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# Detection of Mycobacterium Bovis and Mycobacterium Tuberculosis from Clinical Samples by Conventional and Molecular Techniques in Egypt

*[1]Hazem H. Ramadan, [1]Adel H. N. El-Gohary, [1]Amro A. Mohamed and [2]Essam A. Nasr*

[1]Department of Hygiene and Zoonoses, Fac. Vet. Med., Mansoura University, Egypt
[2]Department of Bacterial Diagnostic Products, Vet. Serum and
Vaccine Research Institute, Abbasia, Cairo, Egypt

**Abstract:** Tuberculosis (TB) remains a global health problem despite near eradication in some developed countries. This study was conducted to compare between conventional and molecular techniques for detection of Mycobacterium bovis (*M. bovis*) and *M. tuberculosis* in Egypt. A prevalence of 0.96% (32/3347) was determined in cattle from different farms in Egypt tested by tuberculin skin test during the period from November 2010 until December 2011. While abattoir surveillance showed a prevalence percentage of 0.21% (15/7235) for suspected tuberculous carcasses (non-tuberculin tested) slaughtered in El-Basateen abattoir, Cairo, Egypt in the period between December 2010 till March 2011. By culturing on Lowenstein Jensen (LJ) media, 20 (62.5%) and 10 (66.7%) *M. bovis* were recovered from tuberculin positive reactors and suspected tuberculous carcasses, respectively. While from sputum samples, the percentages of *M. tuberculosis* isolation were 83.3% (35/42) from clinically diagnosed patients and 8.3% (1/12) from apparently healthy persons. No *M. bovis* was isolated from sputum by conventional culturing method. Using PCR, 618 bp and 185 bp fragments were detected in infected animal samples and human samples, respectively. PCR results were in concordance to that of conventional culturing on LJ except for samples that yielding no isolates. In conclusion, this study suggested that PCR offers the potential for the diagnosis of TB in a few hours.

**Key words:** Tuberculosis · Lowenstein Jensen Media · PCR · Tuberculin Test

## INTRODUCTION

Tuberculosis (TB) is a disease of worldwide occurrence that causes great harm to dairy farms and health risks to the population. The cause of bovine tuberculosis (BTB) in cattle is *Mycobacterium bovis* (*M. bovis*) which is also pathogenic for a large number of other animals and its transmission to human constitutes public health problems [1]. While human TB is mainly caused by *M. tuberculosis* but in regions where BTB is prevalent in animals, human TB cases due to *M. bovis* may occur [2].

With increasing incidence of tuberculosis globally, rapid and reliable diagnostic assay is required not only for detection but also for identification of the pathogenic mycobacteria in clinical samples. This is essential for the promotion of diagnosis, treatment and control of TB [3].

Herd diagnosis based on tuberculin skin testing which has been a hallmark of BTB eradication campaigns and has been effective in reducing the prevalence of BTB in most developed countries but it lacks sensitivity and specificity [4]. Culturing of organisms has a specificity that approach 100% and permits susceptibility testing of the isolates but the main disadvantages that growth of the organism may take 6-8 weeks, culture techniques also require viable organisms and this can be a problem when tissues are inadequately handled [5]. Also, mycobacteria are identified conventionally by phenotypic traits and biochemical profiles but these tests are laborious and time consuming [6]. Polymerase chain reaction (PCR) amplification of the Mycobacterium DNA is a rapid and reliable method for rapid diagnosis [7] and seems to have sensitivity equal or greater than that of the culture method [8].

**Corresponding Author:** Hazem Hassan Ramadan, Department of Hygiene and Zoonoses, Fac. Vet. Med., Mansoura University, Egypt. Mob: +201001094753.

From all above, it is noteworthy that, the rapid detection and identification of clinically important *Mycobacterium* spp. is essential for good management and infection control. Also, TB control programmes should include specific policies and valid analytical system to ensure the prompt identification, triage and referral or management of animal with suspected or confirmed TB diseases. Measures should be taken to minimize the risk of transmission of organism of *M. tuberculosis* complex [9].

Although many control measures against TB is established in many areas of the world, it is still prevalent in many developed and developing countries including Egypt and it represents a major zoonotic and public health problem.

In this study, PCR was developed for direct detection of *M. bovis* and *M. tuberculosis* from animal and human samples, respectively and comparison with the conventional methods was performed. The prevalence of BTB in cattle from different farms in Egypt was also determined and abattoir surveillance was carried out in El-Basateen abattoir, Cairo to detect BTB in slaughtered carcasses.

