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A simple gold nanoparticle probes assay for identification of *Mycobacterium tuberculosis* and *Mycobacterium tuberculosis* complex from clinical specimens

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ABSTRACT

We had previously developed a nested polymerase chain reaction (PCR)–immunochromatography test (ICT) for identification of *Mycobacterium tuberculosis* (MTB) and differentiation of MTB from other members of *M. tuberculosis* complex (MTBC) from clinical sputum samples (Soo P.C. et al., Journal of Microbiological Methods. 2006, 66(3):440–8.). To further improve the detection flexibility, simplicity and efficiency, and reduce the cost, in this study, an alternative molecular diagnosis assay that utilizes gold nanoparticles derivatized with thiol modified oligonucleotides was developed. The gold nanoparticles probes, GP-1/GP-2 for IS*6110* and GP-3/GP-4 for Rv*3618*, were designed to specifically hybridize with target DNAs of MTBC and MTB strains, respectively. Efficacy of the gold nanoparticle probes assay was evaluated by directly and simultaneously detecting not only MTBC but also MTB from 600 clinical sputum specimens. Results were compared with traditional culture and biochemical identification methods together with patients' clinical assessments. This assay showed a 96.6% sensitivity and 98.9% specificity towards detection of MTBC, and a 94.7% sensitivity and 99.6% specificity for detection of MTB. In conclusion, the gold nanoparticle probes assay is a simple, rapid, cost-effective and accurate detection system and shows great potential in clinical application of MTBC and MTB detection, especially in developing countries.

1. Introduction

Tuberculosis (TB) is the major cause of human adult death by infectious agents, accounting for approximately two million deaths annually, mainly in the developing countries [1]. Currently, the global number of TB cases is rising at a rate of 2% per year (World Health Organization tuberculosis fact sheet; http://www.stoptb.org/). While conventional smear microscopy and culture methods are widely used in diagnosis of TB, the former is insensitive [2] and the latter takes up to 6–8 weeks to provide a result, limiting the value of these methods in aiding diagnosis and immediate decisions on treatment. Using IS*6110* and/or 16S rDNA as the detection targets, many commercially available nucleic acid amplification-based detection systems have also been developed as rapid tests for the direct identification of *Mycobacterium tuberculosis* complex (MTBC) from clinical specimens [3–5]. However, although the mycobacteria grouped in the MTBC are closely related, based on DNA–DNA hybridization, multilocus enzyme electrophoresis and 16S rDNA nucleotide acid sequence identity level [6,7], MTBC members differ widely in terms of host tropisms, phenotypes, and pathogenicity. It is intriguing that some species are either exclusively human (*M. tuberculosis*, *Mycobacterium africanum*, *Mycobacterium canetti*) or rodent (*Mycobacterium microti*) pathogens. Others either have a wide host spectrum (*Mycobacterium bovis*) [8] or are used as a vaccine strain (*M. bovis* BCG). Differentiation of *M. tuberculosis* (MTB) from the other members of the MTBC is thus necessary for the treatment of individual patients and for epidemiological study purposes, especially in areas of the world where tuberculosis has reached epidemic proportions, the transmission of *M. bovis* between animals or animal products and humans is a particular problem, or where the vaccine strain *M. bovis* BCG causes pediatric infections [9].

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Sequence-specific methods for detecting polynucleotides are critical to the diagnosis of pathogenic diseases [10,11]. Most detection systems make use of the hybridization of the target polynucleotide with oligo- or polynucleotide probes containing covalently linked reporter groups [12,13]. Although commercial identification systems are available, they cannot be widely applied, especially in developing countries as the identification cost is comparatively high. We had previously developed a nested PCR–ICT assay which was shown to be effective in direct and rapid diagnosis of not only MTBC, but also MTB from clinical sputum samples [14]. To further improve the simplicity of operation procedures and reduce the costs in the performance of MTBC/MTB identification, in the present study, using the thiol group-gold chemistry technique, the oligonucleotide modified Au nanoparticles were developed as probes for DNA detection. Based on the results of our previous study, the IS*6110* and Rv*3618* [15] DNA fragment were selected as the DNA target for diagnosis of MTBC and MTB, respectively [14]. After PCR, the gold nanoparticle probes were added, followed by result reading by either direct observation or spectrophotometry. The detection limit, sensitivity and specificity of this assay were tested. Using clinical sputum specimens as the detection targets, results obtained from this approach were compared with those from conventional culture methods and biochemical identification in combination with clinical assessment. The gold nanoparticle probes assay was simple to use, cost-effective and had a high sensitivity and specificity with respect to the identification of MTBC and MTB directly from sputum samples. It thus has great potential for clinical application, especially in regions of low income and a high MTBC/MTB prevalence rate.

