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MOLECULAR STUDIES ON *MYCOBACTERIUM*

By

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ABSTRACT

Bovine tuberculosis is a chronic bacterial disease of animals and humans caused by *M. 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. 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 the current study: A total of 49 specimens were collected from four major abattoirs (El-Basateen, El-Monieb, Beni-Suef and Al-Fayoum) to be analyzed bacteriologically for: isolation, identification and confirmation of *M. bovis* with molecular methods. Only 19 isolates were found to be positive slow-growers *Mycobacterium* species by conventional cultivation method on solid medium (Lowenstein-Jensen medium). Genotyping detection of MTC by amplification of gene responsible for production of MPB70 secretory protein by conventional PCR and by amplification of extRD9 region by real-time PCR was carried out directly on specimens. Out of 49 DNA templates extracted directly from specimens, 14 specimens were confirmed to be infected by MTC by conventional PCR and 31 specimens were confirmed to be infected by MTC by real-time PCR. The results revealed that real-time PCR assay was the most sensitive, powerful and efficient assay compared with conventional PCR.

Keywords:

Bovine tuberculosis- *M. bovis* - Conventional PCR - Real-time PCR.

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 **(Kate *et al.,* 2011)**. 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 **(FAO, 1993)** and OIE-World Organization for Animal Health **(OIE, 1988 - 1992)** 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 **(Thoen *et al.,* 2006)**. The estimated proportion of human cases infected with *M. bovis* in developing countries accounted to be 3.1% for all forms of tuberculosis **(Leite *et al.,* 2003)**. 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* **(El-Sabban *et al.,* 1992)**. 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 **(Waddington, 2010).** During the past decade, advances in PCR technology have resulted in these molecular diagnostics to become key procedures for TB diagnoses **(Beyrer *et al.,* 1998)**. 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 **(Wagar, 1996)**.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 **(Max, 2012).**The aim of this work was to isolate MTC by traditional culturing method, compare and evaluate the efficiency of conventional PCR, and real-time PCR techniques in the diagnosis of bovine tuberculosis directly from specimens.

MATERIAL AND METHODS

1. Samples:

A total of 49 specimens were collected from 4 abattoirs (El-Basateen, El-Monieb, Beni-Suef and Al-Fayoum) during the period of early winter of 2015 to winter of 2017 to be analyzed bacteriologically for: isolation and confirmation of MTC with molecular methods. The samples were collected from internal organ (lung-liver-spleen) and lymph nodes showing tuberculosis-like lesions (granulomatous changes resembling tuberculosis) from cattle carcasses slaughtered during routine work in the abattoirs previously mentioned. The collected samples were collected aseptically and were transmitted to Animal Health Research Institute, Dokki, and Giza. Each sample was divided into two portions (one for bacteriological lab and the other for biotechnology lab) to be investigated phenotypically and genotypically in the same time.

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 into a set of solid egg-based media (LJ). Cultures were incubated for a minimum of 8 weeks at 37°C with CO2. The media was 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-Neelsen technique. Growth of *M. bovis* generally occurs within 3-6 weeks of incubation. Isolates was identified by determining traditional cultural and biochemical properties **(OIE, 2009)**.

3. DNA Extraction from Bovine Tissue Samples and Mycobacterial Isolates:

The Deoxyribose Nucleic Acid (DNA) extraction from tissue samples and mycobacterial isolates was carried out using GeneJET Genomic DNA Purification Kit, Thermo Scientific (Cat. No. K0721). All strains used for validation were kindly provided by Veterinary Serum and Vaccine Research Institute (VSVRI). The Genomic DNA was extracted from both mycobacterial strains and non-mycobacterial strains by using Gene JET Genomic DNA Purification Kit.

4. Primers synthesis:

For conventional PCR, two oligonucleotide primers were designed and synthesized as *tb*1-F and *tb*1-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 MTC **(Debra *et al.*, 1992).**

For real-time PCR, Primers and probes synthesis for detection of MTC using real-time PCR, two oligonucleotide primers and probe were designed to target a conserved regions that found external to RD9 and present in all members of the MTBC (ext-RD9 F, ext-RD9 R and ext-RD9 P) (Table 2).

5. 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 **(Debra *et al.*, 1992).** 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 MTC). The products were visualized and photographed using a Gel Doc™ XR+ System with Image Lab™ Software (Bio-Rad®).