## MATERIALS AND METHODS

**Sample Collection:** Three thousands and three hundreds forty-seven cattle from different farms in Egypt were examined by skin test in the period between November 2010 until December 2011 and the positive reactor animals were slaughtered. The internal organs (lung, liver, spleen) and lymph nodes were taken from positive tuberculin test reactors upon slaughtering. Suspected tissue samples and lymph nodes showing tuberculous like lesions were collected from cattle carcasses slaughtered during routine work in El-Basateen abattoir, Cairo, Egypt during the period between December 2010 until March 2011. In addition, lymph nodes were taken from five apparently healthy cattle (age >4 years) slaughtered in El-Mansoura abattoir, Dakahlia, Egypt.

Forty two sputum samples were collected from clinically diagnosed patients for isolation and identification of mycobacterium spp. that were admitted at Mansoura and Abassia Chest hospitals, Egypt for isolation and identification of mycobacterium spp. Also, 12 sputum samples were taken from apparently healthy personnel that had occupational contact with infected cattle. Sputum samples were collected in 5ml sterile plastic bottles and placed in 15ml sterile bottles to avoid leakage of infectious samples.

**Isolation and Identification of Mycobacteria:** All bovine samples were processed for isolation and cultivation of mycobacteria using Marks' method and Lowenstein-Jensen media (LJ) with or without pyruvate [10]. Sputum samples were processed by Petroff's method for isolation of mycobacteria [11] and the processed sputum sediments were inoculated onto slopes of LJ medium containing glycerol and pyruvate (0.5%). The inoculated slopes of bovine and human samples were incubated at 37ºC and were observed for growth for at least 8-12 weeks. The cultures showing evidence of growth were examined for presence of mycobacteria. Species level identification of isolates was done by standard biochemical tests as recommended by the Center for Disease Control and Prevention (CDC) with appropriate controls [12].

**DNA Extraction:** DNA was extracted from collected lymph nodes, tissues and sputum samples by using Gene JET Genomic DNA Purification Kit#K0721 (Fermentas) and according to the manufacturer protocol. The strains of *M. bovis* and *M. tuberculosis* isolated from bovine and human were kept at the laboratory of Bacterial Diagnostic Products Research Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abassia, Cairo, Egypt. Five µl of the extracted genomic DNA was then subjected to 0.7% agarose gel electrophoresis for determination of quality [13].

**PCR Amplification:** The primers N (5'-GGA GGG TTG GGA TGA ACA AAG CAG-3') and S (5'-GTA TCC GTG TGT CTT GAC CTA TTT G-3') were used to amplify the hupB gene (N-S PCR) from the DNA extracted from 23 samples (lymph nodes and tissues) for direct detection of *M. bovis* [3]. Also, DNA extracted from 24 sputum samples was amplified for direct detection of PncA gene of *M. tuberculosis* using PncATB-1.2 (5'-ATG CGG GCG TTG ATC ATC GTC-3') and PncAMT-2 (5'-CGG TGT GCC GGA GAA GCG G-3') primers [14].

PCR amplifications were performed in a final volume of 50 µl PCR mixture consisted of 20 µl of 2X Master Mix (GeNeiTM PCR Master Mix Kit), 2.5 µl of each primer, 7 µl template DNA and 18 µl nuclease free H2O. The T100 thermal cycler (BIO-RAD) was programmed for hupB DNA target to initially incubate the samples for 10 min

at 94°C, followed by 35 cycles consisting of 94°C for 90 seconds, 60°C for 90 seconds and 72°C for 110 seconds. Final extension was given at 72°C for 30 minutes. Specific *M. bovis* DNA detection for each sample was identified by specific 618 bp DNA bands on 1.8% agarose gel, stained with ethidium bromide and evaluated under UV transilluminator [3].

In PncATB DNA target, the cycling parameters were 95°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 67°C for 1 min and extension at 72°C for 1 min. Final extension was given at 72°C for 10 min. Specific *M. tuberculosis* DNA detection for each sample was identified by specific 185 bp DNA bands on 1.8% agarose gel, stained with ethidium bromide and evaluated under UV transilluminator.

The estimation of the sizes of PCR products was done according to the migration pattern of a 100-bp DNA ladder (Fermentas).

## RESULTS

**Conventional Diagnosis of Tuberculosis:** Thirty-two out of the 3347 examined animals from different farms in Egypt showed a positive reaction to tuberculin skin test with a prevalence of 0.96%. Also, fifteen carcasses showed tuberculous lesions from a total of 7235 animals slaughtered during routine work in El-Basateen abattoir, Cairo, Egypt with a prevalence rate of 0.21%.