2. Materials and methods

2.1. Specimen collection and processing

A total of 600 sequential clinical sputum specimens were collected from the Mycobacteriology Laboratory, Department of Laboratory Medicine, National Taiwan University Hospital between October 2005 and March 2006. Collection of these clinical samples was approved by the Review Board Committee at the Hospital. Specimens were processed on receipt according to standard routine diagnosis procedures [16]. Briefly, an equal volume of NaOH–citrate–N-acetyl–L-cysteine solution was added to the sputum sample at room temperature for 15 min. After centrifugation, the precipitate was resuspended in 1 ml of phosphate-buffered saline (pH 7.4).

2.2. Culture and biochemical methods for diagnosis of MTBC and MTB

The Lowenstein–Jensen (LJ) slants (Difco, USA) and Middlebrook 7H11 medium plates (Becton–Dickinson, USA) were inoculated with 250 μl of decontaminated sample suspension, incubated at 37 °C with 5% CO_2. An inverted light microscope was used to observe mycobacterial growth during weeks 2–8 after inoculation. The guidelines of the US Center for Disease Control and Prevention [17] were followed for the determination of positive mycobacterial growth. Identification of bacterial strains as MTBC and/or MTB is mainly based on the routine morphological and biochemical assays [9]. Cells were further confirmed to species level by 16S rDNA sequence analysis if ambiguous identification results were obtained by culture methods [18].

2.3. Mycobacterium ATCC reference strains

A total of 23 Mycobacterium ATCC reference strains (Table 1) were used as controls in the gold nanoparticle probes assay.

Table 1
Mycobacterium species tested in this study.

Reference strains[a]	Gold nanoparticle probes assay	
	IS*6110*	Rv*3618*
Mycobacterium spp.		
M. tuberculosis H37Rv (ATCC 27294)	+	+
M. bovis (ATCC 19210)	+	–
M. microti (ATCC 19422)	+	–
M. avium-intracellulare complex (ATCC35761)	–	–
M. kansaii (ATCC 12478)	–	–
M. marinum (ATCC 927)	–	–
M. chelonae (ATCC 35752)	–	–
M. abscessus (ATCC 19977)	–	–
M. fortuitum (ATCC 6841)	–	–
M. smegmatis (ATCC 35798)	–	–
M. xenopis (ATCC 19250)	–	–
M. asiaticum	–	–
M. haemophilum	–	–
M. mucogenicum	–	–
M. malmoense	–	–
M. terrae	–	–
M. triviae	–	–
M. vaccae	–	–
M. flavescence	–	–
M. gastri	–	–
M. gordonae	–	–
M. scrofulaceum	–	–
M. simiae	–	–

+, aggregation.
–, no aggregation.
[a] Reference strains were either ATCC strains or clinically isolated strains identified by biochemical assays and 16S rDNA sequencing.

2.4. Preparation of mycobacterial DNA from sputum specimens and nested PCR

Decontaminated sample suspensions (100–300 μl) were mixed with equal volume of wash buffer (Tris–HCl buffer, pH 8). After vortexing for 20 s, samples were subject to centrifugation at 13,000 rpm for 10 min before discarding the supernatant. The precipitated pellet was resuspended and lysed in lysis buffer (KOH, pH 13.1) at 95 °C for 15 min before being neutralized by neutralization buffer (HCl and acetic acid, pH 1.2) [16]. To evaluate the sensitivity of this assay, the chromosomal DNA of *M. tuberculosis* H37Rv grown on Middlebrook 7H11 agar plates was extracted with the same procedures, and were serially diluted in normal saline before PCR. A 5 μl of crude extract suspension was then transferred to an eppendorf tube containing 50 μl of amplification reagent (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 20 pmol for each primer and 2.5 units Taq DNA polymerase (Takara, Japan)) for nested PCR. Primers (Table 2) used in nested PCR were designed from IS*6110* [19,20] or Rv*3618* belonging to RD9 [15] in the *M. tuberculosis* genome. The nested PCR was carried out in a thermal reactor (Biometra, Germany). After a 5 min incubation at 94 °C, the longer DNA fragments (245 bp for IS*6110* and 326 bp for Rv*3618*) were first amplified by primer pairs INS1–INS2 and Rv3618F–Rv3618R, respectively, in 40 cycles at 94 °C for 30 s, 64 °C for 15 s, and 72 °C for 30 s. Samples were incubated for 1 min at 72 °C after the last cycle. The PCR mixtures without template DNA were used as negative controls. Subsequently, 5 μl of PCR mixture from first PCR reaction was used for the second PCR reaction (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 20 pmol for each primer and 2.5 units Taq DNA polymerase (Takara, Japan)). A total of 14 cycles were performed to amplify the 110 bp (for IS*6110*) and 124 bp (for Rv*3618*) fragments by using the primer pairs G-IS6110F–G-IS6110R and G-3618F–G-3618R, respectively.

