Journal of Microbiological Methods 163 (2019) 105657

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Evaluation of Q Gene Mycobacteria: A novel and easy nucleic acid chromatography method for mycobacterial species identification

Kinuyo Chikamatsu[a,*], Akio Aono[a], Akiko Kawai[b], Hiroyuki Hata[b], Tomotada Iwamoto[c], Yuriko Igarashi[a], Akiko Takaki[a], Hiroyuki Yamada[a], Satoshi Mitarai[a,d]

[a] Department of Mycobacterium Reference and Research, The Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Kiyose, Japan
[b] Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan
[c] Department of Microbiology, Kobe Institute of Health, Kobe, Japan
[d] Basic Mycobacteriology, Graduate school of Biomedical Science, Nagasaki University, Nagasaki, Japan

ARTICLE INFO

Keywords:
Q Gene Mycobacteria
Mycobacterial species identification
Dipstick nucleic acid chromatography
Multiplex PCR
DNA tags

ABSTRACT

Objectives: A simple, rapid, and new diagnostic test for mycobacteria, named Q Gene Mycobacteria, has been developed. It is based on multiplex PCR using primers harbouring DNA tags combined with a dipstick nucleic acid chromatography method, which does not require the denaturation of PCR products for hybridization and can identify five species of mycobacteria including *Mycobacterium tuberculosis* complex (MTC), *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium kansasii*, and *Mycobacterium gordonae*. This study aimed to evaluate Q Gene Mycobacteria for the accurate identification of these five species.

Methods: A total of 340 mycobacterial strains/isolates were tested, of which 159 were type strains (four MTC and 155 non-tuberculosis mycobacteria (NTM) including four subspecies) and 181 were clinical isolates (18 *M. tuberculosis*, two *Mycobacterium bovis* Bacillus Calmette et Guérin (BCG), and 161 NTM comprising 16 species) collected from eight laboratories and hospitals in Japan. Species identification of NTM isolates was performed using the DNA-DNA hybridization method and/or direct sequencing of 16S rRNA, *hsp65*, and *rpoB* genes. Q Gene Mycobacteria was compared with above conventional methods for identifying the five species.

Results: Q Gene Mycobacteria showed excellent concordance for species identification, specifically 99.4% (158/159) for type strains and 99.4% (180/181) for clinical isolates. The two strains that were misidentified as *M. gordonae* were *Mycobacterium paragordonae*. As they are genetically close and there is few case reports of *M. paragordonae*, it might not be a serious critical issue to distinguish *M. paragordonae* from *M. gordonae*.

Conclusions: Q Gene Mycobacteria was able to identify frequently isolated mycobacterial species accurately and easily. Therefore, Q Gene Mycobacteria could be a useful tool for the identification of specific mycobacteria in clinical laboratories.

1. Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is still a major public health threat worldwide and mainly in developing countries. Based on the WHO global TB report 2018, approximately 10.0 million people developed tuberculosis in 2017 and 1.6 million died from the disease including 0.3 million people living with HIV; further, the 30 countries with the highest burden shared 86.9% of the global tuberculosis incidence (WHO, 2018). In contrast, the incidence of non-tuberculosis mycobacterium (NTM), and especially *Mycobacterium avium* and *Mycobacterium intracellulare*, is relatively high in many industrialized countries (Namkoong et al., 2016; Shah et al., 2016). Further, there is a difference in drug susceptibility and disease progression based on the bacterial species (Lucke et al., 2012). Even though *Mycobacterium gordonae* is frequently isolated from clinical specimens, most cases are considered contamination from the environment (Scorzolini et al., 2016). Therefore, mycobacterial diagnostics are necessary to identify NTM species isolated from clinical specimens. The base sequences of the 16S rRNA gene of all type strains are registered in several databases such as NCBI (GenBank), The European Nucleotide Archive/The European Bioinformatics Institute (EMBL-Bank), and The DNA Data Bank of Japan Center (DDBJ), and sequencing is recognized as one standard for the identification of mycobacterial species. However, as several species of mycobacteria such as *Mycobacterium kansasii* and *Mycobacterium gastri* share identical 16S rRNA gene sequences, identification requires the sequencing of

