Biochip System for Rapid and Accurate Identification of Mycobacterial Species from Isolates and Sputum[▽]

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The accurate detection of mycobacterial species from isolates and clinical samples is important for pathogenic diagnosis and treatment and for disease control. There is an urgent need for the development of a rapid, simple, and accurate detection method. We established a biochip assay system, including a biochip, sample preparation apparatus, hybridization instrument, chip washing machine, and laser confocal scanner equipped with interpretation software for automatic diagnosis. The biochip simultaneously identified 17 common mycobacterial species by targeting the differences in the 16S rRNA. The system was assessed with 64 reference strains and 296 *Mycobacterium tuberculosis* and 243 nontuberculous mycobacterial isolates, as well as 138 other bacteria and 195 sputum samples, and then compared to DNA sequencing. The entire biochip assay took 6 h. The concordance rate between the biochip assay and the DNA sequencing results was 100%. In conclusion, the biochip system provides a simple, rapid, reliable, and highly accurate clinical assay for determination of mycobacterial species in a 6-h procedure, from either culture isolates or sputum samples, allowing earlier pathogen-adapted antimicrobial therapy in patients.

Mycobacterium tuberculosis is an important pathogen; it is responsible for 1.3 to 1.7 million mortalities worldwide in 2007 (26). However, the incidence of opportunistic infections by nontuberculous mycobacteria (NTM) has gradually increased, causing a number of different NTM diseases (4, 5, 14). This apparent increase is thought to be the consequence of several factors; the increased use of diagnostic methods that can identify these agents has contributed to this number, while the AIDS epidemic is a principal cause (18). A report on a nationwide random survey in China on the epidemiology of tuberculosis in 2000 revealed that 11.1% of the 441 bacterial strains isolated from patient sputum were NTM (15). The low level of identification of NTM is partially due to the use of conventional methods, which rely on growth characteristics, colony morphology, pigment production, and biochemical tests. Because of the slow growth of mycobacteria, the methods are time-consuming, taking 4 to 8 weeks to complete. Extensive experience in the interpretation of the results of biochemical tests is also required, and the identity of specific species from cultures is often difficult to determine, resulting in possible

misidentification. As a result of these numerous limitations, conventional methods are not widely used by the majority of clinical laboratories in China. However, the correct identification of NTM is clinically important because most NTM are naturally resistant to first-line antituberculosis drugs, and different species of NTM are sensitive to different antibiotics drugs. Thus, there is an urgent need for the development of a rapid, simple, and accurate method for mycobacterium species identification (21).

Recently, different nucleic acid based molecular assays such as DNA sequencing (1), microarray assay (6, 16, 25), PCR-restriction fragment length polymorphism (RFLP) assays (23), and commercial kits such as Accuprobe (Gen-Probe, San Diego, CA) (2), GenoType (Hain, Germany), (19, 20), and INNO-LiPA (Innogenetics, Belgium) (24), have emerged as rapid tools for species identification. The molecular identification methods are based on the polymorphisms in the 16S rRNA (1) or 16S-23S rRNA spacer regions (24) or 23S rRNA (20) or hsp65 genes (12). The 16S rRNA gene sequences of most mycobacterial species are well known and can be found in online databases. Although the molecular methods mentioned above have greatly improved the diagnosis of NTM, there is still a need for a quick, semiautomated or fully automated, total solution-based system to meet the high-throughput demands of busy clinics, particularly for large centers with numerous patients.

We report here a rapid diagnosis solution that includes a biochip, apparatus for sample preparation, chip hybridization, washing and data acquisition, and dedicated software for automated diagnosis. The biochip is designed to detect differ-

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ences in 16S rRNA sequences for mycobacteria species identification. The entire process is semiautomatic and high throughput. The automated determination of species could also eliminate some elements of operator error.

