Visual Format for Detection of *Mycobacterium tuberculosis* and *M. bovis* in Clinical Samples Using Molecular Beacons

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**A real-time polymerase chain reaction (PCR) assay for
the direct identification of *Mycobacterium tuberculosis*
and *M. bovis* using molecular beacons was developed.
The assay was modified for use in regular thermal cy-
clers. Molecular beacons that were specific for *M. tuber-
culosis* (Tb-B) and *M. bovis* (Bo-B) were designed. The
fluorescence of the target PCR product-molecular bea-
con probe complex was detected visually using a tran-
silluminator. The results were then compared with
those of conventional multiplex PCR (CM-PCR) assays
and biochemical identification. The detection limit of
Tb-B and Bo-B beacons was 500 fg and 50 fg by the visual
format and real-time PCR assay, respectively, compared
with 5 pg by CM-PCR assay. Pulmonary and extrapul-
monary samples were examined. The agreement be-
tween culture and the two assays was very good in
sputum samples and fair in extrapulmonary samples.
The agreement between clinical diagnoses with the
two assays was moderate in extrapulmonary samples.
There was very good agreement between CM-PCR
and visual format assays for all samples used in the
study. Concordance in the identification of isolates
by the visual, CM-PCR assay, and biochemical iden-
tification was seen. Hence, the use of molecular
beacon detection of *M. tuberculosis* and *M. bovis* in
clinical samples is feasible by setting up two asym-
metric PCRs concurrently. The assay is sensitive,
specific, simple to interpret, and takes less than 3
hours to complete.** *(J Mol Diagn 2009, 11:430–438; DOI:
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For effective treatment of tuberculosis (TB), rapid and
accurate diagnosis is essential. Conventional polymer-
ase chain reaction (PCR)-based assays designed to de-
tect *Mycobacterium tuberculosis* that involve electro-
phoresis based analysis of amplicons are relatively fast
but are laborious and the potential risk of inadvertent
dispersal of amplicons leading to contamination of un-
tested samples exists. To overcome these difficulties a
number of probes, including TaqMan probes, molecular
beacons, and side-by-side probes, that can report the
amplification of the correct amplicon in sealed tubes
have been developed.[1–3] Several different instruments
that measure increase in fluorescence of these probes
while simultaneously performing amplification have also
become available. Although such real-time PCR assays
are more quantitative than conventional ones, the instru-
ments used are not commonly available in resource-poor
locations. Since such localities are often where tubercu-
losis is more prevalent, it is important to develop sealed
tube assays that can be implemented on conventional
PCR machines but have the simplicity and accuracy of
probe-based detection.

In the previously developed tests different coding and
intergenic regions from the *M. tuberculosis* genome, such
as *devR, IS6110, IS986,* RNA polymerase gene, and
ribosomal RNA gene have been used as targets for ampli-
fication using molecular beacons or fluorescent probes.[4–7]
One of the limitations of these tests is that they have exclu-
sively focused on *M. tuberculosis*. However, other closely

related bacteria such as *M. bovis* have been often associated clinically with human and bovine samples in practice.[8–10] Therefore an ideal test should distinguish between *M. tuberculosis* and *M. bovis*. We used the *mce3* operon that has a differential organization in the *M. tuberculosis* and *M. bovis* genome as a suitable target for the assay.[11]

To develop a simple sealed tube test that can reliably detect *M. tuberculosis* in clinical samples using conventional PCR instrument for amplification and a common lamp for transillumination, we used molecular beacons as probes. However, the signal intensity in PCRs performed with molecular beacons is often limited because the two product strands of the amplicon bind to each other excluding the probe from the target strand. We improved the fluorescence intensity of these reactions by performing asymmetric PCR that produced more of the molecular beacon target strand than of the opposite strand. The optimizations were performed using a real-time instrument. We show that our assay is robust, specific, sensitive, and can be completed in less than 3 hours. The presence of mycobacteria in the sample can be conclusively established by visualizing the fluorescent amplicon in a blue light transilluminator by the naked eye. We also compared and correlated our endpoint visual format assay with acid-fast bacilli (AFB) smear microscopy; a gel-based conventional multiplex PCR (CM-PCR) assay, culture, and clinical diagnosis. The simplified visual format assay was found to be an accurate predictor of the presence of *M. tuberculosis* and *M. bovis* in clinical samples.