* Corresponding author at: 3-1-24 Matsuyama, Kiyose, Tokyo 204-8533, Japan.
E-mail address: chikamatsu@jata.or.jp (K. Chikamatsu).

https://doi.org/10.1016/j.mimet.2019.105657
Received 26 April 2019; Received in revised form 17 June 2019
Available online 19 June 2019

additional genes such as *hsp65*, *rpoB*, and the 16S-23S rRNA internal transcribed spacer (ITS) region. As this procedure is not cost- and time-effective, and also technically difficult, it is difficult for common clinical laboratories to perform sequencing-based identification of mycobacteria. Although the line probe assay GenoType Mycobacterium CM and AS (Hain Lifescience, Nehren, Germany) have been widely used for the identification of 31 mycobacterial species, processing takes as long as 5 h from PCR to the determination, and identification is complicated based on the combination of species-specific probe patterns (Russo et al., 2006). Several commercial nucleic acid amplification tests can also be used to detect *M. tuberculosis* complex (MTC), *M. avium*, and *M. intracellulare* in clinical specimens (Ikegame et al., 2012; Chikamatsu et al., 2016). However, they require dedicated instruments; moreover, NTM diseases other than those caused by these three species have been recently increasing (Fukano et al., 2018; Shojaei et al., 2013). Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry has been used to identify NTM species; however, protein extraction from mycobacterial strains is still complicated, and it might be difficult for ordinary clinical laboratories to introduce the required instruments based on their high cost (Rodriguez-Temporal et al., 2018; Moreno et al., 2018). Therefore, there is a need for simple, easy, and cost-effective mycobacterial species identification technology.

Q Gene Mycobacteria (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan), which will be launched soon, is a dipstick-based nucleic acid chromatography method based on multiplex PCR that employs DNA-tagged primers and DNA–DNA hybridization on a membrane strip. There have been several studies describing detection systems using this technology (Nagai et al., 2016; Yamamuro et al., 2018). In Q Gene Mycobacteria, multiplex PCR is performed using five primer pairs for the identification of five *Mycobacterium* species including MTC, *M. avium*, *M. intracellulare*, *M. kansasii*, and *M. gordonae*, in addition to a primer pair used as an internal amplification control. The DNA tag attached to the forward primer is complementary to a gold nanoparticle-labeled probe, and that of the reverse primer is complementary to a specific probe for each mycobacterial species immobilized on the strip membrane. The DNA tag is connected the primer with a spacer to stop the extension by DNA polymerase. After multiplex PCR, the DNA tag associated with the amplification product and the gold nanoparticle-labeled probe attached to the membrane hybridize and move upstream. Next, the specific DNA probes capture the DNA tags of the reverse primers, and the five species can be identified based on the position of the colored lines (Fig. 1). Q Gene Mycobacteria processing takes 1 h from PCR to the end result.

Here, we describe the first evaluation of Q Gene Mycobacteria for the identification of target species using type strains and clinical isolates.

