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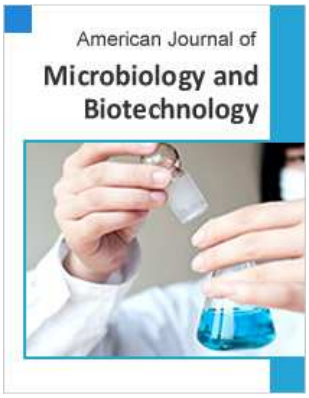

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Evaluation of Different PCR-based Techniques in Diagnosis of Bovine Tuberculosis in Infected Cattle Lymph Nodes

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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

Yearbook [2] and OIE-World Organization for Animal Health [3] both reported that bovine TB is present in the majority of African countries.

Active animal tuberculosis outbreaks represent possible sources of infection to both animal and human populations [4]. The estimated proportion of human cases infected with *Mycobacterium bovis* in developing countries accounted to be 3.1% for all forms of tuberculosis [5]. Moreover, in an analysis on more than 300 *Mycobacterium* strains originating from human sputum, which was conducted in Egypt, approximately five percent of these strains were diagnosed as *M. bovis* [6].

M. bovis infects humans, primarily by the ingestion of unpasteurized dairy products but also via aerosols and through breaks in the skin. Raw or undercooked meat can also be a source of the infection [7].

Bovine TB is responsible for the condemnation of a significant amount of all inspected meat. In 1991, Egypt meat and viscera condemnation estimated at a value of nearly US$ 5 million [8]. Most human tuberculosis cases due to *M. bovis* occur in young individuals resulting from drinking or handling of contaminated milk [9]. Although cattle are considered to be the major hosts of *M. bovis*, the disease has been reported in many domesticated and non-domesticated animals.

In live cattle, tuberculosis is usually diagnosed in the field with the tuberculin skin test. In this test, tuberculin is intradermally injected; a positive test is indicated by a delayed hypersensitivity reaction (swelling). A major drawback for using of this test in wildlife species is the fact that two sedations within a 2-3 days' time interval are required. Besides that, newly infected animals cannot be detected, positive results are primarily seen in animals infected for 1 to 9 weeks. Main argument in favor of the use of the tuberculin skin test in cattle is its cost-effectiveness. False negative responses are sometimes seen soon after infection, in the late stages of the disease, in animals with poor immune responses and in those that have recently calved [10].

During the past decade, advances in PCR technology have resulted in these molecular diagnostics to become key procedures for TB diagnoses [11]. In diagnostic laboratories the use of PCR is limited due to the high cost and sometimes the availability of adequate test sample volume. To overcome these shortcomings and also to increase the diagnostic capacity of PCR, multiplex PCR (mPCR) has been introduced [12]. In multiplex PCR more than one target sequence can be amplified by including more than one pair of primers in the reaction. Multiplex PCR has the potential to produce considerable savings of time and effort within the laboratory without compromising test utility [13].

Moreover, in the past few years, quantitative real-time PCR (qPCR) tests have been extensively developed in clinical microbiology laboratories for routine diagnosis of infectious diseases, particularly bacterial diseases. This molecular tool is well-suited for the rapid detection of bacteria directly in clinical specimens, allowing early, sensitive and specific laboratory confirmation of related diseases [14].

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 CO_2. 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.*

	Sequences	Amplified product
TB1-F	5'-GAACAATCCGGAGTTGACAA-3'	
TB1-R	5'-AGCACGCTGTCAATCATGTA-3'	372 bp
CSB-F	5'-TTCCGAATCCCTTGTGA-3'	
CSB-R1	5'-GGAGAGCGCCGTTGTA-3'	168 bp
CSB-R2	5'-AGTCGCGTGGCTTCTCTTTTA-3'	337 bp

2.7. Monoplex and Multiplex Conventional PCR

DNA amplification was done in 25 µl reaction volume containing 5 µl of Taq master ready–to–use mixes for PCR (Jena Bioscience, Cat No. 102S), 10 µM of each oligonucleotide primers, 5 µl of DNA template and fill up to 25 µl with DNAse-RNAse free water. The optimized PCR program for TB1 primers was as follows: initial denaturation of 5 min at 94ºC; 40 cycles of 30 s at 94ºC, 1 minute at 62ºC and 1min at 72ºC; and final extension step at 72ºC for 5 min [19]. While, The optimized PCR program for mPCR using CSB-F, CSB-R1 and CSB-R2 primers was as follows: initial denaturation of 5 min at 94ºC; 30 cycles of 1 min at 94ºC, 1.5 minute at 52.3ºC and 1 min. at 72ºC; and final extension step at 72ºC for 5 min [17]. After amplification a 5 µl of the reaction product was mixed with 1 µl of 6X gel loading buffer and subjected to electrophoresis on 1.5% agarose gel at 100V for 30 min. The Gel was stained with ethiduim bromide and photographed on UV transilluminator. Samples considered positive when a single band of DNA were observed at 372 bp (for *M. bovis*) or two bands at 168 bp and 337 bp (for *M. bovis* and *M. tuberculosis,* respectively). The products were visualized and photographed using a Gel Doc™ XR+ System with Image Lab™ Software (Bio-Rad®).