Materials and Methods

Samples

Sputum samples were collected from 97 pulmonary tuberculosis patients attending the outpatient department of LRS Institute of tuberculosis and Respiratory Disease, New Delhi. Seventy-one aseptically aspirated pleural fluid samples were obtained from patients registered in the Department of Respiratory Medicine, Safdarjung Hospital, New Delhi. The study was approved by the institutional ethical committee. The clinical criteria as described by Light[12] were adopted for the diagnosis of pleural tuberculosis. The definitive criterion for tuberculous pleural effusion was the demonstration of acid-fast bacilli in sputum and or pleural fluid by microscopy or culture. Suggestive criteria included: 1) patients with clinical history of fever, pleuritic chest pain, cough, breathlessness, and chest radiography with evidence of pleural effusion; 2) cytological examination of the pleural fluid for predominance of lymphocytes and paucity of mesothelial cells; 3) biochemical estimation for protein content (>3 g/dl) in pleural fluid: serum protein ratio (>0.5); and 4) response of patients to antituberculous treatment. Tuberculous pleural effusion was diagnosed if the definitive criterion or all of the suggestive criteria were met. Based on these criteria, 51 patients were classified as patients with tuberculous pleural effusion.

Criteria for including malignant pleural effusion patients (controls) were: clinical history suggestive of rapidly refilling pleural effusion with or without focal malignant lesion elsewhere in the body; pleural fluid being exudative, usually hemorrhagic; and on cytological examination, positive for malignant cells. Based on these criteria 20 patients were classified as nontuberculous pleural effusion patients (non-TB).

The cerebrospinal fluid (CSF) sample was obtained from a clinically suspected case of tuberculous meningitis. The patient was admitted to the pediatric ward at Kalawati Saran Children Hospital, New Delhi.

Processing of Samples

All samples except CSF were processed by the NALC-NaOH method.[13] For processing of CSF, the filtration method was used as described earlier.[14] The sediments were processed for AFB microscopy, isolation of mycobacteria, and target DNA. A total of 118 samples (97 sputum, 20 pleural fluid, and the CSF sample) were inoculated into MGIT tubes (BD BACTEC MGIT 960 system for mycobacteria testing). The remaining 51 pleural fluid samples were inoculated on Lowenstein-Jensen medium and into 7H9 broth. All cultures were examined up to 8 weeks. Species level identification of isolates was done by standard biochemical tests (niacin production, nitrate reduction, catalase and aryl sulfatase activity, Tween hydrolysis, thiopen-2-carboxylic acid hydrazide sensitivity) as recommended by the Centers for Disease Control and Prevention, Atlanta, GA, with appropriate controls.[15]

Mycobacterial Strains

M. tuberculosis (*M. tuberculosis,* H37Rv) DNA was obtained from Tuberculosis Research Material, NIAD, National Institutes of Health. *M. bovis* (AN5) was obtained from National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra, India.

Design and Characterization of the Molecular Beacons

M. tuberculosis beacon (Tb-B) was designed targeting the unique 12.7-kb fragment of the *M. tuberculosis* genome. The *M. bovis* beacon (Bo-B) was designed commencing 1 bp 5′ to the deletion site of the 12.7-kb fragment in the *M. bovis* genome. The positions of the beacons and primers are shown in Figure 1.[11]
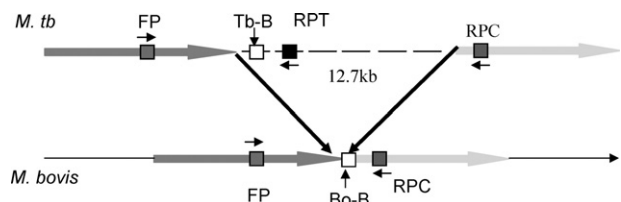
Figure 1. Position of primers and beacons in the *mce3* operon[11] used for the detection and identification of *M. tuberculosis* and *M. bovis*. 12.7-kb insert prevents Taq polymerase-mediated amplification in *M. tuberculosis,* hence amplification seen only in *M. bovis*.

Thermal denaturation profile studies were conducted to determine the optimal annealing temperature for the real-time PCR. Real-time PCR was done as follows: molecular beacon buffer alone (negative control); buffer + Tb-B (negative control); buffer + Tb-B + target oligonucleotide of *M. tuberculosis* (TO-tb, positive control); buffer + Tb-B + target oligonucleotide *M. bovis* (TO-bo). Identical combination of reagents was used with *M. bovis* beacon (Bo-B). For the assay the molecular beacon buffer (3.5 mmol/L $MgCl_2$ and 10 mmol/L Tris-HCl), with 0.3 μmol/L each of molecular beacon (Tb-B and Bo-B) and 0.6 μmol/L each of target oligonucleotide (5′-GTACTATGCT-GACCCATGCGCCCT-3′ and TO-Bo, 5′-CGGAGAGCG-CCGTTGTAGGCC-3′) was used. Target oligonucleotides contained the complementary sequence of the beacon's loop. Each tube was subjected to sequential decrease in temperature (1°C at a time) from 96 to 45°C. Fluorescence was estimated during the 30-second hold period at each temperature in the real-time thermal cycler. The melt curve of the molecular beacons was obtained by plotting a −dF/dT curve.