2. Material and methods

2.1. Mycobacterial strain identification and culture

A total of 340 mycobacterial strains/isolates were tested, of which 159 were type strains (four MTC and 155 NTM including four subspecies; Supplementary Table S1) and 181 were clinical isolates (18 *M. tuberculosis*, two *Mycobacterium bovis* Bacillus Calmette et Guérin (BCG), and 161 NTM comprising 16 species; Table 1). The clinical isolates were collected from commercial laboratories, hospitals, and an institute of health in Japan as follows: Miroku Medical Laboratory Co., Ltd. (Saku, Nagano, Japan), Bio Medical Laboratories, Inc. (Kawagoe, Saitama, Japan), LSI Medience Co. (Itabashi, Tokyo, Japan), SRL Inc. (Hachioji, Tokyo, Japan), National Hospital Organization Tokyo National Hospital (Kiyose, Tokyo, Japan), Kawasaki Municipal Ida Hospital (Kawasaki, Kanagawa, Japan), Fukujuji Hospital (Kiyose, Tokyo, Japan), and Kobe Institute of Health (Kobe, Hyogo, Japan). The type strains and clinical isolates were stored at −80 °C and subcultured in MycoBroth (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) at the appropriate temperatures (25–37 °C, depending on the species) until the OD reached 0.1–0.2 at 530 nm. MTC isolates were identified using Capilia TB-Neo (TAUNS, Izunokuni-shi, Shizuoka, Japan), and additional multiplex PCR analyses were performed to identify the region of difference to distinguish *M. tuberculosis* from *M. bovis* BCG as previously described (Talbot et al., 1997). Species identification of NTM isolates was performed using the DNA-DNA hybridization method (DDH Mycobacteria Kit, Kyokuto Pharmaceutical Industrial Co., Ltd. Tokyo, Japan) and/or direct sequencing of 16S rRNA, *hsp65*, and *rpoB* genes, as previously described (Springer et al., 1996; Brunello et al., 2001; Kim et al., 1999). The strains identified as *M. kansasii* were submitted to direct sequencing of the *hsp65* gene and 16S−23S rRNA ITS to determine subtypes (Iwamoto and Saito, 2006).

2.2. DNA extraction

Bacterial DNA was extracted using the Kaneka Easy DNA extraction kit (KANEKA Co., Osaka, Japan). Briefly, 20 µl of reagent A was added to 20 µl of the bacteria growing in MycoBroth and heated at 98 °C for 10 min. Next, 80 µl of reagent B was added to the suspension. The solution containing extracted DNA was stored at −20 °C until PCR amplification was performed.

2.3. PCR and Q Gene Mycobacteria

The PCR reaction (20 µl in total) contained 5 µl of DNA template, 10 µl of PCR master mix, and 5 µl of primers with a DNA tag mix at 175–300 nM (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan). The target genes of each species were as follows: IS*6110* for MTC and ITS for *M. gordonae*; those of *M. kansasii*, *M. avium*, and *M. intracellulare* are the subject of a patent application. The thermal cycling profile was as follows: 25 °C for 5 min, 94 °C for 1 min, followed by 35 cycles of 94 °C for 5 s, 60 °C for 10 s, and 72 °C for 15 s. After mixing the amplified PCR product with 160 µl of developing solution, the Q Gene Mycobacteria strip was placed into the sample. Test results were available within 10 min and were indicated by red lines in the specific test area for each species including MTC, *M. kansasii*, *M. avium*, *M. intracellulare*, and *M. gordonae* (Fig. 2). If the control line does not develop color within 10 min, it indicates that the amplification process was not successful. When the control line and the specific test lines develop color, five mycobacterial species can be identified.

3. Results

3.1. Type strains

The 159 type strains (62 *Mycobacterium*, 11 *Mycolicibacter*, three *Mycolicibacillus*, five *Mycobacteroides*, and 78 *Mycolicibacterium*) were tested by Q Gene Mycobacteria. MTC (*M. tuberculosis*, *Mycobacterium africanum*, *M. bovis*, and *Mycobacterium microti*), *M. kansasii*, *M. avium* (*Mycobacterium avium* subsp. *avium*, *Mycobacterium avium* subsp. *paratuberculosis*, and *Mycobacterium avium* subsp. *silvaticum*), *M. intracellulare*, and *M. gordonae* were identified correctly. The remaining 148 type strains were negative except for *Mycobacterium paragordonae*, which was identified as *M. gordonae*. *Mycobacterium arosiense*, *Mycobacterium bouchedurhonense*, *Mycobacterium chimaera*, *Mycobacterium colombiense*, *Mycobacterium marseillense*, *Mycobacterium timonense*, *Mycolicibacterium vulneris*, and *Mycobacterium yongonense*, which have been regarded as members of the previous *M. avium–intracellulare* complex (MAC) and are presently independent species, were negative based on the Q Gene Mycobacteria test. Thus, the concordance of species identification was 99.4% (158/159) for type strains using this assay.

