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Comparative Study Between Traditional Methods and Molecular Methods in Diagnosis of Bovine Tuberculosis

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Abstract

Mycobacterium bovis is the major causative agent of bovine tuberculosis (BTB) and part of the *Mycobacterium tuberculosis complex* (MTBC). BTB has an impact on the national and international economy, affects the ecosystem via transmission to wildlife and is of public health concern due to its zoonotic potential. Although still present in some industrialized countries, BTB today mostly affects developing countries lacking the resources to apply expensive test and slaughter schemes. Tuberculosis (TB) remains a global health problem despite near eradication in some developed countries. This study was conducted from early winter of 2015 to winter of 2017 to compare between conventional and molecular techniques for detection of *Mycobacterium bovis* (*M. bovis*) in Egypt. A total of 49 specimens were collected from four major abattoirs (El-Basateen-El-Monieb- Beni-Suef- Al-fayoum) to be analyzed bacteriologically and biochemically 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 (LJ medium and Stone brink) and identified biochemically to 17 *M. bovis* isolates and 2 isolates *M. tuberculosis*. Genotyping detection of *Mycobacterium tuberculosis complex* by amplification of ext-RD9 region by real-time PCR was carried out on positive cultures and directly on specimens. Out of 49 DNA templates extracted directly from specimens, 31 specimens were confirmed to be infected by *Mycobacterium tuberculosis complex* by amplification of ext-RD9 region by real-time PCR. This study reports the development and evaluation of a single-tube, two-targets, real-time PCR assay which can differentiate between *M. bovis* and *M. tuberculosis*. The multiplex real-time PCR target RD1 and RD4 using 2 sets of primers-probes. A 31 MTBC positive DNA from clinical specimens were identified by this assay as, 27 *M. bovis* isolates and 4 *M. tuberculosis* isolates. A 19 positive cultures were confirmed to be *Mycobacterium tuberculosis complex* were identified by this assay as, 17 *M. bovis* isolates and 2 *M. tuberculosis* isolates.

1. Introduction

Tuberculosis (TB) is recognized as one of the most important threats to human and

animal health causing mortality, morbidity and economic losses [1]. It remains a major global health problem and causes ill-health among millions of people each year and ranks as the second leading cause of death from an infectious disease worldwide after the human immunodeficiency virus (HIV) [2]. Tuberculosis is communicable mycobacterial disease caused by members of *Mycobacterium tuberculosis complex* (MTBC) [3]. Although, recent studies indicated that *M. tuberculosis* has been isolated from cattle [4] and *M. bovis* from humans infected with bovine tuberculosis [5], *M. tuberculosis* is specifically adapted to humans while *M. bovis* is most frequently isolated from domesticated cattle [1], In spite of variation in host specificity, the members of MTBC are characterized by 99.9% or greater similarity at nucleotide level and are virtually identical at 16s ribosomal Ribonucleic Acid (rRNA) sequence [6].

Bovine tuberculosis is a chronic bacterial disease characterized by progressive development of tubercles in any tissue/organ of the body [7, 8]. It has been recognized from 176 countries as one of the important bovine diseases causing great economic loss [9]. Bovine tuberculosis is a contagious disease, which can affect most warm-blooded animals, including human being [10]. Organisms are excreted in the exhaled air, in sputum, feaces (from both intestinal lesions and swallowed sputum from pulmonary lesions), milk, urine, vaginal and uterine discharges, and discharges from open peripheral lymph nodes of infected animals [10].

In cattle, exposure to this organism can result in a chronic disease that jeopardizes animal welfare and productivity and in some countries leads to significant economic losses by causing ill health and mortality [11]. Moreover, human TB of animal origin caused by *M. bovis* is becoming increasingly evident in developing countries [12, 13]. Bovine tuberculosis diseased animal loses 10 to 25% of their productive efficiency; direct losses due to the infection become evident by decrease in 10 to 18% milk and 15% reduction in meat production [14]. Apart from effects on animal production, it has also a significant public health importance [15]. Currently, the disease in human is becoming increasingly important in developing countries, as humans and animals are sharing the same micro environment and dwelling premises, especially in rural areas, and HIV infection increases individuals' susceptibility to TB infection [16]. It is estimated that *M. bovis* causes 10 to 15% of human cases of tuberculosis in countries where pasteurization of milk is rare and bovine tuberculosis is common [17].

