Molecular identification of M. bovis BCG by Multiplex PCR

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ABSTRACT

Mycobacterium bovis (*M. bovis*) BCG belongs to *Mycobacterium tuberculosis* complex (MTBC), highly related organisms, which are 99.9 % similar at nucleotide level and phenotypically similar. Its differentiation from other members of MTBC by conventional methods is laborious and time consuming. PCR provides a rapid alternative method for differentiation between members of MTBC. It depends on identification of region of difference (RD) which have been lost during attenuation of *M. bovis*. Two different BCG strains (from two sources) were confirmed as a member of *M. tuberculosis* (MTB) complex (MTC) and as BCG strains by PCR using primers to a region of the 16S rRNA gene that is conserved in all mycobacteria and region of difference (RD1, RD4, RD9 and RD12) respectively. DNA of *Mycobacterium tuberculosis* was used as a control to compare with BCG strains. The results showed that 16S rRNA gene was present in all tested strains, while the RD1, RD4, RD9 and RD12 were absent only in BCG strains.

Keywords: Region of difference, BCG, PCR.

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1. INTRODUCTION:

M. bovis had been attenuated over media containing bile after 230 passages. This attenuation gave the birth to nonpathogenic Bacille Calmette-Gue´rin (BCG) strain. which then used as a vaccine against *M. tuberculosis*, then as a recombinant vehicle for multivalent vaccines against other infectious diseases, and as cancer immunotherapy (Talbot et al., 1997). Immunization of children with BCG is recommended by the world health organization in communities with high prevalence of tuberculosis; however, it is contraindicated in infants with immunodeficiency. Unfortunately, they are vaccinated prior to diagnosis, and immunodeficiency may be diagnosed after development of BCG complication (Monajemzadeh et al., 2010). Usually, BCG complications varied between local lesions or disseminated disease. The local lesions like hypersensitivity, localized lymphadenitis, or fistula formation at site of inoculation are subclinical and regress spontaneously in immunocompetent patients. While, the disseminated BCG mainly occur in children with defective immunity and HIV infection with severe outcomes that may lead to death (Paiman et al., 2006). Additionally, Intravehicular BCG therapy, for treatment of superficial bladder cancer, showed some complications including cystitis, prostatitis, epididymo-orchitis, peritonitis, pneumonitis, hepatitis, arteritis, osteomyelitis, and generalized sepsis (Watts et al., 2011). Identification of the isolates of the MTBC to the species level is very important to epidemiologic and public health considerations and to optimize treatment to avoid emergence of drug resistant BCG (Pinsky and Banaei, 2008). There are 16 genomic regions (named region of difference) were deleted from BCG because of its attenuation. These regions still present in pathogenic strains of *M. tuberculosis* (Teo et al., 2013), which provides the key for the differentiation between members of MTBC.

The current study was aimed to identify 2 BCG strains with multiplex PCR using primer sets for region of difference (RD1, RD4, RD9 and RD12).

2. MATERIAL AND METHODS

2.1. bacterial strains:

M. bovis BCG Connaught strain and *M. bovis* BCG Pasteur obtained from the laboratory culture

repository of the Center for Molecular Medicine and Infectious Disease (CMMID), Virginia-Maryland Regional College of Veterinary Medicine, at Virginia Tech. The strains were kept at -80°C. The same strains, *Mycobacterium bovis* (ATCC® 35745™) (BCG Connaught) and *Mycobacterium bovis* (ATCC® 35734™) (BCG Pasteur), were obtained from American Type Culture Collection (ATCC, USA).

2.2. Bacterial cultures according to NCCLS, 2003:

BCG strains were cultured on middlebrook 7H9 broth (Becton Dickinson and company, USA, Catalog No. 271310) supplemented with 10% Albumin-dextrose-catalase (ADC) enrichment (Becton Dickinson and company, USA, Catalog No. 211887) and incubated for 14 days at 37°C. The mycobacterial cells were spin down at 4000 xg for 15 min. The cells were re-suspended in molecular-grade water (Hyclone®, Utah) and then vortex with 7-10 glass beads of 5mm (diameter). The suspension was centrifuged twice at 500 xg for 5 min to remove bacterial clumps. The sterility of the suspensions was confirmed by plating on tryptic soy agar (TSA).

2.3. Multiplex PCR:

The mycobacterial strains were confirmed as a member of *M. tuberculosis* (MTB) complex (MTC) and as BCG strains by PCR using primers to a region of the 16S rRNA gene (Pinsky and Banaei, 2008) that is conserved in all mycobacteria and region of difference (RD1, RD4, RD9 and RD12) respectively (Warren et al., 2006).

2.3.1. Primer design

Primers were designed as previously described (Warren et al., 2006 and Pinsky and Banaei, 2008). The primers sets included RD1, RD4, RD9, RD12 and primers set for 16S rRNA were synthesized by Sigma - Aldrich (Table 1).

Table 1: PCR primers and corresponding amplification product size (Warren et al., 2006 and Pinsky and Banaei, 2008).

