Detection and Differentiation between *Mycobacterium bovis* and *Mycobacterium tuberculosis* in Cattle Milk and Lymph Nodes Using Multiplex Real-Time PCR

Suzan A. Mohamed, Kh. F. Mohamed, M.G. Aggour, Hanaa A. Ahmed and S.A. Selim

1Tuberculosis Unit, Bacteriology Department, Animal Health Research Institute, Dokki, Giza, Egypt
2Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
3Biotechnology Department, Animal Health Research Institute, Dokki, Giza, Egypt
4Genome Unit, Animal Health Research Institute, Dokki, Giza, Egypt

**Abstract:** A novel multiplex real-time PCR assay was developed and applied directly to biological samples with evidence of bTB in order to differentiate between *M. bovis* and *M. tuberculosis*. The primers and TaqMan probes were designed to target the IS1081 gene, the multi-copy insertion element in the MTC and the 12.7-kb fragment present in *M. tuberculosis*, not in the *M. bovis* genome. The assay was optimized and validated by testing 10 species of mycobacteria including *M. bovis* and *M. tuberculosis* and 3 other bacterial species such as: Escherichia coli, shigella Spp., salmonella Spp. in cattle milk and lymph nodes. The tests identified 96.4% (27/28) as *M. bovis* from the MTC-positive bTB samples using conventional PCR for specific insertion elements IS1081. MTC-negative bTB samples were tested using conventional PCR and the real-time PCR. When comparative analyses were conducted on all bovine samples, using conventional PCR as the gold standard, the relative accuracy of real-time PCR was 99.1% and the relative specificity was 100%. The detection limits of the real-time PCR assays for *M. bovis* and *M. tuberculosis* genomic DNA were 2 DNA copies per PCR reaction. Consequently, this multiplex real-time PCR assay is a useful diagnostic tool for the identification of MTC and differentiation of *M. bovis* and *M. tuberculosis*, as well as the epidemiologic surveillance of animals slaughtered in abattoir.

**Key words:** Real-Time PCR • *Mycobacterium bovis* • *Mycobacterium tuberculosis Complex* • IS1081

**INTRODUCTION**

*Mycobacterium bovis* and closely associated acid-fast bacilli cause diseases in humans. Epidemiologic investigations reveal that the organism may be ingested or inhaled. Extra pulmonary lesions may occur associated with the consumption of infected milk, even though with the practice of boiling milk and the growth of milk pasteurization plants all over the world. The digestive route of infection has become less important. On the other hand, airborne infection continues to occur among meat industry and slaughterhouse workers, in regions where the infection in cattle is still prevalent [1]. Major grouping of *Mycobacterium tuberculosis complex* are: *M. tuberculosis*, *M. bovis*, *M. microti* and *M. africanum* [2].

Because of the slow growth rate of *Mycobacterium tuberculosis*, isolation, identification and drug susceptibility testing of this organism and other clinically important mycobacteria can take several weeks or longer. During the past several years, many molecular methods have been developed for direct detection, species identification and drug susceptibility testing of mycobacteria. These methods can potentially reduce the diagnostic time from weeks to days [3]. Strikingly, the genome sequence of *M. bovis* is > 99.95% identical at the nucleotide level to that of *M. tuberculosis*, showing collinearity and no evidence of extensive translocations, duplications or inversions. But deletion of genetic information that has led to a reduced genome size, revealed 11 deletions from the genome of *M. bovis*, ranging in size from 1 to 12.7 kb. Surprisingly,
the sequence contains only one locus in *M. bovis*, termed TbD1, which is absent from the majority of extant *M. tuberculosis* strains. Therefore, at a gross level, deletion has been the dominant mechanism in shaping the *M. bovis* genome [4]. An oligonucleotide array which could detect and differentiate mycobacteria to the species level by using the internal transcribed spacer (ITS) sequence was developed [5]. Also, microsphere-based multiplex assay was developed, by using the xMAP technology, for the simultaneous rapid detection of the *Mycobacterium tuberculosis complex* (MTC) and the differentiation of *M. tuberculosis* and *M. bovis*. The assay simultaneously detected 4 target sequences, including specific insertion elements IS6110 and IS1081 of MTC, a 12.7-kb fragment specific for *M. tuberculosis* and an uninterrupted 229 bp sequence specific for *M. bovis* [6]. Real-time quantitative PCR has been developed to measure accumulation of PCR product through a dual labeled TaqMan probe and it provided very accurate and reproducible quantitation of gene copies [7]. Real-time PCR offers significant improvements to the quantitation of viral load because of its enormous dynamic range that can accommodate at least eight log10 copies of nucleic acid template[8].