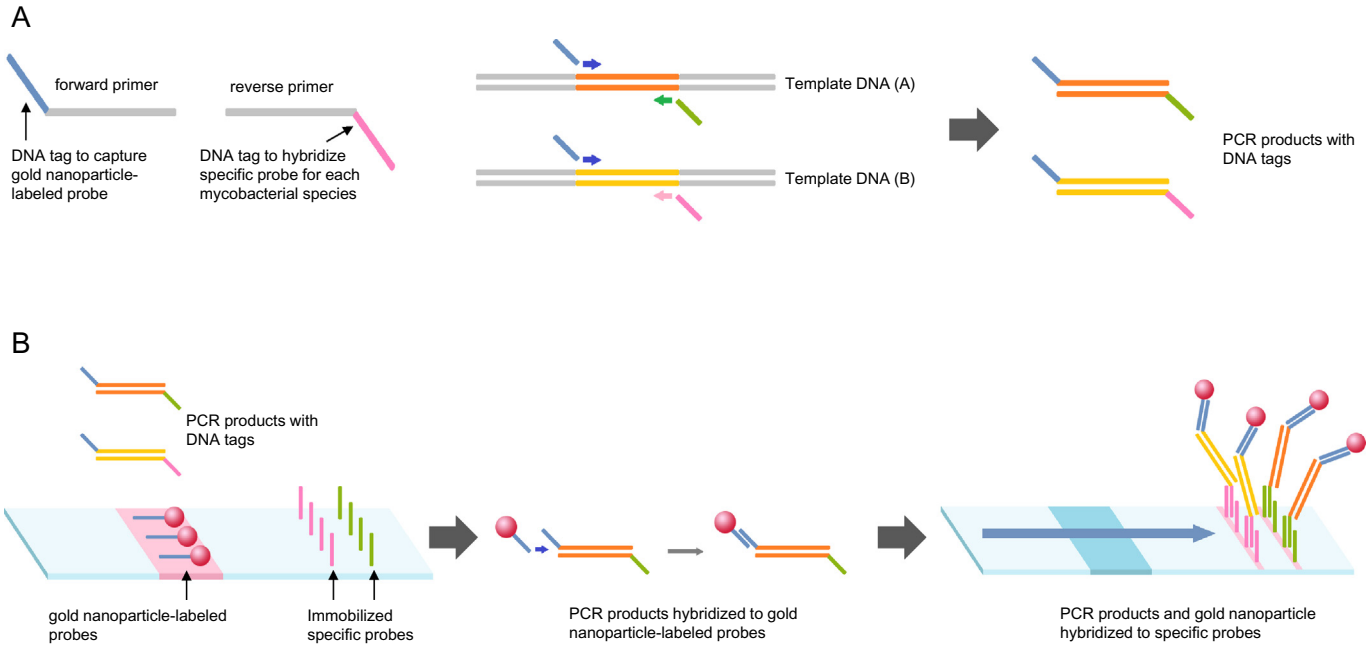

Fig. 1. Mechanism of identification by Q Gene Mycobacteria. (A) Design of primer and multiplex PCR process: A DNA tag to capture a gold nanoparticle-labeled probe and a DNA tag to hybridize a specific probe (MTC, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. gordonae* and internal amplification control) on the strip membrane attach to the forward and the reverse primer, respectively. The DNA tag connect the primer with a spacer to stop the extension of DNA polymerase. The target genes in template DNA amplify by multiplex PCR using the DNA tag-attached primer sets. (B) Identification process: the DNA tag associated with the PCR product and the gold nanoparticle-labeled probe attached to the membrane hybridize and move upstream. The six specific DNA probes immobilized at different positions on the strip membrane hybridize the specific DNA tags of the PCR product, and the five mycobacterial species can be identified based on the position of the colored lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Clinical isolates used in this study and identification by Q Gene Mycobacteria.

