Evaluation of Different PCR-based Techniques in Diagnosis of Bovine Tuberculosis in Infected Cattle Lymph Nodes

Nashwa M. Helmy¹, Abdel-Rasheed F. Abdel-Moghney², Mohamed A. M. Atia³,*

¹Biotechnology Dept., Animal Health Research Institute, ARC, Giza, Egypt
²Microbiology Dept., Animal Health Research Institute, ARC, Giza, Egypt
³Genome Mapping Dept., Agricultural Genetic Engineering Research Institute (AGERI), ARC, Giza, Egypt

Email address
matia@ageri.sci.eg (Mohamed A. M. Atia)

Citation

Abstract
Bovine tuberculosis is a chronic bacterial disease of animals and humans caused by Mycobacterium bovis. In a large number of countries bovine tuberculosis is a major infectious disease among cattle and other domesticated animals. M. bovis can infect humans, primarily by the ingestion of unpasteurized dairy products but also in aerosols and through breaks in the skin. Raw or undercooked meat can also be a source of the infection. In live cattle, tuberculosis is usually diagnosed in the field with the tuberculin skin test which should be performed in the course of the twelve months prior to presentation for slaughter. An additional problem with the skin test is the widespread cross-reactivity with environmental mycobacteria which can limit its use. The diagnosis is confirmed by the isolation and identification of M. bovis on selective culture media or by different polymerase chain reaction (PCR) assays. In this study, initially, 70 cows with the TB symptoms were screened for tuberculosis infection using intradermal tuberculin test (IDTT). The test results revealed that 17 animals (24%) showed positive reaction. The lymph nodes of positive animals were examined for gross lesions and consequently conducted for bacteriological examination, which revealed that only 12 animals were infected with M. bovis and one animal was infected with pathogenic M. tuberculosis. Therefore, three PCR-based assays (monoplex, conventional multiplex and Real Time PCR) were applied to positive cases. The results revealed that real-time PCR assay was the most sensitive, powerful and efficient assay compared with monoplex and multiplex conventional PCR assays.

1. Introduction

The WHO defines zoonosis as infections and diseases which can be transmitted naturally from animals to humans. A recent WHO report highlights the difficulties in the diagnosis of such diseases and suggests that the true incidence of many neglected zoonosis, including zoonotic TB, may be greatly underestimated [1].

Bovine tuberculosis (TB) is classified by FAO and OIE as a "List B" disease. This category includes all animal diseases which are considered important because of their socio-economic and/or public health impacts. The FAO/OIE/WHO Animal Health
The aim of this work was to compare and evaluate the efficiency of monoplex conventional PCR, multiplex conventional PCR and real time PCR techniques in the diagnosis of tuberculosis in lymph nodes specimens from slaughtered skin-test positive animals.

2. Materials and Methods

Seventy cows reared in a private farm at Egypt complaining of low milk yield, emaciation and anorexia, intermittent diarrhea not responding to anthelmintic treatment, irregular febrile episodes, cough and labored respiration were selected for this study. The single intradermal tuberculin test was carried out for screening of tuberculosis infection in animals.

The test was done through intradermal injection of 0.1 ml (0.5 mg/ml) of bovine PPD (Central Lab. for Evaluation of Vet. Biologics, Abbassia, Egypt) corresponding to 5000 i.u./dose and examined the site after 72 hours by measuring the thickness of skin fold. The interpretation of the results was performed according to [15], i.e. a positive animal has more than 3.0 mm of swelling at site of injection.

2.1. Postmortem Examination

From all examined animals, presumably infected tissues were collected for bacteriological examination. It included the right and left bronchial lymph nodes. These lymph nodes were sliced in situ and examined visually. When histologically granulomatous changes were observed in the lymph nodes lesions resembling tuberculosis were found, they were submitted for bacteriological examination [16].

2.2. Bacteriological Examination

To process specimens for culture, the tissues were first homogenized using a mortar and pestle, followed by decontamination with 2–4% sodium hydroxide. The mixture was shaken for 10–15 minutes at room temperature and then neutralized. The suspension was centrifuged, then supernatant was discarded, and the pellet was used for culture and microscopic examination. The pellet was inoculated to a set of solid egg-based media, such as Lowenstein–Jensen. Cultures were incubated for a minimum of 8 weeks (and preferably for 10–12 weeks) at 37°C with or without CO₂. The media should be in tightly closed tubes to avoid desiccation. Slopes were examined for macroscopic growth at intervals during the incubation period. When growth was visible, smears were prepared and stained by the Ziehl–Nelson technique. Growth of M. bovis generally occurs within 3–6 weeks of incubation depending on the media used. Isolates can be identified by determining traditional cultural and biochemical properties [7].

