Global Veterinaria 13 (5): 794-800, 2014
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2014.13.05.86179

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

[1]Suzan A. Mohamed, [2]Kh. F. Mohamed, [3]M.G. Aggour, [4]Hanaa A. Ahmed and [2]S.A. Selim

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

Abstract: A novel multiplex real-time PCR assay was developed and applied directly to biological samples with evidence of bTB in order to differentiate between *M. bovis* and *M. tuberculosis*. The primers and TaqMan probes were designed to target the IS*1081*gene, the multi-copy insertion element in the MTC and the 12.7-kb fragment present in *M. tuberculosis*, not in the *M. bovis* genome. The assay was optimized and validated by testing 10 species of mycobacteria including *M. bovis* and *M. tuberculosis* and 3 other bacterial species such as*: Escherichia coli*, *shigella Spp., salmonella Spp*. in cattle milk and lymph nodes. The tests identified 96.4% (27/28) as *M. bovis* from the MTC-positive bTB samples using conventional PCR for specific insertion elements IS*1081*. MTC-negative bTB samples were tested using conventional PCR and the real-time PCR. When comparative analyses were conducted on all bovine samples, using conventional PCR as the gold standard, the relative accuracy of real-time PCR was 99.1% and the relative specificity was 100%.The detection limits of the real-time PCR assays for *M. bovis* and *M. tuberculosis* genomic DNA were 2 DNA copies per PCR reaction. Consequently, this multiplex real-time PCR assay is a useful diagnostic tool for the identification of MTC and differentiation of *M. bovis* and *M. tuberculosis*, as well as the epidemiologic surveillance of animals slaughtered in abattoir.

Key words: Real-Time PCR · *Mycobacterium bovis* · *Mycobacterium tuberculosis Complex* · IS*1081*

INTRODUCTION

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

Because of the slow growth rate of *Mycobacterium tuberculosis*, isolation, identification and drug susceptibility testing of this organism and other clinically important mycobacteria can take several weeks or longer. During the past several years, many molecular methods have been developed for direct detection, species identification and drug susceptibility testing of mycobacteria. These methods can potentially reduce the diagnostic time from weeks to days [3]. Strikingly, the genome sequence of *M. bovis* is > 99.95% identical at the nucleotide level to that of *M. tuberculosis*, showing collinearity and no evidence of extensive translocations, duplications or inversions. But deletion of genetic information that has led to a reduced genome size, revealed 11 deletions from the genome of *M. bovis*, ranging in size from ˜1 to 12.7 kb. Surprisingly,

Corresponding Author: Khaled Al Amry, Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.

the sequence contains only one locus in *M. bovis*, termed TbD1, which is absent from the majority of extant *M. tuberculosis* strains. Therefore, at a gross level, deletion has been the dominant mechanism in shaping the *M. bovis* genome [4]. An oligonucleotide array which could detect and differentiate mycobacteria to the species level by using the internal transcribed spacer (ITS) sequence was developed [5]. Also, microsphere-based multiplex assay was developed, by using the xMAP technology, for the simultaneous rapid detection of the *Mycobacterium tuberculosis complex* (MTC) and the differentiation of *M. tuberculosis* and *M. bovis*. The assay simultaneously detected 4 target sequences, including specific insertion elements IS*6110* and IS*1081* of MTC, a 12.7- kb fragment specific for *M. tuberculosis* and an uninterrupted 229 bp sequence specific for *M. bovis* [6]. Real-time quantitative PCR has been developed to measure accumulation of PCR product through a dual labeled TaqMan probe and it provided very accurate and reproducible quantitation of gene copies [7]. Real-time PCR offers significant improvements to the quantitation of viral load because of its enormous dynamic range that can accommodate at least eight $\log_{10}$ copies of nucleic acid template[8].