In developing countries, the socio economic situation and low standard living area for both animals and humans are more contributing in TB transmission from human to human and from human to cattle or vice versa [18]. Human infection due to *M. bovis* is thought to be mainly through drinking of contaminated or unpasteurized raw milk and under cooked meat. The high prevalence of TB in cattle, close contact of cattle and humans, the habit of raw milk and meat consumption, and the increasing prevalence of HIV may all increase the potential for transmission of *M. bovis* and other Mycobacteria between cattle and humans [16]. 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 [19].

During the past decade, advances in Polymerase Chain Reaction (PCR) technology have resulted in these molecular diagnostics to become key procedures for TB diagnoses [20]. 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 [21]. 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 [22]. 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 [23].

The aim of this work was to compare between traditional methods and molecular methods in diagnosis of tuberculosis in cows and evaluate the efficiency of real-time PCR and multiplex real-time PCR techniques in the diagnosis of tuberculosis directly from biological specimens of slaughtered cows.

2. Materials and Methods

A total of 49 specimens were collected from 4 abattoirs (El-Basateen- El-Monieb- Beni-suef- Al-fayoum) during the period of early winter of 2015 to winter of 2017 to be analyzed bacteriologically and biochemically for: isolation, identification and confirmation of *M. bovis* with molecular methods.

The samples were collected from internal organ (lung-liver- spleen) and lymph nodes showing tuberculosis-like lesions 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, Giza.

Each sample was divided to two portions (one for

bacteriological lab and the other for biotechnology lab) to be investigated phenotypically and genotypically in the same time.

2.1. Samples Decontamination

Organs and tissues showing gross lesions or congestion were transferred to a sterile mortar containing washed sterile sand. The fat was trimmed and the suspected material was cut into small pieces. Two ml of sterile distilled water were added to the crushed tissue, homogenized and ground till suspension was obtained.

An equal amount of 2 ml of suspension sample and NAOH-NALC solution was placed into a 15 ml centrifuge tube. The amount was capped tightly and was vortexed for 20 seconds. The tube was inverted to ensure NAOH-NALC solution contacts the entire interior surface of the tube and lid. The tube was stranded for a minimum of 15 minutes which was prolonged by a few minutes if the sample was particularly thick but did not exceed 20 minutes. The tube was filled to 14 ml with phosphate buffer (pH 6.8) to neutralize alkali and terminate the decontamination process. The tube was centrifuged at 3000 g for 15 minutes. Supernatant was carefully poured off into a liquid waste container with 10% sodium hypochlorite and the pellet was retained.

2.2. Lowenstein–JENSEN Culturing

Only 0.2 – 0.4 ml (2 – 4 drops) of centrifuged sediment of decontaminated specimen was inoculated on slopes of Lowenstein–Jensen (LJ) medium. They were distributed over the surface, incubated at 37°C in a slant position with the screw cap loose for at least a week until the sediment has been absorbed. Then, they were incubated upright until growth is observed or discarded negative after eight weeks. 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 depending on the media used. Isolates can be identified by determining traditional cultural and biochemical properties [24].

2.3. Niacin Test

Only 1 ml of sterile saline was added to the culture slant (at least 3 weeks old containing at least 50 colonies). After puncturing the medium, the slant was placed horizontally (so fluid cover all surface of the media) for 30 minutes at room temperature for niacin extraction then raised upright for 5 minutes. Then, only 0.5 ml of the fluid extract was removed in screw cap tube. The niacin strip was inserted for 15 – 20 minutes until yellow color of fluid is observed or discarded as negative [25].

2.4. Catalase Test

Only 1 ml of freshly prepared Tween-peroxide mixture was added to 14-day old LJ. The formation of bubbles appearing on the surface was observed or discarded as negative after 20 minutes [26].

2.5. PNB Test

An amount of 0.2 – 0.4 ml (2 - 4 drops) of centrifuged sediment of decontaminated specimen was inoculated. Slopes of LJ media containing ParaNitroBenzoic acid (PNB) at a concentration of 500 mg/litre were distributed over the surface, incubated at 37°C, and then examined at 3, 7, 14 and 21 days [27].