Table 2
DNA sequences of primers and gold nanoparticle probes used for PCR amplification and DNA hybridization respectively.

Primer sequence (5′→3′)	Applification characteristics	Reference
INS1: CGTGAGGGCATCGAGGTGGC INS2: GCGTAGGCGTCGGTGACAAA	1st PCR for amplification of IS*6110*	[14]
G-IS6110F: CTCGTCCAGCGCCGCTTCGG G-IS6110R: GCGTCGGTGACAAAGGCCAC	2nd PCR for amplification of IS*6110*	[14]
Rv3618F: ATTGCACATCCGCCCC Rv3618R: GGACAAACCCTGCCGC	1st PCR for amplification of Rv*3618*	This study
G-3618F: CGACTGGTTCACCCTG G-3618R: TAACAGCGACGTGCCCAG	2nd PCR for amplification of Rv*3618*	This study
Gold nanoparticle probes sequence		
GP-1: SH-A10-CACCCATCGTCTGGAGTGG GP-2: CCAAGCGGATGCACCGG-A10-SH	Hybridization to target DNA for IS*6110*	This study
GP-3: SH-A10-AGCCATGATTTCGCCATCG GP-4:CTGGGCACGTCGCTGTT-A10-SH	Hybridization to target DNA for Rv*3618*	This study

2.5. Au nanoparticles preparation

Au colloids were prepared by the Natan method [21]. Briefly, 39.37 mg of $HAuCl_4 \cdot 3H_2O$ was dissolved in 100 mL of distilled, deionized water by heating and vigorously stirring. Then 10 mL of 38.8 mM sodium citrate solution was added as the solution was boiling. Finally the tetrachloroauric solution turned claret, with particle concentration of approximately 20 nM, and with the mean diameter of Au at 13.7 ± 0.8 nm.

2.6. Preparation of oligonucleotide/Au conjugates

Au particles capped with 3′- and 5′-thiol terminated oligonucleotides were prepared following Mirkin's strategy [22]. The synthesized gold nanoparticles were centrifuged for 7 min at 10 000 rpm to remove the excess citrate and brought into 10 mM phosphate buffer (pH 7). The Au nanoparticles were derivatized by using 1 mL of a colloidal solution with 5 OD_{260} (165 μg) of oligonucleotides (5′-HS-A_{10}-oligomer-3′ or 5′-oligomer-A_{10}-SH-3′) to age for 16 h, The solution was then transferred to 0.1 M NaCl, 10 mM phosphate buffer (pH 7), and allowed to stand for an additional 42 h. The resulting aged solution was centrifuged for 20 min at 13 000 rpm twice to attain red precipitates. The resulting precipitates were then washed with 1 mL of a 0.1 M NaCl, 10 mM phosphate buffer (pH 7) solution and then re-suspended in 1 mL of 0.3 M NaCl, 10 mM phosphate buffer (pH 7). All procedures were carried out at room temperature (25 °C).

2.7. Result development and reading

Detection of the existence of target DNA was achieved by the addition of 100 μl of each probe at the final concentration of 20 nM. The mixture suspensions were denatured at 95 °C for 10 min, and cooled down to 55 °C for DNA hybridization for 2 h. Due to a red shift in the surface plasmon resonance of Au nanoparticles [22], the positive reaction means a change of color from red to purple and the aggregation of gold nanoparticle probes. The cut-off point was 1.698 determined by the average absorbance values minus three standard deviations of 20 control samples without target DNA.

