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ORIGINAL RESEARCH

Validity of bioconjugated silica nanoparticles in comparison with direct smear, culture, and polymerase chain reaction for detection of *Mycobacterium tuberculosis* in sputum specimens

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Background: Tuberculosis is a public health problem worldwide, and new easy to perform diagnostic methods with high accuracy are necessary for optimal control of the disease. Recently, fluorescent silica nanoparticles (FSNP) has attracted immense interest for the detection of pathogenic microorganisms. The aim of this study was to detect *Mycobacterium tuberculosis* in clinical samples using bioconjugated FSNP compared with microscopic examination, polymerase chain reaction (PCR), nested PCR, and culture as the gold standard.

Methods: In total, 152 sputum specimens were obtained from patients who were suspected to have pulmonary tuberculosis. All samples were examined by the four techniques described.

Results: The assay showed 97.1% sensitivity (95% confidence interval [CI] 91–99.2) and 91.35% specificity (CI 78.3–97.1). Furthermore, assays using variable bacterial concentrations indicated that 100 colony forming units/mL of *M. tuberculosis* could be detected. There were no differences between the results obtained from two types of mouse monoclonal antibody against Hsp-65 and 16 KDa antigens.

Conclusion: We performed this assay in a large number of clinical samples to confirm the diagnostic specificity and sensitivity of the test and can recommend its application for diagnosis of *M. tuberculosis.* We believe that this method is more convenient for routine diagnosis of *M. tuberculosis* in sputum and will be more easily applicable in the field, and with sufficient sensitivity.

Keywords: *Mycobacterium tuberculosis*, fluorescent silica nanoparticles, bioconjugation, IS6110

Introduction

Tuberculosis is a public health problem of global importance. *Mycobacterium tuberculosis* is the main etiology of the disease. According to the World Health Organization, in 2009 there were an estimated 9.4 million incident cases of tuberculosis globally (equivalent to 137 cases per 100,000 population), and approximately 1.7 million people died of tuberculosis in that year. The absolute number of cases continues to increase slightly every year. Most of the cases in 2009 (55%) occurred in Asia.[1,2] Multidrug-resistant and extensively drug-resistant strains of *M. tuberculosis* are spreading rapidly, so this bacterium poses a serious dilemma for public health. There were an estimated 440,000 cases of multidrug-resistant tuberculosis in 2008. Drug-resistant isolates of *M. tuberculosis* have been found in all countries. The four countries that had the largest number of estimated cases of multidrug-resistant tuberculosis

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in 2008 were China, India, the Russian Federation, and South Africa.[3] Rapid detection of tuberculosis is extremely important for early therapy and designing control measures for prevention of the spread of bacteria in the human population and inhibiting emergence of multidrug-resistant and/or extensively drug-resistant isolates. The gold standard for diagnosis of tuberculosis is the cultivation of *M. tuberculosis*. Cultivation can detect 100 bacilli/mL of sputum in comparison with the 5,000–10,000 bacilli/mL needed for microscopy.[4] Commercial biochemical kits are available for the detection and phenotypic characterization of *M. tuberculosis*, but molecular methods developed in recent years are more rapid and accurate for this purpose.[5] Generally, molecular methods offer several advantages over conventional techniques for rapid detection and identification of mycobacterium. However, molecular methods are expensive and require more skilled personnel, so that implementation of these methods is difficult, especially in regions where *M. tuberculosis* infection is more common.[6] Rapid and accurate detection of trace amounts of organisms, such as pathogenic bacteria, is important for clinical diagnosis and prevention of accidental outbreaks. Recently, nanoparticles have been successfully used for detection of micro-organisms. Unlike traditional methods for the detection of bacteria, which require amplification or enrichment of the target bacteria in samples, fewer numbers of bacteria are needed for detection by the nanoparticle method in a shorter time.[7] Some of the different nanomaterials developed can be used as a convenient surface for the attachment of biomolecules. They can be used as a convenient surface for molecular assembly and may be composed of inorganic or polymeric materials.[8] Stöber et al first reported a method for synthesis of a dye-doped nanoparticle which encapsulates many thousands of dye molecules inside silica nanoparticles.[9,10] There are several advantages to using dye-doped silica nanoparticles, including their small size (20–70 nm in diameter) brightness, photostability, and ease of functionalization. Using surface modification, it is possible to conjugate a dye-doped silica nanoparticle to a particle, so that thousands of dye molecules can be attached to a biomolecule. Consequently, a significantly greater signal is emitted from signal binding events, which leads to increased sensitivity in comparison with a single fluorophore-labeled assay.[11] Recently, a study was reported for detection of *M. tuberculosis* using a reference strain of *M. tuberculosis*.[12] The aim of the present study was to evaluate the validity of the FSNP assay in comparison with culture as the gold standard, acid-fast staining, and polymerase chain reaction (PCR) in order to develop a simple, rapid, and sensitive method for detection of *M. tuberculosis* in sputum specimens.

