The use of multiplex PCR for Identification of *Mycobacterium bovis* isolates in Egypt.

Ashraf, A. Abd El Tawwab [1], Ahmed, M. Ammar [2], Essam, A. Nasr [3],Aisha, R. Ali [4], Fatma, I. El Hofy [1] and Mohammed, E. E. Sayed Ahmed [5]

[1] Bacteriology, Immunology and Mycology Dep., Fac. of Vet. Med., Benha Univ., Egypt.

[2] Bacteriology, Mycology and Immunology Dep., Fac. of Vet. Med., Zagazig Univ., Egypt.

[3] Veterinary Serum and Vaccine Research Institute, Abbasia.

[4] Serology Unit -Animal Health Research Institute, Dokki – Giza, Egypt.

[5] Animal Health Research Institute (Mansoura branch) Dokki – Giza, Egypt.

ABSTRCT

The cause of bovine tuberculosis in cattle mainly is *Mycobacterium bovis* which is pathogenic for a large number of other animals, and its transmission to human constitutes a public health problem. The control and eradication of *Mycobacterium bovis* in Egypt depend on developing rapid and accurate detection techniques. Control of bovine tuberculosis in Egypt is mainly depending on test and slaughter policy which consume the economy and yet there is no evidence of reducing the prevalence of tuberculosis. We need rapid and accurate method for identification of *M.bovis.* The aim of this study, establishment of multiplex PCR for Identification of *Mycobacterium bovis* isolates in Egypt and comparing it with the Conventional gold standard mycobacterial culture techniques.

Ten samples, collected from different areas of Egypt, were positive using conventional Mycobacterial culture technique, were subjected to multiplex PCR by using two sets of primers. The primer set one amplified 123bp DNA segment located within insertion element IS6110 characteristic of Mycobacterium tuberculosis complex (MTBC).The primer set two, amplified

470bp fragment which species-specific for *M.bovis.* The results of multiplex PCR showed that seven samples gave two specific PCR product (123 and 470 bp) indicating that they were positive for M.bovis. While two samples identified as MTBC gave only one 123 bp DNA band. Only one sample gave negative result. Multiplex PCR results were in concordance to the conventional culturing techniques results with high sensitivity and specificity (100%). It can be done with one day work in comparison with routine culture methods which take several weeks (6-8 weeks) by using culture methods.

Keywords: Bovine tuberculosis, Mycobacterial culture, Multiplex PCR, *Mycobacterium bovis.*

1. **INTRODUCTION**

Tuberculosis (TB) is an ancient disease and remains important in humans and animals worldwide. The human tubercle bacillus infects an estimated two billion persons or approximately one-third of the world's population, and it is estimated that 1.5 to two million people die from human tuberculosis annually.

Bovine tuberculosis (TB) is a contagious disease of both animals and humans. Prior to the advent of eradication measures, bovine TB existed as a major disease of man and domestic animals. *M. bovis* persists in humans, causing pulmonary and extra-pulmonary disease. Unlike transmission of *M. bovis* from cattle to humans, the role of human-to-human airborne transmission in the spread of *M.bovis* is controversial. The predominant view has been that human-to-human transmission is rare (Clarke, 2017).

Bovine tuberculosis (BT) is caused by *Mycobacterium bovis* and it considers a great problem in many countries, and cause significant economic and public-health hazards. The detection of the disease in susceptible animals especially in cattle usually depends on case history,

clinical and Post mortem findings, intradermal tuberculin skin tests, and slaughtering house meat inspection. Definitive diagnosis is based on culture with morphological appearance and biochemical tests for differential identification of Mycobacterium genus members (Mishra *et al.,* 2005 and Santhil *et al.,* 2014).

However, these conventional methods need special skills, and time consuming. The molecular biology techniques such as PCR have the advantage as alternative diagnostics methods (Rodriguez, *et al*., 1995).

Several PCR-based protocols have been established for mycobacteria identification belonging to the *Mycobacterium tuberculosis* complex (MTC), which comprises five species (*Mycobacterium tuberculosis, M. bovis, M.africanum, M. microti* and *M. Canetti*). One of the most applicable protocols uses primers that amplify segments of the IS6110 genomic insertion characteristic of MTC (Ruggiero*, et al*. 2007).

