

Detection of *vspL* gene of *Mycoplasma bovis* field strains using a PCR assay

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Abstract

Mycoplasma bovis is an important bovine pathogen and can evade the host immune system using an extensive antigenic variation of the highly immunogenic surface lipoprotein antigens (Vsps). A family of 13 *vsp* genes was recently found in the chromosome of *M. bovis*. Among them a lympho-inhibitory peptide (Mb-LIP) encoded by the *vspL* gene of *M. bovis* was found to be able to inhibit naïve lymphocyte activation during the respiratory infection. In this study, a PCR assay was developed to help detect the *vspL*, *vspA*, *vspO*, and *vspC* genes from some 70 *M. bovis* strains. The VspL protein could also be detected by western blotting with a vspL-specific antiserum. Test results by PCR assays indicated that the most common *vsp* genes among 70 *M. bovis* field strains isolated in the U.S. were *vspA* and *vspL*. The less common gene was *vspC* gene.

Introduction

Mycoplasma bovis is an important bovine pathogen that can rapidly change its surface antigenic repertoire to evade host immune responses. It contains multiple related but divergent genes that encode variable surface lipoproteins (Vsps). These *vsp* genes undergo random and spontaneous on/off switching at a high frequency using diverse genetic mechanisms. Through these events, Vsps can be turned off and the antigenic phenotype will be altered (1,2,3).

In addition to immune evasion through antigenic modulation, the Vsps of *M. bovis* have been characterized as adhesion factors that can suppress host lymphocyte function (4,5). Recently, Rosenbusch et al had isolated a *M. bovis* which can produce immuno-suppressive peptide homologous to the C-terminus of Vsp-L (Mb-LIP, *M. bovis* inhibitory peptide) and also shown that this mycoplasma product is able to inhibit naïve lymphocyte activation (5).

In this study, a PCR assay was developed to detect the genes that encode VspL, VspA, VspO, and VspC from some 70 field strains of *M. bovis*. This PCR assay is a useful tool for the differentiation of *M. bovis* field isolates, and also will help vaccine scientists in formulating customer made *M. bovis* vaccines.

Materials and Methods

DNA extraction

1 ml of the liquid culture was centrifuged at 14000 r.c.f. for 3 minutes at ambient temperature. The pellet was resuspended in 200 µl sterile filtered PBS. The solution was then boiled for 10 minutes and then placed at -20 °C for 10 minutes. Samples were then centrifuged at 13000 r.c.f. for 3 minutes at ambient temperature and the supernatant was removed and placed in a DNase/RNase free microcentrifuge tube.

PCR protocol

In order to detect the presence of the *vspL* gene, A 538 bp DNA amplicon was amplified using the following reagents in a 25 µl reaction: 15.3 µl nuclease free water (Ambion, Austin, TX); 2.5 µl 10X Amplitaq PCR buffer II (Applied Biosystems, Foster City, CA); 2.0 mM MgCl₂ (Applied Biosystems, Foster City, CA); 200 µM each deoxynucleotide triphosphate (dNTP)(Applied Biosystems, Foster City, CA); 0.8 µM each primer (FWD-TGAAGCCGCTATCAAAGAAGC; RVS-TCATATGGTGCCTTTGTTCCCAGG; Integrated DNA Technologies, Iowa City, IA); 0.5 U of Amplitaq DNA polymerase (Applied Biosystems, Foster City, CA); and 2.5 µl of the previously extracted DNA template. The template was amplified using the following thermal cycling protocol: One cycle at 95 °C for 3 minutes, 58 °C for 90 seconds, 72 °C for 2 minutes, then 30 cycles at 95 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 90 seconds, then one cycle at 72 °C for 10 minutes and hold at 4 °C. The resulting samples were then run at 70 volts for 1 hour on a 1% agarose gel containing ethidium bromide and TBE (Bio-Rad Laboratories, Hercules, CA). The samples were run alongside the Amplisize Molecular Ruler (Bio-Rad Laboratories, Hercules, CA) in order to verify the correct size amplicon was present. The resulting gels were photographed using ultraviolet transillumination and Polaroid type 667 black and white film. PCR to verify the presence of the *M. bovis* species (6) and to detect the *vspA*, *vspO* and *vspC* genes (3) was carried out as described previously.

Western blotting

The VspL protein of *M. bovis* can be detected by running the whole culture of the test *M. bovis* isolate through a 4-20% SDS-PAGE gel (BIO-RAD). After protein separation, proteins were transferred to a nitrocellulose paper (BIORAD) and then blotted with an antiserum against VspL of *M. bovis* (obtained from Dr. R.F. Rosenbusch) diluted at 1:50 for 2 hours at room temperature. After washing with PBS-Tween buffer, the nitrocellulose paper was blotted with Protein G-Peroxidase (Sigma P8170) diluted at 1:50 for 2 hours at room temperature. After washing, nitrocellulose paper was incubated with a peroxidase substrate (KPL) for about 5 minutes or until the bands are visible and then washed with distilled water to stop color reaction.