Materials and methods

Tris (2,2′-bipyridyl) dichlororuthenium(II) hexahydrate(RuBpy), Triton X-100, protein A from *Staphylococcus aureus,* acetonitrile, glycine, N acetyl-L-cysteine (NALC), cyanogen bromide, tetraethyl orthosilicate (TEOS), *n*-hexanol, cyclohexane, acetone, ammonium hydroxide (NH_4OH, 28–30 wt%) were purchased from Sigma-Aldrich (St Louis, MO). All other chemicals were of analytical reagent grade. Mouse IgG (IgG2a) monoclonal antibodies to *M. tuberculosis* were purchased from AbCam (Cambridge, UK) against Hsp-65 antigen and AbD Serotec (Oxford, UK) against 16 KDa antigen. Lowenstein-Jensen medium (Merck, Darmstadt, Germany) was used for culture of *M. tuberculosis*. Primer oligonucleotides for conventional and nested PCR were purchased from TaG Co (Copenhagen, Denmark). Distilled deionized water was used in the preparation of all aqueous solutions. A standard strain of reference *M. tuberculosis* (H37Rv) and *Escherichia coli* (American Type Culture Collection 2532) were obtained from the Pasteur Institute in Iran.

Instrumentation

An atomic force microscope (SPM probe scanner DS 95–50 E, Danish Microengineering A/S, Herlev, Denmark) and particle size analyzers (Analystte 22-Nano Tec, Fritsch, Germany) were used for visualization of the morphology and estimation of the size of the silica nanoparticles. Protein concentration was evaluated using a ultraviolet-visible spectrophotometer (Biphotometer Plus, Eppendorf, Germany). A spectrofluorometer (RF-5301 PC, Shimadzu, Japan), and an ultraviolet-visible spectrophotometer (Varian Carry-100, Australia) was used for evolution of emission and absorbance of nanoparticles after synthesis. The presence of an amide group after conjugation of protein A was confirmed using an infrared spectrometer (Bomem MB 155S FT-IR spectrometer, ABB Bomem Inc, Canada). A Techne TC thermal cycler (Techne Co Ltd, Leicester, UK) for amplification of extracted DNA, and a fluorescent microscope (Olympus BX51 and DP70 digital camera system, Olympus America Inc, Center Valley, PA) was used for visualization of microscopic slides and their imaging.

Sputum collection and processing

One hundred and fifty-three sputum samples were obtained from the reference mycobacteriology laboratory at Ahvaz Jundishapur University of Medical Sciences from March

2009 to April 2010. All specimens were collected from patients who were suspected to have pulmonary tuberculosis. Specimens were examined by four techniques, ie, microscopic examination, culture, PCR, and FSNP assay. Specimens were processed immediately according to standard routine diagnostic procedures using the NALC method.[13] Mycobacteria were concentrated for culture and smear preparation, and 250 µL of each concentrated sample were inoculated into Lowenstein–Jensen media. All cultures were incubated at 37°C with 5% CO_2 and humidity. After 8 weeks of incubation, cultures showing no growth were reported as negative and discarded. For microscopic examination, using a disposable Pasteur pipette, one drop of the sediment of the sputum specimen was spread onto a clean microscopic slide and, after air-drying and fixation by heat, the smears were stained using the Ziehl–Neelsen method.[14]