The multiplex PCR may be viable diagnostic method in endemic Tuberculosis areas, where both of human and bovine TB co-occurs and differentiation between them is necessary for monitoring the spreads of M.bovis to human (Bakshi, *et al.,* 2005). Them-PCR can detect *M. bovis* DNA and represents a valid additional tool for the post-mortem diagnosis of BTB. Multiplex PCR is faster than culture-based detection, reducing diagnosis time from 120 to approximately 2 days, even when automated culturing with broth medium is used (Figueiredo, *et al,* 2012).

2. **MATERIALS AND METHODS**

2.1. Samples collection

Thirteen bacterial isolate samples, (previous microbiological characterization with gold standard mycobacterial culture), were used in this study. Two positive control samples (reference MTBC and *M.bovis*) and one sample brucella bovis used as negative control were gratefully obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt.

Ten samples were collected from different areas of Egypt were subjected to routine conventional techniques for isolation and identification of mycobacteria species.

Seven out of 10 samples were positive for *M.bovis*, two samples were positive for MTBC and one sample gave negative result as saprophytic bacteria.

All The DNA was isolated by using GF-1 Bacterial DNA Extraction Kit, (Catalog No. GF-BA-050), Vivantis Technologies, Malaysia).

2.2. DNA Extraction

According to the standard protocols of the kit.Briefly, one loop full of bacterial isolates was well mixed in 200 µl TE buffer. The suspended bacterial isolates were centrifuged at 12000 xg for 5 min. The supernatant was decanted and the resultant pellet was washed twice using TE buffer till be clean. 200µl of PBS was Add and was resuspended completely by pipetting. 20µl of Proteinase K and 2µl of Lysis Enhancer were added to the sample and mixed immediately. 200µl of Buffer TB was added and mix thoroughly by pulsed-vortexing. Incubate at 65°C for 10 min.

200µl of absolute ethanol was added &mixed immediately and thoroughly by pulsed- vortexing to obtain a homogeneous solution. 650µl of sample was transferred into a column assembled in a clean collection tube and centrifuged at 5,000 x *g* for 1 min. the column was washed with 650µl Wash Buffer and centrifuged at 5,000 x *g* for 1 min. column washing was repeated once again. The column was placed into a clean micro centrifuge tube. 200µl of preheated Elution Buffer was added, TE buffer or sterile water directly onto column membrane and stand at room temperature for 2 min. and Centrifuged at 5,000 x

g for 1 min to elute DNA, and Stored DNA at -20°C. The extracted DNA was evaluated by spectrophotometer and agarose gel electrophoresis.

2.3. Multiplex PCR Amplification

The purified DNA was subjected to multiplex PCR by using two sets of primers, were synthesized by *Bio basic Canada Inc.,* as shown in table (1).

The primer set one amplify 123bp DNA segment located within insertion element IS6110 characteristic of MTBC.The primer set two, amplify 470bp fragment which species-specific for M.bovis .

Table (1): Primers sequences and PCR products size.

Primer set	primer	Target	Primer sequence (5'-3')	Product size (bp)	References
one	TB1	MTBC	CCTGCGAGCGTAGGCGTCGG	123	***Eisenach ,et al (1990)***
	TB2		CTCGTCCAGCGCCGCTTCGG		
Two	**MTBF**	M.bovis	CCCGCTGATGCAAGTGCC	470	***Romero, et al., (1999)***
	MTBR		CCCGCACATCCCAACACC		

The Multiplex PCR reaction was performed according to (Figueiredo, *et al.*, 2012) with modification, using EmeraldAmp® MAX PCR Master, (Cat no. RR320A), Takara Bio USA, as following:-

EmeraldAmp MAX PCR Master Mix (2X) 25 µl

DNA Template 3 µl (< 500 ng)

Primer TB1 0.5 µl 50pmol (final conc.)

Primer TB2 (MTC) 0.5 µl "

Primer MTBF 0.5 µl "

Primer MTBR (M. bovis) 0.5 µl "

DNAse, RNAase free water 20ul"

PCR reaction was carried out in a GeneAmp PCR System 9600 (Applied Bio systems), using an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 68°C for 1 min, and

extension at 72°C for 1 min. Followed by a final extension step at 72°c for 7 min. Amplified PCR products generated in samples were compared with standard MTBC and *M.bovis* positive controls. After amplification, PCR products were electrophoreses on 1.5% agarose gel containing ethidium bromide staining (0.5ug/ ml) using VC 100bp DNA Ladder which Serves as molecular weight standard for electrophoresis agarose Product No : NL1401,(Vivantis Technologies Malaysia), and the stained gel visualized under UV light.