2.6. TCH Test

An amount of 0.2 – 0.4 ml (2 - 4 drops) of centrifuged sediment of decontaminated specimen was inoculated. Slopes of LJ media containing Thiophene-2-Carboxylic acid Hydrazide (TCH) at a concentration of 2 mg/ml were distributed over the surface, incubated at 37°C [28].

2.7. Methods Used for 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 GeneJET Genomic DNA Purification Kit.

2.8. Primers and Probes Synthesis

For detection of *Mycobacterium tuberculosis complex* 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 1).

For multiplex real-time PCR, another 2 sets of primers and their probes were used for characterization and differentiation between *M. bovis* and *M. tuberculosis*: RD1 F, RD1 R, RD1 P and RD4 F, RD4 R, RD4 P. Based on primer design scheme, *M. tuberculosis* is expected to reveal positive results with 2 sets of primers and probes RD1 and RD4; while, *M. bovis* is expected to reveal positive result only with RD1 and negative with RD4.

Table 1. *Oligonucleotide sequences of primers and probes.*

Target, primer and probe	Sequence (5' ⇒ 3')	Fluorophore	r^2 value	Calculated efficiency (%)
RD1				
F	CCC TTT CTC GTG TTT ATA CGT TTG A	FAM-BHQ	0.98	90
R	GCC ATA TCG TCC GGA GCT TCAC			
P	TCT GAG AGG TTG TCA			
RD4z				
F	CCA CGA CTA TGA CTA GGA CAG CAA	HEX-BHQ	0.98	97
R	AAG AAC TAT CAA TCG GGC AAG ATC			
P	ACC AGT GAG GAA ACC			
ext-RD9				
F	GCC ACC ACC GAC TCA TAC	FAM-BHQ	0.99	88
R	CGA GGA GGT CAT CCT GCT CTA			
P	G+TT +CTT CAG +CTG GT+C C			

2.9. Real-Time PCR for Detection of *Mycobacterium tuberculosis complex*

All genomic DNA directly extracted from tissue samples, isolates cultures and references strains, were included in real-time polymerase chain reaction analysis in order to detect whether they belong to *Mycobacterium tuberculosis complex* 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 MTBC-RD real-time PCR was FAM-BHQ.

2.10. Multiplex Real-Time PCR for Differentiation Between *M. tuberculosis* and *M. bovis*

Only positive cases identified by MTBC real-time PCR from tissue samples, isolates cultures and references strains were used for further validation by using multiplex real-time PCR in order to differentiate between *M. bovis* and *M. tuberculosis*.

The multiplex real-time PCR was conducted as following: 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 RD1 forward and reverse, 0.5 µl (5 Pmol) of probe, 1 µl (10 Pmol) of each primer RD4 forward and reverse, 0.5 µl (5 Pmol) of probe, 2.5 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. The fluorophore which was used in RD1 was FAM-BHQ and the fluorophore which was used in RD4 was HEX-BHQ in multiplex real-time PCR.

3. Results and Discussion

3.1. Conventional Test Cultivation on Solid Media LJ

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 and stone brink).

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 [29].

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 [30].

3.2. Microscopical Examination of Isolates

A total of 19 isolates smears were prepared from positive culture on LJ media and was stained with Ziehl-Neelsen stain. All positive smears were identified as straight or slightly curved, non-motile and non-sporing acid-fast rods.

3.3. Biochemical Identification

With the goal of differentiation between *Mycobacterium tuberculosis complex* and other mycobacterial members, PNB test was developed, based on inhibition growth of *Mycobacterium tuberculosis complex* members on PNB medium. TCH, Niacin test and Catalase test were used for differentiation between *M. bovis* and *M. tuberculosis* [29] as 17 isolates gave negative result for these test that mentioned before and was suggested to be *M. bovis*. On other hand, 2 isolates gave positive results with these tests that mentioned before and was suggested to be *M. tuberculosis*.

3.4. Real-Time PCR for Detection of *Mycobacterium tuberculosis complex*

Out of 49 DNA templates extracted directly from specimens, 31 specimens were confirmed to be *Mycobacterium tuberculosis complex* by amplification of ext-RD9 region by real-time PCR. 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*) [33].

