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Multicenter evaluation of the biochip assay for rapid detection of mycobacterial isolates in smear-positive specimens

Hong Fang, Yanwan Shangguan, Hao Wang, Zhongkang Ji, Jundan Shao, Ruihong Zhao, Shuting Wang, Lin Zheng, Xiuyuan Jin, Shujuan Huang, Kaijin Xu*, Jifang Sheng*

State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, No. 79 Qingchun Road, Shangcheng District, Hangzhou 310003, Zhejiang, China

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ABSTRACT

Objectives: The objective of this study was to conduct a multicentre evaluation of the performance of the biochip assay in the rapid identification of mycobacteria in smear-positive sputum specimens.
Methods: A total of 1751 sputum specimens were obtained from 7 cities in Zhejiang, China. All of the specimens were used for the discrimination of Mycobacterium species using the biochip assay, and the results were compared to the golden standard method of culture, hsp65, 16S rRNA and *rpoB* sequence analysis.
Results: In the 1751 sputum specimens, 1685 samples were cultured successfully; among these samples, 1361 were *Mycobacterium tuberculosis*, 323 were NTM and 1 was *Nocadia farcinica*. Of the 323 NTM, most of them were *Mycobacterium intracellulare*(52.5%) followed by *Mycobacterium abscessus* (20.7%), *Mycobacterium avium (11.7%), Mycobacterium kansasii (9.6%) and Mycobacterium fortuitum* (1.9%). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the biochip assay to differentiate TB and NTM from AFB positive specimens were 99.8%, 99.7%, 99.9%, 99.1%, 98.8%, 1, 1, and 99.7%, respectively. The concordance between the biochip assay and mycobacterial culture for the identification of NTM species was 95.4%.
Conclusions: The biochip assay is a reliable tool for the rapid identification of most mycobacteria in clinical sputum specimens. This assay can be helpful for physicians in the early diagnosis and treatment of mycobacterium infections.

Introduction

Tuberculosis has bounced back in the past several decades and became the main cause of death from one single infectious disease, mainly due to the epidemic of HIV (World_Health_Organization, 2017). Meanwhile, the incidence and prevalence of nontuberculous mycobacteria (NTM) are increasing worldwide (Yu et al., 2016; McGrath and Anderson, 2007), especially NTM lung diseases (NTMPD) in both immunocompetent and immunodeficient patients. In some developed countries, NTM are even more prevalent than tuberculosis (Deshpande et al., 2017; Pasipanodya et al., 2017). NTM includes all kinds of mycobacteria other than *Mycobacterium tuberculosis* complex and *Mycobacterium leprae* (Primm et al., 2004). Most NTM are opportunistic pathogens and commonly exist in the environment in water, soil, and animals and can even colonize the human body. There are more than 190 kinds of NTM identified to date, including 191 species and 15 subspecies (http://www.bacterio.net/mycobacterium.html (Anon, 2018)). Some of the NTM are pathogenic, while others are not (Griffith et al., 2007). Different species of *nontuberculous mycobacteria* have completely different habitus, viability and pathogenicity. The precise and timely identification of NTM is critically important because different species of NTM possess entirely different antibacterial spectra, and most of them are resistant to first-line anti-*tuberculosis* drugs (Haworth et al., 2017). Thus, a rapid and accurate method is warranted for the distinction of TB and NTM and the discrimination of NTM species. The most conventional methods are smear and culture. Smear is a rapid way to identify mycobacteria but is unable to differentiate NTM from *M. tuberculosis*. Cultures can make up for this deficiency, but it can take several weeks to obtain the results. Recently, some molecular diagnostic methods were published. However, most molecular methods require isolated strains from clinical samples. In this

* Corresponding authors.
E-mail addresses: zdyxyxkj@zju.edu.cn (K. Xu), jifang_sheng@zju.edu.cn (J. Sheng).