3. RESULTS

In this study, The results of multiplex PCR : Ten samples collected from different areas of Egypt were positive using conventional Mycobacterial bacterial culture technique, As shown in table (2) and figure (1) revealed that seven samples gave two specific PCR product (123 and 470 bp) indicating that they were positive for *M .bovis.* While two samples identified as MTBC gave only one 123 bp DNA band. Only one sample gave negative result as saprophytic bacteria.

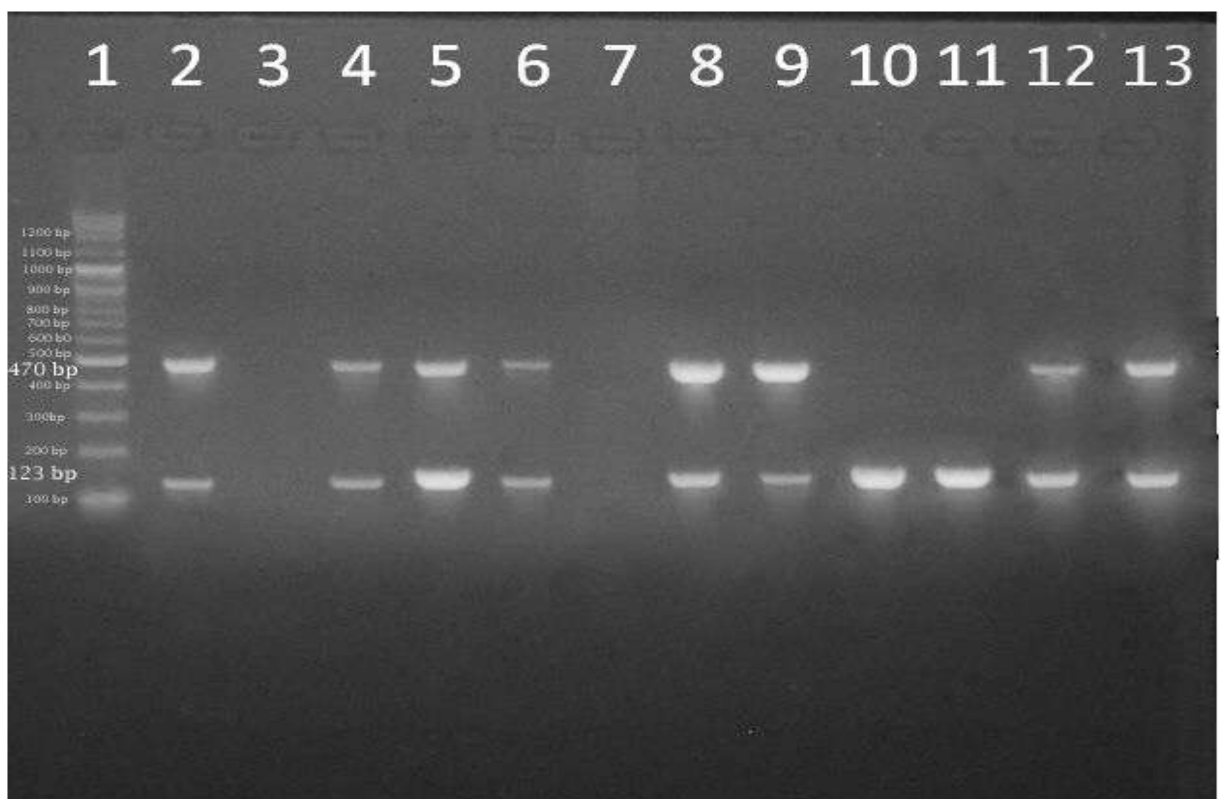

Figure (1): The use of multiplex PCR for identification of M.bovis isolates in Egypt.

Lane (1): VC100 bp ladder DNA marker used as DNA size standard (The sizes were3000,2500,2000,1500,1200,1000,900,800,700,600,500,400,300,200,100bp)

Lane (2): Sample (1) positive control PCR product (123bp) for standard MTBC &Sample (2) Positive control PCR product (470bp) for standard *M.bovis*.