MATERIALS AND METHODS

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

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

Real-time PCR:

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

Real-time Amplification:

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

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

Table 1: Target species and their gene sequences

Target species	Oligonucleotide	Sequence (5"?3')	Target gene/sequences
MTC	IS*1081*_650F(p1)	CGGACTGGCTGCTGCAGC	IS*1081*
	IS*1081*_851R(P2)	AGCTCTTTGGCCATGATCGA	
	IS*1081*_712Pa	FAM-TGCTACCTGCTGGGAGTATCCACTCGBHQ1	
M.bovis	THB_312851F(P3)	TGTGCGAGCTGAGCGATGTC	
	Tbovis_825R(P4)	AAATGGCTATTGACCAGCTAAGATAT	The229-bpcontiguous sequences
M.tuberculosis	THB_312940Pb	JOE-CCGTAGTCGTGCAGAAGCGCAACAC-BHQ1	
	THB_312851F(P5)	TGTGCGAGCTGAGCGATGTC	
	TH_313003R(P6)	GCGCCCTATTTGATCTCTGCAA	
	THB_312940Pc	JOE-CCGTAGTCGTGCAGAAGCGCAACAC-BHQ1	The 12.7-kb fragment

FAM (6, carboxy fluorescein;JOE (6-carboxy 4", 5" dichloro-2",7"dimethoxyfluroresceinBHQ (Black hole quencher 1) according to[9].

- The reaction mixture for *M. bovis* (reference strains) and field isolates consisted of 6.25µ| (maxima)ready to use mix containing(PCR buffers, Taq polymerase, dNTPs), 1µ| P3,1µ| P4, 0.5µ| Probe of *M.bovis*,0.075 µ| ROX, 3.6 µ| DNase/RNase free water. The PCR mix was vortexed. The PCR mix was transferred into real –time plate.
- The reaction mixture for the PCR mix for *M. tuberculosis* reference strain and field isolates consisted of 6.25µ| maxima ready to use mix,(1µl P1, 1µ| P2, 1µ| P5,1µ| P6). 0.25µl probe for *M. tuberculosis*, 0.25µl probe for *M. complex*, 0.15 µ| ROX, 1.6µ| DNase/RNase free water. PCR mix was vortexed. The PCR mix was transferred to real-time plate. Only 2.5µ| of target DNA extracted was added. Reaction mixture for other non mycobacterial strain consisted of 6.25µl (maxima) mix, 0.5µl P3, 0.5µl P4, 2.425 µlDNase/RNase free water, 0.075 µl ROX, 0.25µl probe *M. tuberculosis*. The PCR mixture was vortexed and spinned. PCR mixture was transferred into real-time plate. Only 2.5 µl DNA extracted was added.

Amplification for Sensitivity of Multiplex Real-Time Probe and Primers: All DNA samples included in this method extracted from *M.tuberculosis* reference strains and previously quantified DNA copies using control positive with known DNA copies (MTplex Genetic pcr solutions ™, Spain) for qPCR detection of *Mycobacterium tuberculosis complex* (Ready to use). Also this method include sensitivity and repeatability of multiplex real-time PCR.

Standard curve was performed for *M. tuberculosis* and *M. complex* The run was performed in 12.5µl reaction mixture, The reaction mixture consisted of 6.25µ| maxima mix, 1µ| P5,1µl P6, 3.6µ| DNase/RNase free water,0.075 µl ROX, 0.5µ| probe specific for *M.tuberculosis*. The reaction mixture used for *M.tuberculosis complex* primers and probes included 6.25µ| maxima mix, 1µ| P1,1µ|P2,0.5µ|pa,3.6 µ DNase/RNase free water, 0.075µl ROX, the two PCR mix was vortexed and spinned. The PCR mix was transferred into real-time plate. Only 2.5 µl of genomic DNA were extracted.

Detection of *M. tuberculosis* Complex at Tissue and Milk Samples Extracted DNA: All DNA extracted from tissue and milk samples were involved in these reaction to detect *Mycobacterium spp.* in these extracted DNA samples.

These method was performed using MTplex Genetic pcr solutions ™, Spain)for qPCR detection of *Mycobacterium tuberculosis complex* (Ready to use).

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

This method was carried out to detect and differentiate *Mycobacterium spp.* using specific primers and probes for *M. bovis* and *M. tuberculosis*. The run was performed in 12.5µl reaction mixture. A.1stReaction mixture consisted of 6.25 µ| maxima mix, 0.5 µ| P5, 0.5 µ| P6, 4.9µ| DNase/RNase free water, 0.075 µ| ROX, 0.25 µ|probe (*M. tuberculosis*). B. 2ndmixture consisted of the same content but replace P5,P6 with P3, P4 and probe *M. tuberculosis* with probe (*M. bovis)*. 3rdmixure consisted of same content but replace P5, P6 with P1, P2 and probe of *M. bovis* with probe of *(M. tuberculosis complex)*. The PCR mixture was vortexed and spinned, Reaction mixture was transferred into real-time plate, Only 2.5 µ| of extracted DNA was added. Thermal profile Table 2.