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study, we conducted a multicenter experiment to evaluate a commercial diagnostic kit (CapitalBio, Beijing, China) in a large-scale population in Zhejiang, China. This method is based on the 16S rRNA gene sequences, which can identify 17 mycobacterial species (*M. tuberculosis*, *M. intracellulare*, *M. chelonae/M. abscessus*, *M. kansasii*, *M. avium*, *M. gordonae*, *M. fortuitum*, *M. scrofulaceum*, *M. gilvum*, *M. terrae*, *M. phlei*, *M. nonchromogenicum*, *M. marinum/M. ulcerans*, *M. aurum*, *M. szulgai/M. malmoense*, *M. xenopi*, and *M. smegmatis*) directly in acid-fast bacilli (AFB) smear-positive respiratory specimens within 6 h (Zhu et al., 2010). The results obtained with the biochip assay were compared with the results obtained with culture, which is currently considered the standard diagnostic assay.

Material and methods

Study populations

Our study was conducted at 8 research sites (hospitals) from 7 cities in Zhejiang Province, China. All of the specimens were collected from patients who were under suspicion of *M. tuberculosis* or NTM according to clinical manifestations or imaging investigations. AFB smear-positive sputum samples were confirmed by the Ziehl-Neelsen method and were collected consecutively from each hospital between January 2016, and June 2018. For each patient, only one specimen was collected in the study.

Laboratory methods

Direct smears from each sputum specimen were identified by Ziehl-Neelsen staining for acid fast bacilli (AFB) (van Ingen, 2015). All sputum specimens were digested and decontaminated with Nacetyl-Lcysteine 2% and sodium hydroxide (NALC-NaOH) for 15 min. The mixture was separated into two parts, a minimum of 1.5 ml for biochip assay (Capital Bio, Beijing, China) (Zhu et al., 2010), and the remaining part was diluted with a PBS buffer to 10 mL and centrifuged at 3000 g for 15 min. The supernatant was discarded completely, and the sediment was resuspended in 0.5 mL PBS buffer. The suspension was inoculated into 4 ml of 7H9 broth (Becton Dickinson, Cockeysville, MD) for culturing and further species identification.

Nucleic acid extraction

The initial 1.5 ml specimen mixture obtained for the biochip assay above was centrifuged at 12000 rpm for 5 min. The supernatant was discarded, and the sediment was diluted to 1 ml with sterile saline solution and centrifuged at 12000 rpm for 5 min. The supernatant was discarded, and 100 μl nucleic acid extract solution (Capital Bio, Beijing, China) was added to the residue. The suspension was transferred to a specific tube (Capital Bio, Beijing, China) and then vortexed for 10 min. The suspension was then placed in a 95° C dry bath for 8 min before centrifuging again at 12000 rpm for 5 min. The nucleic acid was in the supernatant and stored at −20° C.

Biochip hybridization

The nucleic acid extracted above was subjected to asymmetric PCR before hybridization. The specific operating procedures and amplification conditions are described in a previous article (Zhu et al., 2010).

The amplification products were maintained at 95 °C for 5 min and then placed in ice-water immediately (in order to obtain single stranded DNA). Next, 6 μl amplification products were mixed with 9 μl hybridization buffer, and 13.5 μl of this mixture was transferred to a prepared slide (Capital Bio, Beijing, China). Chip hybridization was performed in a three-dimensional tilting agitator BioMixer II (Capital Bio, Beijing, China) hybridization oven and an automated SlideWasher (Capital Bio, Beijing, China). Microarrays on the slides were analyzed using a LuxScan 10K-B confocal laser scanner; the fluorescent intensities were quantified by use of dedicated software called the mycobacteria identification array test system (both from Capital Bio).

DNA sequencing and quantitative PCR

PCR sequencing of hsp65 was performed using the primers 5′-ACCAACGATGGTGTGTCCAT-3′ and 5′-CTTGTCGAACCGCATACCCT-3′ and that of 16S rRNA using the primers 5′-AGAGTTT-GATCMTGGCTCAG-3′ and 5′-CCGTCAATTCMTTTRAGTTT-3′ (Telenti et al., 1993; Springer et al., 1995). The hsp65 and 16S rRNA sequences were compared with those in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/). If the hsp65 and 16S rRNA sequences were unable to differentiate NTM species, then the *rpo*B gene sequence was employed as previously suggested (Ben et al., 2008; Adekambi et al., 2003).

