

## Rapid detection of *Mycobacterium bovis* in clinical samples using THP-1 cells

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### ABSTRACT

The rapid isolation of *M. bovis* was attempted in THP-1 cells (Macrophage cell line). THP-1 cells infected with *M. bovis* showed characteristic cytopathic effect 48 hr after infection. The bacteria had grown very well in the first passage itself, without any blind passages in cell culture. The presence of bacteria in the cell culture fluid was confirmed by Ziehl-Neelsen staining and nested PCR amplification of insertion sequences.

**Key words:** *M. bovis*, THP-1 cells, nested PCR, Ziehl-Neelsen stain, decontamination

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### Introduction

Bovine tuberculosis (TB) is caused by *M. bovis*, which can infect a wide range of animal species and cause approximately 2000 human deaths per annum (6%) world wide. Bovine tuberculosis has severe implications for animal health since it causes reduced productivity and premature death in cattle in affected farms, causing severe economic losses. The isolation of *M. bovis* from suspected cases is the gold standard to be adopted as a refined procedure for bovine TB diagnosis (ADAMS, 2001; TAYLOR et al., 2001).

Following preliminary screening of suspected samples using Ziehl-Neelsen staining, isolation can be carried out in a bacteriological medium. However, cross-contamination among bovine carcasses, improper decontamination procedure and duration of isolation procedure (often 3 weeks and up to 8-10 weeks in liquid medium) jeopardizes the isolation of *M. bovis*. The lengthy duration of isolation procedure imposes an unavoidable delay in important decisions about outbreaks and of suspected herds put under restriction.

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Shorter time-span isolation procedures are required for quicker diagnosis. In this study, THP-1 cells will be used for rapid isolation of *M. bovis* from suspected samples. Subsequently, the nested PCR will be used for confirmation of presence of *M. bovis* genome in the infected cell culture.

### Materials and methods

*Mycobacterium bovis*. Fifteen suspected clinical samples (lung nodules) were collected from Kanchipuram and Madurai District of Tamil Nadu, India. Before culturing, the samples were decontaminated by mixing an equal volume of sodium hydroxide 40 g/l (4% w/v) solution for 20 min. Similarly, samples were decontaminated with antibiotic solution (Polymyxin B 2000 U/mL, Amphotericin B 200 µg/mL, Nalidixic acid 800 µg/mL, Trimethoprim 200 µg /mL) at 4 °C overnight. Samples were then centrifuged to pellet the bacteria. The pellet was washed twice with sterile PBS. Finally, the pellet was dissolved in sterile RPMI medium. *Mycobacterium tuberculosis* reference strain DNA obtained from the Tuberculosis Research Centre (TRC), Chennai, was used as positive control for PCR amplification.

*Cells*. THP-1 cells (human monocytic leukemia cells) were obtained from the National Center for Cell Science, Pune, India. They were maintained as suspension cells in RPMI 1640 medium supplemented with 2 mM L-Glutamine, 4.59 g L- Glucose, 10 mM HEPES buffer, 1.0 mM Sodium pyruvate and 10% foetal bovine serum at 37 °C in a 5% CO<sub>2</sub> humidified incubator. The cells have Fc and C3b receptor, lack surface and cytoplasmic immunoglobulins and are phagocytic (TSUCHIYA et al., 1980). The cells were subcultured at the split ratio of 1:3 and passaged every 3 days.

*Treatment of cells with PMA*. After complete growth of cells in a culture flask, the cells were treated with 20 nM phorbol 12 - myristate 13 - acetate (PMA, Himedia) in order to halt proliferation and to allow the cells to adhere and express a macrophage-like phenotype (MONAHAN et al., 2001). After overnight incubation in a CO<sub>2</sub> incubator at 37 °C, the monolayer of macrophage cell line was formed, which was used as substrate for infection of *M. bovis*.