The signal-to-noise ratio of the molecular beacons was determined by measuring the fluorescence at 491 nm excitation and 515 nm emission in a spectrofluorimeter (Spectra MAX Gemini XS, Molecular Devices). Background fluorescence of the buffer and beacons was assessed and deducted from the fluorescence estimated following target hybridization. The signal-to-noise ratio (STNR) of the molecular beacons was determined as follows: $STNR = F_{open} − F_{buffer}/F_{closed} − F_{buffer}$, where F_{buffer} = fluorescence of molecular beacons buffer (containing 1 mmol/L $MgCl_2$ + 20 mmol/L Tris-HCl, pH 8); F_{closed} = fluorescence of 0.3 μmol/L molecular beacons in the molecular beacons buffer; F_{open} = fluorescence of molecular beacon buffer containing 0.3 μmol/L molecular beacons and 0.6 μmol/L target oligonucleotide.

The readings obtained with *M. tuberculosis* beacon were as follows: molecular beacon buffer was F_{buffer} = 5.4, for *M. tuberculosis* beacon with buffer F_{closed} = 136.2, *M. tuberculosis* beacon with target oligonucleotide of *M. tuberculosis* F_{open} = 3055.1. Similarly for *M. bovis* beacon the reading obtained was as follows: buffer alone F_{buffer} = 4.4, for *M. bovis* beacon with buffer F_{closed} = 38.1, *M. bovis* beacon with target oligonucleotide of *M. bovis* F_{open} = 905.6. The signal-to-noise ratio of the respective molecular beacons was 27 and 23 for *M. bovis* and *M. tuberculosis* beacons, respectively. These characterizations were performed at the room temperature. The signal to noise ratio for the beacons were determined with twofold increase in target oligonucleotide by the fluorometric and visual format methods.

PCR Assay

The assay was first standardized in a real-time format (FP, forward primer common to both *M. tuberculosis* and *M. bovis*; RPT, reverse primer from 12.7-kb fragment specific for *M. tuberculosis*; RPC, reverse primer from the sequence present in both *M. tuberculosis* and *M. bovis*). For *M. tuberculosis* the primers used were FP (5′-ATGACGCCTTC-CTAACCAGAA-3′) and RPT (5′-ATGCATCGGAATAAGAT-GTCAGGC-3′) with Tb-B (FAM-5′-*CCGCGG*AGGGGCG-CATGGGTCAGCATAGTA*CCGCGG*-3′-BHQ2) and for *M. bovis* the primers used were FP and RPC (5′-ACCGGA-TATCTTAGCTGGTCAA-3′) with Bo-B (FAM-5′-*CCGCGC*-TGGCCTACAACGGCGCTCTCC*GCGCGG*-3′–BHQ2). Sequences in italic demarcate the beacon stem. Subsequently the real-time format assay was modified for the visual format. The visual format assay was compared with the CM-PCR assay.

Real-Time PCR Assay

The experiments were performed with an iCycler iQ real-time detection system (Bio-Rad). For the asymmetric PCR assay, two reaction mixtures were set up for detection of *M. tuberculosis* and *M. bovis.* The first mixture (50 μl reaction) contained the primer FP (0.1 μmol/L) and RPT (0.5 μmol/L) and the Tb-B (0.6 μmol/L). The second mixture (50 μl reaction) contained the FP and RPC (0.5 μmol/L) and the Bo-b (0.6 μmol/L). The PCR reaction mixture contained 1X AmpliTaq Gold PCR buffer, 4 mmol/L $MgCl_2$, 0.25 mmol/L dNTPs (Eppendorf), and 2.5 U AmpliTaq Gold enzyme (Applied Biosystems). The thermal cycle parameters were 95°C for 10 minutes and 40 cycles of each for 45 seconds at 95°C, 45 seconds at the optimized annealing temperature, 45 seconds at 72°C, and final extension at 72°C for 10 minutes. Annealing temperature for asymmetric PCR assay was determined by the melt curve analysis of the beacons with their target oligonucleotides.

Visual Format Using Molecular Beacons

The asymmetric PCR parameter and conditions in the visual format were identical to the real-time PCR assay with the exception of the Taq polymerase enzyme (MBI Fermentas) in place of AmpliTaq Gold enzyme.

Multiplex PCR

Three primers were used: FP common to *M. tuberculosis* and *M. bovis* and two reverse primers, RPT and RPC, specific for *M. tuberculosis* and *M. bovis,* respectively. The assay mixture (25 μl reaction) contained FP (0.625 μmol/L), RPT and RPC (0.325 μmol/L each), 1X PCR buffer (100 mmol/L Tris/HCl, pH 8.8, 500 mmol/L KCl, 0.8% Nonidet P-40), 2.5 mmol/L $MgCl_2$, 0.3 mmol/L dNTPs, and 1.25 U TaqDNA polymerase. The thermal cycle parameters were 95°C for 10 minutes and 40 cycles of each, 45 seconds at 95°C, 45 seconds at 58°C, 45 seconds at 72°C, and final extension at 72°C for 10 minutes. The standardized assay was used for detection of *M. tuberculosis* as well as *M. bovis* in clinical samples. The amplified product obtained in CM-PCR assay is 162 and 127 bp for *M. tuberculosis* and *M. bovis,* respectively.