6. Real-time PCR:

All genomic DNA directly extracted from tissue samples and references strains were included in real-time polymerase chain reaction analysis in order to detect whether they belong to MTC

or not. This assay was performed in a 25-µl final reaction volume. The reaction mixture consisted of 12.5 µl of SensiFAST™ Probe Hi-ROX 2x Mix, 1 µl (10 Pmol) of each primer forward and reverse (ext-RD9), 0.5 µl (5 Pmol) of probe (ext-RD9), 5 µl of PCR grade sterile water and 5 µl of DNA template. The real-time PCR tube was tightly closed, vortexed, centrifuged and placed into the block/rotor of light thermocycler. Thermocycling conditions were as follows: 1 cycle at 95°C for 10 min, followed by 50 cycles at 95°C for 15 sec and 60°C for 1 min. Fluorophore was used in MTBCRD real-time PCR was FAM-BHQ.

RESULTS AND DISCUSSION

Out of 49 isolates were originating from cattle showing tuberculosis like lesion in PM finding, only 19 isolates were found to be positive slow-growers *Mycobacterium* species showing rough, crumbly, waxy and non-pigmented (Cream colored) colonies after the bacteriological examination using conventional cultivation method on solid medium (LJ medium) Fig. (1). 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 **(OIE, 2009)**.The failure of detecting mycobacteria in the samples giving no isolates may be related to the low number of mycobacteria present in the sample and perhaps the uneven distribution throughout the body or even accumulation within a single lymph node as stated by **(Thomson, 2006)**. A total of 19 isolates smears were prepared from positive culture on LJ media and were stained with Ziehl-Neelsen stain. All positive smears were identified as straight or slightly curved, non-motile and non-spring acid-fast rods. Conventional PCR for detection of *M. bovis* and *M. tuberculosis*, giving a 372 bp amplified product. Only 14 out of 49 tissues samples had a positive PCR produced a 372 bp DNA amplified product which was detected in ethidium bromide stained agarose gels Fig. (2).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 (*tb*1-F and *tb*1-R) to amplify the gene segments, this method was considered to confirm the

TB diagnosis in tissues samples but showed low sensitivity **(Ciro *et al.*, 2004).** 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. However, PCR is now being used on a routine basis in some laboratories to detect the *M. tuberculosis* group in paraffin-embedded tissues **(Miller *et al.*, 2002)**.Out of 49 DNA templates extracted directly from specimens, 31 specimens were confirmed to be MTC by amplification of extRD9 region by real-time PCR Fig.(3). The obtained results are in agreement with the sensitivity of the real-time PCR assay which was clarified by performing 10-fold dilutions using 2 ng of purified DNA for both strains (*M. bovis* and *M. tuberculosis*) **(Suheir *et al.*, 2010)**. The ease of use, decrease in hands-on time, and decreased potential for amplicon contamination found with this assay compared to conventional PCRs are invaluable **(Tanya *et al.*, 2011)**. Real-time PCR has been widely evaluated for the detection of *M. tuberculosis* complex in clinical samples (mainly sputum) in human patients and has recently been used for the diagnosis of tuberculosis in animals. A, number of commercially available kits and various'in-house' methods have been evaluated for the detection of the *M. tuberculosis* complex in fresh and fixed tissues. Although direct PCR can produce a rapid result, it is recommended that culture be used in parallel to confirm a viable *M. bovis* infection and all these are in agreement with this study as this study was depended on culturing, confirming the results with conventional PCR and real-time PCR directly on clinical specimens (Table 3).

CONCLUSION

The study presented here focuses on accurately diagnosis of the MTC, 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 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.

Table (1): Oligonucleotide sequences of primers specific for MTC for conventional PCR.

Primer	Sequences	Amplified product
tb1-F	5'-GAACAATCCGGAGTTGACAA-3'	372 bp
tb1-R	5'-AGCACGCTGTCAATCATGTA-3'	

Table (2): Oligonucleotide sequences of primers and probes specific for MTC for real-time PCR.