Data analysis

Sensitivity, specificity, positive predictive value and negative predictive value were calculated using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA). Microsoft Office Excel 2016 (Microsoft Corporation., Redmond, WA, USA) was used to evaluate the performance of the biochip assay compared to the culture results and the median and interquartile range. Adobe Illustrator CS6 (Adobe Inc., San Jose, California, USA) was used to generate the figures.

Results

Basic characteristics

A total of 1751 specimens (1700 sputum and 51 bronchoalveolar lavage fluid) were obtained from 1751 patients. Of the 1751 samples, 66 specimens were unable to be diagnosed, neither by the biochip assay nor by culture. Three were unable to be diagnosed by the biochip assay, but were successfully cultured and identified as TB by the sequencing method. Thus, a total of 1685 culture-positive sputum samples from 7 cities of Zhejiang Province were included in the analysis. As shown in Table 1, the age of the patients ranged from 15 to 93 years with a median age of 55 years IQR (interquartile range (34, 69)). The proportion of males was 54.2%. The AFB smears were classified into 5 levels: 367 (21.8%) samples had a smear grade of scanty, 617 (36.6%) had a smear grade of 1+, 287 (17.0%) were 2+, 179 (10.6%) were 3+, and 235 (14.0%) were 4+.

Table 1
Demographic and clinical characteristics of the population studied.

Characteristics	n (%)
Age 15–93 year	1685 (100)
Median 55	IQR (34,69)
Sex	
Male	914 (54.2)
Female	771 (45.8)
Microbiological smear characteristics	
Scanty	367 (21.8)
1+	617 (36.6)
2+	287 (17.0)
3+	179 (10.6)
4+	235 (14.0)

AFB = acid-fast bacilli; IQR = interquartile range.

Table 2
Performance of biochip assay in the detection of TB and NTM from AFB positive specimens.

		Sequencing Positive	Negative	Sensitivity (95%CI)	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)
Biochip	TB	n (%)					
	Positive	1358 (80.6)	1 (0.1)	99.8 (99.3–99.9)	99.7 (98.0–1)	99.9 (99.5–1)	99.1 (97.1–99.8)
	Negative	3 (0.2)	323 (19.2)				
	NTM						
	Positive	319 (18.9)	0 (0)	98.8 (96.6–99.6)	1 (99.7–1)	1 (98.5–1)	99.7 (99.2–99.9)
	Negative	4 (0.2)	1362 (80.8)				

CI = confidence interval; PPV: positive predictive value; NPV: negative predictive value.

Comparison between the biochip assay and sequencing for the detection of TB and NTM

Of the 1685 samples, 1361 were *M. tuberculosis*, 323 were NTM and 1 was *Nocadia farcinica* according to sequencing results. According to the biochip assay, there were 1358 cases of *M. tuberculosis*, 316 cases of NTM, 3 cases were mycobacterium-free (no mycobacterium detected), 5 cases were nonidentifications (belonging to mycobacterium but unable to identify the species by biochip assay) and 3 cases were mixed infection. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of biochip assay to differentiate TB and NTM from AFB positive specimens were 99.8%, 99.7%, 99.9%, 99.1% and 98.8%, 1, 1, 99.7% respectively (Table 2). There was a good concordance between biochip assay and sequencing in the differentiation of TB and NTM from mycobacterium. It is worth mentioning that the biochip assay was unable to differentiate *M. chelonae* and *M. abscessus*, but in our study, the *M. chelonae/M. abscessus* isolates detected by biochip were all identified as *M. abscessus* through sequencing, which means that all of the *M. chelonae/M. abscessus* detected by biochip can be taken as *M. abscessus* in this study, which may reflect the status of *M. chelonae/M. abscessus* incidence in Zhejiang Province.