Detection of PCR-Amplified Product

For the gel-based CM-PCR, amplicon(s) were detected by standard ethidium bromide staining and ultraviolet

illumination of 2.2% agarose gels. For the visual format using molecular beacons, tubes were positioned over a dark reader box (Clare Chemical Research). Tubes were illuminated with a source of visible excitation blue light devoid of ultraviolet radiation. An amber screen separated the light source from the viewer. The fluorescence was viewed and assessed in the dark by at least two individuals.

Sensitivity of the Assays

The limit of sensitivity of the PCR assays (real-time PCR, CM-PCR, and molecular beacons using visual format) was determined by serial dilutions of *M. tuberculosis* and *M. bovis* DNA.

Statistical Analysis

All statistical analyses were done using STATA software, version 9.2 (StataCorp, College Station, TX). The agreement between the assays used was assessed using the kappa coefficient (κ). The strength of agreement was defined as: poor, $\kappa \leq 0.2$; fair, $\kappa = 0.21–0.4$; moderate, $\kappa = 0.41–0.6$; good, $\kappa = 0.61–0.8$; very good, $\kappa = 0.81–1$.[16]

Results

Rationale for the PCR Target Used in the Study

Sequence analysis of *M. tuberculosis* and *M. bovis* has shown that *M. bovis* lacks the 12.7-kb fragment containing the *mce3* operon.[11] All *M. tuberculosis* isolates examined showed the presence of the 12.7-kb fragment, while all of the *M. bovis* strains lacked this fragment.[11] We exploited the differences in the organization of the *mce3* operon in the two species.[11] Primers were designed as follows: FP was common to both *M. tuberculosis* and *M. bovis*; RPT was derived from the 12.7-kb fragment and RPC from the region adjacent to the 12.7-kb fragment (Figure 1).[11] RPT primer was specific for *M. tuberculosis*, while the sequence of the RPC reverse primer was present in both *M. tuberculosis* and *M. bovis*. However, amplification occurs in case of *M. bovis* with the FP and RPC exclusively and not in the case of *M. tuberculosis*, as the 12.7-kb insert prevents Taq polymerase-mediated amplification.

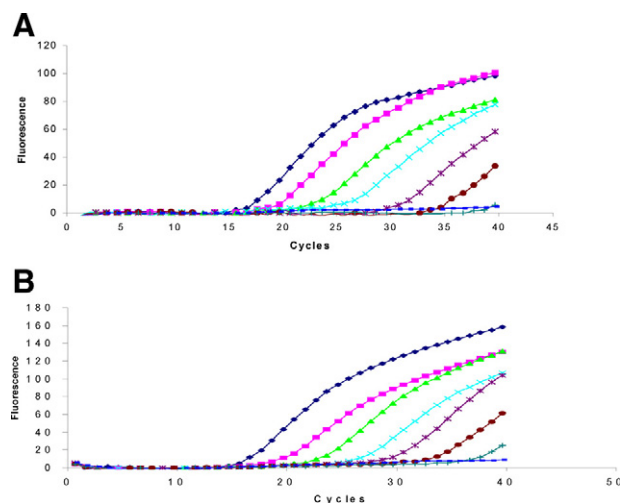#### Characterization of Molecular Beacons

The beacons were characterized by visual and fluorometric techniques. The specificity of molecular beacons was assessed by adding excess of the target oligonucleotides and an aliquot of each molecular beacon. Photographs were taken. Figure 2A shows that *M. tuberculosis* target oligonucleotide elicited fluorescence with Tb-B and *M. bovis* target oligonucleotide elicited fluorescence with Bo-B and not vice versa. In absence of the target oligonucleotides, no fluorescence was generated by the
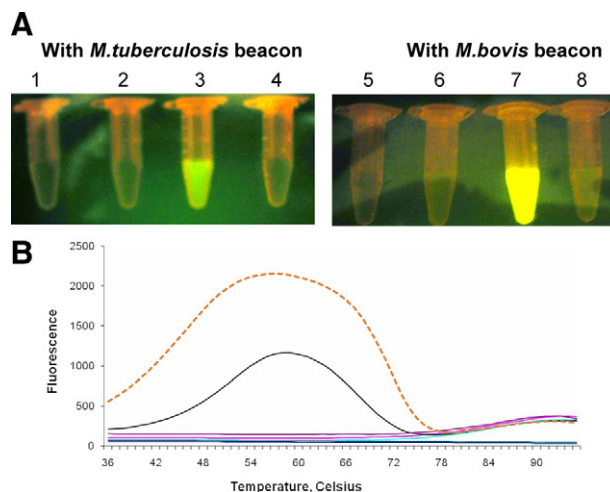
Figure 2. A: Signal-to-noise ratio of molecular beacons. The signal-to-noise ratio of *M. tuberculosis* and *M. bovis* beacons was determined by the visual format assay. Tube 1, buffer (negative control); tube 2, buffer + *M. tuberculosis* beacon (negative control); tube 3, buffer + *M. tuberculosis* beacon + target oligonucleotide of *M. tuberculosis* (positive control); tube 4, buffer + *M. tuberculosis* beacon + target oligonucleotide of *M. bovis*; tube 5, buffer (negative control); tube 6, buffer + *M. bovis* beacon (negative control); tube 7, buffer + *M. bovis* beacons + target oligonucleotide *M. bovis* (positive control); tube 8, buffer + *M. bovis* beacon + target oligonucleotide *M. tuberculosis*. **B:** Thermo-denaturation profile for molecular beacons. Dotted line denotes *M. bovis* beacon with its target oligonucleotide melt curve. Continuous line denotes *M. tuberculosis* beacon with its target oligonucleotide melt curve. All other lines denotes various controls.