2.8. Real Time PCR

Real time DNA amplification was done according to the manufacturer's protocol of MTB Real-TM Real Time PCR kit for the detection of *Mycobacterium tuberculosis* complex (MTC) (REF: TB15-50FRT, Sacace Biotechnologies®). The reaction was conducted in 25 µl total volume containing of 15 µl of reaction mixture consist of: 10 µl of PCR-mix-1, 5 µl of PCR Buffer Flu, 0.5 µl of TaqF DNA Polymerase and 0.5µl of UDG-Enzyme, and 10 µl of extracted DNA. The optimized PCR program for using TB1 primer was as follow: initial denaturation of 15 min at 95ºC; 45 cycles of 15 s at 95ºC, 30 s at 65ºC and 15 s at 72ºC. The results were interpreted according to the presence of fluorescence FAM curve with the threshold line.

3. Results and Discussion

The results of field test represented in IDTT and laboratory tests represented by culture examination of all tissues samples of infected animals, conventional PCR, multiplex PCR, and real time PCR are summarized in table (2).

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

Test	No. of examined animals	No. of positive cases	Percentage* %
Field Test (IDTT)	70	17	24
Isolation and identification	17	13	76
Monoplex PCR	17	3	18
Multiplex PCR	17	3	18
Real time PCR	17	13	76

* = 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].

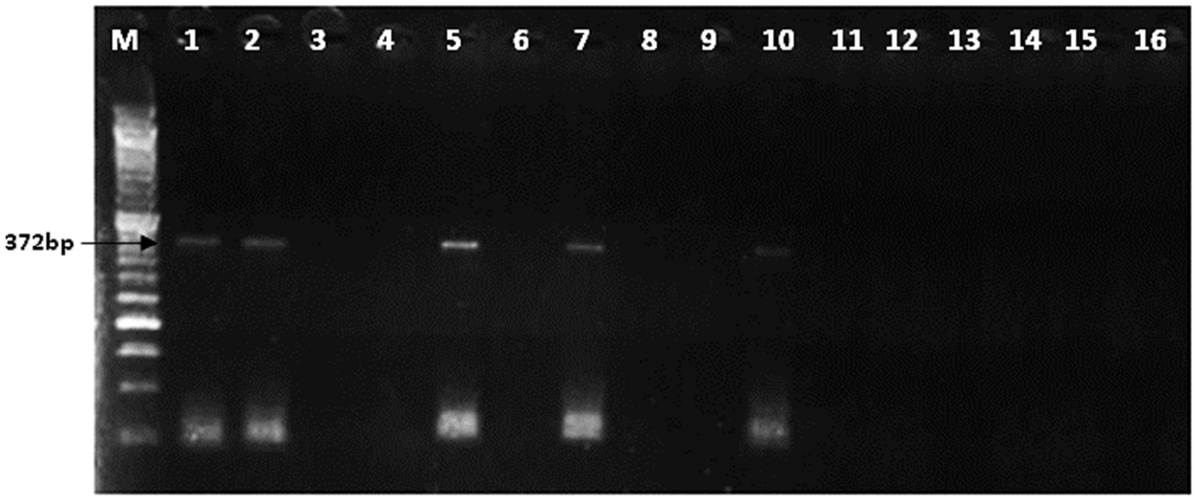

Fig. 1. *Profile generated by monoplex PCR assay. Ethedium bromide stained 1.5% agarose gel electrophoresis showing only the five positive amplified product of 372 bp resulting from species-specific primer TB1. Where, lane M: 50 bp DNA ladder, lanes 1-2: M. bovis and M. tuberculosis positive controls,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

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 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].