No. of isolates	Organism	Q gene Mycobacteria result	
		Hybridization patterns	Interpretation
18	*Mycobacterium tuberculosis*	AC, MTC	*M. tuberculosis* complex
2	*Mycobacterium bovis* BCG[a]	AC, MTC	*M. tuberculosis* complex
19	*Mycobacterium avium*	AC, AV	*M. avium*
20	*Mycobacterium intracellulare*	AC, IN	*M. intracellulare*
29	*Mycobacterium kansasii*[b]	AC, KA	*M. kansasii*
19	*Mycobacterium gordonae*	AC, GO	*M. gordonae*
10	*Mycobacteroides abscessus*	AC	No identification
10	*Mycobacteroides chelonae*	AC	No identification
10	*Mycolicibacterium fortuitum*	AC	No identification
2	*Mycobacterium gastri*	AC	No identification
10	*Mycobacterium lentiflavum*	AC	No identification
5	*Mycobacterium marinum*	AC	No identification
13	*Mycolicibacterium mucogenicum* or *Mycolicibacterium phocaicum*	AC	No identification
1	*Mycobacterium paragordonae*	AC, GO	*M. gordonae*
10	*Mycolicibacterium peregrinum*	AC	No identification
3	*Mycobacterium triplex*	AC	No identification

AC, amplification control; MTC, *M. tuberculosis* complex; AV, *M. avium*, IN, *M. intracellulare*; KA, *M. kansasii*; GO, *M. gordonae*.

[a] Substrain Tokyo (1), substrain Connaught (1).

[b] Subtype I (19), intermediate subtype I (2), subtype II (2), atypical subtype II (2), subtype III (1), subtype VI (3).

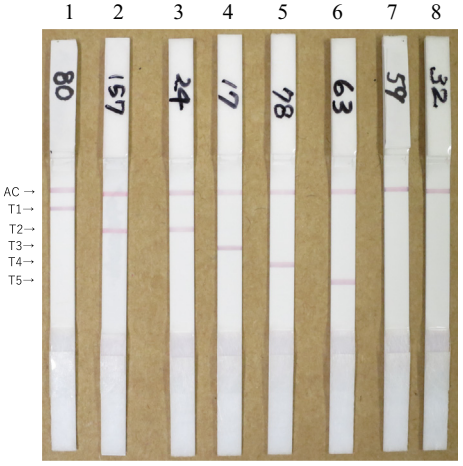

Fig. 2. Hybridization patterns observed with Q Gene Mycobacteria. AC, amplification control; T1 to T5, specific identification lines. Typical hybridization patterns are shown in the figure as follows: lane 1 = *Mycobacterium kansasii* (ATCC12478); lane 2 = *Mycobacterium tuberculosis* (ATCC27294); lane 3 = *Mycobacterium bovis* (ATCC19210); lane 4 = *Mycobacterium avium* subsp. *avium* (ATCC25291); lane 5 = *Mycobacterium intracellulare* (ATCC13950); lane 6 = *Mycobacterium gordonae* (ATCC14470); lane 7 = *Mycobacterium gastri* (ATCC15754); lane 8 = *Mycobacterium chimaera* (JCM14737).

3.2. Clinical isolates

Using Q Gene Mycobacteria, 107 of 181 clinical isolates (16 species), comprising MTC including *M. bovis* BCG, *M. kansasii, M. avium*, *M. intracellulare*, and *M. gordonae* were correctly identified. Further, for 73 of the remaining 74 isolates, amplification probes were positive, but all species probes were negative. Finally, one *M. paragordonae* isolate was misidentified as *M. gordonae*. Thus, the concordance of species identification was 99.4% (180/181) for clinical isolates using Q Gene Mycobacteria (Table 1).

4. Discussion

In this study, Q Gene Mycobacteria was evaluated for its ability to identify mycobacterial species. The originality of Q Gene Mycobacteria is based on the fact that it can specifically distinguish five species that are commonly isolated in clinical settings, specifically MTC, *M. avium*, *M. intracellulare*, *M. kansasii*, and *M. gordonae*, from other mycobacterial species (Henkle et al., 2015; Morimoto et al., 2017). This assay is based on a simple nucleic acid chromatography technique that can use the PCR product directly without denaturation.