DNA extraction and PCR assay

Chromosomal DNA was extracted using proteinase K and phenol:chloroform, then precipitated by ethanol and isopropanol according to a method described elsewhere.[15] Purified DNA was amplified with a pair of primers specific to the *M. tuberculosis* complex strains (*IS6110*) and two specific pairs of external and internal primers for this bacterium (ie, nested PCR). Conventional and nested PCR for detection of *M. tuberculosis* were performed as described by Eisenach et al[16] and Wilson et al, respectively[17] (Table 1 and Figure 1).

Synthesis of dye-doped silica nanoparticles

RuBpy-doped silica nanoparticles were synthesized using a microemulsion method as described by Stöber et al.[9,18] In brief, a microemulsion was prepared by addition of 20 mM RuBpy dye to water (0.48 mL), TEOS 100 µL, cyclohexane 7.5 mL, *n*-hexanol 1.8 mL, and Triton X-100 1.77 mL. After mixing for 20 minutes, 60 µL of NH_4OH was added to initiate polymerization. The reaction was allowed to continue for 24 hours. The nanoparticles were isolated by destabilizing the water-in-oil microemulsion system using acetone, followed by centrifuging and washing with ethanol and water several times to remove any surfactant molecules. The fluorescent intensity, size, and shape of the synthesized nanoparticles was evaluated using a spectrofluorometer, atomic force microscope, and particle size analyzer, as described previously. The shape and size of the silica nanoparticles are shown in Figures 2 and 3.

Surface modification of FSNP and covalent immobilization of protein A

There are several methods available to biofunctionalize silica nanoparticles, including amino group cross-linkage, avidin-biotin linkage bridge, disulfide coupling, chemical binding, and cyanogen bromide modification. In this assay, we used cyanogen bromide for surface modification of silica nanoparticles in order to immobilize protein A covalently on FSNP. Protein A is a highly stable cell surface receptor produced by several strains of *Staphylococcus aureus*. This protein is capable of binding to the Fc portion of immunoglobulins, especially IgGs, from a large number of species. One molecule of this protein is capable of binding to at least two molecules of IgG simultaneously.[19] Using this technique, FSNP with a natural surface sianol group is activated by cyanogen bromide reagent, forming a reactive –OCN derivative of FSNP. Briefly, a solution of cyanogen bromide in acetonitrile is added to a suspension of silica nanoparticles to yield an –OCN group on the particle surface.[20] The particles are then available for bioconjugation to the protein A biomolecules containing free amino groups. To block the protein A-immobilized nanoparticles, 6 mL of 0.3 M glycine

Table 1 Characteristics of DNA oligonucleotide primers used for IS6110-based *Mycobacterium tuberculosis* complex polymerase chain reaction and nested polymerase chain reaction for detection of *M. tuberculosis*

Primer name	Target DNA	Sequences (5′–3′)	Length	Target position	Amplicon size (bp)	Reference
PCR						
IS1	IS6110	CTCGTCCAGCGCCGCTTCGG	20-mer	762–781	123	18
IS2		CCTGCGAGCGTAGGCGGTGG	20-mer	856–884		
Nested PCR						
Outer set						
TB 294	IS6110	GGACAACGCCGAATTGCGAAGGGC	24-mer	295–318	580	19
TB 850		TAGGCGTCGGTGACAAAGGCCACG	24-mer	851–874		
Inner set						
TB 505	IS6110	ACGACCACATCAACC	15-mer	505–515	181	19
TB 670		AGTTTGGTCATCAGCC	16-mer	670–685		

Abbreviation: PCR, polymerase chain reaction.