Ability of biochip assay to detect species of NTM

There were 319 NTM identified by the biochip system and 323 by culture (Table 3). Most of these species were *M. intracellulare* (52.5%) followed by *M. abscessus* (20.7%), *M. avium* (11.7%), *M. kansasii* (9.6%) and *M. fortuitum* (1.9%). Of the 5 nonidentification samples, 4 were identified as *M. shimoidei*, and 1 was *N. farcinica* by culture. The 15 misjudged or unable-to-identify isolates by biochip assay are shown in Table 4. Three mixed infections (*M. tuberculosis* and *M. chelonae/M. abscessus*, *M. chelonae/M. abscessus* and *M. intracellulare, M. chelonae/M. abscessus* and *M. avium*) were all finally identified as *M. abscessus* by culture. The signals obtained in these samples in the biochip assay were notably clear and strong, showing them to be co-infections (Figure 1). *M. abscessus* is a rapidly growing NTM, while *M. intracellulare*, TB and *M. avium* are slow-growing mycobacterium; thus, the culture method is less able to differentiate mixed infections. The clinical information and follow-up data of these three patients were unavailable, however, we regard those 3 mixed infections identified by biochip assay as being incorrectly diagnosed with regard to culture. Thus, the resulting concordance between biochip and mycobacterial culture was 95.4%. The proportion of mycobacteria and geographical distribution, as shown in Figure 2, range from 14.7% to 28.0%. Among these 7 cities, Taizhou city represented the highest prevalence of NTM (28.0%), which was considerably greater than that of any other city. This finding could be observed because Taizhou is a coastal city that is warm and humid; these characteristics are suitable for the propagation of NTM. In total, NTM accounted for 19.2% of all smear-positive clinical specimens in Zhejiang, China.

Table 3
Prevalence of NTM species detected by biochip assay and sequencing with clinical samples.

Identification of mycobacterial species			
Biochip assay		Sequencing	
NTM	Number (%)	NTM	Number (%)
M. intracellulare	169 (52.3)	*M. intracellulare*	170 (52.5)
M. chelonae/M. abscessus	64 (19.8)	*M. abscessus*	67 (20.7)
M. kansasii	31 (9.6)	*M. kansasii*	31 (9.6)
M. avium	40 (12.4)	*M. avium*	38 (11.7)
M. fortuitum	8 (2.5)	*M. fortuitum*	6 (1.9)
M. terrae	1 (0.3)	*M. terrae*	2 (0.6)
M. gilvum	2 (0.6)	*M. porcinum*	1 (0.3)
M. szulgai/M. malmoense	1 (0.3)	*M. colombiense*	1 (0.3)
M. chelonae/M. abscessus and M. avium	1 (0.3)	*M. shimoidei*	4 (1.2)
		M. virginiense	1 (0.3)
M. chelonae/M. abscessus and M. intracellulare	1 (0.3)	*M. paraense*	1 (0.3)
M. chelonae/M. abscessus and TB	1 (0.3)	*M. aubagnense*	1 (0.3)
Non-identification	5 (1.5)	*N.farcinica*	1 (0.3)

NTM = *nontuberculous mycobacteria*; TB = *tuberculosis*.

Discussion

China is one of the top 3 countries in the world with a high burden of TB. In the last several decades, the incidence of

Table 4
Fifteen inconsistent results compared with biochip and sequence.

Patient number	Biochip assay	Sequencing	Number
P177	*M. fortuitum*	*M. virginiense*	1
P1589	*M. avium*	*M. intracellulare*	1
P1573	*M. avium*	*M. colombiense*	1
P225	*M. gilvum*	*M. porcinum*	1
P586	*M. gilvum*	*M. aubagnense*	1
P1028	*M. szulgai/M. malmoense*	*M. paraense*	1
P669	*M. fortuitum*	*M. terrae*	1
P207, P90, P856, P916	Non-identification	*M. shimoidei*	4
P1232	Non-identification	*N. farcinica*	1
P777	*M. chelonae/M. abscessus and M. avium*	*M. abscessus*	1
P176	*M. chelonae/M. abscessus and M. intracellulare*	*M. abscessus*	1
P1221	*M. chelonae/M. abscessus and TB*	*M. abscessus*	1

TB = tuberculosis.