The 19 isolates cultures showed positive results to *Mycobacterium tuberculosis complex* by real-time PCR assay. This assay was evaluated on culture isolates that resulted from conventional culturing method and also on direct clinical specimens to compare between efficacy of traditional method and molecular method in diagnosis of *Mycobacterium tuberculosis complex.* 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 [31].

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. 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. However, PCR is now being used on a routine basis in some laboratories to detect the *M. tuberculosis* group in paraffin-embedded tissues [32]. 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 real-time PCR and application of real-time PCR directly on clinical specimens.

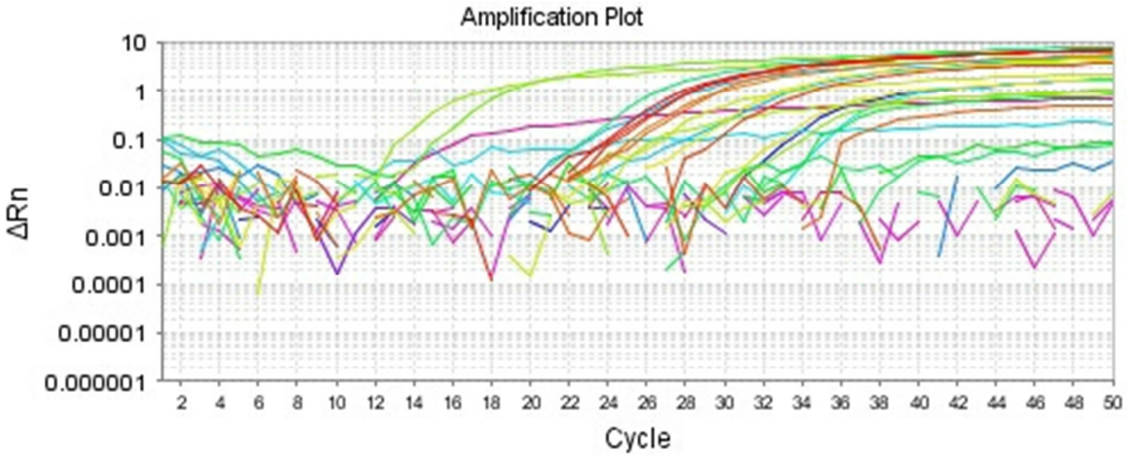

Figure 1. *Amplification plot of Mycobacterium tuberculosis complex by ext-RD9 real-time PCR.*

3.5. Multiplex Real-Time PCR for Differentiation Between *M. bovis* and *M. tuberculosis*

The multiplex real-time PCR target RD1 and RD4 in order to differentiate between *M. bovis* and *M. tuberculosis*. The analysis of 31 MTBC positive DNA which resulted from pervious real-time PCR directly on clinical specimens were identified by this assay as, 27 *M. bovis* isolates and 4 *M. tuberculosis* isolates. Furthermore, 19 positive cultures which were confirmed to be *Mycobacterium tuberculosis complex* by pervious real-time PCR were identified by this assay as, 17 *M. bovis* isolates and 2 *M. tuberculosis* isolates.

A single step multiplex real-time PCR was developed to evaluate specificity of primers and probes which were used to distinguish between members of MTC at species level. This PCR reaction was applied on other non-mycobacterial strains (*E. coli, Shigella* Spp., *Listeria* Spp.) and gave negative results with this assay which proved the specificity of used primers and probes. Furthermore, a multiplex real-time PCR using RD1 and RD4 primers and probes 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 [34]. This study describes the evaluation of a multiplex real-time PCR assay that can accurately differentiate between *M. bovis* and *M. tuberculosis* in 2.5 hrs and can be introduced in the molecular diagnosis of mycobacteria. This assay provides added value over the multiple single-target conventional PCR assays and other published assays because of its high level of specificity and sensitivity, short turnaround time, cost-effectiveness, and performance on clinical specimens determined to be positive for MTBC. The use of the MTBC-RD real-time PCR assay may save up to 8 weeks of time to differentiate species of the MTBC and may provide information to achieve proper drug therapy and early insight into TB transmission.