	Primer sequence 5'- 3'	*M. tuberculosis*	BCG
RD1	AAGCGGTTGCCGCCGACCGACC CTGGCTATATTCCTGGGCCCGG GAGGCGATCTGGCGGTTTGGGG	Present 150bp	Absent 200bp
RD4	ATGTGCGAGCTGAGCGATG TGTACTATGCTGACCCATGCG AAAGGAGCACCATCGTCCAC	Present 172bp	Absent 268 bp
RD9	CAAGTTGCCGTTTCGAGCC CAATGTTTGTTGCGCTGC GCTACCCTCGACCAAGTGTT	Present 235bp	Absent 108bp
RD12	GGGAGCCCAGCATTTACCTC GTGTTGCGGGAATTACTCGG AGCAGGAGCGGTTGGATATTC	Present 369bp	Absent 306 bp
16SrRNA	Forward: CAACGCGAAGAACCTTACCT Reverse: TGCACACAGGCCACAAGGGA	Present 78 bp	Present 78 bp

PCR = polymerase chain reaction; RD = regions of difference; BCG = bacille Calmette-Guérin.

2.3.2. DNA extraction (Lea˜o et al., 2004):

Three hundred µL of the 1 McFarland mycobacterial suspension was transferred to a micro-centrifuge tube and the bacteria was killed by boiling the suspension for 20 minutes. After cooling, 300 µL of chloroform was added and vortex briefly (10 sec). The tubes were incubated for 20 min at 80°C and then stored at -20°C for 20 minutes. Further, the samples were thawed and centrifuged for 3 min at 14,000 x g. The aqueous phase supernatant was collected in a clean micro-centrifuge tube and stored at –20°C until use. The purity of DNA was determined by 260/280 ratio using Nano-drop spectrophotometer and a ratio of 1.8 to 2 was considered as the cut off. Chromosomal DNA of *M. tuberculosis* H37RV (ATCC) was used as positive control. It was obtained from the laboratory culture repository of the Center for Molecular Medicine and Infectious Disease (CMMID), Virginia-Maryland Regional College of Veterinary Medicine, at Virginia Tech.

2.3.3. PCR amplification (Warren et al., 2006):

Five DNA samples from the mentioned microorganisms were done using multiplex PCR. For each 50 µL reaction mixture contained 25 µL GoTaq® Green Master mix, 2X (Promega, Madison WI, USA) 1 µL of each primer (1X, 10 µM), 1 µL of extracted DNA and 10 µL nuclease free water. Amplification was initiated by denaturation at 95 °C for 2 min, followed by 45 cycles of 94 °C for 1 min, 62 °C for 30 sec of annealing, and 72 °C for 30 sec. of extension. After the last cycle, the samples were incubated at 72 °C for 10 min (final extension). PCR amplification products were electrophoretically fractionated in 2.0% agarose gel in 1xTBE (Tris Borate EDTA) pH 8.3 at 2 V/cm for first ten minutes then increase for 7 V/cm for 50-60 min, and visualized under UV light.

3. RESULTS:

3.1. Spectrophotometer reading of DNA:

The chromosomal DNA 260/280 ratio using Nano-drop spectrophotometer was varied between 1.8 - 2.1 among BCG strains.

3.2. PCR:

A specific product of 78 bp using primer set for 16S rRNA was amplified from all the five organisms tested in the present study. With RD primer sets, specific product of 108, 200, 268 and 306 bp was amplified using BCG strains' DNA as a template. While 150, 172, 235 and 369 bp amplicon were produced using DNA from *M. tuberculosis* as a control (Fig.1).