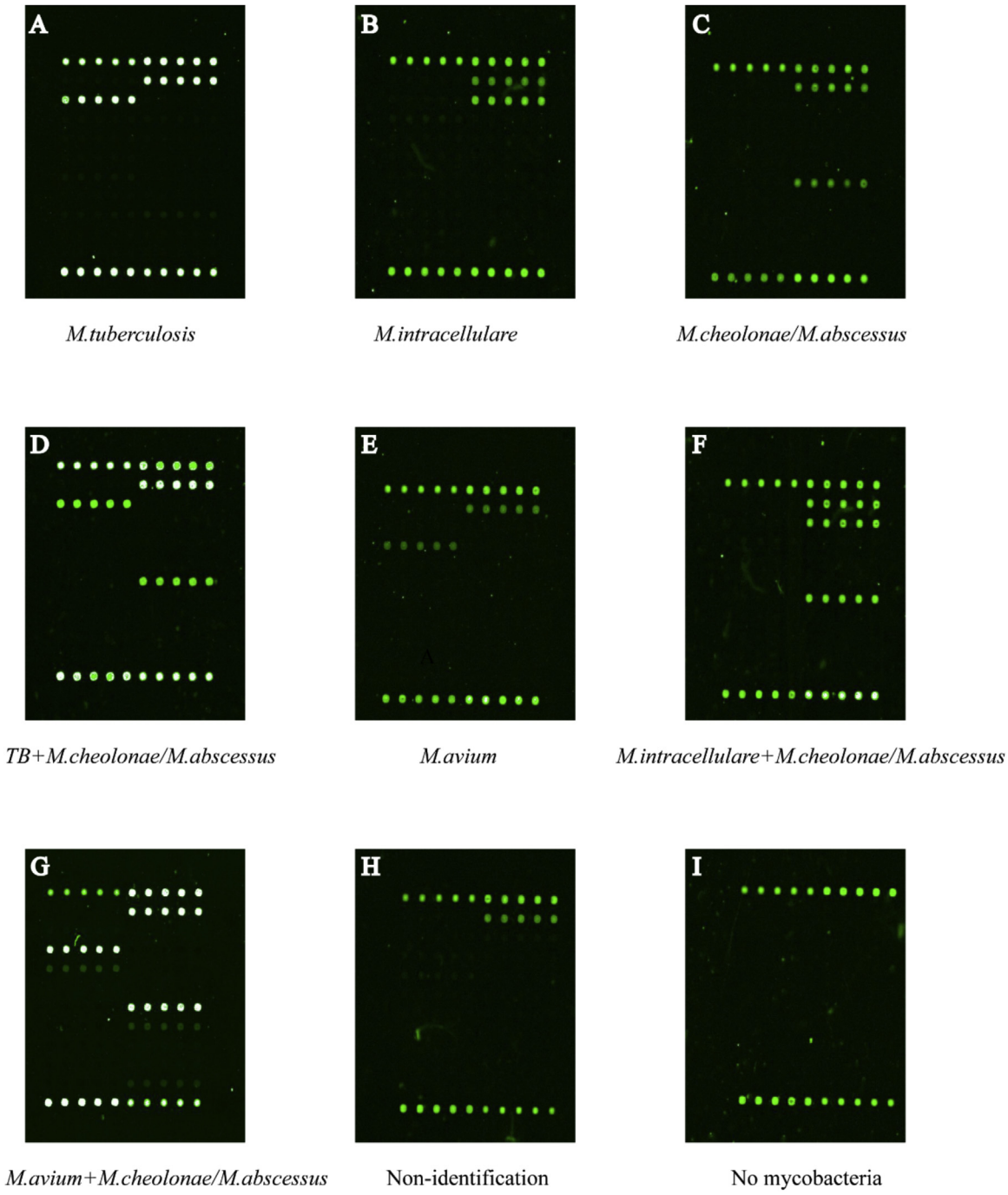

Figure 1. Different images of different mycobacteria detected by biochip assay. (A) *M. tuberculosis*. (B) *M. intracellulare*. (C) *M. chelonae*/*M. abscessus*. (D) co-infection of *M. tuberculosis* and *chelonae*/*M. abscessus*, TB = *M. tuberculosis*. (E) *M. avium*. (F) co-infection of *M. intracellulare* and *M. chelonae*/*M. abscessus*. (G) co-infection of *M. avium* and *M. chelonae*/*M. abscessus*. (H) Non-identification, nontuberculous mycobacteria that can not be detected by biochip assay. (I) no mycobacteria.