2.3. Mycobacteria Strains

M. tuberculosis and M. bovis strains used in this study were isolated from tissues of infected animal.
2.4. DNA Extraction Method for Isolated Mycobacterial Strains

For the extraction of DNA, a loop-full of mycobacterial colonies were suspended in a microfuge tube containing 100 µl of Tris-EDTA buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) containing 1% Triton X 100. The suspension was subjected to boiling for 10 min followed by a brief centrifugation and the supernatant was directly used as a template for PCR [17, 18].

2.5. DNA Extraction Method for Bovine Infected Tissue Samples

DNA was extracted from tissue samples according to the manual of MTB Real-TM Real Time PCR kit which used for the detection of Mycobacterium tuberculosis complex (Sacace Biotechnology REF, TB15-50FRT).

2.6. Primers Synthesis

For conventional PCR, two oligonucleotide primers were designed and synthesized as TB1-F and TB1-R (Table 1). The 20 bp primers were constructed to target a 372 bp region of the gene responsible for production of MPB70 secretory protein which is considered to be specific for M. bovis [19]. For multiplex PCR, another three oligonucleotide primers (Table 1) were used for characterization and differentiation between M. bovis and M. tuberculosis: the common forward primer, CSB-F and two reverse primers, including M. bovis-specific, CSB-R1 which give 168 bp and M. tuberculosis-specific, CSB-R2 which give 337 bp PCR yield [17].

Table 1. Oligonucleotide sequences of primers specific for M. bovis and M. tuberculosis.

<table>
<thead>
<tr>
<th>Sequences</th>
<th>Amplified product</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB1-F</td>
<td>5'-GAACAATCCGGAGTGTGAC-3'</td>
</tr>
<tr>
<td>TB1-R</td>
<td>5'-AGCACCGTCTCAATCTGTA-3'</td>
</tr>
<tr>
<td>CSB-F</td>
<td>5'-TTCGGAATCCTTGTGTA-3'</td>
</tr>
<tr>
<td>CSB-R1</td>
<td>5'-GGAGAGCGCCGCTTGTGTA-3'</td>
</tr>
<tr>
<td>CSB-R2</td>
<td>5'-AGTCGCGGTGGCTTCTTCCA-3'</td>
</tr>
</tbody>
</table>

Table 2. Comparative studies between field and laboratory Techniques in the diagnosis of tuberculosis in cows.

<table>
<thead>
<tr>
<th>Test</th>
<th>No. of examined animals</th>
<th>No. of positive cases</th>
<th>Percentage* %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field Test (IDTT)</td>
<td>70</td>
<td>17</td>
<td>24</td>
</tr>
<tr>
<td>Isolation and identification</td>
<td>17</td>
<td>13</td>
<td>76</td>
</tr>
<tr>
<td>Monoplex PCR</td>
<td>17</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>Multiplex PCR</td>
<td>17</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>Real time PCR</td>
<td>17</td>
<td>13</td>
<td>76</td>
</tr>
</tbody>
</table>

* = Percent calculated according to No. of examined animals.

3.1. Intradermal Tuberculin Test

The intradermal tuberculin test results revealed that 17 animals (24%) showed positive reaction, while 53 animals (76%) showed negative reaction. In this context, this results are similar to [15] records, who recorded that the IDTT was the official test in the eradication and detection of infected animals in the field. While, Francis et al., [20] stated that the...
sensitivity of the IDTT was moderate and in more recent work the IDTT was found to have a sensitivity of 65.61% [21]. Moreover, Nwanta et al., [22] reported that the tuberculin test is valuable in the control of zoonotic tuberculosis because early recognition of pre-clinical infection in animals intended for food production and early removal of infected animals from the herd eliminates a future source of infection for other animals and for humans. In case of cattle, a tuberculin test should be performed in the course of the twelve months prior to presentation for slaughter. However, the use of this test in Africa has traditionally been limited by tuberculin availability, the need for repeat visits and the lack of suitable handling facilities. An additional problem with the skin test is the widespread cross-reactivity with environmental mycobacteria which can limit its use. Therefore, the use of more sensitive and specific tests become necessary to confirm the results obtained from the IDTT.