3. Results

3.1. Principles of gold nanoparticle probes assay

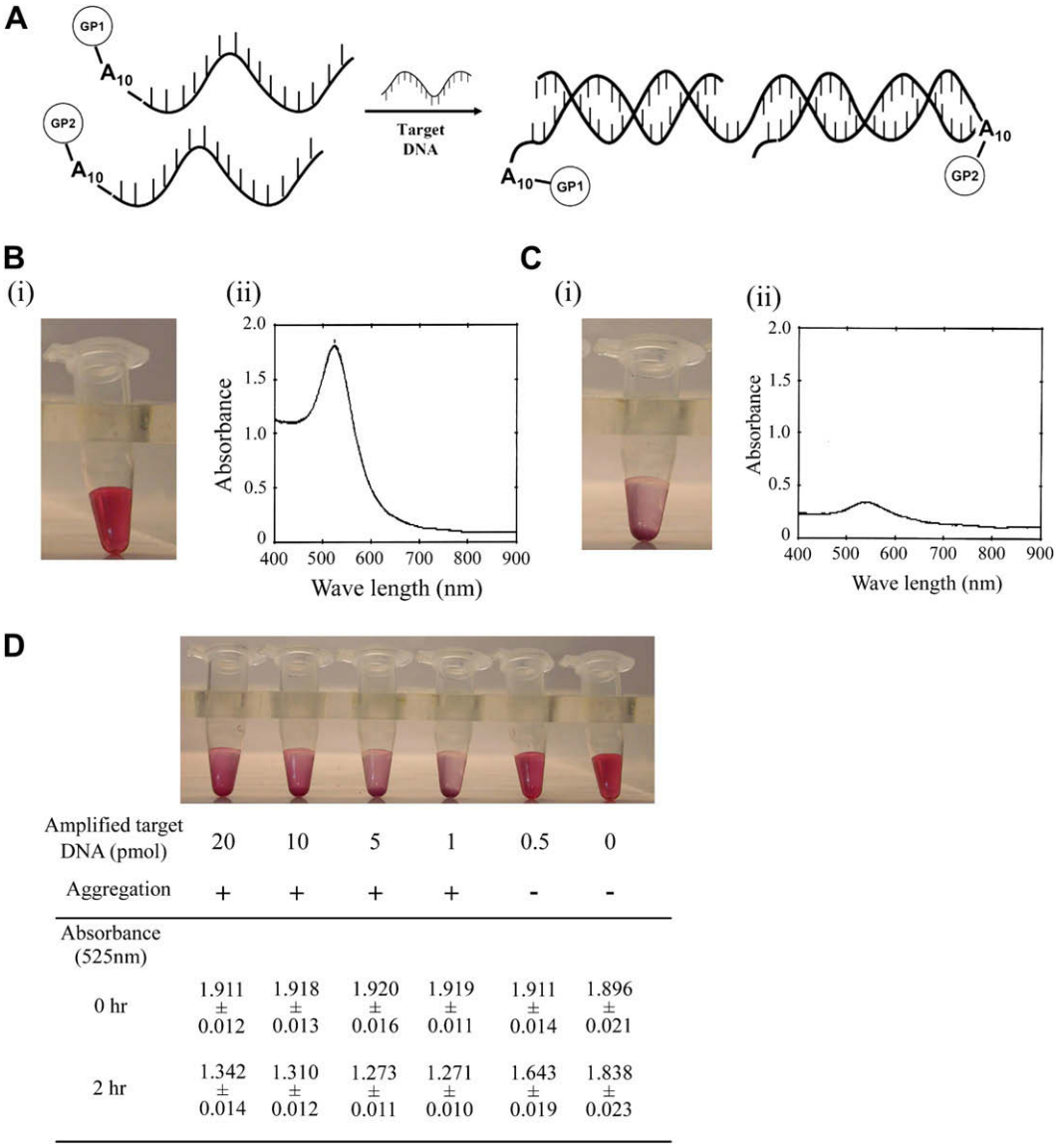
The gold nanoparticle probes assay mainly comprised of two steps: target DNA amplification by (nested) PCR and the addition of gold nanoparticles probes for detection. The principle of the gold nanoparticle probes assay is summarized in Fig. 1. This assay comprise two main steps, firstly, the target DNA amplification by single or nested PCR, secondly, nanoparticle detection. Briefly, when using DNA prepared from a single bacterial colony as a template, a single PCR is enough for subsequent assay development. In comparison, for the detection of target DNA prepared from clinical sputum samples, nested PCR is suggested [14]. The gold nanoparticle probes were added to the heat denatured PCR products, and incubated at 55 °C for DNA hybridization for 2 h. The absorbance of the solution was measured by spectrophotometer. When single-stranded target DNA was present in the solution, the gold nanoparticle probes (for example, GP-1/GP-2 herein, Table 2) aggregated through hybridization to target DNA, resulting in decrease in absorbance of the solution at 525 nm (OD_{525nm}) measured by spectrophotometry (Fig. 1C). On the contrary, the color and absorbance pattern were not changed if specific target DNAs were absent in the solution (Fig. 1B). The positive reaction which means aggregation of gold nanoparticles is accompanied by a dynamic change of suspension color from red to reddish purple. Although color shift could be detected by spectrophotometry as early as 2 h after the addition of probes, a significant difference could also be observed at about 4 h by direct observation.