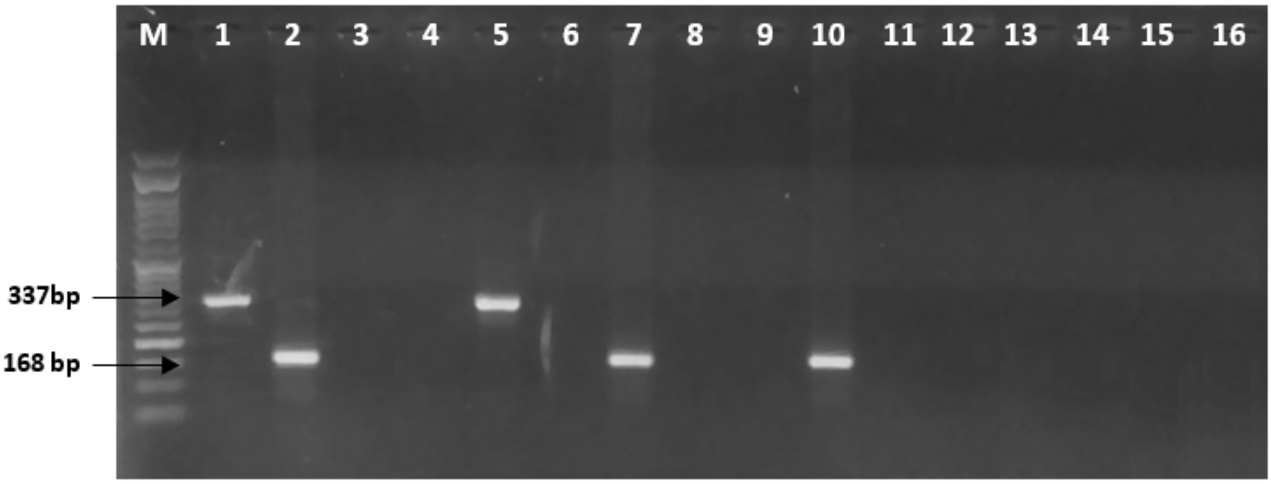

Fig. 2. *Profile generated by multiplex PCR assay. Ethedium bromide stained 1.5% agarose gel electrophoresis showing amplified product of 337bp and 168 bp for M. tuberculosis and M. bovis resulting from amplification generated from species-specific oligonucleotide primers CBS-F with CBS-R1 and CBS- R2. Lane M: 50 bp DNA ladder, lane 1-2: M. tuberculosis and M. bovis positive controls, lanes 3-15: samples of the 13 IDTT positive animals, and lane 16: negative control.*

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 tissues 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. IS*6110* 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].

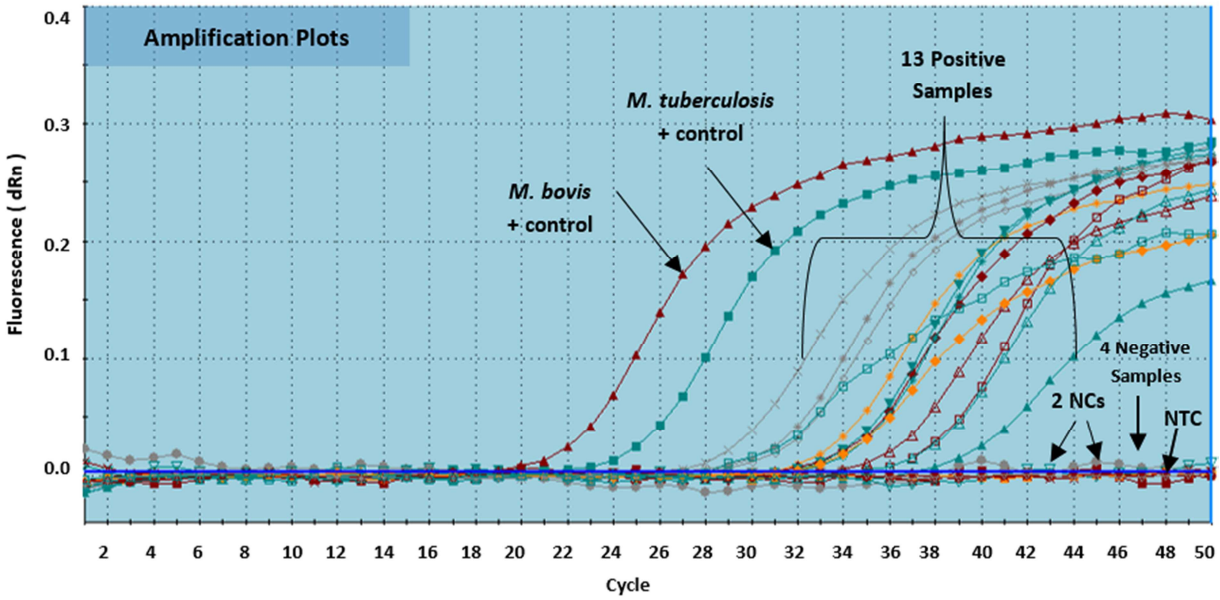

Fig. 3. *Amplification plots for real-time PCR assay. Showing amplification curves of 13 tested samples and two positive controls (M. bovis and M. tuberculosis), curves below the threshold of 4 negative tested samples, two negative controls (NCs; M. bovis and M. tuberculosis) and Non-Template Control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

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