3.2. Bacteriological Examination

Bacteriological examination revealed the isolation of *M. bovis* from 12 animals and *M. tuberculosis* from 1 animal. At the same time, no mycobacteria was isolated from the rest of tuberculin positive cases.

Traditional mycobacterial culture remains the gold standard method for routine confirmation of infection. However, microbiological diagnosis of *M. bovis* is an extremely slow procedure, which may take as long as 2 to 3 months. An additional 2 to 3 weeks is required for biochemical identification and typing [7].

The development of a reliable, sensitive and rapid screening test would be of great help in the control of the disease. Polymerase chain reaction (PCR) methods offer great potential in this respect. Therefore, three PCR tests (monoplex, multiplex conventional PCR and Real Time PCR) were applied to DNA of all positive tissues cases, *M. bovis* strain and *M. tuberculosis* strains isolated from tissues samples.

3.3. Monoplex Conventional PCR Analysis

The first of these was a classical PCR for detection of *M. bovis* and *M. tuberculosis*, giving a 372 bp amplified product. Three out of 17 tissues samples collected from IDTT positive cows had a positive PCR produced a 372 bp DNA amplified product which was detected in ethidium bromide stained agarose gels (Fig. 1).

The use of molecular tests permits the identification of specific sequences of *M. tuberculosis* complex including *M. bovis* and *M. tuberculosis*. This primers corresponding to the gene that codified the secretion of MPB70 protein, the most abundant antigen found in culture supernatant in vaccine and virulent strains of *M. bovis*. MPB70 stimulates the immune cell and humoral response during the infection in bovine and humans. By virtue of the specificity of the MPB70 antigen and the previous description of the oligonucleotides (TB1-F and TB1-R) to amplify the gene segments, this method was considered to confirm the TB diagnosis in tissues samples but showed low sensitivity [23].

![Fig. 1. Profile generated by monoplex PCR assay. Ethedium bromide stained 1.5% agarose gel electrophoresis showing only the five positive amplified product of 372 bp resulting from species-specific primer TB1. Where, lane M: 50 bp DNA ladder, lanes 1-2: *M. bovis* and *M. tuberculosis* positive controls, lanes 3-15: samples of the 13 IDTT positive animals and lane 16: negative control.](image)

### 3.4. Multiplex Conventional PCR Analysis

The second PCR test was mPCR which used to differentiate between *M. bovis* and *M. tuberculosis* giving 168 bp and 337 bp amplified product with *M. bovis* and *M. tuberculosis*, respectively. The amplification primers for single tube m-PCR, designed in this study, were based on the previously described sequences by [17]. A common forward primer, CSB-F, was designed to hybridize the 229-bp sequence found in both *M. bovis* and *M. tuberculosis*, and complemented bases 50–66. The *M. bovis*-specific reverse primer, CSB-R1, complements bases 217–202 of the 229-bp sequence and is also expected to hybridize to the 229-bp sequence.
sequence from both organisms, but should generate a unique 168-bp PCR product in the case of *M. bovis* only and not in *M. tuberculosis*. This is expected since the 229-bp sequence, present in *M. tuberculosis*, is interrupted at position 197 by a unique 12.7-kb fragment. In principle, in a PCR reaction, the 12.7-kb size insertion in *M. tuberculosis* is beyond the amplification limits of Taq DNA polymerase and, hence, cannot be amplified using primer CSB-R1. In contrast, *M. tuberculosis* specific reverse primer CSB-R2, which complements bases 23,729–23,708 of the 12.7-kb fragment, is designed to hybridize to the 12.7-kb fragment and is expected to generate a unique 337-bp PCR product specific to *M. tuberculosis*.

The m-PCR assay was initially tested with genomic DNA from isolated strain *M. bovis* and *M. tuberculosis*. After several rounds of amplification and testing different annealing temperatures, adequate conditions were found to distinguish two species in a single reaction. Three out of 17 tissue samples were revealed a positive results (two tissue samples give 168 bp DNA amplified product confirming that this tissues were infected with *M. bovis*, while one tissue sample give 337 bp DNA amplified product confirming that this tissues were infected with *M. tuberculosis*) (Fig. 2).