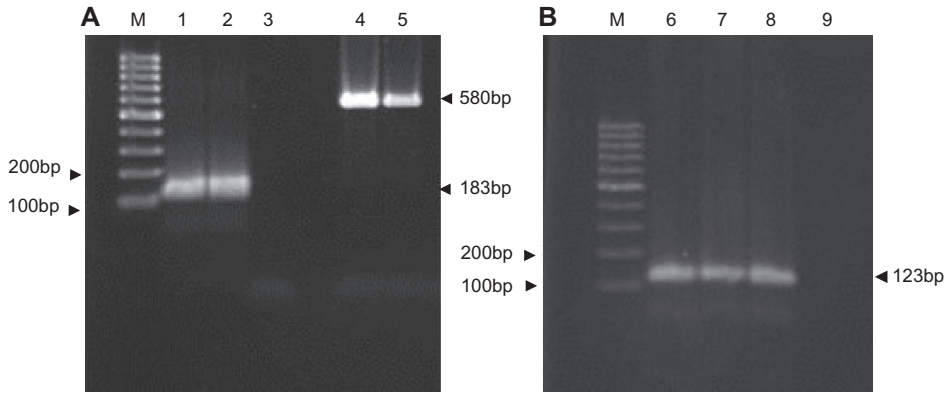

Figure 1 Conventional and nested polymerase chain reaction products of *Mycobacterium tuberculosis* IS6110 fragment by agarose gel electrophoresis (2%). (**A**) Nested polymerase chain reaction, M 100 bp DNA size marker, (second round) (1) positive control, (2) clinical positive sample, (3) negative control (first round), (4) positive control, (5) clinical positive sample. (**B**) Conventional polymerase chain reaction, (6) positive control, (7, 8) positive clinical samples, (9) negative control.

solution (pH 8.0) is added and incubated for 16 hours at 4°C. For quality control, the amount of immobilized protein A on the nanoparticles is measured using the Bradford method.[21] The presence of an amide group (–NHCO–) is confirmed by infrared spectra assay.

Bioconjugation of a specific monoclonal antibody to *M. tuberculosis*

In the next step, mouse monoclonal anti-*M. tuberculosis* antibody was conjugated to the agents in the sputum specimen. Briefly, mouse monoclonal antibody was added to 500 µL of a sediment suspension of sputum in phosphate-buffered saline following processing (final antibody concentration 5 µg/mL), and after 1 hour of incubation at 37°C, the suspension was washed twice with phosphate-buffered saline. Afterwards, 0.1 mg/mL of protein A-conjugated nanoparticles were added, and the suspension was incubated for 1 hour at 37°C. Free FSNP protein A-conjugates which did not bind to bacteria were removed by centrifugation (8000 rpm per 2 minutes). The primary amine group of the *M. tuberculosis* monoclonal antibody was reacted with the activated FSNP, forming antibody-conjugated FSNP.

Microscopic examination

The fluorescent microscope used in this project was equipped with a 450–490 nm band pass excitation and a 515 nm long pass emission filter. The pellet was spread on a glass slide and observed by fluorescent microscopy. Figure 5 is a schematic representation of this method for detecting *M. tuberculosis*. To remove the fluorescence background during microscopic examination, quartz microscope slides (UQG Optics, Cambridge, UK) were used (Figure 4).

Determination of limit of bacterial detection by FSNP assay

In this step, the ability of culture and FSNP assay for detection of different *M. tuberculosis* concentrations was evaluated. A single cell suspension was prepared by harvesting *M. tuberculosis* (H37Rv strain) in phosphate-buffered saline