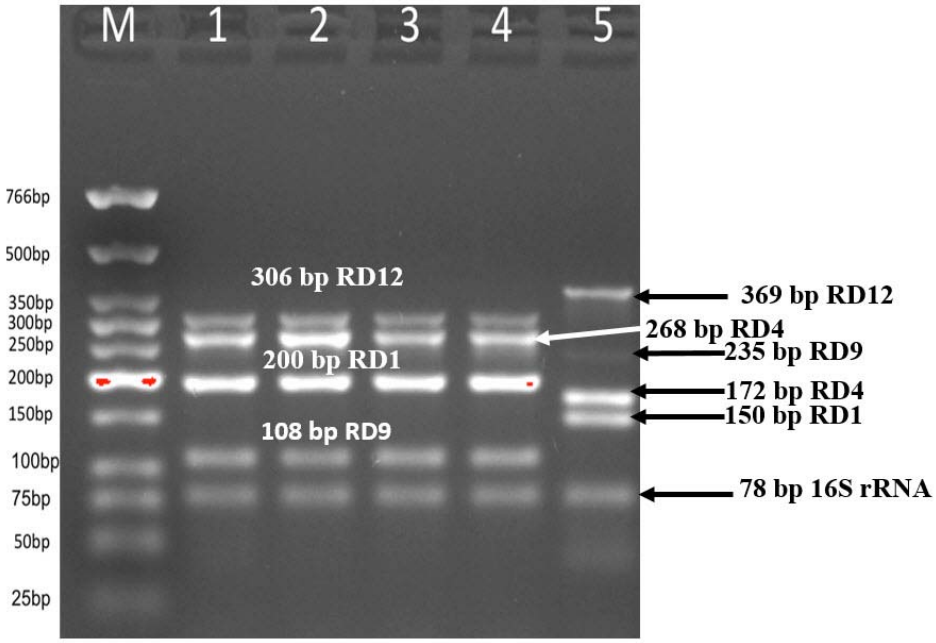

Figure 1: Electrophoretic fractionation of PCR products on a 2% agarose gel. Lane M, low molecular weight DNA Ladder (New England Biolabs® Inc), Lane 1, *M. bovis* BCG Connaught, Lane 2, ATCC® 35745™, Lane 3, *M. bovis* BCG Pasteur strain, Lane 4, ATCC® 35734 *and* Lane 5, *M. tuberculosis* H37RV.

4. DISCUSSION:

M. bovis BCG belongs to Mycobacterium tuberculosis complex (MTC) of highly related organisms, which are 99.9 % similar at nucleotide level and phenotypically similar (Pinsky and Banaei, 2008). Its identification by the combination of biochemical and growth features is laborious and time-consuming (Talbot et al., 1997). Also high-performance liquid chromatography (HPLC) can be used to identify BCG from *M. tuberculosis* and *M. bovis*. But it is not practically applied for the routine diagnostic laboratory use, due to requirement of well-trained personnel, specialized equipment and protracted turnaround time (Teo et al., 2013). PCR provides a rapid alternative method for differentiation between members of MTC.

The spectrophotometric quantification of DNA determines the purity of DNA depending on ratio between DNA/protein, which represented by A260/A280 ratio. Pure double stranded DNA has an A260/A280 ratio of 1.8 and ratios of 1.7 to 2.0 are acceptable (Helden et al., 2001). The current results of DNA samples were between 1.8 and 2.1, showing that they were of high quality. The method used for extraction of DNA was simple, inexpensive, rapid and produced highly pure

double stranded DNA. The 16S ribosomal RNA gene is genus specific that is conserved in all mycobacteria. If nucleic acid from any mycobacterial species is present, the primer set of 16S rRNA should amplify a specific product, thus controlling for extraction efficiency and reaction inhibitors (Pinsky and Banaei, 2008 and Monajemzade et al., 2010). All tested strains amplified the specific product with primer set for 16S rRNA, which confirmed that the tested DNA belonged to genus mycobacterium.

Comparative genomics with the complete DNA sequence of *M. tuberculosis* H37Rv has resulted in the demonstration of 16 regions of the genome (regions of difference [RD]) with deletions in *M. bovis* and *M. bovis* BCG strains; subsequent studies found that some of these regions were also deleted in other members of the MTC (Parsons et al. 2002). Warren et al. (2006) found that RD1, RD4, RD9 and RD12 could differentiate *M. canettii, M. tuberculosis, M. caprae, M. bovis* and BCG stains. The RD1 was found in all strains tested except BCG strains, while RD4 was absent only in *M. bovis* and BCG strains. They found that RD9 amplicon was present only in *M. canettii* and *M. tuberculosis*, while RD12 present only in *M. tuberculosis*. Talbot et al. (1997) used RD1 for the differentiation of BCG strains from 152 MTC isolates from 24 countries, using 3 primers ET1, ET2 and ET3 for RD1. They explained that primers ET1 and ET3 are complementary to regions flanking the RD1, while ET2 is complementary to DNA within the RD1 sequence. The strains without RD1, ET1 and ET3 bind and amplify a 200-bp region. While strains with RD1, these two primers bind but the 9,650-bp sequence is too large to efficiently amplified. But primers ET2 yielded a 150-bp product with ET3 primer only in strains with part or all of the RD1 sequence present. They found that RD1 was absent in all BCG stains tested. Another study using RD1 and RD9 primers identified 16 mycobacterial isolates obtained from children with BCG vaccine adverse reaction (Teo et al., 2013). Thus PCR-amplification of RDs has been used for differentiation of members of MTC (Lea˜o et al., 2004).

In this study the primer sets for RD1, RD4, RD9 and RD12 were used to confirm the test strains as BCG strains. DNA of *M. tuberculosis* H37RV was used as positive control. Comparing the results of PCR amplification products with previously published results (table 1), it was observed that the four RD were absent in both tested strains, which confirmed that they were BCG strains. All of them were present in the positive control *M. tuberculosis* H37RV as previously mentioned (Warren et al., 2006 and Pinsky and Banaei, 2008).

Thus, the identification of the tested strains as BCG was done in one working day. So, it was considered effective method for rapid identification of MTC to strain level, which could facilitate rapid diagnosis and that may lead to effective treatment of mycobacterial infections.

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