molecular beacons in the tubes. The signal-to-noise ratio of the respective molecular beacons was determined as described in *Materials and Methods*. The signal-to-noise ratio of the respective molecular beacons was 27 and 23 for *M. bovis* and *M. tuberculosis* beacons, respectively.

Further, the thermal denaturation profile for each molecular beacon was determined by real-time PCR. As shown in Figure 2B, the probe target hybrid for *M. tuberculosis* beacon melts at 58°C and that of *M. bovis* melts at 56°C, and the stems of each molecular beacon melt at a

Figure 3. Amplification plots show the detection of the serially diluted DNA of *M. tuberculosis* (**A**) and *M. bovis* (**B**) by real-time PCR using molecular beacons. Tenfold serial dilution of the mycobacterial DNA, ranging from 5×10^6 to 5 bacilli/reaction (from left to right) and the negative control were amplified in individual tubes. PCR cycles have been plotted against fluorescence intensity.
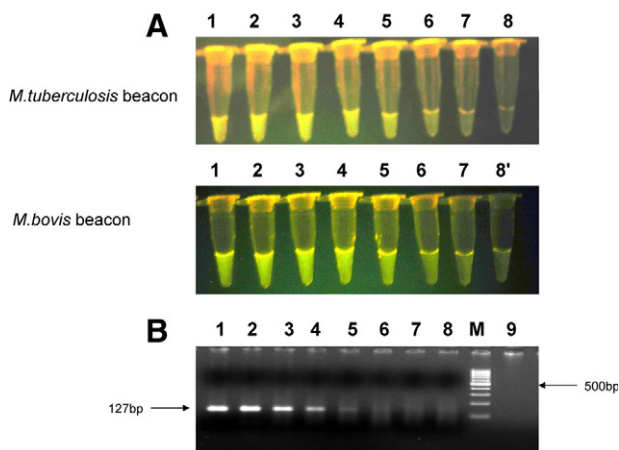
Figure 4. Limit of detection of *M. tuberculosis* and *M. bovis* DNA by PCR assay using molecular beacons by the visual format (**A**) and gel-based assays (**B**). *M. tuberculosis* and *M. bovis* DNA were added as follows: tube 1, 50 ng (5×10^6 bacilli); tube 2, 5 ng (5×10^5 bacilli); tube 3, 500 pg (5×10^4 bacilli); tube 4, 50 pg (5×10^3 bacilli); tube 5, 5 pg (5×10^2 bacilli); tube 6, 500 fg (5×10^1 bacilli); tube 7, 50 fg (5 bacilli); tube 8, negative control in visual format; tube 8′, 5 fg (~1 bacilli) in gel-based; tube 9, negative control.

range between 74 and 76°C. These features indicate that the detection temperatures can be below 60°C, at which the probe-target hybrids would be allowed to form when the target is produced in the reaction and the stems would remain closed when the target is absent.