Results

Of the 86 samples that tested positive for *M. bovis* via species specific PCR (6), 74 (86%) contained the *vspA* gene, 45 (52%) were positive for the *vspO* gene, 3 (3%) were positive for the *vspC* gene and 59 (69%) were positive for the *vspL* gene.

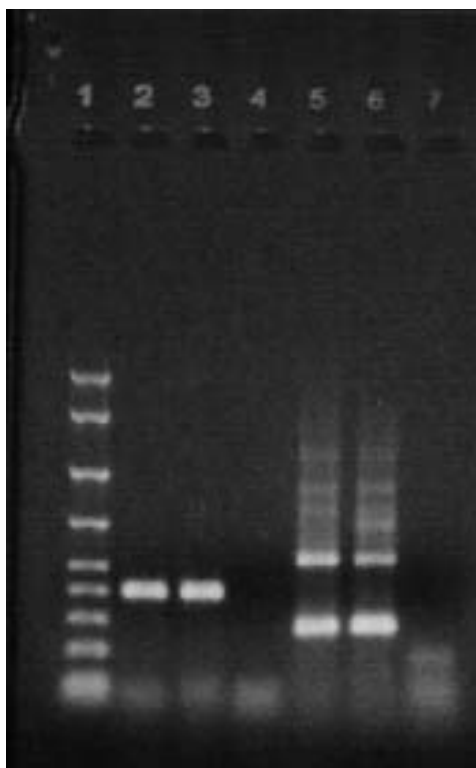


Figure 1. PCR analysis of an *M. bovis* culture. The presence of a 537 bp amplicon indicates the presence of the *vspL* gene. Lane 1: Amplisize Molecular Ruler (from top: 2,000 bp, 1,500, 1,000, 700, 500, 400, 300, 200, 100, 50); Lane 2: *M. bovis* sample culture tested with the species specific primers; Lane 3: Species specific positive control; Lane 4: Negative control (water); Lane 5: *M. bovis* sample culture tested with the *vspL* primer set; Lane 6: *vspL* positive control; Lane 7: Negative control (water).

Due to the redundant nature of the sequences of many of the *vsp* genes, it was difficult to design primers that would utilize a nucleotide sequence that is unique compared to the rest of the genes in the family. In order to design primers that would fit the criteria necessary for efficient PCR amplification, the forward primer had to be placed on a redundant region of the gene. This region shares sequence similarity with the *vspF* and *vspM* genes as well as being duplicated on each of these genes. Because of this there are bands at 271 (*vspF* and *vspM*) and 278 (*vspL*) that are caused by the forward primer annealing in two different locations. The reverse primer however is located in a unique sequence that results in its binding in one specific location on the *vspL* gene alone. This results in a 537 bp amplicon that is only amplified when the *vspL* gene is present.

In order to ascertain if Mb-LIP is expressed on *M. bovis* isolates that had been determined by PCR to carry the *vspL* gene, antibody specific for the Mb-LIP was used in the Western blot analysis. Test results indicated that antiserum against Mb-LIP can detect Vsp-L on the isolates that carry *vsp-L* gene (Fig. 2).

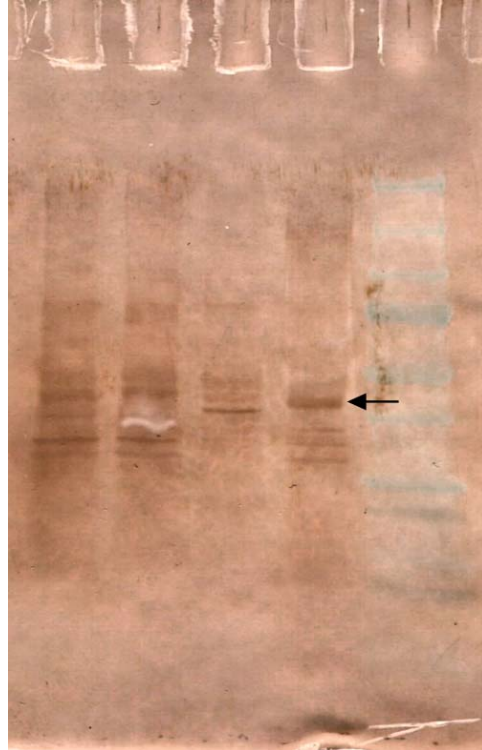


Figure 2. Western blot analysis of an *M. bovis* culture using a known serum from *M. bovis*-infected calf containing antibodies against Mb-LIP. Lane 1: Molecular weight markers (from top: 250 kd, 150, 100, 75, 50, 37, 25, 20); lane 2: Known *M. bovis* isolate that is positive for the presence of *vspL* gene; Lane 3: a field strain of *M. bovis* that was found to be positive for *vspL* gene by PCR; Lane 4: a field strain of *M. bovis* that was found negative for *vspL* gene by PCR; Lane 5: a field strain of *M. bovis* that was found negative for *vspL* gene by PCR.

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