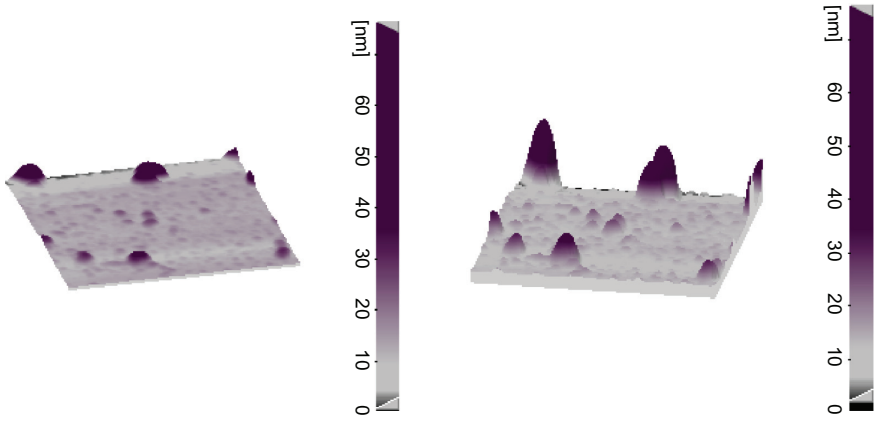

Figure 2 Atomic force microscopic image of the dye-doped silica nanoparticles.

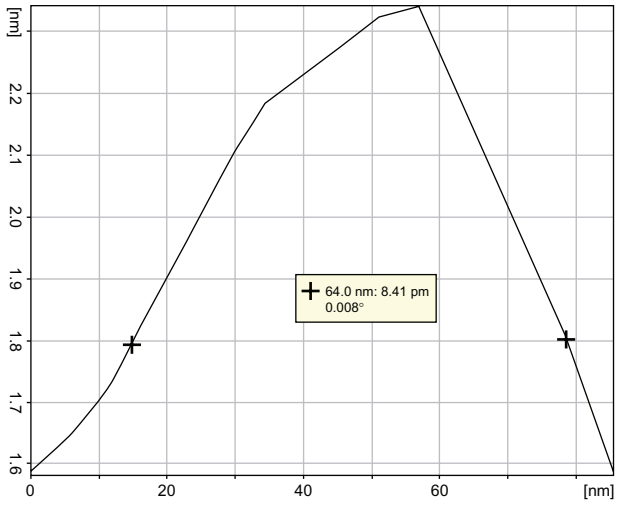

Figure 3 Size particle image profile on atomic force microscopy.

(pH 7.4) using a previously described method.[22] The bacterial suspension was counted in a Petroff-Hausser chamber. Different concentrations of bacteria (10,000, 5000, 1000, 500, 100, 50, and 10 colony forming units/mL) were prepared. Distilled water was used as a negative control in the process, and a suspension of *M. tuberculosis* adjusted to 0.5 McFarland turbidity (about 10^9 colony forming units/mL) was used as a positive control. All experiments were performed in duplicate.

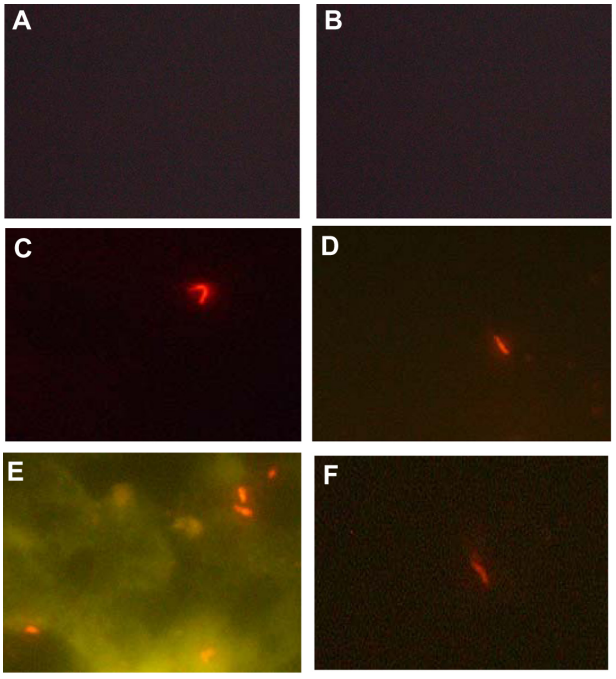

Figure 4 Fluorescence images (100× oil). (**A**) negative control, phosphate-buffered saline in place of the specific monoclonal antibody, (**B**) negative control, *Escherichia coli* in place of the *Mycobacterium tuberculosis*. (**C**, **D** and **F**) Specific interaction of bioconjugated nanoparticles with *M. tuberculosis*; (**E**) the nonspecific interaction (autofluorescence) despite displaying *M. tuberculosis* with a bright fluorescence.