*Infection of macrophage cell line*. *M. bovis* was propagated in THP-1 cells as per the method described by ORME et al., (1994). Briefly, the decontaminated sample was layered over the monolayer and kept for 2 hr at 37 °C in CO<sub>2</sub> incubator for adsorption. After adsorption, the inoculum was removed and maintenance medium was added. Cytopathic changes were observed after 48 hr post-infection. The intra- and extra-cellular bacteria were collected and checked for the presence of bacteria by Ziehl-Neelsen staining.

*DNA extraction and polymerase chain reaction*. An ultraclean microbial DNA isolation kit, obtained from MoBio laboratories, USA, was used for extraction of DNA

from infected cell culture fluid. The DNA was suspended in 50 µL of elution buffer provided in the kit. The DNA was stored at -20 °C until used for PCR amplification.

PCR kit (Bangalore genei, India) was used. A 200 bp sequence of the TB-complex specific insertion sequence IS 6110 was amplified using semi-nested PCR. The primer and the temperature profile were followed as described by RITELLI et al. (2003).

Primer sequence

5' IS Ext-1CCCGGACAGGCCGAGTTT 3

5' IS Ext-2CCGGCATGTCCGGAGATC 3'

5' IS Int- 1CCCCATCGACCTACTACG 3'

The PCR mixture consisted of 10 × buffer containing 1.5 mM MgCl<sub>2</sub>, 200 µM each dNTP mix, 0.2 µM each primer and 2.5 U Taq DNA polymerase. The mixture was amplified by initial denaturation for 5 min at 95 °C and 30 cycles of 30 s at 95 °C; 30 s at 68 °C; 60 s at 72 °C followed by 3 min. of final extension at 72 °C. Primers IS 6110 Ext-1 and Ext-2 were used in the first step.

In the second step, the first step PCR product was used as a template. The PCR mixture was submitted to 40 cycles of amplification (30 s at 95 °C; 30 s at 50 °C; 60 s at 72 °C) followed by 5 min. of final extension at 72 °C. Primers Ext-1 and Int-1 were used in this step. Reference positive and negative control were used each time under the same conditions. The final amplicon was run in 1.5% agarose gel electrophoresis at 100V for 45 min. and ethidium bromide-stained gel was viewed through UV transilluminator (Fotodyne, USA).

## Results

*Sample collection.* A total of 15 samples was collected. All samples were found positive by initial screening using Ziehl-Neelsen staining. Of these, 5 samples were propagated in THP-1 cells.

*Propagation of M. bovis in THP-1 cells.* The PMA-treated THP-1 cells infected with *M. bovis* after 48 hrs of post-infection showed characteristic cytopathic effects (CPE) (Fig. 1). Uninfected culture showed no CPE. The infected culture fluid was stained with Ziehl -Neelsen staining and the acid fast organism appeared as a bright red colour. The infected culture grown in L-J medium confirmed the presence of *M. bovis* in the cell culture.

*PCR.* The insertion sequence was amplified using nested PCR and the expected amplicon size of 200 bp was observed in the infected culture fluid and positive control. No amplification was noticed in the uninfected culture fluid (Fig. 2).

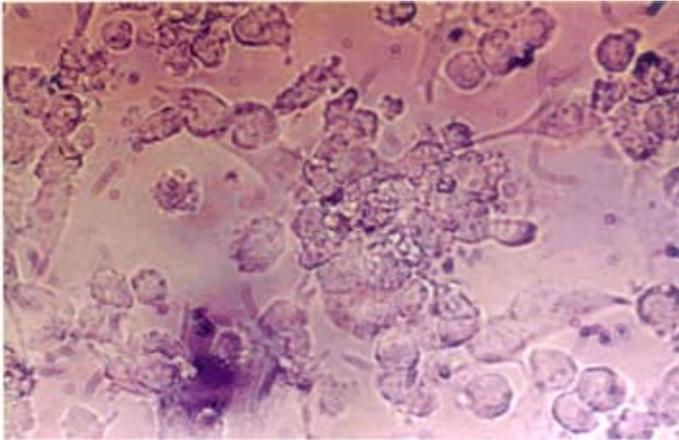


Fig. 1. Infected THP-1 cells showing cytopathic effect (CPE), such as grouping of cells. H&E;  $\times 400$ .