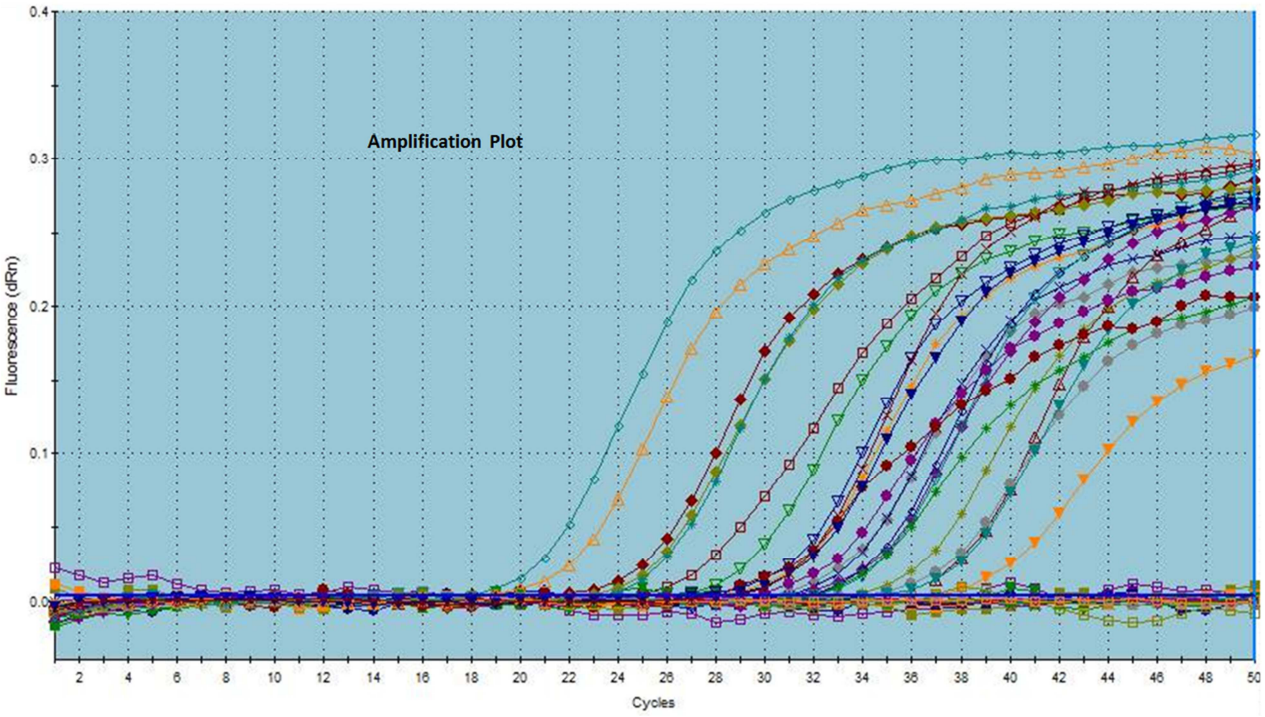

Figure 2. *Amplification plot for multiplex real-time PCR for differentiation between Mycobacterium bovis and Mycobacterium tuberculosis (For RD1 as detected by FAM).*

This study reports the development and evaluation of a single-tube, two-target, real-time PCR assay which can differentiate between *M. bovis* and *M. tuberculosis* in approximately 2.5 hrs directly on clinical specimens. The ease of use, decrease in hands-on time, and decreased potential for amplicon contamination found with this assay compared to separate PCR are invaluable [31]. Additional benefit was reported by a substantial savings in both technician time and consumable costs when comparing this new assay to the existing assay, which requires multiple conventional PCRs and post amplification analysis. Turnaround time was also significantly reduced by the run time of the assay combined with its use on clinical specimens positive for MTBC DNA.