Thermal profil of multiplex real-time primers and probes

Initial denaturation	1 Cycle	95°C for10 minutes
Denaturation	45 Cycle	95°Cfor 15 seconds
anneling/extention:		60°Cfor one minute

***E. Comparing* the Sensitivity of Real-time and Conventional Polymerase Chain Reaction:** Sensitivity of real-time and conventional PCR were compared convetional PCR 270bp for *M. tuberculosis* and 270bp470bp for *M. bovis* according to[10].

RESULTS

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

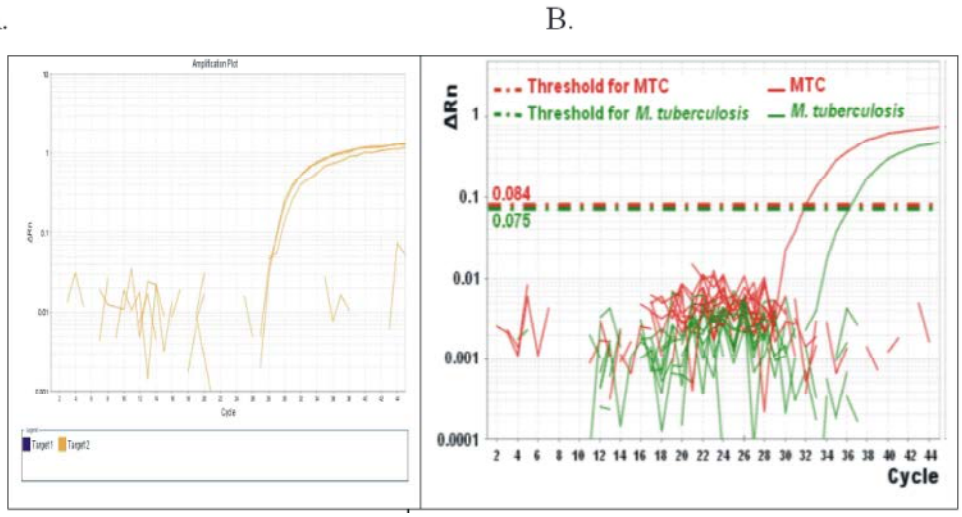

Fig. 1: Amplification curves of multiplex TaqMan real-time assays for detection MTC using IS*1081* gene and 12.7-kb insertion/deletion fragment primers and TaqMan probe.
(A) *M. bovis* DNA was amplified simultaneously by the MTC and *M. bovis*-specific primers (THB_312851F and Tbovis_825R) and TaqMan probe.
(B) *M. tuberculosis* DNA was amplified together in one tube by the MTC and *M. tuberculosis*-specific primers (THB_312851F and TH_313003R) and TaqMan probe. Figures 2 and 3 illustrate that the TaqMan probe and primers can detect till 2 DNA copies per PCR reaction.