MATERIALS AND METHODS

Culture strains and clinical specimens. A control panel including 64 reference strains (consisting of mycobacteria and bacteria; Table 1) for sensitivity and specificity analysis was obtained from the National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). Samples comprising 677 clinical isolates (296 *M. tuberculosis*, 243 NTM, 138 other bacteria) and 195 sputum specimens were obtained from the National TB Reference Laboratory, Beijing Hospital, Beijing Tiantan Hospital, Beijing Tongren Hospital, 309th Hospital of the PLA, and 302th Hospital of the PLA (all Beijing, China). Among the isolates, a set of 460 previously well-characterized isolates was used for threshold determination, including 146 *M. tuberculosis* and 176 NTM isolates (characterized by sequencing of the 16S rRNA gene) and 138 other bacteria (identified by the Vitek-2 [bioMérieux, France]) or by the MicroScan Autoscan-4 system (Dade Behring, Inc., West Sacramento, CA). A further 217 clinical mycobacterial isolates and 195 clinical sputum specimens, which were used for chip validation, were consecutively collected (from August 2007 to May 2008) by the National TB Reference Laboratory. The mycobacterial isolates were cultured either on solid Lowenstein-Jensen media or in Bactec-MGIT (BD Biosciences) or MB/BacT (bioMérieux, France) liquid media. For sputum samples, 0.5-ml portions of sputum samples were used for mycobacterial nucleic acid extraction using the same procedure as reported (9). The 17 mycobacterial reference strains, used for sensitivity evaluation, were cultured in 4 ml of 7H9 broth (Becton Dickinson, Cockeysville, MD) until a concentration of $\sim 10^8$ CFU/ml was obtained, estimated spectrophotometrically at 600 nm. Colony counts were performed by plating serial dilutions of a fresh culture in duplicate onto 7H9 agar.

Biochip preparation. The biochip could identify 17 mycobacterial species: *M. tuberculosis*, *M. intracellulare*, *M. avium*, *M. gordonae*, *M. kansasii*, *M. fortuitum*, *M. scrofulaceum*, *M. gilvum*, *M. terrae*, *M. chelonae*, *M. abscessus*, *M. phlei*, *M. nonchromogenicum*, *M. marinum*, *M. ulcerans*, *M. aurum*, *M. szulgai*, *M. malmoense*, *M. xenopi*, and *M. smegmatis*. Oligonucleotide probes were designed by multiple-sequence alignment analysis of the sequences available in GenBank by using the DNAMAN (version 4.0) program. Probes were chosen in several species-specific sequence regions of the 16S rRNA gene for differentiation among different *Mycobacterium* species. For some species that are difficult to differentiate, an artificial mismatch was introduced into specific oligonucleotide probes to provide better thermal differentiation between the sequences. The lengths of these probes were between 15 to 30 nucleotides, with melting temperatures (T_m) between 60 and 65°C. The 5' end of each probe was modified by adding a spacer with 25 consecutive thymines and an amino-linker group for covalent immobilization on the aldehyde-coated glass surface. Oligonucleotide probes were contact printed onto OPALdehydeSlide aldehyde-activated slides at a concentration of 10 μ M in DNA spotting solution using a SmartArrayer-48 microarrayer (both from CapitalBio, Beijing, China) and were covalently immobilized on the slides via an amino group at their 5' ends (8–10) to create the biochips (layout shown in Fig. 1). All oligonucleotide probes and primers listed in Table 2 were obtained from Invitrogen (Beijing, China). In each array, five types of control were printed, including a fluorescent dye HEX-labeled oligonucleotide as a surface chemistry control, an oligonucleotide complementary to a synthetic template included in the hybridization mixture as a hybridization positive control to monitor the hybridization process, an oligonucleotide with the consensus sequence of 16S rRNA gene of genus *Mycobacterium* as mycobacteria and PCR control, an oligonucleotide designed to not hybridize to any sequences present in the hybridization mixture as the negative control for background signal corrections, and DNA spotting solution without any oligonucleotide probe as the blank control to monitor the spotting process.

Asymmetric PCR. Asymmetric PCR was performed as described previously (9), with some minor modifications. Uracil DNA glycosylase (UNG, 0.02 U/20 μ l) and dUTP (400 nM) was used to prevent carryover contamination during amplification. PCR was performed in a Peltier PTC225 thermal cycler (MJ Research, Watertown, MA) in two amplification rounds, with an initial activation step at 37°C for 10 min and then DNA denaturation at 94°C for 10 min, followed by a first round of exponential amplification of 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 40 s; a second round of linear amplification of 10 cycles of 94°C for 30 s and 72°C for 60 s; and a final extension step at 72°C for 5 min. PCR products (2 μ l) were analyzed by electrophoresis in 2.0% agarose.