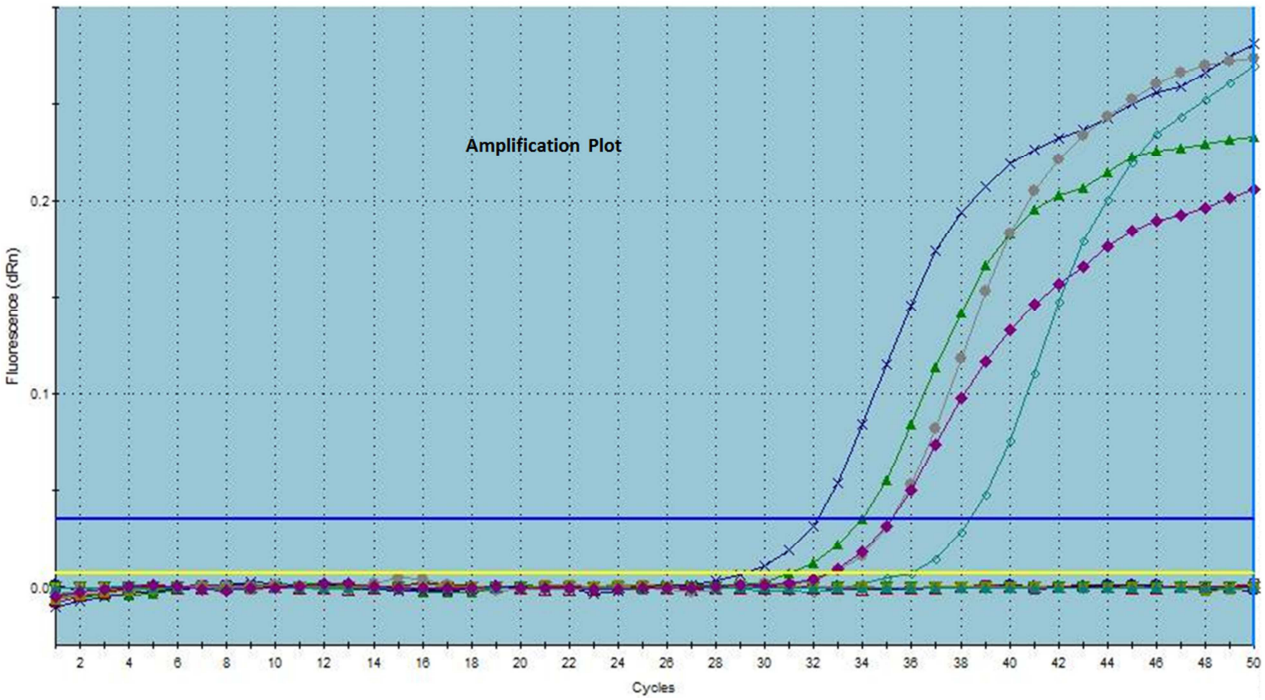

Figure 3. *Amplification plot for multiplex real-time PCR for differentiation between Mycobacterium bovis and Mycobacterium tuberculosis (For RD4 as detected by HEX).*

4. Conclusion

From all pervious results, this study reports that molecular method in detection of *Mycobacterium tuberculosis complex* was more accurate and sensitive than traditional method in detection of *Mycobacterium tuberculosis complex.* When comparing results of both, 12 specimens gave false negative with culturing and gave positive results with direct real-time PCR on clinical specimens. The use of ext-RD9 real time PCR assay to detect *Mycobacterium tuberculosis complex* in tissue samples may provide a more rapid method for diagnoses in veterinarian field than culture.

The multiplex real-time PCR was used to differentiate between *M. bovis* and *M. tuberculosis* using 2 sets of primers-probes RD1 and RD4. This assay was applied on both positive culture isolates and other biological samples. The samples which gave positive curves with RD1 and RD4 were considered to be *M. tuberculosis* and the samples which gave positive curves only with RD1 and negative with RD4 were considered to be *M. bovis.*

References

[1] Smith, N. H., Gordon, S. V., dela Rua-Domenech, R., Clifton-Hadley, R. S. and Hewinson, R. G. (2006) Bottle necks and broomsticks: the molecular evolution of Mycobacterium bovis. NRM. 4: 670-681.

[2] Birhanu, T., Mezgebu, E., Ejeta, E. and Gizachew, A. (2015) Review on Diagnostic Techniques of Bovine Tuberculosis in Ethiopia. Rep Op. 1: 7-14.

[3] Pal, M. (2007) Zoonoses. 2nd ed. Satyam publishers, Jaipur, India. Pp. 124-125.

[4] Ameni, G., Vordermeier, M., Firdessa, R., Aseffa, A., Hewinson, G., Stephen, V. G. and Berg, S. (2011) Mycobacterium tuberculosis infection in grazing cattle in central Ethiopia, The Vet J. 188: 359-361.