tuberculosis has decreased annually according to China's potent *tuberculosis* control policy (World_Health_Organization, 2017). Meanwhile, the prevalence of nontuberculous mycobacteria has increased greatly from 4.3% in 1979 to 11.1% in 2000 (National Technical Steering Group of the Epidemiological Sampling Survey for Tuberculosis and Office of the Nationwide Epidemiological Sampling Survey for Tuberculosis, 2002), and 22.9% in 2010 (Technical Guidance Group of the Fifth National TB Epidemiological Survey and The Office of the Fifth National TB Epidemiological Survey, 2012).

It is well-known that the treatment regimens for TB and NTM are basically different. Thus, the timely distinction of TB and NTM and the discrimination of NTM species can significantly reduce medical expenditure, treatment turnaround times, psychological burden and patient morbidity. In this study, we evaluated the performance of the biochip system on smear-positive sputum specimens from patients with suspected mycobacterial infection at multiple sites in Zhejiang, China. The sensitivity, specificity, PPV and NPV are notably high in the in the discrimination of TB and NTM from AFB-positive specimens. Moreover, the overall concordance between biochip and culture in

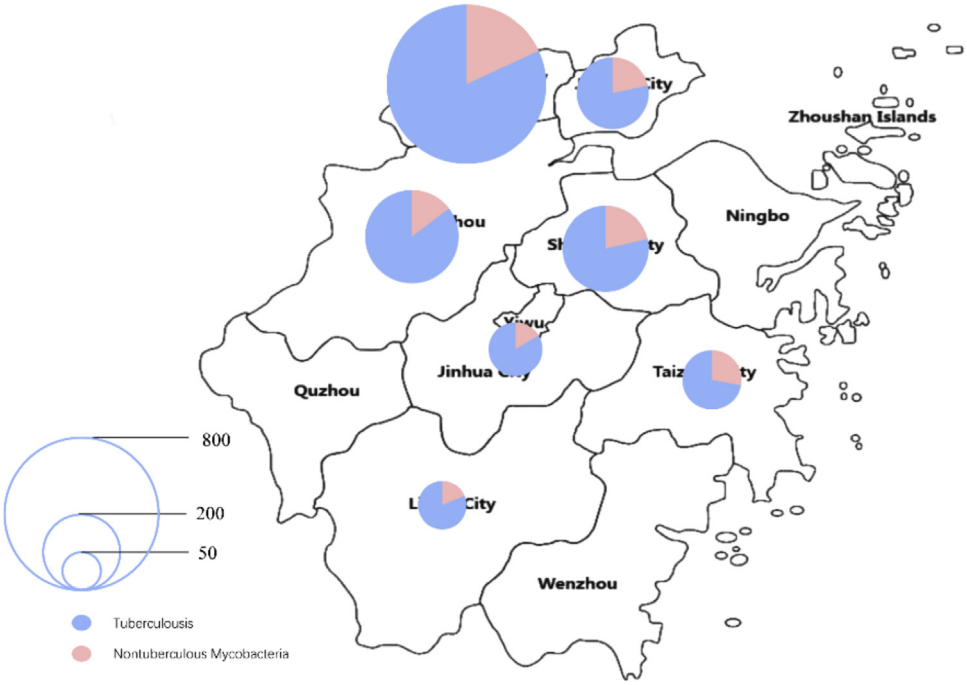

Figure 2. Distribution of cities of mycobacteria in Zhejiang, China. The size of the circles represents the number of mycobacteria infectious cases, stratified by *tuberculosis* (blue) and nontuberculous mycobacteria (pink).

the differentiation of NTM species was remarkable. The biochip assay is suitable for application to smear-positive sputum specimens in clinical settings.