**MATERIALS AND METHODS**

**DNA Extraction:** All strains used for validation were kindly provided by Veterinary Serum and Vaccine Research Institute (VSVRI) was extracted using ready to use kit, Thermo Scientific GeneJET Genomic DNA Purification Kit Also, the other non-mycobacterial strains *E.coli, Listeria Spp., Salmonella, Shigella Spp.* extracted using the same kit.

**Samples:** A total number of fifty milk and tissue samples were collected during 2014 from tuberculous animals from Elsharquia governorate and Bassatine slaughter house. Thirty three milk samples were collected from apparently healthy cattle and seventy tissue samples with suspected tuberculosis lesions and DNA extracted using Thermo Scientific GeneJET Genomic DNA Purification Kit (N.B in milk samples prewash step using PBS).

**Real-time PCR:**

- Using Maxima Probe qPCR Master Mix, Primers and Taq man probes in table (1). For multiplex real-time PCR to differentiate between members of MTC at species level.
- Detection of *Mycobacterium Complex* in DNA extracted from tissue and milk samples using MTplex Genetic pcr solutions TM, Spain) qPCR *tuberculosis complex* detection (Ready to use)

**Real-time Amplification:**

- Amplification of real-time primers and probes of MTC, *M. bovis, M. tuberculosis* and other non-mycobacterial strains.

All reference strains and field isolates for *M. bovis* and *M. tuberculosis* and other non-mycobacterial strains as *E. coli* were included in this Polymerase Chain Reaction to differentiate between these Mycobacterium species and validate sensitivity and specificity of multiplex real-time primers and probes used. The run was performed in 12.5µl total reaction mixture.

<table>
<thead>
<tr>
<th>Target species</th>
<th>Oligonucleotide</th>
<th>Sequence (5’?3’)</th>
<th>Target gene/sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>MTC</em></td>
<td>IS1081_650F(p1)</td>
<td>CGGACTGGCTGCTGCAGC</td>
<td>IS1081</td>
</tr>
<tr>
<td></td>
<td>IS1081_851R(P2)</td>
<td>AGCTCTTTGGCCATGATCGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IS1081_712Pa</td>
<td>FAM-TGCTACCTGCTGGAGTATACCTCAGBH1</td>
<td></td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>THB_312851F(P3)</td>
<td>TGTCGGAGCTAGCGGATGTCT</td>
<td>The229-bp contiguous sequences</td>
</tr>
<tr>
<td></td>
<td>THbovis_825R(P4)</td>
<td>AATAGCTATTTGACCAAGCTAAGATAT</td>
<td></td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>THB_312940Pb</td>
<td>JOE-CCGTAGTCGTGCGAGAGCGCAACAC-BHQ1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>THB_312851F(P5)</td>
<td>TGTCGGAGCTAGCGGATGTCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TH_313003R(P6)</td>
<td>GCCGCTATTTGATCTCTGCAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>THB_312940Pc</td>
<td>JOE-CCGTAGTCGTGCGAGAGCGCAACAC-BHQ1</td>
<td>The 12.7-kb fragment</td>
</tr>
</tbody>
</table>

FAM (6, carboxy fluorescein, JOE (6-carboxy 4’, 5’ dichloro-2’,7’ dimethoxyfluorescein BHQ (Black hole quencher 1) according to[9].
• The reaction mixture for *M. bovis* (reference strains) and field isolates consisted of 6.25 µl (maxima ready to use mix containing [PCR buffers, Taq polymerase, dNTPs], 1 µl P3, 1 µl P4, 0.5 µl Probe of *M. bovis*, 0.075 µl ROX, 3.6 µl DNase/RNase free water. The PCR mix was vortexed. The PCR mix was transferred into real-time plate. The run was performed in 12.5 µl reaction mixture. A. 1st reaction mixture consisted of 6.25 µl maxima mix, 0.5 µl P5, 0.5 µl P6, 4.9 µl DNase/RNase free water, 0.075 µl ROX, 0.25 µl probe (*M. tuberculosis*). B. 2nd mixture consisted of the same content but replace P5, P6 with P3, P4 and probe *M. tuberculosis* with probe (*M. bovis*). 3rd mixture consisted of same content but replace P5, P6 with P1, P2 and probe of *M. bovis* with probe of (*M. tuberculosis* complex). The PCR mix was vortexed and spin. Reaction mix was transferred into real-time plate, 2.5 µl of target DNA extracted was added. Thermal profile Table 2.