Lane (3): Sample (3) negative control (brucella DNA).

Lane (4, 5, 6, 8, 9, 12, and 13): Sample (4, 5, 6, 8, 9, 12, and 13) were positive *M.bovis* isolate, gave 123 bp and 470 bp respectively.

Lane (7): sample (7) Negative for multiplex PCR (saprophytic bacteria).

Lane (10 and 11): Sample 10 and 11 gave (123 bp) indicating positive *M. tuberculosis* complex isolate.

Table (2) : Comparison analysis between Conventional gold standard mycobacterial culture and multiplex PCR results in mycobacteria isolates in Egypt.

Sample No.	**Conventional gold standard mycobacterial culture**		**Multiplex PCR**	
	(MTBC)	***M .bovis***	**(MTBC) 123bp PCR product**	**(*M.bovis*) 470 bp PCR product**
1**	+*	-	+	-
2***	-	+	-	+
3****	-	-	-	-
4,5,6,8, 9,12& 13	-	+	+	+
7	-	-	-	-
10	+	-	+	-
11	+	-	+	-
Total No.	13		13	
Total positive	2 isolate 1 reference	7 isolate 1 reference	2 isolate 1 reference	7 isolate 1 reference

100 % specificity and sensitivity result between Conventional gold standard mycobacterial culture and Multiplex PCR

- * + = positive, - = Negative
- ** = Reference MTBC sample
- *** = Reference *M.bovis* sample
- **** = Brucella abortus as negative control.

Multiplex PCR results were in concordance to the conventional culturing techniques results with high sensitivity and specificity (100%).

4. DISCUSSION

The accurate precise diagnosis of bovine tuberculosis remains, up till now, an achieve goal. Traditional methods of culture and post mortem examination are very effective procedures for diagnosing tuberculosis. However, these procedures take long time. A PCR-based assay could be used to detect the presence of *M. bovis* isolates within three days and thus become helpful tool for the control and subsequent eradication of the disease (Ruggiero, *et al.* 2007).

The results in this study in agreement with Eisenach, *et al* (1990) used PCR techniques for identification MTBC and (Romero *et al*, 1999) added that PCR assay can be successful technique for identification of *M.bovis* and can be used in the epidemiological characterization of animal infected with bovine tuberculosis. (Figueiredo,*et al.*,2009) suggested that The molecular assay a single -step multiplex PCR o which have two set primers identify bacteria as MTC members as well as to distinguish *M.bovis* isolates from the other members of this complex. Hazem, *et al.,* (2012) showed that PCR results were in concordance to that of conventional culturing on LJ except for samples that yielding no isolate.

Lobna *et al.,* 2015 concluded that PCR results were compatible with that of conventional culturing but PCR considers rapid and screening test for detection of *M. bovis.* The multiplex PCR can be done

with one day work in comparison with routine culture methods which take several weeks (6-8 weeks) by using culture methods. These results coincides with results of Samaha *et al.* (2009) , Sabry, M. and Elkerdasy, A. (2014) which concluded that the use of PCR in the diagnosis of TB in clinical samples is as rapid, more reliable, sensitive and specific techniques. Moreover, (Figueiredo,*et al*., 2012) found that molecular diagnosis using multiplex PCR , combined with ante mortem and post mortem inspection, appeared to be a promising technique to improve the surveillance of bovine tuberculosis and contributing to the success of the bovine tuberculosis eradication program.

This study aimed to establish multiplex PCR for confirmation of the identification of *M.bovis*. It is highly sensitive, accurate, highly specified diagnostic technique. The definitive diagnosis is time consuming, laborious which render the eradication strategy of the disease. So, we recommend the use of molecular biology technique especially multiplex PCR besides tuberculin test and conventional culture techniques to overcome and verify the infection of cattle with mycobacterium species. other than *M.bovis*. These strategies will improve the eradication bovine tuberculosis program in Egypt and subsequent give a good impact on human public health.

5. REFERENCES

Clarke, R. (2017): Bovine TB: An old disease, a modern enigma. Livestock, Vet advice January 4, 2017 Bovine TB: An old disease, a modern enigma. Livestock, Vet advice Canadian cattle men a beef magazine, January 4, 2017 .