The culture results from animals showed that out of 32 processed tissue samples from tuberculin positive cows, 20 (62.5%) were recovered by conventional culture method. A total of 18 (72%) *M. bovis* isolates were recovered from 25 tuberculin positive cows showing visible lesions on post-mortem findings. Whilst, only 2 (28.5%) *M. bovis* isolates were detected from seven tuberculin positive cows with no visible lesions. From non tuberculin tested animals with suspected tuberculous

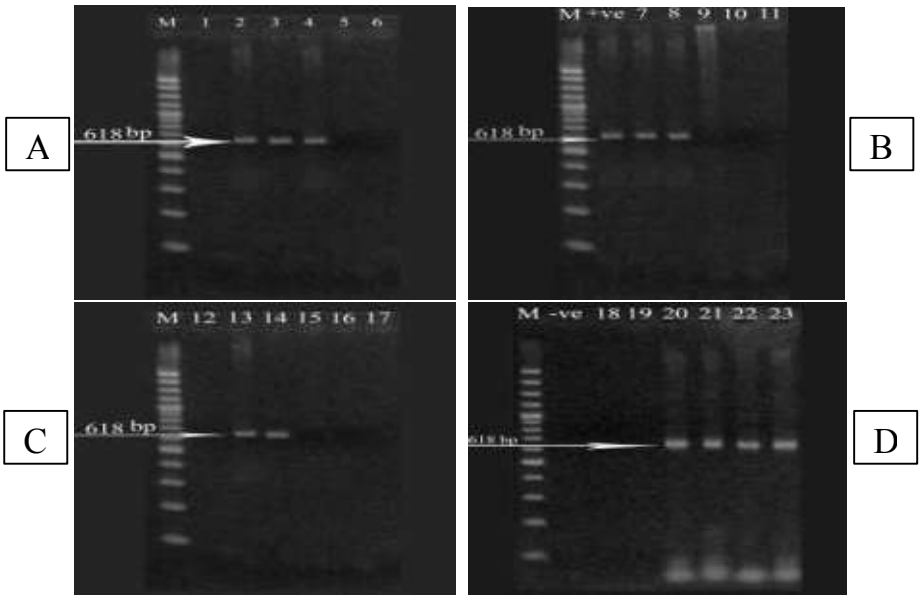


Fig. 1: PCR amplification results of the examined samples for *M. bovis*

A. Suspected tuberculous samples from slaughtered cattle (non-tuberculin tested). M: Marker, 100 bp ladder. Lanes 1, 5, 6: Suspected tuberculous samples which gave no isolates upon culturing on L.J. media. Lanes 2, 3, 4: Suspected tuberculous samples which gaves *M. bovis* isolates upon culturing on L.J. media.
B. Samples from tuberculin +ve reactors showing no visible lesions. Lane +ve: M. bovis (AN5). Lanes 7, 8: Samples which gave *M. bovis* isolates upon culturing. Lanes 9, 10, 11: Samples which give no isolates upon culturing.
C. Lane 12: Sample from tuberculin +ve reactor showing no visible lesions and giving no isolate on culturing. Lanes 13, 14: Samples from tuberculin +ve reactors showing visible lesions and giving no isolates upon culturing. Lanes 15, 16, 17: Samples from apparently healthy animals that giving no isolates on culturing.
D. Lane -ve: Distilled water. Lanes 18, 19: Samples from tuberculin +ve reactors showing visible lesions and giving no isolates upon culturing. Lanes 20, 21, 22, 23: Samples from tuberculin +ve reactors showing visible lesions and giving *M. bovis* isolates upon culturing.