TABLE 1. Sixty-four reference strains used for biochip hybridization specificity analysis

No.	Species	Strain no.
1	<i>Corynebacterium pseudodiphtheriticum</i>	38203
2	<i>Corynebacterium xerosis</i>	1.1919
3	<i>Neisseria subflava</i>	29110
4	<i>Proteus mirabilis</i>	49005
5	<i>Proteus vulgaris</i>	1.1527
6	<i>Citrobacter freundii</i>	1.1732
7	<i>Enterobacter cloacae</i>	1.181
8	<i>Enterobacter aerogenes</i>	1.2021
9	<i>Serratia marcescens</i>	1.1857
10	<i>Escherichia coli</i>	1.2463
11	<i>Klebsiella pneumoniae</i>	1.1526
12	<i>Stenotrophomonas maltophilia</i>	1.1788
13	<i>Pseudomonas aeruginosa</i>	1.2464
14	<i>Acinetobacter calcoaceticus</i>	1.2004
15	<i>Staphylococcus epidermidis</i>	1.2429
16	<i>Staphylococcus aureus</i>	1.2386
17	<i>Streptococcus salivarius</i>	1.2498
18	<i>Streptococcus mutans</i>	1.2499
19	<i>Alcaligenes faecalis</i>	1.924
20	<i>Rhodococcus rhodochrous</i>	4.1147
21	<i>Nocardia asteroides</i>	4.1165
22	<i>Nocardia otitidiscaviarum</i>	4.1168
23	<i>Actinoplanes italicus</i>	4.1065
24	<i>Micrococcus luteus</i>	1.1848
25	<i>Enterococcus faecalis</i>	1.2025
26	<i>Enterobacter faecium</i>	1.2024
27	<i>Streptococcus mitis</i>	32232
28	<i>Streptococcus pyogenes</i>	32067
29	<i>Nocardia brasiliensis</i>	4.1128
30	<i>Candida albicans</i>	2.2086
31	<i>Neisseria gonorrhoeae</i>	29802
32	<i>Fusobacterium nucleatum</i>	1.2526
33	<i>Mycobacterium intracellulare</i>	ATCC 13950
34	<i>Mycobacterium avium</i>	ATCC 25291
35	<i>Mycobacterium gordonae</i>	ATCC 14470
36	<i>Mycobacterium kansasii</i>	ATCC 12478
37	<i>Mycobacterium fortuitum</i>	ATCC 6481
38	<i>Mycobacterium scrofulaceum</i>	ATCC 19981
39	<i>Mycobacterium gilvum</i>	ATCC 43909
40	<i>Mycobacterium terrae</i>	ATCC 19619
41	<i>Mycobacterium chelonae</i>	ATCC 14472
42	<i>Mycobacterium abscessus</i>	ATCC 19977
43	<i>Mycobacterium phlei</i>	ATCC 11758
44	<i>Mycobacterium nonchromogenicum</i>	ATCC 19530
45	<i>Mycobacterium marinum</i>	ATCC 927
46	<i>Mycobacterium ulcerans</i>	ATCC 19423
47	<i>Mycobacterium aurum</i>	ATCC 23366
48	<i>Mycobacterium szulgai</i>	NCTC 10831
49	<i>Mycobacterium xenopi</i>	ATCC 19250
50	<i>Mycobacterium smegmatis</i>	ATCC 19420
51	<i>Mycobacterium aichiense</i>	ATCC 27280
52	<i>Mycobacterium asiaticum</i>	ATCC 25276
53	<i>Mycobacterium austroafricanum</i>	ATCC 33464
54	<i>Mycobacterium chubuense</i>	ATCC 27278
55	<i>Mycobacterium diernhoferi</i>	ATCC 19340
56	<i>Mycobacterium duvalii</i>	ATCC 43910
57	<i>Mycobacterium malmoense</i>	ATCC 29571
58	<i>Mycobacterium gadium</i>	ATCC 27726
59	<i>Mycobacterium gastri</i>	ATCC 15754
60	<i>Mycobacterium neoaurum</i>	ATCC 25795
61	<i>Mycobacterium simiae</i>	ATCC 25275
62	<i>Mycobacterium thermoresistibile</i>	ATCC 19527
63	<i>Mycobacterium triviale</i>	ATCC 23292
64	<i>Mycobacterium tuberculosis</i>	ATCC 27294

FIG. 1. Images of each of the 17 mycobacterial microarray hybridization results.