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

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

DISCUSSION

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

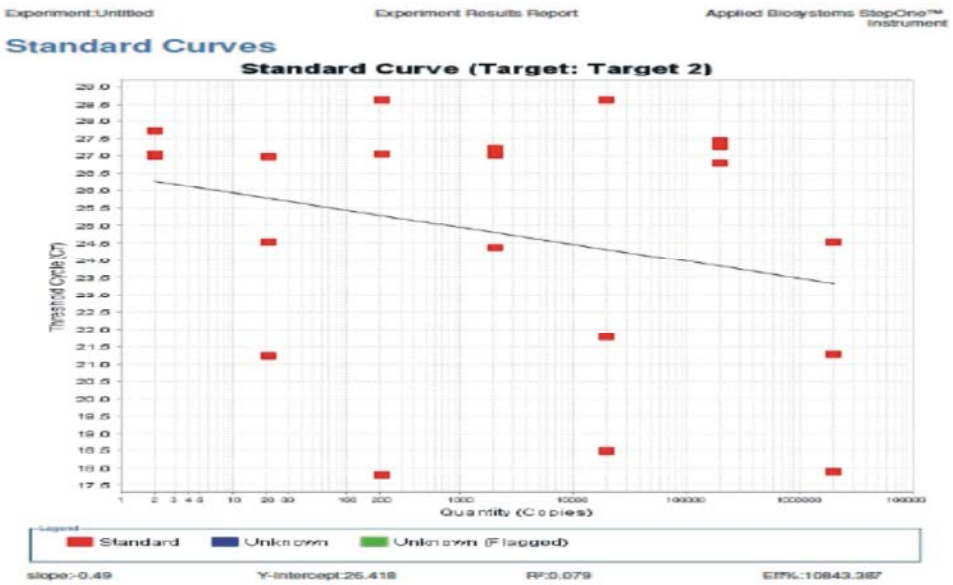

Fig. 2: Standard curves of TaqMan real-time PCR to detect *M. tuberculosis* using 12.7-kb fragment primers and TaqMan probe. A minimum of 2 DNA copies could be detected after 45 cycles.

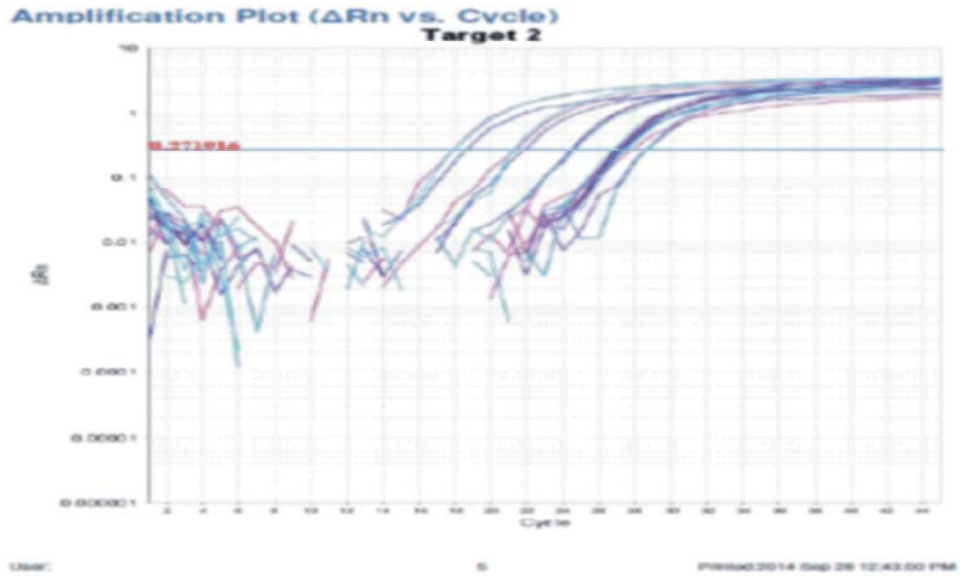

Fig. 3: Amplification curves of Taq Man real-time PCR to detect *M.tuberculosis complex* using IS*1081* primers and TaqMan probe. Aminimum of 2DNA copies can be detected after 45cycles.

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

Table 3: Target gene/sequence of strains

	Target gene/sequence		
Strain	IS*1081*	The 229-bp contiguous sequences	The 12.7-kb fragment
M. bovis	+	+	-
M. tuberculosis	+	-	+
M. avium	-	-	-
M. phlei	-	-	-
E. coli	-	-	-
Salmonella Spp.	-	-	-
Listeria Spp.	-	-	-

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

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

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

CONCLUSION

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

ACKNOWLEDGEMENTS

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

REFERENCES

1. Thoen, C., P. Lobue and I. de Kantor, 2006. The importance of *Mycobacterium bovis* as a zoonosis. Vet Microbiol., 112: 339-345.
2. Forbes, B.A., F.S. Daniel and S.W. Alice, 1998. Mycobacteria. In: Diagnostics Microbiology. Bailey and Scotts(eds), C.60P.715-747. 10th edition 3. H. Soini and J.M. Musser, 2001. Molecular Diagnosis of Mycobacteria.Clin.Chem., 47: 809-814.
3. Hanna Soini and James M. Musser, 2001. Molecular Diagnosis of Mycobacteria.Clin. Chemist., 47(5): 809-814.
4. Garnier, T., K. Eiglmeier, J.C. Camus, N. Medina, H. Mansoor, M. Pryor, S. Duthoy, S. Grondin, C. Lacroix, C. Monsempe, S. Simon, B. Harris, R. Atkin, J. Doggett, R. Mayes, L. Keating, P.R. heeler, J. Parkhill, B.G. Barrell, S.T. Cole, S.V. Gordon and R.G. Hewinson, 2003. The complete genome sequence of *Mycobacterium bovis*. Proc. Natl. Acad. Sci. USA., 100: 7877-7882.
5. Park, H., H. Jang, E. Song, C.L. Chan, M. Lee, S. Jeong, J. Park, B. Kang and C. Kim, 2005. Detection and genotyping of Mycobacterium species from clinical isolates and specimens and specimens by oligonucleotide array. J. Clin. Microbiol., 43(4): 1782-1788.