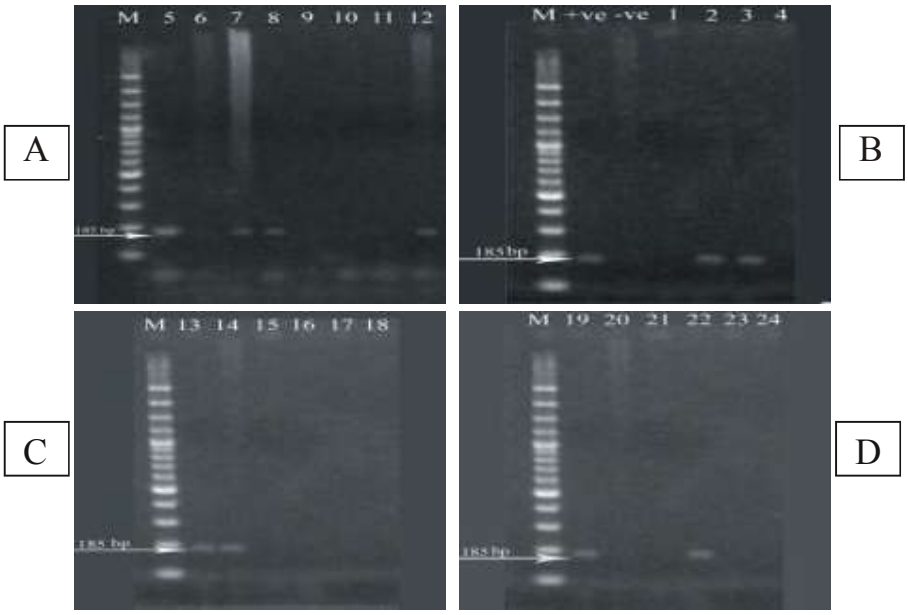


Fig. 2: PCR amplification results of the examined samples for *M. tuberculosis* A. Sputum samples from clinically diagnosed patients. Lane +ve: M. tuberculosis (H37Rv). Lane -ve: Distilled water. Lanes 1, 4: Sputum samples from patients which give no isolates upon culturing on L.J. media. Lanes 2, 3: Sputum samples that gave *M. tuberculosis* isolates upon culturing.

Photograph 2: B. Lanes 5, 7, 8, 12: Sputum samples that gave *M. tuberculosis* isolates upon culturing. Lanes 6, 9, 10, 11: Sputum samples from patients which give no isolates upon culturing on L.J. media.

Photograph 2: C. Lanes 13, 14: Sputum samples from patients which give isolates upon culturing on L.J. media. Lanes 15, 16, 17, 18: Sputum samples from apparently healthy personnel that gave no *M. tuberculosis* isolates upon culturing.

Photograph 2: D. Lanes 19, 20: Sputum samples from patients that gave no *M. tuberculosis* isolate upon culturing. Lanes 21, 22, 23, 24: Sputum samples from apparently healthy personnel that gave no *M. tuberculosis* isolates upon culturing

lesions, the percentage of *M. bovis* isolation was 66.7% (10/15) but from apparently healthy animals, no isolates were obtained.

From sputum samples, results showed that 35 (83.3%) of 42 clinically diagnosed patients gave *M. tuberculosis* through conventional culture method but only 1 (8.3%) *M. tuberculosis* isolate was recovered from 12 sputum samples taken from apparently healthy persons who had an occupational contact with cattle in farms examined by tuberculin skin test in this study. No *M. bovis* was isolated.

**Molecular Diagnosis of Tuberculosis (PCR):** From twenty three animals examined by N-S PCR, nine samples that were positive by culturing showing also specific band of hupB gene. Also two animal samples were positive by N-S PCR although yielding no isolates upon culturing. From human samples, twenty four sputa were tested by PncA PCR and ten samples giving the specific band. In addition, six samples from apparently healthy were subjected to N-S PCR and only one sample showing the specific band that targeting hupB gene of *M. bovis*. The amplified products of hupB gene obtained with representative bovine samples were shown in Fig. 1 and the target for positive samples was 618 bp. Also, PncA primers were used for direct detection of *M. tuberculosis* in DNA extracted from sputum samples with 185-bp specific band for positive samples as in Fig. 2.

The molecular sizes and extents of migration of the amplified PCR products generated in the samples were compared with those of the positive controls, namely *M. bovis* (AN5; VSVRI, Abassia, Cairo- Egypt) and *M. tuberculosis* (H37Rv; VSVRI, Abassia, Cairo- Egypt).

## DISCUSSION

For BTB control, infected cattle need to be identified accurately and in the early stages of the disease. The failure in achieving this will allow continuing transmission of the disease with subsequent public health hazards due to transmission of *M. bovis* from animals to human.

In this study, the prevalence of BTB determined by tuberculin test was 0.96% in the examined farms in Egypt during the period from November 2010 until December 2011. Our result is lower than that recorded in Egypt by Mossad *et al.* [15] 4.6% and Moussa *et al.* [16] (2.46%). Tuberculin skin testing is universally considered as a method for the diagnosis of BTB in live cattle and forms the basis for national test and slaughter programmes [17]. From this study, it was revealed that BTB is still present in farms in the examined areas in Egypt reflecting its economic and zoonotic importance.