**Detection of *M. bovis* and *M. tuberculosis in Tissue and Milk Samples***: All DNA samples extracted from tissue and milk samples which gave positive results in method (c) were involved in these reaction.

E. Comparing the Sensitivity of Real-time and Conventional Polymerase Chain Reaction: Sensitivity of real-time and conventional PCR were compared convensional PCR 270bp for *M. tuberculosis* and 270bp-470bp for *M. bovis* according to[10].

**RESULTS**

This method based on simultaneous amplification of two target sequence: a *M. tuberculosis* complex specific gene and amplified with P1 and P2 and probe labeled with FAM and 229pb target sequence specific to *M. bovis* and amplified with P3 and P4 and probe labeled with JOE and also 12.7kb target sequence specific to *M. tuberculosis* and amplified with P5, P6 and probe labeled with JOE. Both primers of *M. tuberculosis* complex and *M. tuberculosis* with their probes in single tube were assigned as target 1 and target 2 in Step One® real-time PC machine thermal profile preparation and primers of *M. bovis* and its probe in another tube assign as target 2. The reference strain and field isolate of *M. tuberculosis* gave two curves one; confirming that they were *M. tuberculosis* complex and another indicating that they were *M. tuberculosis*. Therefore, strain and field isolate of *M. bovis* gave only one curve indicating that they were *M. bovis*, as shown in Table (2).
Fig. 1: Amplification curves of multiplex TaqMan real-time assays for detection MTC using IS\textsubscript{1081} gene and 12.7-kb insertion/deletion fragment primers and TaqMan probe.

(A) \textit{M. bovis} DNA was amplified simultaneously by the MTC and \textit{M. bovis}-specific primers (THB\_312851F and Tbovis\_825R) and TaqMan probe.

(B) \textit{M. tuberculosis} DNA was amplified together in one tube by the MTC and \textit{M. tuberculosis}-specific primers (THB\_312851F and TH\_313003R) and TaqMan probe. Figures 2 and 3 illustrate that the TaqMan probe and primers can detect till 2 DNA copies per PCR reaction.

The amplification was performed to estimate specificity of primers and probes of multiplex real-time PCR to differentiate between members of \textit{Mycobacterium complex}. The PCR was carried out on strains other than \textit{Mycobacterium complex}, such as: \textit{E.coli}, \textit{Salmonella Spp.} And \textit{Shigella Spp}. The samples with positive curve confirmed that they were \textit{M. tuberculosis} and negative curves confirmed that the samples DNA belong to strains other than \textit{Mycobacterium}, as shown in table(2).

Analysis of amplification was carried out in Step One® Real-Time PCR System. Figure (1) illustrate the results of analysis. All DNA tissue samples gave positive curve with target 2 only confirming it is \textit{M. bovis}. All DNA tissue samples that gave two positive curves: the first curve confirmed it was \textit{M. tuberculosis} complex and the second one indicating \textit{M. tuberculosis}.