3.2. Detection limit

The detection limit of gold nanoparticle probes assay was measured using IS*6110* DNA amplified from prepared *M. tuberculosis* H37Rv chromosome as the target. After PCR amplification, the amount of amplified DNA was quantified and serially diluted from 20 pmol to 0.5 pmol. Subsequently, 20 nM of each GP-1 and GP-2 gold nanoparticle probe was added for detection. Spectrophotometry (OD_{525nm}) analysis indicated the absorbance was measured to be around 1.913 at 0 h in each sample, including the negative control samples containing no target DNA. In comparison, 2 h after probes addition, while the absorbance of negative control samples remained basically unchanged, those of samples containing 0.5 pmol target DNA were reduced to around 1.643. Absorbance was further reduced to 1.271–1.342 for samples containing 1–20 pmol DNA (Fig. 1D). Briefly, target DNA as low as 0.5 pmol shows a positive reaction by spectrophotometry analysis 2 h after probes addition. Results with a similar detection limit could also be obtained by direct observation 4 h after probes addition (Fig. 1D).

3.3. Identification and distinction of MTBC and MTB strains

Based on the results of our previous study, two DNA fragments IS*6110* and Rv*3618* specific to MTBC and MTB, respectively [14] were used as the detection targets in this assay. Chromosomal DNAs prepared from standard strains *M. tuberculosis* strain H37Rv (ATCC



Amplified target DNA (pmol)	20	10	5	1	0.5	0
Aggregation	+	+	+	+	-	-
Absorbance (525nm)						
0 hr	1.911 ± 0.012	1.918 ± 0.013	1.920 ± 0.016	1.919 ± 0.011	1.911 ± 0.014	1.896 ± 0.021
2 hr	1.342 ± 0.014	1.310 ± 0.012	1.273 ± 0.011	1.271 ± 0.010	1.643 ± 0.019	1.838 ± 0.023

Fig. 1. Schematic illustration of the concept for detecting target DNA by gold nanoparticle probes. (A) Two oligonucleotides probes GP1 and GP2 labeled with gold nanoparticles were annealed to complementary target DNA (IS*6110*) after which gold nanoparticles were aggregated. (B) When no target DNAs were present, the solution appeared red and UV–visible spectrum analysis showed a maximum absorbance at wavelength 525 nm. (C) In the presence of target DNAs, gold nanoparticles aggregated through hybridization between probes and target DNAs, leading to color change from red to purple/blue and decrease in absorbance at wavelength 525 nm. (D) Detection limit of gold nanoparticle probes assay by direct observation and spectrophotometry. The target DNA IS*6110* ranging from 20 pmol to 0 pmol were prepared from PCR and mixed with 20 μM gold nanoparticle probes. After denaturation for 5 min at 80 °C, the mixtures were incubated at 55 °C for 2 and 4 h, when results were recorded by direct observation and spectrophotometry (OD 525 nm), respectively. + and −, positive and negative reaction for gold nanoparticle probes assay. Results shown were averages from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

27294), *M. bovis* ATCC19210 and *Mycobacterium avium* ATCC35761 grown on Middlebrook 7H11 plates were used as templates in PCR. The primer pairs, G-IS6110F/G-IS6110R and G-3618F/G-3618R (Table 2) were used in amplification of specific DNA fragments of MTBC and MTB, respectively. After DNA amplification, a specific 110 bp and a 124 bp DNA fragment were respectively amplified from IS*6110* and Rv*3618* [14]. These PCR products were used for subsequent result development of gold nanoparticle probes assay.

Detection of target DNA existence was achieved by addition of two pairs of gold nanoparticle probes GP-1/GP-2 and GP-3/GP-4 specifically designed for hybridization with IS*6110* and Rv*3618* target DNA respectively. 4 h after probes addition, results were read by direct observation. Positive color change was observed in DNA samples prepared from *M. tuberculosis* strain H37Rv which contains both IS*6110* and Rv*3618* (Fig. 2). For *M. bovis* ATCC19210 which is a member of MTBC, positive reaction was observed in IS*6110*, but not in Rv*3618* probe. No color change was observed in *M. avium* ATCC35761 which is not an MTBC member (Fig. 2). In concordance with color change, gold nanoparticles aggregation was clearly observed under light microscopy (Fig. 2). These results showed that while no reaction was obtained against *M. avium*, the gold nanoparticles assay could detect MTBC and further distinguish MTB from *M. bovis*.

