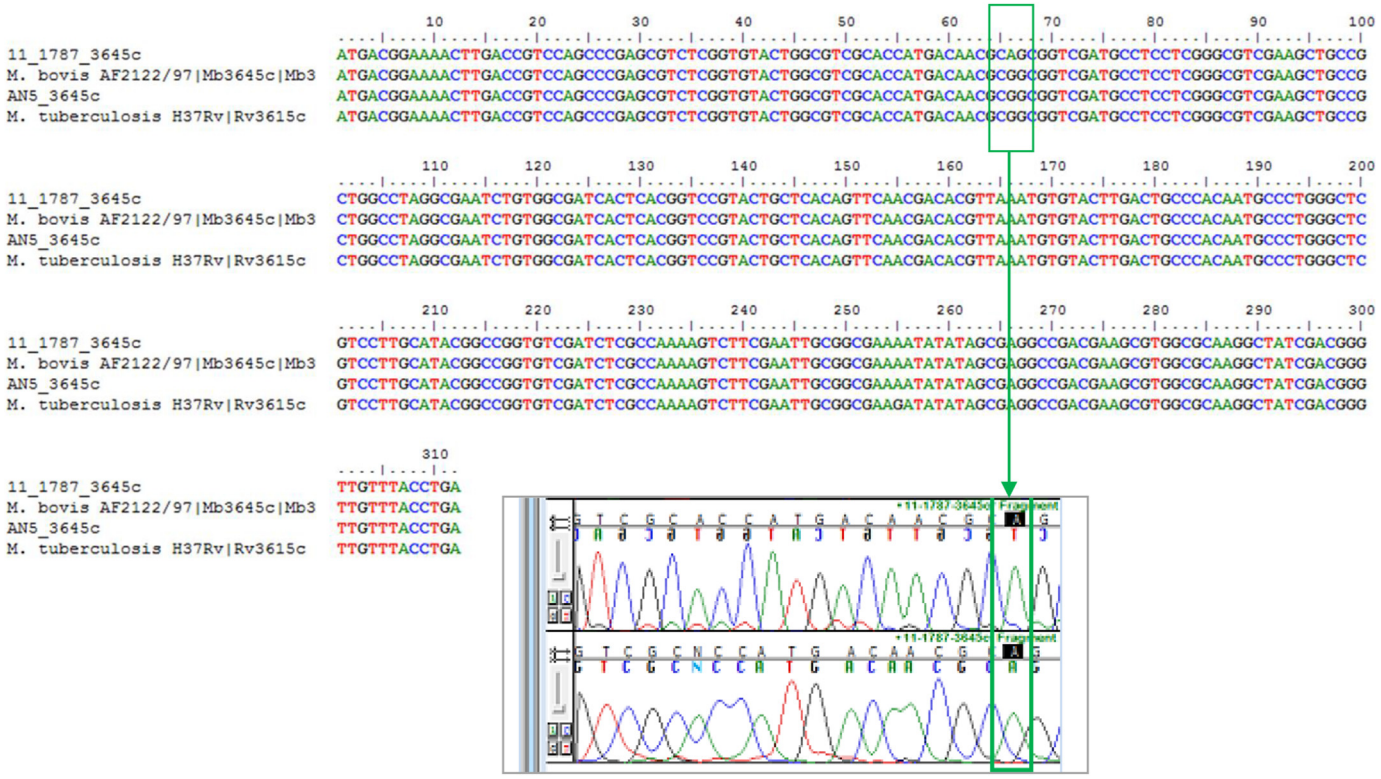

Fig. 1. *M. bovis* alignment of complete coding sequence for the Mb3645c gene. A. Alignment of complete nucleotide coding sequence for *mb3645c* gene (312bp) including the only one field isolate (11–1787, GeneBank accession no. **MG655484**) exhibiting a SNP at the 66bp nucleotide position. The alignment also included two *M. bovis* reference sequences (AN5 and AF2122/97) and *M. tuberculosis* H37Rv reference strain. The arrow indicates the chromatogram obtained for 11–1787 isolate encompassing the region where the SNP was detected. The window shows the overlapping regions of sequencing the gen in both senses.