6. Chen, R., Y. Bi, G. Yang, Z. Liu, Z. Liu, B. Zeng and T. Tong, 2010. Development of a fluorescent microsphere-based multiplex assay for simultaneous rapid detection of *Mycobacterium tuberculosis complex* and differentiation Of *M. tuberculosis* and *M. bovis* in clinical samples. Diagn. Mol. Pathol. 19(3): 172-9.
7. Heid, C.A., J. Stevens, K.J. Livak and P.M. Williams, 1996. Real time quantitative PCR. Genome Res., 6: 986-994.
8. Ishiguro, T., J. Saitoh, H. Yawata, H. Yamagishi, S. Iwasaki and Y. Mitoma, 1995. Homogeneous quantitative assay of hepatitis C virus RNA by polymerase chain reaction in the presence of a fluorescent intercalater. Anal.Biochem., 229: 207-213.
9. Ba-Ra-Da Koh, Young-Boo Jang1, Bok-Kyung Ku1, Ho-Seong Cho2, Seong-YeolBae, Ho-Myung Na, Seong-Do Park, Yong-Hwan Kim and Yong-Un Mun, 2011. Development of real-time PCR for rapid detection of *Mycobacterium bovis* DNA in cattle lymph nodes and differentiation of *M. bovis* and *M. tuberculosis*. Korean J. Vet. Serv., 34(4): 321-331.
10. Mohamed, Kh. F., 2001. Differentiation of *Mycobacterium Tuberculosis* and *Mycobacterium Bovis* on Moulecular Basis. M.V.Sc. thesis, microbiology Dept. Fac. Vet. Med., Cairo Univ.
11. Thoen, C.O. and D.E. Williams, 1994. Tuberculosis, tuberculoidosis and other mycobacterial infections. In: handbook on zoonosis. Ed. G.W. Beran, pp: 41-60. Boca Raton, Fla: CRC PRESS.
12. Karlson, A.G. and D.T. Carr, 1970. Tuberculosis caused by *Mycobacterium bovis*. Ann. Intern. Med., 73: 979-983.
13. Cormican, M.G. and J. Flynn, 1992. Tuberculosis in the west of Ireland 1986-1990. Ir. J. Med. Sci., 161: 70-72.
14. Brett, J.L. and M.W. Humble, 1991. Incidence of human tuberculosis caused by Mycobacterium Bovis. N.Z. MED. J., 104(904): 13-14.
15. Brosch, R., S.V. Gordon, M. Marmiesse, P. Brodin, C. Buchrieser, K. Eiglmeier, T. Garnier, C. Gutierrez, G. Hewinson, K. Kremer, L.M. Parsons, A.S. Pym, S. Samper, D. van Soolingen and S.T. Cole, 2002. A new evolutionary scenario for the Mycobacterium tuberculosis complex. Proc Natl. Acad. Sci. USA., 99: 3684-3689.
16. Mostowy, S., J. Inwald, S. Gordon, C. Martin, R. Warren, K. Kremer, D. Cousins and M.A. Behr, 2005. Revisiting the evolution of *Mycobacterium bovis*. J Bacteriol., 187: 6386-6395.
17. OIE., Manual, Standard Curve Preparation to Validate real-time Primers and Probes.
18. Kurabachew, M.1., Ø. Enger, R.A. Sandaa, R. Skuce and B. Bjorvatn, 2004. A multiplex polymerase chain reaction assay for genus, group-and species-specific detection of mycobacteria. Diagn. Microbiol. Infect. Dis., 49(2): 99-104.
19. 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*. J. Microbiol. Methods, 78: 339-343.