To confirm the color change and aggregations are not artificial phenomena, after results reading, reaction tubes were heated to 80 °C to denature the DNAs. The suspension color turned from purple to red and the aggregated particles were resuspended in the solution, as indicated by direct observation from above the eppendorf tubes (Fig. 3). When the tubes were cooled down again on bench, the same color shift and gold nanoparticles aggregation

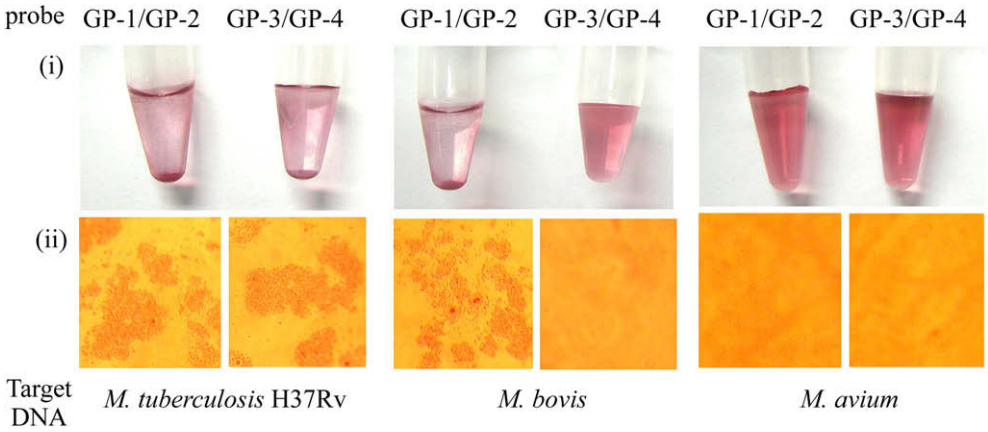


Fig. 2. Use of gold nanoparticle probes assay for detection of IS*6110* and Rv*3618*. Chromosomal DNAs prepared from MTB, *M. bovis*, and *M. avium* were used as templates for PCR amplification by GP-1/GP-2 and GP-3/GP-4 primers, respectively. The gold nanoparticle probes assay for detection of IS*6110* or Rv*3618* DNA fragments were then performed. IS*6110* and Rv*3618* were detected from *M. tuberculosis* H37Rv. IS*6110*, but not Rv*3618* was detected in *M. bovis*. In comparison, no positive reaction was observed from *M. avium*. (i) direct observation; (ii) Aggregation of gold nanoparticles under the 400X light microscopy (Leica, Germany).

were observed (Fig. 3). These results indicated color shift observed in this assay is reversible and is a nature of hybridization between template and probe DNAs.

3.4. Specificity of gold nanoparticle probes assay

To evaluate the specificity of gold nanoparticle probes assay, a total of 23 *Mycobacterium* spp. reference strains grown on Middlebrook 7H11 plates were used for identification. Positive reactions for both IS*6110* and Rv*3618* probes were obtained in identifying *M. tuberculosis* H37Rv. In comparison, IS*6110*-positive, but Rv*3618*-negative reaction was present in detecting *M. bovis* ATCC19210 and *M. microti* ATCC 19422 which are MTBC members. For the other non-tuberculous *Mycobacterium* (NTM) strains, no positive reactions were observed from both probes (Table 1). Briefly, this assay specifically identified MTBC and further differentiated MTB from MTBC, and no cross-reaction with the other NTM bacterial strains was observed.

3.5. Clinical evaluation

A total of 600 consecutive clinical sputum specimens subjected to routine mycobacteria identification in National Taiwan University Hospital were analysed. Besides traditional culture and biochemical identification, the spent sediments of specimens were also subject for chromosomal DNA extraction [14]. Purified DNAs were amplified by nested PCR and detected for presence of MTBC and MTB by gold nanoparticle probes assay. The primer pairs, INS1/INS2 and Rv3618F/Rv3618R (Table 2) were used in amplification of the 1st DNA fragments of MTBC and MTB, respectively. This is followed by amplification of the 2nd DNA fragments using the primer pairs G-IS6110F/G-IS6110R and G-3618F/G-3618R (Table 2). After DNA amplification, results were measured by spectrophotometer and compared with those obtained from traditional culture methods. Among the 600 specimens identified by culture and biochemical methods, a total of 58 specimens were identified to contain MTBC organisms (Table 3A). In comparison, 56 specimens