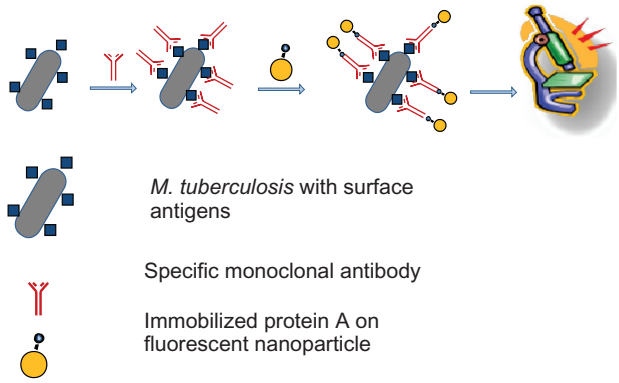

Figure 5 Schematic illustration for detecting *Mycobacterium tuberculosis* based on fluorescent silica nanoparticle assay.

Data analysis

Analysis of the data was performed using the SPSS 15 (SPSS Inc, Chicago, IL), and diagnostic sensitivity and specificity and other statistical parameters were calculated using Stats-Direct statistical software (Version 2.7.2). The investigators were blinded to the clinical data and samples used during the experiment for detection of *M. tuberculosis*.

Results

Smear of sputum, culture, PCR, and FSNP assays were performed for all the 152 specimens taken from patients. Reference strains were treated similarly to the field samples. By acid-fast staining, 63.2% (96/152) of the specimens were positive for *M. tuberculosis*, while 69.7% (106/152) were culture-positive. Of 152 samples, 86 (56.6%) were positive by PCR and 100 (65.8%) were positive by nested PCR for *M. tuberculosis*.

Of the 152 specimens, 107 (70.3%) were positive by FSNP assay. Of 107 specimens, 103 (96.2%) were culture-positive and 88 (82%) were positive by both acid-fast staining and culture. Thus, four samples that were positive by FSNP assay were reported negative by the culture method. Furthermore, of these 107 samples, 78 (72.8%) were positive by both PCR and nested PCR techniques, and 10 samples were negative by both techniques (Table 2).

Comparison of limit of bacterial detection by FSNP and culture methods

The limits of the FSNP assay against culture for the bacterial agent was calculated in this section. As described previously, all bacterial solutions were tested in duplicate for confirmation. Limits of detection for both methods were 100 colony forming units/mL, ie, there was no difference between the two methods in ability to detect bacteria in solution.

Table 2 Comparison of four techniques versus culture method for diagnosis of *Mycobacterium tuberculosis*

Techniques	Culture (gold standard)		Values	
	Positive n	Negative n	Sensitivity %	Specificity %
FSNP assay				
Positive	103	4	97.1	91.3
Negative	3	42		
Total	106	46		
Acid-fast staining				
Positive	90	6	86	84.9
Negative	16	40		
Total	106	46		
PCR				
Positive	78	8	82.6	73.5
Negative	28	38		
Total	106	46		
Nested PCR				
Positive	94	6	86.9	88.6
Negative	12	40		
Total	106	46		

Abbreviations: PCR, polymerase chain reaction; FSNP, fluorescent silica nanoparticles.