Real-Time PCR and Multiplex PCR Sensitivity

As the signal intensities generated by real-time PCR were inadequate, asymmetric PCR was performed to intensify and maximize the fluorescence. To demonstrate the limits of detection of *M. tuberculosis* and *M. bovis*, serial dilutions of the DNA ranging from 500 ng to 10 fg were added to the asymmetric PCR reaction. Two separate reactions were set up using Tb-B and Bo-B. The limit of detection by real-time PCR is shown in Figure 3, A and B. The detection limit with Tb-B and Bo-B beacons was 500 fg (50 bacilli) and 50 fg (5 bacilli), respectively. The sensitivity of the visual format using molecular beacons was similar to that detected by real-time PCR (Figure 4A).
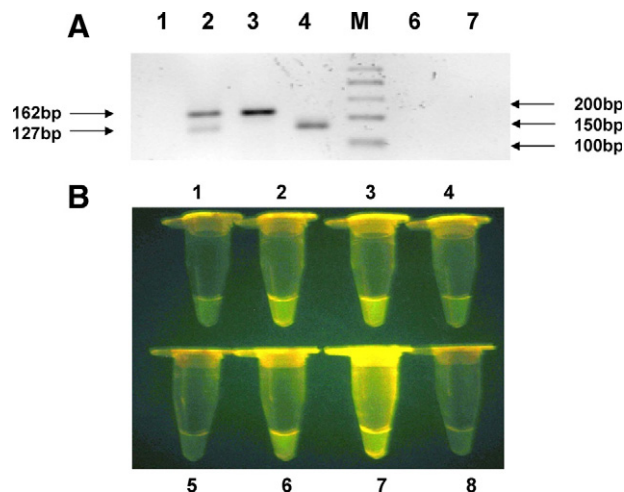
Figure 5. A: CM-PCR for detecting and differentiating *M. tuberculosis* and *M. bovis* in the CSF sample, CS-112. The ethidium bromide stained amplification products of *M. tuberculosis* and *M. bovis* generated by using FP, RPT, and RPC primers were electrophoresed on 2.2% agarose gel. The 162- and 127-bp products obtained in *M. tuberculosis* and *M. bovis* respectively, are indicated. Lane 1, PF-7; lane 2, CSF; lane 3, *M. tuberculosis* (positive control, H37Rv); lane 4, *M. bovis* (positive control AN-5); lane M, 50-bp molecular weight marker; lanes 6 and 7, negative control. **B:** Visual format using molecular beacons for detecting and differentiating *M. tuberculosis* and *M. bovis* in the CSF sample, CS-112. Tubes 1–4 showing fluorescence obtained using primer pair FP and RPT with *M. tuberculosis* beacon and tubes 5–8 showing fluorescence obtained using primer pair FP and RPC with *M. bovis* beacon. Tubes 1 and 5, PF-7; tubes 2 and 6, CSF; tube 3, *M. tuberculosis* positive control; tube 7, *M. bovis* positive control; tubes 4 and 8, negative control.

Gel-based CM-PCR assay showed lower sensitivity of detection of mycobacterial DNA (5 pg, equivalent to 500 bacilli; Figure 4B).

Comparative Analysis of the Four Methods for Detection of Mycobacteria in 97 Sputum Samples

Four methods were used for detecting mycobacteria in sputum samples: AFB smear microscopy, isolation by culture, visual format using molecular beacons, and CM-PCR assays (Table 1). The results of the detection of AFB by smear microscopy were compared with culture,

Table 1. Comparative Analysis of AFB Smear Microscopy of Sputum Samples with Isolation of Mycobacteria by Culture, Visual Format, and CM-PCR Assay

| | AFB | | | κ (P value)* | | |
	P ($n = 45$)	N ($n = 52$)	Total ($n = 97$)	Culture versus assays	Culture versus AFB	Visual versus CM-PCR
Assays						
Culture[†]						
P	45	33	78		0.35 (<0.001)	0.89 (<0.001)
N	0	19				
Visual format[‡]						
P	45	30	75	0.91 (<0.001)		
N	0	22				
CM-PCR						
P	45	26	71	0.80 (<0.001)		
N	0	26				

*Agreement between the assays calculated using STATA software version 9.2.
[†]Inoculated in MGIT tubes, incubated at 37°C for 6–8 weeks.
[‡]Detection of *M. tuberculosis* and *M. bovis* using beacons.
P, positive; N, negative.

Table 2.   Comparative Analysis of Clinical Diagnosis and Isolation of Mycobacteria from Samples Derived from Pleurisy Patients with AFB Smear Microscopy, Visual Format, and CM-PCR Assay

| | Clinical diagnosis* | | | | κ (P value)[†] | | | |
| | TB patients (*n* = 51) Culture[‡] | | Non-TB patients (*n* = 20) Culture | | | | | |
Assay method	P 13	N 38	P 0	N 20	Culture versus assays	Clinical diagnosis versus assays	Culture versus clinical diagnosis	Visual versus CM-PCR
Visual format[§]								
P	11	24	0	0	0.26 (<0.01)	0.55 (<0.001)	0.16 (<0.01)	0.89 (<0.001)
N	2[¶]	14	0	20				
CM-PCR								
P	11	20	0	0	0.33 (<0.001)	0.47 (<0.001)		
N	2[¶]	18	0	20				
AFB								
P	3	4	0	0	0.20 (<0.04)	0.08 (<0.04)		
N	10	34	0	20				

*Clinical diagnosis of TB and non-TB patients based on criteria detailed in *Materials and Methods*.
[†]Agreement between the assays calculated using STATA software version 9.2.
[‡]Culture: sample inoculated into MGIT/LJ/7H9, incubated at 37°C for 6–8 weeks.
[§]Detection of *M. tuberculosis* and *M. bovis* using beacons.
[¶]MOTT isolates *M. fortuitum* and *M. chelonae*.
P, positive; N, negative.

visual format, and CM-PCR assays. Of the 97 sputum samples processed, in 45 samples (45/97, 46.3%) AFB was detected. All 45 AFB smear positive samples were positive by the three methods used, namely culture, visual format, and CM-PCR assays. Among the 52 AFB negative samples, in 33 samples mycobacteria were isolated by culture. By the visual format assay 30 of the 52 (57.6%) and by the CM-PCR assay 26 of the 52 (50%) samples were positive for *M. tuberculosis*. On comparing these assay results with culture, in the visual format assay using molecular beacons and CM-PCR assay a very good agreement was seen (κ = 0.91 and 0.80 respectively; Table 1). Between the visual format and CM-PCR assays there was near perfect agreement (κ = 0.89). However, between culture and AFB detection by smear microscopy, the agreement was limited (κ = 0.35).