[5] Zeweld, S. W. (2014) Cultural and molecular detection of zoonotic tuberculosis and its public health impacts in selected districts of Tigray region, Ethiopia. Sokoto J Vet Sc. 12: 1-12.

[6] Brosch, R., Gordon, S. V., Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., Garnier, T., Guitierrez, C., Hewinson, G., Kremer, K., Parsons, L. M., Pym, A. S., Samper, S., van Soolinge, D., Cole, S. T. (2002) A new evolutionary scenario for the Mycobacterium tuberculosis complex. Proc. Natl. Acad. Sci. USA 99: 3684-3689.

[7] Hlokwe, T. M. van Helden, P. and Michel, A. (2013) Evaluation of the Discriminatory Power of Variable Number of Tandem Repeat Typing of Mycobacterium bovis Isolates from Southern Africa. Tran Em Dis. 60: 111–120.

[8] Pal, M., Zenebe, N. and Rahman, M. T. (2014) Growing significance of mycobacterium bovis in human and health. Microb H. 3: 29-34.

[9] Awah-Ndukum, J. Kudi, A. C., Bradley, G. Ane-Anyangwe, I. P. Titanji, V. K, FonTebug, S. and Tchoumboue, J. (2013) Prevalence of bovine tuberculosis in cattle in the highlands of Cameroon based on the detection of lesions in slaughtered cattle and tuberculin skin tests of live cattle. Vet Med. 57: 59–76.

[10] Radostits, O. M., Gay, C. C., Hinchelift, K. W. and Constabel, P. D. (2007) Veterinary Medicine. A text book of the disease of cattle, sheep, pig, goat and horses. 10th ed., Elsevier, London. Pp. 1007-1040.

[11] Ewnetu, L., Melaku, A. and Birhanu, A. (2012) Bovine Tuberculosis Prevalence in Slaughtered Cattle at Akaki Municipal Abattoir Based on Meat Inspection Methods. Glob Vet. 5: 541-545.

[12] Russel, D. G. (2003) Highlighting the parallels between human and animal tuberculosis. J of vet med edu. 30: 140-142.

[13] Mamo, G., Abebe, F., Worku, Y., Hussein, N., Legesse, M., Tilahun, G., Medhin, G. Bjune, G. and Ameni, G. (2013) Bovine tuberculosis and its associated risk factors in pastoral and agro-pastoral cattle herds of Afar Region, Northeast Ethiopia. JVMAH. 6: 171-179.

[14] Radostits, O. M., Blood, D. C. and Gay, C. C. (1994) Diseases caused by mycobacterium. In: veterinary medicine, a text book of disease of cattle, sheep, pigs, goats and horses. 8th ed. London, Bailliere tindu. Pp. 748-785.

[15] Müller, B. Dürr, S. Alonso, S. Hattendorf, J., Laisse, C. J. M., Sven D. C. Parsons, Paul, D. and Zinsstag, J. (2013) Zoonotic Mycobacterium bovis induced Tuberculosis in Humans. Eme Infec Dis. 19: 899-908.

[16] Shitaye, J. E., Getahun, B., Alemayehu, T., Skoric, M., Treml, F., Fictum, P., Vrbas, V. and Pavlik, I. (2006) A prevalence study of bovine tuberculosis by using abattoir meat inspection and tuberculin skin testing data, histopathological and IS6110 PCR examination of tissues with tuberculous lesions in cattle in Ethiopia. Vet Med. 51: 512-522.

[17] Ashford, D. A., Whitney, E., Raghunathan, P. and Cosivi, O., (2001) Epidemiology of selected mycobacteria that infect humans and other animals, Scientific and Technical Review, OIE. 20: 325–337.

[18] Ejeh, E. F., Markus, I. F., Ejeh, A. S., Musa, J. A. Lawan, F. A., Ameh, J. A., Kudi, A. C. and Cadmus, S. I. B. (2013) Seasonal prevalence of bovine tuberculosis lesion in cattle slaughtered in Yola abattoirs. Bangl JVet Med. 11: 113-120.

[19] Waddington K (2004) To stamp out 'so terrible a malady': bovine tuberculosis and tuberculin testing in Britain, 1890–1939. Med. Hist., 48, 29–48.