Eisenach, K.D.; Cave, M.D.; Bates, J.H.; Crawford, J.T. (1990): Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis*. *J. Infect. Dis.* 161, 977-981.

Figueiredo, E.E.S.; Silvestre, F.G.;Campos,W.N.; Furlanetto,L.V.,Medeiros, L.; Lilenbaum, W.; Fonseca, L. S.; Silva, J.T.; Paschoalin, V. (2009): Identification of *Mycobacterium bovis* Isolates by a multiplex PCR. *Braz. J. Microbiol*., v.40: 231-233, 2009.

Figueiredo, E. E. S., Junior, A. C. C., Furlanetto, L. V., Silva, F. G. S., Duarte, R. S., Silva, J. T., Lilenbaum, W. &Paschoalin, V. M. F. (2012): Molecular techniques for identification of species of the Mycobacterium tuberculosis complex: The use of multiplex PCR and an adapted HPLC method for identification of Mycobacterium bovis and diagnosis of bovine tuberculosis. In: Understanding Tuberculosis– Global Experiences and Innovative Approaches to the Diagnosis, 411–432.

Hazem H. Ramadan, Adel H. N. El-Gohary, Amro A. Mohamed and Essam A. Nasr (2012): Detection of *Mycobacterium Bovis* and *Mycobacterium Tuberculosis from Clinical* Samples by Conventional and Molecular Techniques in Egypt Global Veterinaria 9 (6): 648-654, 2012.

Juan G. Rodriguez, Gloria A. Mejia, Patricia Del Portillo, Manuel E. Patarroyo and Luis A. Murillo (1995): Species-specific identification of Mycobacterium bovis by PCR Microbiology (1995), 141, 2131-2138.

Lobna, M.A. Salem1 and Nasr, E.A.(2015): Conventional and molecular detection of *Mycobacterium bovis* in milk of cows and its public health hazard. International Journal of Advanced Research (2015), Volume 3, Issue 6, 693-701.

Mishra, A.A., Singhal, D.S., Chauhan,V.M., Katoch., Parsad, H.K.(2005):

Direct detection and identification of *Mycobacterium Tuberculosis* and *Mycobacterium bovis* by a Novel Nested PCR Assay: Correlation with Conventional Techniques. J. Clin.Micro., 43(11):5670-5678.

Moussa, I.M.;Mohamed, K.H.F.; Marwah, M.; Nasr,E.A.; Shibl, A.M.; Salem-Bekhit ,M.M. and Hatem,M.E. (2011): Comparison between conventional and modern techniques used for identification of Mycobacterium tuberculosis complex. African Journal of Microbiology Research, 5: 4338-4343

Romero, R.E., Garzon, D.L., Mejia, G.A.,Monroy, W., Patarroyo, M.E., Murillo, L.A.(1999) : Identification of *M.bovis* in bovine clinical samples by PCR species-specific primers.Canadian J. Vet. Res., 63(2):101-106.

Ruggiero, A.P.; Ikuno, A.A.; Ferreira, V.C.A.; Roxo, E. (2007): Tuberculose bovina: alternativaspara o diagnóstico. Arq. Inst. Biol. 74 (1), 55-65.

Sabry,M. and Elkerdasy,A. (2014): A polymerase chain reaction and enzyme linked immunosorbent assay based approach for diagnosis and differentiation between vaccinated and infected cattle with Mycobacterium bovis. J Pharm. Bioallied Sci. 2014 Apr-Jun; 6(2): 115–121.

Samaha, H.A. ; Haggsg Y.N. and Noseir, M.A. (2009): Tuberculosis among cattle and man with using PCR test for its diagnosis. Alex.J. Vet .Science, 28 (1):215-225.

Senthil,N.R.; Ranjani,M.R.and Vasumath, K. (2014): Comparative diagnosis of Mycobacterium bovis by Polymerase chain reaction and Ziel-Neilson staining technique using Milk and nasal washing .Journal of Research in Agriculture and Animal Science Volume 2 ~ Issue 5 pp: 01-03.

Yumi U.; Tooru M. (2007): Tuberculosis as a zoonosis from a veterinary perspective.Compar. Immun., Microbiol. & Infec. Dis. 30, 415-425.