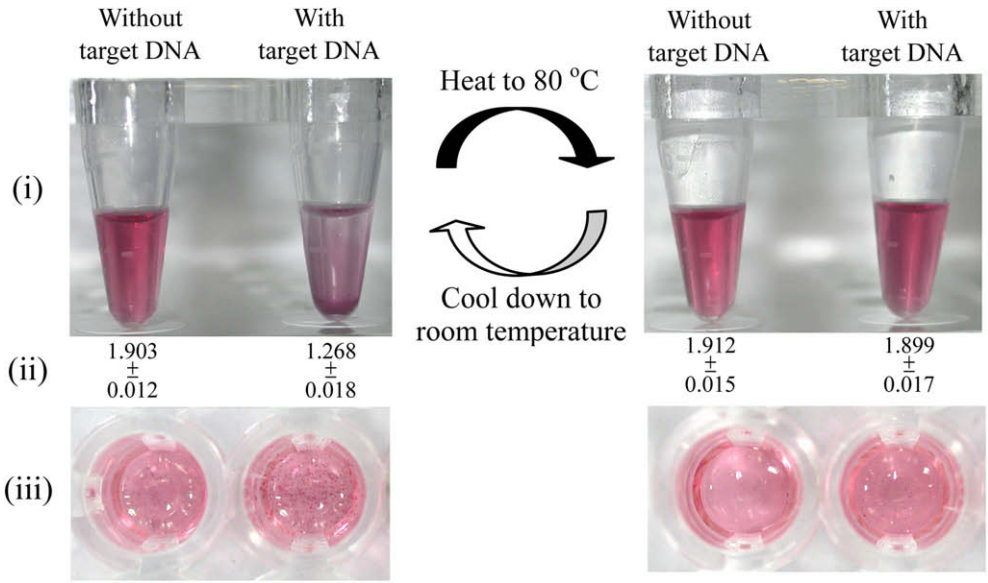


Fig. 3. Reversible reaction of gold nanoparticle probes assay. The positive and negative result of gold nanoparticle probes assay were shown in the left. After heating the reaction tubes to 80 °C to denature the DNAs, the color of suspension turned from purple to red and the aggregated particles were dispersed in the solution, as shown in the right. When tubes were cooled down again on bench, the same color shift and gold nanoparticles aggregation were again observed. Results were shown as (i) the color change of reaction solution in eppendorf tubes, (ii) the absorbance value at 525 nm and also (iii) color change in 96 well microtiter plate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Comparison of MTBC diagnosis results from consecutive 600 clinical sputum specimens by culture and gold nanoparticle probes (A) GP-1/GP-2 assay and (B) GP-3/GP-4 assay.

(A) GP-1/GP-2 assay		
Gold nanoparticle probes (IS*6110*) assays (no. of samples)	Culture[a]	
	Positive	Negative
Positive(62)	56	6
Negative(538)	2	536
Overall (600)	58	542
(B) GP-3/GP-4 assay		
Gold nanoparticle probes (Rv*3618*) assays (no. of samples)	Culture[b]	
	Positive	Negative
Positive(56)	54	2
Negative(544)	3	541
Overall (600)	57	543

[a] Culture and biochemical diagnosis results were from mycobacteriology laboratory, National Taiwan University Hospital (NTUH). Sensitivity: 96.6%, Specificity: 98.9%; positive predictive value: 90.3%, negative predictive value: 99.6%. The results were read 2 h after probes addition by spectrophotometry at OD_{525nm}.

[b] Culture and biochemical diagnosis results were from mycobacteriology laboratory, NTUH. Sensitivity: 94.7%, Specificity: 99.6%; positive predictive value: 96.4%, negative predictive value: 99.2%. The results were read 2 h after probes addition by spectrophotometry at OD_{525nm}.

were detected IS*6110* positive by gold nanoparticle probes assay. For the 2 IS*6110*-negative specimens, subsequent PCR using genomic DNA extracted from cultured bacteria followed by gold nanoparticle probe assay analysis confirms the two bacterial strains to be MTBC (IS*6110* positive). At the same time, a total of 62 specimens were identified to be gold nanoparticle probes assay positive. Thus 6 specimens that were MTBC culture-negative were detected as IS*6110* positive. Further clinical assessment showed that 2 out of the 6 patients showed significant clinical syndromes of MTB infection. In brief, a sensitivity of 96.6% and specificity of 98.9% were obtained in MTBC detection using the gold nanoparticle probes assay.