In the case of *mb3645c* gene, only one isolate (named 11–1787) exhibited a synonymous SNP, encompassing a G to A SNP, located at position 66bp of the codifying sequence, when compared to the *M. bovis* AN5, *M. bovis* AF2122/97 and *M. tuberculosis* H37Rv reference strains (Fig. 1). In addition, all *M. bovis* sequences including field isolates and reference strains showed a synonymous substitution (G to A SNP) in the position 255bp for the gene *mb3645c* when compared to *M. tuberculosis* H37Rv (Fig. 1).

4. Discussion

In this study, *esxA*, *esxB* and *mb3645c* complete codifying sequences of local *M. bovis* field isolates were analyzed to identify polymorphisms that could potentially impact the immunity associated with ESAT-6, CFP-10 and EspC antigens. Our results suggest that both, the coding sequences of the genes *esxA*, *esxB* and *mb3645c* are highly stable and consequently their deduced ESAT-6, CFP-10 and EspC protein sequences. These antigens remained invariant despite the 58 M. *bovis* isolates were classified as twelve different molecular spoligotypes. Although previous works performed in the human adapted counterpart *M. tuberculosis* reported non-synonymous SNPs in the *esxB* gene encompassing a cellular epitope in CFP-10 [8], other studies carried out in different geographical areas showed that *esxA* and *esxB* are highly conserved among *M. tuberculosis* isolates [6,7].

Regarding EspC protein, this is part of the *espACD* operon, which is restricted to pathogenic mycobacteria. A protein alignment reported previously, including one sequence of *M. tuberculosis*, one of *Mycobacterium marinum* and one of *Mycobacterium leprae* EspC proteins, have shown that 45 out 103 amino acid residues of EspC are conserved including the YxxxD/E motif (Y87-D91), which is involved in the interaction with other proteins [12]. However, there are no previous reports for searching polymorphisms of the gene or even at the protein level among *M. tuberculosis* and *M. bovis* isolates from humans or tuberculous bovines, respectively. In the present study, all 58 isolates analyzed did not exhibit polymorphisms compared to *M. bovis* AN5 and *M. bovis* AF2122/97 references. However, all *M. bovis* sequences evaluated (including field isolates and reference strains) showed a synonymous substitution in the nucleotide position 255 for the gene *mb3645c* when compared to *M. tuberculosis* H37Rv. This position corresponding to the K-83 amino acid, nearby the functional Y87-D91 motif [12], constitutes a difference between *M. tuberculosis* H37Rv and all the *M. bovis* strains included in this study.

Navarro and coworkers showed that the persistence of *M. bovis* infection could facilitate clonally complex infections owing to the emergence of clonal variants like those described for *M. tuberculosis* in humans. They found that genetic changes were located within the coding region of the PPE protein family, proving that microevolution events of *M. bovis* isolates occurs in naturally infected herds even in a short period of infection time [9].

In the present study it was found that, despite the genomic variability of the *M. bovis* isolates, *esxA* and *esxB* remained invariant. In case of *mb3645c* exhibited a synonymous SNP only in one isolate. Considering the entirety of the results obtained, the lack of allelic variation in the genes codifying the three important immunogenic proteins supports the use of the ESAT-6, CFP-10 and EspC antigens as diagnostic reagents for bTB diagnosis, regardless of the advancement of the disease or even of the molecular spoligotype associated among *M. bovis* field isolates. Pathogen and host factors determine the progression of tuberculous lesions. As Esat-6, Cfp-10 and EspC are recognized virulence factors that probably play a role in the pathogenesis of tuberculosis infection, in the present study the total of 58 M. *bovis* field strains analyzed were selected based on their variable profile considering not only the genomic variability (spoligotyping) but also the virulence level (pathological visible lesion in organs) observed at the

slaughterhouse inspection [10]. However, this preliminary study included 58 M. *bovis* local strains coming from infected animals in natural conditions, which could differ in age, production type, and stage of infection or even other factors that influence the outcome of bTB (and the pathology score). Finally, further studies should be performed considering all these facts, including a higher number of animals to minimize differences not related to the strain and spoligotypes predominating from other geographical locations to globally extend the these findings.

5. Conclusions

This study confirms the high conservation level at the nucleotide and epitope sequence in the immunodominant ESAT-6, CFP-10 proteins and reveals the highly conserved protein sequence of EspC among local *M. bovis* field isolates. The results reinforce the use of these antigens in the diagnosis of bTB.

Conflicts of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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