Comparative Analysis of the Four Methods for Detection of Mycobacteria in Pleural Fluid Samples

Mycobacteria in pleural fluid samples were detected by the four methods described (Table 2). The results obtained by these methods were compared, with isolation of mycobacteria by culture and clinical diagnosis. The distribution of these 71 samples is detailed in Table 2.

Of the 51 clinically diagnosed pleural tuberculosis patients, mycobacteria were detected in 17 samples (33.3%) either by AFB microscopy or by culture. No mycobacteria were detected in non-TB patients by any of the assays used. By the visual format and CM-PCR assays *M. tuberculosis* was detected directly in 11 of the 13 culture positive samples. In two samples negative by both the visual format and CM-PCR assays, the mycobacteria isolated from these samples were identified as *M. fortuitum* and *M. chelonae*, respectively. In culture negative samples, 24 (24/38, 63.1%) samples were positive for *M. tuberculosis* by the visual format assay, compared

with 20 (20/38, 52.6%) samples that were positive by the CM-PCR assay (Table 2). There was fair to moderate agreement among the results obtained by the two assays (visual format and CM-PCR assays) compared with culture and clinical diagnoses except with AFB smear microscopy. Fair agreement between culture and visual format and culture and CM-PCR assays was observed (κ = 0.26 for visual format and 0.33 for CM-PCR assay, respectively). Between clinical diagnosis and the assays used, moderate agreement was observed (κ = 0.55 for visual format and 0.47 for CM-PCR assay, respectively). In 35 of the 51 (68.6%) samples mycobacteria were detected by the visual format assay compared with 31 (60.7%) by the gel-based assay, 13 (25.5%) by culture, and 7 (13.7%) by AFB smear microscopy. Poor agreement was seen between AFB detection and culture and AFB detection and clinical diagnosis (κ = 0.20 and 0.08, respectively). Agreement between the visual format and CM-PCR assays was very good (κ = 0.89).

Detection of Mixed Infection in CSF Sample Clinically Suspected of Tuberculous Meningitis

The CSF sample was processed as described previously.[14] The CSF sample was filtered, and the residue on the filter (0.45 μm) membrane was directly used for detection of *M. tuberculosis* and *M. bovis* by culture (BACTECMGIT960) as well as by the assays described herein. Using molecular beacons (visual format assay) (Figure 5B) and CM-PCR assay, the sample was positive for *M. tuberculosis* as well as *M. bovis* (Figure 5A). The dual PCR-amplified products seen in the sample align to the 162- and 127-bp products obtained in the standard strains of *M. tuberculosis* and *M. bovis* (positive controls), respectively. Two weeks later, the MGIT culture of the CSF sample was similarly positive for both pathogens. The assays have been performed three times to confirm the results. Efforts are under way to

separate the AFB obtained in MGIT media on 7H11 Dubos agar.

Agreement between Direct Detection of Mycobacteria in Clinical Samples by Multiplex PCR Assay and the Visual Format Beacon Assay Versus Classical Identification of Isolates

Seventy-eight isolates were obtained from 97 sputum and 13 from 51 pleural tuberculosis patients. All isolates were AFB positive and were identified by biochemical criteria.[15] Of the 91 isolates, 89 were identified as *M. tuberculosis* with the exception of two isolates from pleural tuberculosis patients. These two isolates were identified as *M. fortuitum* and *M. chelonae*. The mixed culture obtained from the CSF sample in MGIT media has been subcultured on 7H11 Dubos agar to isolate and characterize the strain(s) by standard biochemical tests. There was complete concordance in the direct identification of mycobacteria present in the clinical sample before isolation by the visual format and CM-PCR assays. *M. fortuitum* and *M. chelonae* was isolated from the pleural tuberculosis patients that were negative by both assays. The 20 DNA samples derived from *M. bovis* isolates (courtesy Dr. Niamh Corbally) were identified as *M. bovis* by both assays.