[20] Beyrer C, Jitwatcharanan K, Natpratan C, Kaewvichit R, Nelson K E, Chen C Y, Weiss J B, Morse S A (1998) Molecular methods for the diagnosis of genital ulcer disease in a sexually transmitted disease clinic population in Northern Thailand: predominance of the herpes simplex virus infection. J. Infect. Dis., 178: 243–246.

[21] Wagar E A (1996) Direct hybridization and amplification applications for the diagnosis of infectious diseases. J. Clin. Lab. Anal., 10: 312–325.

[22] Wolcott M J (1992) Advances in nucleic acid-based detection methods. Clin. Microbiol. Rev., 5: 370–386.

[23] Max M (2012) Real-time PCR as a diagnostic tool for bacterial diseases. Expert Rev. Mol. Diagn., 12 (7), 731–754.

[24] Springer, B., Wu, W. K., Bodmer, T., Hasse, G., Pfyffer, G. E., Kroppensted, R. M., Schroer, K. H., Emler, S., Kilbrn, J. O., Kirschner, P., Telenti, A., Coyle, M. B. and Bottger E. C. (1996) Isolation and characterization of a unique group of slowly growing mycobacteria: description of mycobacterium lentiflavum sp. J. Clinical Microbial. 34: 1100-1107.

[25] Baron, E. J., Peterson L. R. and Finegold, S. M. (1994) Mycobacteria. In: Bailey and Scotts (eds): diagnostic Microbiology, 9th edition. P. 590.

[26] Vincent, V., Brown Elliott, B. A., Jost, K. C., Jr & Wallace, R. C., Jr (2003) Mycobacterium: Phenotypic and genotypic identification. In: Manual of Clinical Microbiology, 8th edn, pp. 560-584. Edited by: P. R. Murray, E. J. Baron, M. A. Pfaller, J. H. Jorgensen & R. H. Yolken. Washington, DC: American Society for Microbiology.

[27] Leao, S. C., Martin, A., Mejia, G. I., Palomino, J. C., Robledo, J., Telles, M. A. S. and Portaels, F. (2004) Practical handbook for the phenotypic and genotypic identification of mycobacteria. Brugges, Vanden BROELLE; 2004.

[28] Beverly, G. M., Fredrick, S. N., and Richard, J. W. (1999) Mycobacterium. In Manual of clinical microbiology, Patrick R. M., Ellner J. B., Michael A. P., Fred C. T., and Robert H. Y. (ed.). Chapter. 25 p. 399-410, 7th ed. American society for microbiology. Washintgton, D. C.

[29] OIE (2009): Biotechnology in the diagnosis of infectious diseases and vaccine development. Chapter 1. 1.7.

[30] Thomson, B., (2006) Polymerase Chain Reaction detection of Mycobacteria tuberculosis complex in formalin fixed tissues. In: Mycobacterium bovis Infection in Animals and Humans, 2 ed. Eds., Thoen C. O., F. H. Steele and M. J. Gilsdorf. Iowa State University Press, Ames, Iowa, USA, pp: 63-67.

[31] Tanya A. Halse, Vincent E. Escuyer, Kimberlee A. Musser (2011) Evaluation of a Single Tube Multiplex Real-Time PCR for Differentiation of the 2 Mycobacterium tuberculosis Complex in Clinical Specimens. J. Clin. Microbiol. doi: 10.1128/JCM.00467-11.

[32] Miller J., Jenny A. & Payeur J. (2002) Polymerase chain reaction detection of Mycobacterium bovis and M. avium organisms in formalin-fixed tissues from culture-negative organisms. Vet. Micro., 2328, 1–9.

[33] Suheir E, Gila K B, Abedelmajeed K A, Sharif E Q, Charles L G, Mark S, Ziad A (2010) Rapid Differentiation of Mycobacterium tuberculosis and M. bovis by High-Resolution Melt Curve Analysis. J. Clin. Microbiol. 2010, 48 (11): 4269.

[34] Zhu, R. Y., K. X. Zhang, M. Q. Zhao, Y. H. Liu, Y. Y. Xu, C. M. Ju, B. Li and J. D. Chen, (2009) Use of visual loop-mediated isotheral amplification of rim sequence for rapid detection of Mycobacterium tuberculosis and Mycobacterium bovis.