**DISCUSSION**

Bovine tuberculosis caused by \textit{Mycobacterium bovis} remains one of the most prevalent and devastating diseases of cattle in developing countries throughout most of the world [11]. Bovine tuberculosis is currently an important zoonosis worldwide and the possibility of human infection with \textit{Mycobacterium bovis} cannot be ignored. Although scarce epidemiological information is available, \textit{M. bovis} has been reported to have caused between 6 and 30 % of the cases of human tuberculosis (TB) in the USA before milk pasteurization [12]. It is also the cause of 6.3% of the bacteriologically confirmed cases of tuberculosis in western Ireland [13] and Brett and Humble [14]. Brosuch et al. [15] evaluated variable regions resulting from insertion-deletion events in the genomes of the tubercle bacilli the majority of these polymorphisms did not occur independently in the different strains of the \textit{M. tuberculosis} complex. Based on the presence or absence of an \textit{M. tuberculosis} specific deletion (TbD1). A rapid and robust real-time PCR assay based on genomic deletion analysis was developed to distinguish between members of the MTC. This assay uses melting-curve analysis in two PCRs to detect the presence or absence of regions of difference (RD) RD9, RD4 and RD1 allowing definitive identification of \textit{M. tuberculosis} and \textit{M. bovis} [16]. A microsphere-based multiplex assay was developed, by using the xMAP technology, for the simultaneous rapid detection of the \textit{Mycobacterium tuberculosis complex} (MTC) and the differentiation of \textit{M. tuberculosis} and \textit{M. bovis}. The assay simultaneously detected 4 target sequences, including specific insertion elements IS\textsubscript{6110} and IS\textsubscript{1081} of MTC, a 12.7- Kb fragment specific for \textit{M. tuberculosis} and an uninterrupted 229 bp sequence specific for \textit{M. bovis} [5].
Fig. 2: Standard curves of TaqMan real-time PCR to detect *M. tuberculosis* using 12.7-kb fragment primers and TaqMan probe. A minimum of 2 DNA copies could be detected after 45 cycles.

According to OIE manual [17], validation was performed for primers and probes used in multiplex real-time PCR to differentiate between members of MTC at species level according to control positive of ready-to-use-kit with known DNA copies $10^7$. This real time reaction was applied on 6 serial dilution of control positive of ready-to-use-kit and reference strains belong to *M. bovis* and *M. tuberculosis*. DNA concentration in reference strain was detected. Reference strain (*M. tuberculosis*) with suitable DNA concentration was used to perform the standard curve to validate primers and probes and estimate its sensitivity to DNA copies. The results observed in Fig. (2) revealed that primers and probes used in multiplex real-time can detect till 2 copies of DNA in samples and repeatability of primers and probes. A single step multiplex real-time PCR was developed to evaluate

Fig. 3: Amplification curves of Taq Man real-time PCR to detect *M. tuberculosis complex* using IS1081 primers and TaqMan probe. A minimum of 2 DNA copies can be detected after 45 cycles.
Table 3: Target gene/sequence of strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Target gene/sequence</th>
<th>The 229-bp contiguous sequences</th>
<th>The 12.7-kb fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. bovis</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M. avium</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M. phlei</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E. coli</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Salmonella Spp.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Listeria Spp.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*positive results(+),negative results(-)

specificity of primers and probes which were used to distinguish between members of MTC at species level. This PCR reaction was applied on M. tuberculosis strain and other non-mycobacterial strains (E.coli, Shigella Spp, Listeria Spp.) using P1,P2 probe 1. Results observed in Table (3) giving positive curve with M. tuberculosis and negative one with other non-mycobacterial strains, which proves the specificity of used primers and probes [18]. A conventional multiplex PCR was developed using P7, P9, L1 and L2. The aim of this PCR reaction was to compare sensitivity of real-time and conventional one. This multiplex PCR reaction was applied on the same 6 M. tuberculosis serial dilution used for estimation of sensitivity of real-time primers and probes according to OIE manual (2013). The results showed that conventional multiplex real-time PCR can detect till (202 DNA copies) although real-time can detect till (2 DNA copies) by using conventional multiplex PCR primers used by [10].

Furthermore, a multiplex real-time PCR using designated primers and probes table(1) was developed for the first time. PCR assay was applied directly to biological samples with evidence of bTB and it was allowed to differentiate between M. bovis and M. tuberculosis for a simple, time saving and a single PCR mixture reaction which can be suitable for routine use [19].

**CONCLUSION**

Finally, this real-time multiplex PCR was applied on samples previously detected by ready-to-use kit used for detection of MTC. To differentiate between M. bovis and M. tuberculosis on biological samples;P1, P2, P3, P4, P5, P6 and Probe1, Probe 2, Probe 3 were used. So Results showed positive curves with P3, P4 and probe amplify (M. bovis) represented by target 2 consider to be M. bovis strain and positive samples gave positive curve with P1, P2, P5, P6 and probe M. tuberculosis, probe M. Tuberculosis complex represented by (target 1) and M. tuberculosis(target 2) on Step One® real-time machine consider to be M. tuberculosis complex sub species M. tuberculosis.

**ACKNOWLEDGEMENTS**

Thanks are dedicated to Prof. Dr. Emad Mokhtar Riad, Head of TB Unit, Bacteriology Department, Animal Health Research Institute, for his great help and support.

**REFERENCES**