Discussion

The molecular beacon-based PCR assay described has the potential for direct detection of specific mycobacterial pathogens, namely *M. tuberculosis* and *M. bovis,* that could potentially be present in clinical samples. In the visual format assay, a sample was considered positive if the fluorescence intensity was more than the negative control (without DNA). In comparison with culture, the visually configured assay using molecular beacons described in this study showed the sensitivity of 96.1% compared with earlier reports of 86.7% for *devR* and 88.3% for *IS6110* PCR.[4] To the best of our knowledge, this is the first report of molecular beacons used in combination with asymmetric PCR for detection of dual infection due to *M. tuberculosis* and *M. bovis* in a sample. This confirms our earlier report using the *hupB*-based assay[17] describing dual detection of *M. tuberculosis* and *M. bovis* in clinical samples. Similar reports of mixed mycobacterial infection in individual clinical samples have been described.[18–20]

The classical microbiological methods for diagnosing tuberculosis have poor sensitivity and specificity.[21] Traditionally, the diagnosis of tuberculosis depends on demonstration of mycobacteria by AFB smear microscopy and growth on Lowenstein-Jensen medium. AFB microscopy is rapid; the results are obtained within a few minutes compared with the 2- to 6-week time period required by the MGIT-960 culture system.[22,23] AFB microscopy is inexpensive, easy to perform, and requires limited training and equipment. The major disadvantage of smear microscopy is its sensitivity, as a smear to be considered positive should have a concentration of 10,000 AFB/ml.[24,25] The low sensitivity is often reflected by the high rates of smear-negative TB cases.[4] On the other hand, there have been some improvements in the culture-based detection systems, but the requirement of expensive instrumentation (such as BACTEC-460, MGIT-960) has restricted their use to specialized laboratories. Mycobacterial culture is more sensitive compared with smear microscopy, as 10 to 100 bacilli/ml are required for culture-based detection.[24,25] Our method is similar in its simplicity compared with smear microscopy but has improved sensitivity and specificity, requiring 5 to 50 bacilli for detection by the visual format assay.

Due to the inherent paucibacillary nature of pleural fluid samples, the isolation of mycobacteria by culture has been inefficient.[26,27] The rate of isolation of mycobacteria from pleural fluid has been reported to range from 12% to 33%.[26,28,29] Besides pleural fluid, inclusion of sputum samples has been considered appropriate to improve the demonstration of mycobacteria by smear microscopy and its isolation[28] (submitted for publication by P Kumar, MK Sen, DS Chauhan, VM Katoch, S Singh, HK Prasad). In the present study in 17 of the 51 (33.3%) samples derived from pleural TB patients, AFB was detected in seven samples and mycobacteria were isolated from 13 (Table 2). These patients meet the definitive criterion for tuberculous pleural effusion. Fifteen of these 17 samples (88.2%) were positive by the visual as well as the CM-PCR assay for *M. tuberculosis*. Besides the definitive criterion, 34 patients met the suggestive criteria for tuberculous pleural effusion, such as clinical history of fever, chest pain, cough, breathlessness, and chest radiography with evidence of pleural effusion and pleural fluid cytology commensurate with pleural TB and responded to antituberculous treatment. In 70% of these (24/34) patients *M. tuberculosis* was detected by the visual format assay. PCR-based detection of *M. tuberculosis* in smear negative samples has been reported.[30] Further, improvement of detection and isolation of mycobacteria in pleural effusion patients has been reported with inclusion of pleural biopsies.[29,31] However, this approach is disadvantaged owing to its invasiveness, complications of the procedure, and the inability to sample infectious foci of the pleural tissue.

Besides demonstration of mycobacteria in patient-derived samples, the detection of mycobacterial DNA in clinical samples using PCR has been widely adopted as a rapid, sensitive, and specific diagnostic method.[32] Use of the nested PCR assay has provided a remarkable increase in sensitivity and specificity of DNA amplification compared with the conventional single-step PCR assay.[18,33,34] However, the nested PCR assay is laborious and time-consuming, which carries the risk of contamination in clinical samples. Hence it has yet to be widely used in tuberculosis diagnosis.[33,34] To overcome the problems of sample contamination and to reduce detection time, real-time PCR technique has been developed as an alternate rapid and sensitive assay for detection of *M. tuberculosis* in clinical samples.[35,36] Although real-time PCR assay has qualitatively advanced microbial detection assays, owing to their technological sophisti-

cation, equipment and reagent costs have restricted its utility in the diagnosis of infectious diseases for specialized laboratories.

The simplicity of the assay and visual interpretation makes it ideally suited for routine use in clinical laboratories with limited resources, such as in developing countries. The assay could be further simplified from the present format by using molecular beacons tagged with different fluorophores in a multiplex assay.[37] Such a multiplex assay would reduce the number of tubes, time, labor, and cost required to set up an assay for a clinical sample. However, the clinical samples suspected of dual infection must necessarily be investigated and analyzed using the visual format assay as outlined in this study. Cross-contamination with PCR products does not occur, as assay tubes are not opened after amplification. The study shows that simple end point detection using molecular beacons and a blue light transilluminator has a great potential in the clinical diagnosis of tuberculosis and that of other pathogens.

Acknowledgments

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