Results of MTB identification out of 600 clinical specimens were analysed. Among the 58 MTBC culture-positive clinical specimens, 57 specimens were culture confirmed to contain MTB, and one specimen with *M. bovis* (Table 3B). The specimen which contained *M. bovis* was also detected Rv*3618* (MTB) negative by gold nanoparticle probes assay. As shown in Table 3B, a total of 54 (54/57) specimens were detected Rv*3618* positive by gold nanoparticle probes assay. Thus 3 specimens were culture-positive for MTB but were Rv*3618*-negative by the gold nanoparticle probes assay. Detailed analysis of the 3 specimens indicated two were also IS*6110*-negative by gold nanoparticle probes assay. For the remaining one specimen which is IS*6110* positive, the bacterial cells might be deficient in RD9 region which contains Rv*3618*. To see whether this is possible, PCR using the RD9-flanking primers [14] followed by agarose gel electrophoresis was performed using chromosomal DNA template prepared from this clinical isolate, *M. tuberculosis* H37Rv and *M. bovis* ATCC19210. Results showed that RD9 were indeed absent in this clinical isolate and *M. bovis*, while present in *M. tuberculosis* H37Rv (data not shown). At the same time, two samples that were Rv*3618* positive were culture-negative (Table 3B). Further analysis showed these two samples were also IS*6110* positive, indicating MTB organisms were indeed contained these two samples. Briefly, a sensitivity of 94.7% and specificity of 99.6% were obtained in MTB detection from this assay.

4. Discussion and conclusions

The nested PCR combined with gold nanoparticle probes developed in this laboratory is efficient in the direct identification of MTBC and MTB from clinical sputum samples. The main characteristics of this system include being easy to operate, sophisticated detection equipment is not necessary, low cost, time saving, and at the same time, maintaining high sensitivity and specificity. However, similar to other molecular detection systems, the sensitivity of this assay is dependent on whether there is enough DNA to be used for interaction with the gold nanoparticles probes. Once enough target DNA is available, the subsequent detection steps are straightforward and simple. Besides detection of MTBC and MTB cells, this assay can also be developed as a technique platform to be applied to any other samples that need specific detection of DNA fragment(s). Furthermore, compared with conventional DNA detection systems whose specificity is mainly determined by specific DNA primer sequences used in DNA amplification, specificity of the gold nanoparticle probes assay is further improved due to addition of specific gold nanoparticle probes for detection. This significantly reduced backgrounds caused by non-specifically amplified DNA fragments during PCR. It was reported that gold nanoparticle probes detected Mycobacterium with clinical samples by using single probe to specifically anneal the RNA polymerase β-subunit gene [23]. Due to these characteristics, the sensitivity and specificity of this assay for clinical diagnosis of MTBC organisms were 96.6% and 98.9%, respectively, and for MTB they were 94.7% and 99.6%, respectively, as compared with conventional methods. Meanwhile, a possibility for developing this method for automation is considered, as a spectrophotometer can be used for result reading.

For the 2 specimens containing gold nanoparticle probes assay (IS*6110*)-negative MTBC specimens, PCR and 16S rDNA sequencing using genomic DNA extracted from cultured bacteria confirmed the two strains belong to MTBC. It was reasoned that either only a little amount of bacterial DNA was extracted, or PCR inhibitors were contained in sputum specimens. Also, a total of 3 specimens were shown to be MTB culture-positive but Rv*3618*-negative by this assay. Detailed analysis of the 3 specimens indicated two were also IS*6110*-negative by gold nanoparticle probes assay. For the remaining one specimen, one possibility was that the bacterium was deficient in RD9 region (containing Rv*3618*) although it harbored the biochemical characters of MTB [14]. To confirm this Rv*3618*-negative strain was indeed RD9-deficient, PCR using the RD9-flanking primers [14] followed by agarose gel electrophoresis was performed using chromosomal DNA templates prepared from this clinical isolate, *M. tuberculosis* H37Rv and *M. bovis* ATCC19210. Results showed that RD9 was indeed absent in this clinical isolate and *M. bovis*, while present in *M. tuberculosis* H37Rv (data not shown). Thus the RD9 DNA region might not be conserved from all MTB strains. On the other hand, among the samples tested, 6 MTBC culture-negative and 2 MTB culture-negative samples were detected as IS*6110* and Rv*3618* positive, respectively, by this assay (Table 3). Retrospective clinical assessment of the medical records from the 6 patients was carefully performed. Results indicated that 2 out of the 6 patients had an MTB infection before coming to Taiwan University hospital, suggesting they were very possibly infected by MTB when diagnosed. For the remaining 4 samples, currently it is difficult to make a conclusion based on the results obtained.

In conclusion, the gold nanoparticles probe assay developed in this laboratory demonstrates a strong applicability in the diagnosis of MTBC and MTB infections directly from sputum samples. The reading of the results can be flexibly achieved by either direct observation or by spectrophotometry. Thus this system is very suitable for use in regions of high MTB/MTBC prevalence rate where smear-positive sputum samples are common.

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