Biochip hybridization and data analysis. Chip hybridization was performed as described previously (9) in a three-dimensional tilting agitator BioMixer II hybridization oven and an automated SlideWasher –8 (both from CapitalBio) was used to wash and dry the hybridized slides to reduce the dye blemishes that occur

frequently during manual posthybridization slide washing. Microarrays on the slides were analyzed by using a LuxScan-10K confocal laser scanner, and the fluorescent intensities were quantified by use of dedicated software called the mycobacteria identification array test system (both from CapitalBio). For thresh-

TABLE 2. Oligonucleotide probes and primers used in this study and the probe threshold and detection limits of the biochip assay for the 17 mycobacteria

Oligonucleotide probe and primer	Sequence (5'–3') ^a	Detection limit (CFU/PCR) in:	
		Saline solution	Sputum
QC	NH ₂ -(T) ₂₅ -GCAAGACAAGTGGAAAGTGTG-HEX		
EC	NH ₂ -(T) ₂₅ -GCAACCACCACCGGAGG		
NC	NH ₂ -(T) ₂₅ -CCTCTCTCGGACTAATCGCC		
BC	DMSO		
<i>Mycobacterium</i> spp.	NH ₂ -(T) ₂₅ -GCGGGCTCATCCCACAC		
<i>M. tuberculosis</i>	NH ₂ -(T) ₂₅ -ACAAGACATGCATCCCGT	100	100
<i>M. intracellulare</i>	NH ₂ -(T) ₂₅ -TAAAGACATGCGCTAAA	100	100
<i>M. avium</i>	NH ₂ -(T) ₂₅ -GACATGCGTCTTGAGGTC	200	200
<i>M. gordonae</i>	NH ₂ -(T) ₂₅ -CTTGTGTCCTGTGGTCCT	50	100
<i>M. kansasii</i>	NH ₂ -(T) ₂₅ -TTATGCCGGTGTGTCAG	50	100
<i>M. fortuitum</i>	NH ₂ -(T) ₂₅ -ATGAAGCGCGTGGTCATA	50	100
<i>M. scrofulaceum</i>	NH ₂ -(T) ₂₅ -CAACCCACAAAGTGAGCC	200	200
<i>M. gilvum</i>	NH ₂ -(T) ₂₅ -CACACACCATGAAGCATG	1,000	1,000
<i>M. terrae</i>	NH ₂ -(T) ₂₅ -CAGAACATGCATCCCA	500	1,000
<i>M. chelonae-M. abscessus</i>	NH ₂ -(T) ₂₅ -TGGACCACTCACCATGAAGTGTGTG	1,000	1,000
<i>M. phlei</i>	NH ₂ -(T) ₂₅ -TCCCAGCCTCGGGGTCAG	1,000	1,000
<i>M. nonchromogenicum</i>	NH ₂ -(T) ₂₅ -CACACCATGCAGCATG	200	200
<i>M. marinum-M. ulcerans</i>	NH ₂ -(T) ₂₅ -CAGAGGACATGAATCCCGT	200	200
<i>M. aurum</i>	NH ₂ -(T) ₂₅ -GACATGCATCGCGTAG	50	50
<i>M. szulgai</i>	NH ₂ -(T) ₂₅ -CATACGCCTCGGGGTCCT	100	100
<i>M. xenopi</i>	NH ₂ -(T) ₂₅ -CCTCCGGTGGTGGTTGC	200	200
<i>M. smegmatis</i>	NH ₂ -(T) ₂₅ -CGACCAGCAGGGTGTATT	1,000	1,000
16S UT primer	TAMRA-TCACTTGCTTCCGTTGAGGTGGCTCAGGACGAACG		
	(T _m , 66.2; 48.0°C; 0.4 μmol/liter)		
16S SS primer	AGCCGTGAGATTTACGAACA (T _m , 50.9°C; 0.2 μmol/liter)		

^a HEX, hexachloro-6-carboxy-fluorescein; TAMRA, 6-carboxy-tetramethyl-rhodamine; (T)₂₅, 25 consecutive thymines.

old determination, a set of 460 cultures (146 *M. tuberculosis*, 176 NTM, and 138 other bacterial strains) was used. All of the hybridization signals were processed by using the following statistical method. The *M. tuberculosis* threshold was determined by the ROC (receiver operating characteristic) method (146 *M. tuberculosis* as positive specimens and 176 NTM plus 138 other bacteria as negative specimens). All of the 16 NTM thresholds were determined by the "mean + three standard deviations" method (146 *M. tuberculosis* and 138 other bacteria as negative specimens) for each probe. For sensitivity determination, the biochip was hybridized by using diluted mycobacteria samples (10^4 , 10^3 , 500, 200, 100, 50, 25, or 10 CFU). The detection limit was the lowest concentration of mycobacteria with a hybridization signal above the threshold.