Analytical specificity and sensitivity

In the present study, the overall diagnostic sensitivity, specificity, efficiency, predictive value for a positive test, and predictive value for a negative test using the FSNP assay versus culture as a gold standard method were 97.1% (95% CI 91.3–99.2), 91.3% (95% CI 78.3–97.1), 95.5% (efficiency), 96.2% (95% CI 90.1–98.7), and 93.3% (95% CI 80.6–98.2), respectively. The sensitivity and specificity of the other techniques performed in this study are shown in Table 2. As previously described, two types of mouse monoclonal antibody against Hsp-65 and 16 kDa antigen from the *M. tuberculosis* surface antigen group were used for confirmation of the FSNP method. No differences were found in the results obtained with the two different monoclonal antibodies for detection of *M. tuberculosis.*

Discussion

In spite of considerable advances in the diagnosis of *M. tuberculosis*, the gold standard method for laboratory diagnosis is culture. Initiation of appropriate therapy is the main reason for developing a rapid test for diagnosis of pulmonary tuberculosis. In this era of molecular biology, efforts are focused on development of more rapid and sensitive tests. Recent advances in the field of nanoscience have provided new opportunities for rapid laboratory diagnosis of tuberculosis. The specificity, sensitivity, and rapidity of this method encouraged us to trial this technique for detection of *M. tuberculosis* in sputum. In this paper, we have reported the feasibility of the FSNP assay for detection of *M. tuberculosis* and compared this assay with other detection methods.

Recently, two studies were performed to detect bacteria using bioconjugated silica nanoparticles. Qin et al showed the ability of bioconjugated RuBpy-doped nanoparticles to detect *M. tuberculosis* in pure and mixed bacterial samples in comparison with commercial FITC-conjugated antirabbit *M. tuberculosis* antibody.[12] The other study, reported by Zhao et al, described the detection of *E. coli* O157:H7 in ground beef samples.[7] They reported a high intensity of fluorescence signals, a decrease in the amount of primary antibody, and detection of a single bacterium within 20 minutes as major advantages of this technique in their research.

It is noteworthy that the above studies were performed on standard strains, whereas in our study we used the FSNP assay in clinical specimens. Moreover, based on our data, the sensitivity and specificity values were both more than 90%, confirming the accuracy of this method.

In another study, these nanoparticles were used by Zhou et al as probes for DNA/microarray detection.[23] They reported a higher sensitivity for target DNA compared with fluorophores. The sensitivity and specificity of the molecular detection system and nanoparticle probe method is dependent on the amount of DNA or the primer sequence. DNA or primer sequences were not used as the template in our method, and thus did not play any role in sensitivity and specificity.

Despite the use of a definite filter for microscopic imaging, an interface by autofluorescent signals was exhibited by a few samples during our experiments (Figure 4E). All four samples that were negative by FSNP assay and positive by culture showed autofluorescent signals during microscopic examination. This problem has not been described in previous reports.[9,7,12] It seems that there is a specific problem when clinical samples are examined. In general, interface by autofluorescence can result from natural factors induced by some reagents and unspecific binding of antibodies to Fc receptors.[24,25]

In addition to increased sensitivity and specificity, another advantage of the FSNP assay compared with the conventional and PCR methods is the short duration of the procedure. Indeed, only 2–3 hours are needed to perform the FSNP assay compared with 6–8 weeks for the culture and 5 hours for the nested PCR assay.

The limit of the FSNP assay for detection of *M. tuberculosis* was performed on pure bacteria and found to be 100 colony forming units/mL. Less than 100 colony forming units/mL was not detected by either FSNP assay or culture. However, in clinical samples, we found some cases that were positive in culture but negative on FSNP assay, and the overall

sensitivity of the FSNP assay versus culture was not 100% but 97%. The FSNP assay in comparison with PCR is cost-effective ($1.0–$1.5 per test) and the results come out in a shorter time. We believe the sensitivity and specificity of this method will enable it to become a routine technique for detection of *M. tuberculosis* in clinical specimens.

Conclusion

Compared with the gold standard method of culture, the total assay time and ease of performing the FSNP assay suggests that this technique can be of great use in the future. We performed the FSNP assay on a large number of clinical samples to confirm its diagnostic specificity and sensitivity, and consider it reliable enough for routine use in laboratories.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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