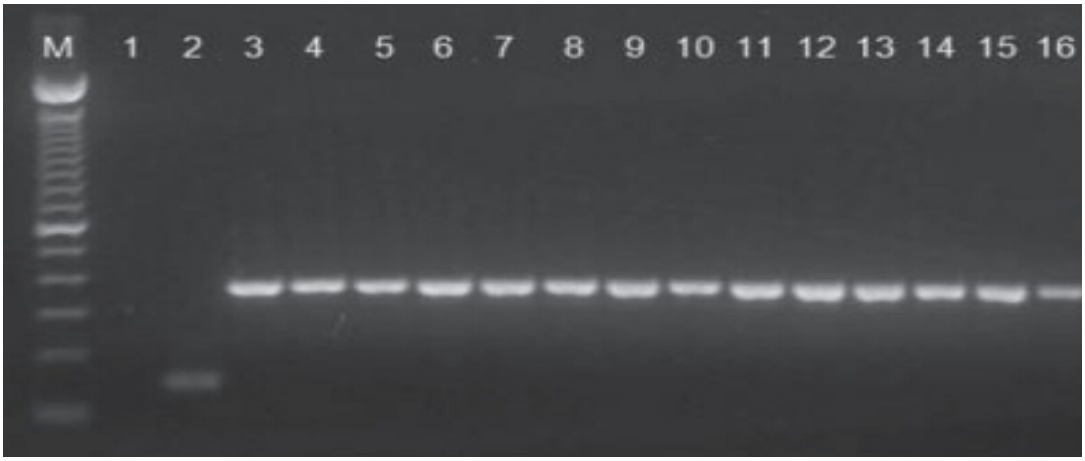
Target, primer and probe	Sequence 5' 3' →	Fluorophore	r^2 value	Calculated efficiency (%)
ext-RD9 F R P	GCC ACC ACC GAC TCA TAC CGA GGA GGT CAT CCT GCT CTA G+TT +CTT CAG +CTG GT+C C	FAM-BHQ	0.99	88

Table (3): Comparative studies between traditional cultivation method, conventional PCR and real-time PCR.

Test	No. of specimens	No. of positive samples	Percentage* %
Traditional cultivation method	49	19	38.77 %
Conventional PCR	49	14	28.57 %
Real-time PCR	49	31	63.26 %

*** =Percent calculated according to No. of specimens.**

Fig. (1): Culture positivity of *M. tuberculosis* complex on LJ medium.



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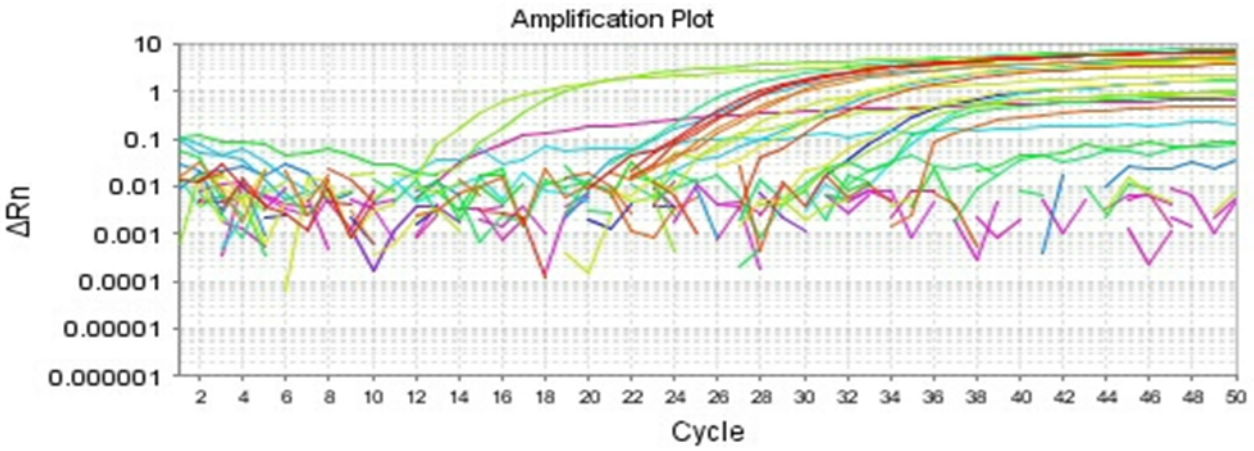

Fig. (2): Profile generated by conventional PCR assay. Ethedium bromide stained 1.5% agarose gel electrophoresis showing only 13 positive amplified for MPB70 secretory protein gene with product of 372 bp resulting from species-specific primer *tb*1. Where, lane M: 100 bp DNA ladder, lane 1: Negative control, lane 2: Negative sample, lanes 3-15: Positive sample for *Mycobacterium tuberculosis* complex, lane 16: Positive control.

Fig. (3): Amplification plot of *Mycobacterium tuberculosis* complex by *ext*-RD9 real-time PCR.

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