DNA sequencing and quantitative PCR. The sequences of the PCR products of the first 500 bp of the 5' region of the 16S rRNA gene from individual patient samples were determined by standard Sanger DNA sequencing. A China state food and drug administration (SFDA)-approved quantitative PCR was performed by following the protocol provided by the manufacturer (PG Biotech, Shenzhen, China).

RESULTS

Sensitivity, reproducibility, and specificity of the biochip system. The detection limits (Table 2) were determined by using a graduated concentration series of the 17 *Mycobacterium* suspensions in saline or spiked-in negative sputum. The results showed that the detection limits of different species varied because of detection differences of the species-specific probes, where 12 of the 17 species can be detected at a concentration above 200 CFU per PCR in saline or negative sputum, the exceptions were *M. gilvum*, *M. terrae*, *M. chelonae-M. abscessus*, *M. phlei*, and *M. smegmatis*, which had a detection limit above 1,000 CFU per PCR. The reproducibility was evaluated with each of negative, low positive (200 CFU per PCR) and high positive (10^5 CFU per PCR) *M. tuberculosis* and *M. intracellulare* samples. Each sample was repeatedly tested (ten times) to evaluate variation. All of the negative samples were determined as a negative result, and all low- and high-positive samples yielded coincident results. To determine the specificity, 64 species of reference strains (*M. tuberculosis* and 31 types of NTM and 32 types of other bacteria; see Table 1) were tested. None of the 32 species of other bacteria produced signals on the *M. tuberculosis*- and NTM-specific probes. Only specific *M. tuberculosis* and NTM samples produced signals on the corresponding probes. *M. nonchromogenicum* and *M. fortuitum* (only when the concentration was higher than 10^7 CFU) had cross-hybridization signals with *M. gilvum*, respectively, but each NTM had a specific signal pattern, which made for clear identification. For *M. marinum* and *M. ulcerans*, *M. chelonae* and *M. abscessus*, *M. szulgai* and *M. malmoeense*, each of the paired NTM strains were detected by the same array probes. In addition, the species (*M. aurum*, *M. aichiense*, *M. asiaticum*, *M. austroafricanum*, *M. chubuense*, *M. diernhoferi*, *M. duvalii*, *M. gadium*, *M. neoaurum*, *M. simiae*, *M. thermoresistibile*, and *M. triviale*) were hybridized in the array to evaluate the genus probe. The results showed that each of these species could hybridize with the genus probe. The images of the microarray hybridization result for *M. tuberculosis* and 16 NTM are shown in Fig. 1.

Results of the biochip test using culture isolates and clinical sputum. The biochip assay was further evaluated with 217 mycobacteria isolates and 195 clinical sputum samples. The identity of each of these samples was conclusively determined by sequencing. The 217 clinical culture isolates included 150 *M. tuberculosis* and 67 NTM samples. In 150 (100%) of the 150 *M.*

tuberculosis isolates and 67 (100%) of the 67 NTM isolates, the biochip assay results were in agreement with sequencing. Among the 67 NTM culture isolates, *M. intracellulare* (39 isolates) and *M. chelonae-M. abscessus* (12 isolates) were in the majority, 6 isolates were *M. kansasii*, 5 isolates were *M. fortuitum*, 3 isolates were *M. avium*, and 2 isolates were *M. terrae*.

A total of 195 sputum samples from suspected tuberculosis cases were collected and tested by using the biochip system. The smear results for acid-fast bacilli (AFB) and culture results had been determined for these samples. Among the 195 tested samples, 116 (59%) were culture positive (82 positive for AFB smear and 34 negative for AFB smear), and 79 (41%) were culture negative (all negative for AFB smears). All 116 culture-positive samples were successfully detected by the biochip, and one culture negative was detected as *M. tuberculosis* by the biochip. All of the samples were confirmed by the China SFDA-certified *M. tuberculosis* quantitative PCR kit, and direct DNA sequencing of 16S rRNA gene was performed for further identification. All of the sequencing results were consistent with the biochip results. Three cases which had been initially clinically diagnosed as lung tuberculosis were identified as *M. intracellulare* using the biochip, which were also confirmed by sequencing.