It is well-accepted that culture is the gold standard of identifying TB and NTM. However, culture takes a long time, requiring 1-8 weeks or even 12 weeks to yield results (Brown-Elliott et al., 2012). Furthermore, even in mycobacterial reference laboratories, it is quite difficult to perform this assay routinely. Many molecular diagnostic methods have been introduced for the rapid identification of mycobacterial species, such as the line probe assay (LPA), DNA sequencing, PCR restriction fragment length polymorphism analysis and real-time PCR. However, most of these methods still require the isolation of clinical specimens. There are several commercial kits that can directly detect mycobacteria from clinical specimens. REBA Myco ID (YD Diagnostics, Yongin, South Korea) is based on the PCR-reverse blot hybridization assay, which can rapidly identify 20 mycobacterial species in AFB smear-positive respiratory specimens with 98.6% concordance with culture (Wang et al., 2014). Anyplex plus MTB/NTM MDR-TB assay (Seegene Inc, Seoul, South Korea) is a new molecular method based on a multiplex real-time PCR system that can detect TB, NTM and genetic determinants of drug resistance in less than 3 h (Sali et al., 2016). However, the Anyplex assay can only detect 75.0% of the NTM directly in clinical samples. Genotype CM/AS (Hain Lifescience) is one of the most widely utilized LPAs and can offer direct detection of up to 13 NTM species. Although such tests exhibit good concordance with culture, they are not clinically validated due to rather small study sizes; therefore, they have not achieved widespread acceptance to date. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry has been introduced to differentiate NTM species recently (Tudó et al., 2015; Rodríguez-Sánchez et al., 2016). This technique is regarded as a promising tool to identify NTM due to its low price, high consistency and short waiting time. However, recently, it is reported that MALDI-TOF is unable to differentiate NTM from primary cultures of respiratory samples, which ultimately limits the widespread adoption of this new technique (van Eck et al., 2016).

In this study, we not only verified the ability of biochip assay in the distinction of TB and NTM and the discrimination of NTM species but also depicted the high prevalence of NTM in Zhejiang Province. The advantages of biochip assay are as follows: first, the biochip assay is notably easy and time-saving, since it can be completed in 6 h in contrast to the 1–8 weeks required for the culture method. Second, this assay is based on testing nucleic acid of mycobacteria and not a large quantity of live bacteria. This property means there are fewer biosafety concerns compared to culture. Thirdly, the price is quite acceptable (USD 21.82 per person) compared to culture (USD 11.64 per person), which makes biochip assay a good substitute for culture, especially in poor counties and less developed areas, where mycobacterial culture cannot be routinely performed. Furthermore, in high epidemic areas, the biochip assay can be a superior way to conduct large-scale screening for TB and NTM. However, there is one major disadvantage of this study in that we only tested the performance of the biochip assay in AFB-positive specimens; we do not know the accuracy of the biochip in AFB-negative patients. Another limitation is that we only included 7 cities in this study, which may not completely represent the whole population of Zhejiang Province.

Conclusions

NTM is growing rapidly around the world, the accurate and timely identification of NTM is critically important because different species of NTM possess entirely different antibacterial spectra, and most of them are resistant to first-line anti-*tuberculosis* drugs. We conducted a multicenter and large-scale study that validated the notable ability of the biochip assay for the identification of TB and NTM. The biochip assay can be a reliable instrument for the rapid diagnosis of mycobacteria to the species level. The biochip assay may be a promising alternative to culture in the discrimination of TB and NTM species and is a method worth expanding to clinical settings across China.

Conflict of interest

The authors have no conflicts of interest to declare. This study was supported by grants from the National Natural Science Foundation of China (No. 81670567) and the National Science and Technology Major Project of the 13th Five-Year Plan of China (2017ZX10105001006002, 2017ZX10105001006001).

Authors' contributions

FH, SJF, XKJ, SGYW: conception and design of the study; ZL, SGYW, JZK, WH: acquisition of data and analysis of data; FH, SJD, WST, SSS: drafting the article; ZRH, XKJ, SJF: critical revision of the manuscript; all authors approved the final manuscript.

Ethics

This protocol was approved by the Ethics Committee of our hospital. We obtained informed consent from all patients whose sputum specimens were studied in the research.

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