The test (mPCR) can be applied to isolated colonies of suspected mycobacteria instead of using conventional biochemical and drug sensitivity tests, which can take between 2 and 4 weeks. Because of the test’s exquisite sensitivity, we have found that a single colony is sufficient to perform the test and a result can be available within 4 hours [24]. Bakshi et al., [17] reported that the m-PCR assay was sensitive, as the amplicons of 168 bp and 337 bp could be visualized when the PCR was performed with as little as 20pg of genomic DNA from *M. bovis* and *M. tuberculosis* strains, respectively.

False-positive and false negative results, particularly in specimens containing low numbers of bacilli, have reduced the reliability of this test. Variability in results has been attributed to the low copy number of the target sequence per bacillus combined with a low number of bacilli. Variability has also been attributed to decontamination methods, DNA extraction procedures, techniques for the elimination of polymerase enzyme inhibitors, internal and external controls and procedures for the prevention of cross-contamination. Improvement in the reliability of PCR as a practical test for the detection of *M. tuberculosis* complex in fresh clinical specimens will require the development of standardized and robust procedures. Cross contamination is the greatest problem with this type of application and this is why proper controls have to be set up with each amplification. Although direct PCR can produce a rapid result, it is recommended that culture be used in parallel to confirm a viable *M. bovis* infection [7].

3.5. Real Time PCR Analysis

In this study, real time PCR was used by MTB Real-TM Real Time PCR kit for the detection of Mycobacterium tuberculosis complex (Sacace Biotechnology REF, TB15-50FRT). The first step was to optimize the assay, which was carried out on the *M. bovis* and *M. tuberculosis* isolate. The results of the real time PCR as shown in the normalized melt curves. After optimization, 13 out of the 17 tissue samples which subjected to the real time PCR test were giving positive results (Fig. 3).

These real time technique allowed to increase the sensitivity of the diagnosis and enabling detection of small amounts of *Mycobacterium tuberculosis* complex DNA by targeting the multicopy target IS6110 insertion element. IS6110 is a genomic insertion element containing 1,361 bp that is found only in organisms of the *Mycobacterium tuberculosis* complex. *M. tuberculosis* has been shown to contain between 0 and 25 copies of this element [25].

Moreover, real-time PCR monitors the accumulation of
PCR product during the amplification reaction, thus enabling identification of the cycles during which near-logarithmic PCR product generation occurs. In other words, the assay can be used to reliably quantify the DNA content in a given sample. In contrast to conventional PCR, real-time PCR requires less manipulation, is more rapid than conventional PCR techniques, has a closed-tube format therefore decreasing risk of cross-contamination, is highly sensitive and specific, thus retaining qualitative efficiency, and provides quantitative information. In many cases, the real-time PCR assays have proved to be more sensitive than existing reference methods [7, 26].

![Amplification Plots](image)

**Fig. 3.** Amplification plots for real-time PCR assay. Showing amplification curves of 13 tested samples and two positive controls (M. bovis and M. tuberculosis), curves below the threshold of 4 negative tested samples, two negative controls (NCs; M. bovis and M. tuberculosis) and Non-Template Control (NTC).

The obtained results are in agreement with Suheir et al., [27] reported that the sensitivity of the real time PCR assay was clarified by performing 10-fold dilutions using 2 ng of purified DNA for both strains (M. bovis and M. tuberculosis). The melting curve was shown as the DNA level reached 2 pg/reaction, while, Kate, et al., [1] reported that by amplifying species-specific DNA sequences using conventional PCR, 5 fg of mycobacterial DNA (corresponding to one mycobacterium) can be detected in clinical samples.

### 4. Conclusion

The study presented here focuses on accurately diagnosis of the Mycobacterium tuberculosis complex, most commonly associated with bovine TB infection. The use of a PCR assay to detect M. bovis in tissue samples may provide a more rapid method for providing diagnostic test results to field veterinarians than culture. Although direct PCR can produce a rapid result, it is recommended that culture be used in parallel to confirm a viable M. bovis infection.

The real-time PCR assay was more specific and sensitive than conventional and multiplex PCR. There is a need to a large-scale study for development of multiplex real-time PCR diagnostics assay for the accurate identification and differentiation of all members of the MTC.

### References


Nashwa M. Helmy et al.: Evaluation of Different PCR-based Techniques in Diagnosis of Bovine Tuberculosis in Infected Cattle Lymph Nodes