DISCUSSION

The importance of the accurate diagnosis of NTM and tuberculosis lies in the different treatments of patients (7, 22). The majority of NTM disease patients should not be treated with first-line anti-TB drugs because of the resistance of NTM to most of them. Further, different NTM species are sensitive to different antibiotics. Therefore, the rapid discrimination of NTM from *M. tuberculosis*, and then the identification of the NTM species are both necessary to ensure correct diagnosis. The test could also facilitate the earlier provision of proper treatment for patients and avoid unnecessary side effects.

Here, we present a newly designed biochip system for the rapid identification of *M. tuberculosis* and 16 NTM isolates. The instrumentation and microarray components of the platform have also been independently evaluated previously for detection of genotypic rifampin and isoniazid resistances (9), severe acute respiratory syndrome (27), staphylococcal isolates (28), and *Enterobacteriaceae* (29). The present biochip method was accurate, sensitive, and able to identify 17 mycobacterial species and more particularly involved a simple semiautomatic protocol which could be completed within 6 h. This biochip procedure is much simpler to execute than conventional biochemical methods and yields results significantly faster.

Several studies have suggested that sequence analysis, especially that based on the 16S rRNA sequences, could obtain accurate information for the identification of most mycobacteria (3, 11), and sequencing has been widely accepted as the "gold standard" (1). We therefore confirmed the biochip result by sequencing rather than the conventional biochemical methods because of the inadequacy of the phenotypic tests. However, a deficiency in 16S rRNA sequence array-based identification was also observed in the present study. For *M. marinum-M. ulcerans* and *M. chelonae-M. abscessus*, each of the paired NTM have the same 16S rRNA sequences, so the two pairs of NTM could not be distinguished by our biochip

TABLE 3. Comparison of the content of the mycobacterial-species biochip assay with other species tests

Organism or region detected ^a	Detection by:			
	CapitalBio mycobacterial-species biochip	INNO-LiPA	GenoType	AccuProbe
16S rRNA gene region	16S rRNA gene region	16S-23S rRNA spacer regions	23S rRNA gene region	rRNA gene region
<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i>
<i>M. intracellulare</i>	<i>M. intracellulare</i>	<i>M. intracellulare</i>	<i>M. intracellulare</i>	<i>M. intracellulare</i>
<i>M. avium</i>	<i>M. avium</i>	<i>M. avium</i>	<i>M. avium</i>	<i>M. avium</i>
<i>M. gordonae</i>	<i>M. gordonae</i>		<i>M. gordonae</i>	<i>M. gordonae</i>
<i>M. kansasii</i>	<i>M. kansasii</i>	<i>M. kansasii</i>	<i>M. kansasii</i>	<i>M. kansasii</i>
<i>M. fortuitum</i>	<i>M. fortuitum</i>	<i>M. fortuitum</i> complex	<i>M. fortuitum</i>	
<i>M. scrofulaceum</i>	<i>M. scrofulaceum</i>	<i>M. scrofulaceum</i>	<i>M. scrofulaceum</i>	
<i>M. chelonae-M. abscessus</i> *	<i>M. chelonae-M. abscessus</i>	<i>M. chelonae-M. abscessus</i>	<i>M. chelonae</i>	
<i>M. marinum-M. ulcerans</i> *	<i>M. marinum-M. ulcerans</i>	<i>M. marinum-M. ulcerans</i>	<i>M. marinum-M. ulcerans</i>	
<i>M. malmoense-M. szulgai</i> *	<i>M. malmoense-M. szulgai</i>	<i>M. malmoense</i>	<i>M. malmoense</i>	
<i>M. xenopi</i>	<i>M. xenopi</i>	<i>M. xenopi</i>	<i>M. xenopi</i>	
<i>M. smegmatis</i>	<i>M. smegmatis</i>	<i>M. smegmatis</i>		
<i>M. phlei</i>	<i>M. phlei</i>			
<i>M. nonchromogenicum</i>	<i>M. nonchromogenicum</i>			
<i>M. gilvum</i>	<i>M. gilvum</i>			
<i>M. terrae</i>	<i>M. terrae</i>			
<i>M. aurum</i>	<i>M. aurum</i>			
<i>M. genavense</i>		<i>M. genavense</i>		
<i>M. simiae</i>		<i>M. simiae</i>		
<i>M. celatum</i>		<i>M. celatum</i>		
<i>M. haemophilum</i>		<i>M. haemophilum</i>		
MAIS		MAIS		
<i>M. abscessus</i>			<i>M. abscessus</i>	
<i>M. interjectum</i>			<i>M. interjectum</i>	
<i>M. peregrinum</i>			<i>M. peregrinum</i>	