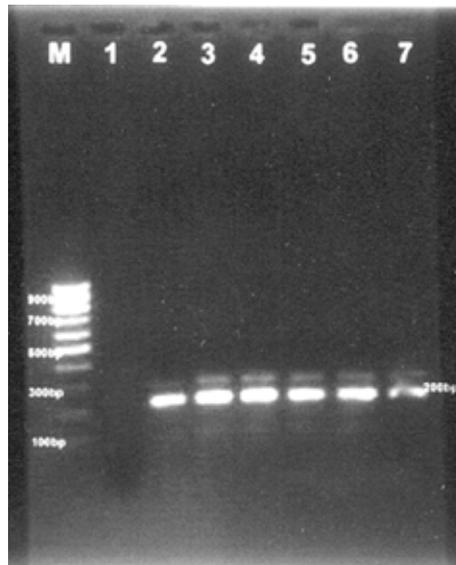


Fig. 2. Agarose gel electrophoresis showing amplified PCR products of insertion gene sequences of *M. bovis*. Lane M - 100 bp ladder; Lane 1 - Negative control (uninfected culture fluid); Lane 2 - *M. tuberculosis* reference strain (Positive control); Lane 3, 4, 5, 6 and 7 - *M. bovis* infected culture fluid (Field strains).

## Discussion

Bacteriological media from suspected cases of tuberculosis demand a laborious and time consuming procedure. Conventionally, there are different methods used for diagnosis of tuberculosis. In the Ziehl-Neelsen smear studies, the presence of a minimum of 10000 organisms per mL of samples is needed to visualize bacilli under a microscope. Isolation of bacteria with the culture method takes 4-6 weeks to obtain visual colonies on L-J medium. In the PCR method, the direct use of PCR on clinical samples, sensitivity may be poor (LIEBANA et al., 1995; WARDS et al., 1995). The variation in results was observed by *M. bovis* isolation in bacteriological media and direct detection of organism by PCR (AMADORI et al., 2002). Currently, radiometric and fluorescence-based protocols for isolation of *M. bovis* in a short span are expensive and are currently subsidiary to bacteriological procedure. However, by using the macrophage cell line the detection of tuberculosis from suspected clinical samples could be carried out within 48 hr, as reported by RITELLI et al., 2003. Human THP-1 cell line has proved to be a powerful and reliable means of *M. bovis* amplification on the first passage itself, and no blind passages are required. Decontamination procedures using antibiotics, as well as NaOH treatment, were adopted in this study. In both methods, the infected culture did not show any contamination. Antibiotics are costlier. Hence, the cheapest NaOH treated samples can be used very well as an inoculum for infecting the cells.

TB complex mycobacterium displays distinct survival strategies in macrophages. This property of mycobacterium is exploited to grow this organism in macrophage cell line. The THP-1 cell is chosen because of convenient cultural conditions and because of their extensive characterisation as a suitable vehicle for amplification of TB complex Mycobacteria. Hence, THP-1 cells can be used as alternative to bacteriological isolation of *M. bovis*.

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**SAŽETAK**

Brzo izdvajanje vrste *Mycobacterium bovis* pokušano je na staničnoj liniji makrofaga THP-1. Stanice THP-1 zaražene tom vrstom pokazivale su karakterističan citopatski učinak 48 sati nakon infekcije. Bakterije su se vrlo dobro razmnožavale već u prvoj pasaži bez potrebe za daljnjim uzgojem. Prisutnost bakterija u hranjivoj tekućini bila je potvrđena bojenjem po Ziehl-Nelseenu i umnožavanjem specifičnih sekvencija ugniježđenom lančanom reakcijom polimerazom.

**Ključne riječi:** *Mycobacterium bovis*, stanice THP-1, ugniježđena PCR, Ziehl-Nelseenovo bojenje

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