The surveillance in El-Basateen abattoir, Cairo, Egypt during the period from December 2010 to March 2011 showed a prevalence rate of 0.21%. This result is higher than that detected by Shitaye *et al.* [18] (0.052%) in a ten years surveillance in Ethiopia. However, it is lower than that recorded by Ameni and Wudie [19] (5.2%), Teklu *et al.* [20] (4.5%), Demelash *et al.* [21] (10.2%) and Tschopp *et al.* [22] (4%). The proper meat inspection may act as a method for lowering the incidence of zoonotic tuberculosis as human indirectly acquires the disease from animal sources by ingestion of meat and meat products from slaughtered infected cattle [23].

By culturing on L.J. media, 20 *M. bovis* isolates were recovered from 32 processed tissue samples of tuberculin positive cows. A total of 18 (72%) isolates was recovered from 25 tuberculin positive cows showing visible lesions on post-mortem findings. 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 [24]. On the other hand concerning non-visible reactors, 2 out of 7 (28.6%) were identified as *M. bovis* and this is due to that infection was in early stage or only microscopic lesions were found in lymph nodes of reactors and thus *M. bovis* could be recovered only on culture [25].

From non tuberculin tested animals with suspected tuberculous lesions, the percentage of *M. bovis* isolation was 66.7% (10/15). This would suggest that the presence of caseous and/or calcified lesions resembling tuberculous lesions may not always found to be of mycobacterial origin as these lesions could be caused by any other intracellular organisms or parasites that might mislead a veterinarian to consider the non tuberculous cattle as being tuberculous [18].

The overall isolation rate of *M. tuberculosis* from sputum of clinically diagnosed patients was 83.3% (35/42) while from apparently healthy persons it was 8.3% (1/12). Our results are in agreement with those of Mendoza *et al.* [26] and Ang *et al.* [27].

The inability to isolate *M. bovis* from sputum samples in this study doesn't exclude the importance of zoonotic tuberculosis. As WHO reported in 1998 that 3.1% of tuberculosis cases in humans worldwide are attribu to *M. bovis* and that 0.4-10% of sputum isolates from patients in African countries could be *M. bovis* [23].

Table 1: Results of PCR of representative samples from animal lymph nodes, tissues and human sputum samples in relation to culturing

| Animals (N-S PCR)….618 bp | No. examined | Culture results | PCR (+ve results) |
|---|---|---|---|
| A- Tuberculin +ve Reactors: | | | |
| 1- Visible Lesions (VL): | 8 | +ve Culture: 4 | 4 |
| | | -ve Culture: 4 | 2 |
| 2- Non Visible Lesions (NVL): | 6 | +ve Culture: 2 | 2 |
| | | -ve Culture: 4 | 0 |
| B- Non tuberculin tested animals: | 6 | +ve Culture: 3 | 3 |
| | | -ve Culture: 3 | 0 |
| C- Apparently healthy animals: | 3 | -ve Culture: 3 | 0 |
| Human (PncA PCR)….185 bp | | | |
| A- Clinically diagnosed patients: | 16 | +ve Culture: 8 | 8 |
| | | -ve Culture: 8 | 1 |
| B- Apparently healthy personnel: | 8 | +ve Culture: 1 | 1 |
| | | -ve Culture: 7 | 0 |

A PCR based assay was optimized to screen cattle lymph nodes and sputum samples according to Table 1. It has been confirmed that species-specific 618 bp fragment that targeting hupB gene is well conserved in animal samples infected with *M. bovis*. These results are in full accordance with Prabhakar *et al.* [3]. Also, 185 pb specific fragment is well conserved in sputum samples from human infected with *M. tuberculosis* and the target was PncA gene. This is in agreement with the results of Shah *et al.* [14].

We compared the PCR results to the findings with the gold standard method of conventional diagnosis using culture on LJ media. It was found that PCR results matched the culture results in all screened samples except that of -ve culture samples from tuberculin positive reactors with visible lesion and -ve culture sputum samples from clinically diagnosed patients that were yielding no isolates. PCR amplification of specific target sequences may perhaps be considered one of the most sensitive approaches to the detection of even small amounts of Mycobacterium species DNA or non viable organisms in biological samples [28].

From this study it was clearly evident that PCR is a sensitive screening assay for the detection of *M. bovis* DNA in lymph nodes of cattle [29-32] and also it can generally be used to diagnose BTB in field condition. Also, PCR assay for the direct detection of *M. tuberculosis* from sputum took few hours to complete with high sensitivity and specificity [33].

In conclusion, prompt diagnosis of TB especially in countries where the disease is still endemic as in Egypt is of great importance to detect and identify infectious cases for strengthening control measures. As PCR technique is much faster than culture and reduce the time for diagnosis from several weeks to two days it can be used for large scale screening of TB in cattle and hence controlling the disease in cattle herds and also reduce the risk of its transmission to human.

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