^a *, species pair detected. MAIS, *Mycobacterium avium-M. intracellulare-M. scrofulaceum* complex.

method. Although sequence variation occurs between *M. malmoense* and *M. szulgai* in two areas of the amplified PCR fragment, the sequences were not readily distinguished from those of *M. tuberculosis* (data not shown). Because of this, we designed a probe to distinguish *M. malmoense* and *M. szulgai* from *M. tuberculosis*, but this probe does not distinguish between *M. malmoense* and *M. szulgai*. These findings indicate a deficiency in the content of the current array. This deficiency could be resolved by the design combined probes based on both the 16S rRNA and the 23S rRNA genes (or the 16S-23S rRNA spacer regions or the hsp65 gene). The information and identification capability should increase accordingly with increased biochip content.

Among the established nucleic acid-based assays for mycobacterial identification, e.g., sequencing, PCR-RFLP, microarray, GenoType, INNO-LiPA, and AccuProbe, the last three technologies are sold as CE-marked or U.S. Food and Drug Administration-approved kits and used in clinical practice. The species detected by these three technologies and by our method are compared in Table 3. Similar to our biochip, both the GenoType and the INNO-LiPA assays detect several pairs of NTM species as one signal. Both the GenoType assay and the INNO-LiPA assay are line probe assays. Because of the space limitations of the strip, relatively few probes can be printed on the membrane, and it is difficult to present more or alternative probes for further species identification on a single assay strip. In contrast, the biochip can readily accommodate a large number of probes and could easily increase the information content to resolve more species. Considering the preva-

lence of mycobacterial species found in clinical samples in China, the present biochip can readily meet the requirements of clinical diagnosis. However, with further development, it could include additional probes to detect more species to progress the scope of CDC epidemiological surveys. We also used an advanced asymmetric PCR process in the amplification step (9) to obtain a higher sensitivity. Troesch et al. (25), Fukushima et al. (6), and Park et al. (16) pioneered the identification of mycobacterium species using microarrays. Moreover, the developed biochip system presented here has several major advantages that aid clinical practice greatly, in particular the ample quality controls (five types of control [see the discussion of biochip preparation in the Materials and Methods]), the global signal uniformity which produces higher quality hybridization results (see Fig. 1; also see references 13 and 17), and the semiautomated, total solution-based procedures that meet the high-throughput demands of busy clinics.

In addition to the high accuracy, sensitivity, and information content, we believe that our assay system has several other critical advantages. We have simplified the procedure for nucleic acid extraction from mycobacteria and described the system option for rapid sample processing of up to 36 samples in less than 30 min for culture isolates and about 50 min for sputum samples, reducing the demands on lab staff. The automatic chip washing procedures and biochip scanner and software were introduced in our platform to reduce the manual work burden of the operator and to ease the implementation of biochip techniques into routine work flows. The amplified target DNA fragments detected in our assay were fluorescently

labeled, and only a single washing step was needed after the chip hybridization incubation. Furthermore, the biochip wash step was processed automatically with a chip cleanup instrument, while the INNO-LiPA Rif TB assay and the genotype MTBDR assay are both based on manual operations. For the data acquisition step of our system, the biochip was analyzed by dedicated software, and the result was printed automatically. For both the INNO-LiPA Rif TB assay and the Genotype MTBDR assay, the operator must manually paste the strip onto an evaluation sheet and read the result by eye using an interpretation chart, which could potentially introduce operator error. Regarding the kit costs, the price for detection of mycobacteria by the biochip is about \$30 per sample (May 2010), which is similar to the cost of the GenoType CM strip.

Although the newly designed biochip system has a number of obvious advantages, we also recognize that there is scope for further development. We propose that an increase in the number of discriminatory loci assessed to ensure that individual species are unequivocally identified, and an increase in the number of mycobacterial species assayed by the chip system could be considered. Further development of the workstation to make the procedure fully automatic would also have direct benefit to workflows, particularly for larger test centers. In conclusion, the biochip system provides a rapid (6-h procedure), simple (semiautomatic), accurate and reliable diagnostic tool for the identification of 17 of the most common mycobacterial species from culture isolates or sputum samples. The system should be useful for the future assay and management of mycobacterial infections for busy hospitals and centers for disease control and prevention.

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