The cross-reactivity of this method was investigated using type strains and clinical isolates. Of 159 type strains, one, namely *M. paragordonae*, was misidentified as *M. gordonae*. Sequence similarities of 16S rRNA, *hsp65*, and *rpoB* genes between *M. gordonae* and *M. paragordonae* were previously reported to be 99.0%, 95.9%, and 95.1%, respectively (Kim et al., 2014). As the two species are genetically close, *M. paragordonae* was misidentified as *M. gordonae* by Q Gene Mycobacteria. In addition, there is few case reports of *M. paragordonae*, it might not be a serious critical issue to distinguish *M. paragordonae* from *M. gordonae* (Cheung et al., 2017). However, it has been reported that several commercial kits sometimes misidentify genotypically close species. GenoType NTM–DR can distinguish *M. chimaera* from *M. intracellulare*; however, *M. arosiense*, *M. timonense*, *M. bouchedurhonense*, and *M. marseillense* were all found to be misidentified as *M. intracellulare* (Mok et al., 2017). Similarly, we observed that *M. arosiense* (DSM 45069), *M. chimaera* (JCM 14737), *M. colombiense* (JCM 16228), *M. marseillense* (JCM 17324), *M. vulneris* (JCM 18115), and *M. yongonense* (DSM 45126) were misidentified as *M. intracellulare* using the TRCReady® MAC test, which is based on a transcription reverse-transcription concerted reaction targeting the 16S rRNA gene (Chikamatsu et al., 2016). *Mycobacterium lentiflavum* was also misidentified as *M. intracellulare* by the COBAS TaqMan MAI (Tomita et al., 2014). We also reported that *M. arosiense*, *M. chimaera*, and some other *M. intracellulare* complex species are misidentified. It has been reported that these species are isolated from clinical specimens, which respiratory samples, osteomyelitic bone lesion and blood (Tortoli et al., 2004; Murcia et al., 2006; Ben Salah et al., 2009). The identification for the isolates will take an extended time for sequencing in a reference laboratory. In contrast, with Q Gene Mycobacteria, this species did not cross-react with the target probe of *M. intracellulare*, and it would be more useful to screen out the real *M. intracellulare* and then the others could be sequenced to determine the clinical relevance of the other species. Therefore, it is suggested that the identification of *M. intracellulare* by Q Gene Mycobacteria is more reliable compared to that with other methods used to identify these five species.

We observed weak color formation for species-specific probes when using seven clinical isolates, specifically five *M. kansasii* (belonging to four subtypes: one intermediate type I, one type II, one atypical type II, and two type VI), one *M. intracellulare*, and one *M. paragordonae*. As the intensity of color was determined by the naked eye, it was difficult to recognize weak color when assaying these isolates. Further, the color intensity when using species-specific probes for these strains was paler than that with the control probes, which served as an internal control to confirm whether the amplification was properly performed. Thus, for these cases, the control probes could not be used as a reference for interpretation. Generally, multiplex PCR combines an enzyme with optimal speed and primers that have high annealing specificity. Therefore, when a single nucleotide polymorphism is present at the priming site of the template, the amplification efficiency decreases, and the amount of PCR product could be reduced. It has been demonstrated that the presence of a single mismatch in the second half of the primer extension sequence can result in an underestimation of gene copy number by up to 1000-fold (Bru et al., 2008). It was believed that the priming sites for these strains differ by a few bases compared to the primers of Q Gene Mycobacteria. It has been reported that *M. kansasii* subtype I, II, III, and IV, but not subtype VI, can be detected using AccuProbe (Richter and Niemann, 1999). In this study, we found that even if the species-specific probe is slightly colored, it must be interpreted as positive.

There already is a similar nucleic chromatography technology to identify several mycobacterial species. SPEED-OLIGO Mycobacteria (Vircell, Spain) is a method based on conventional nucleic acid chromatography (Ramis et al., 2015). The kit requires heating for denaturation of PCR products to convert double-stranded DNA into single-stranded products and hybridization to amplified DNA with species-specific probes immobilized on the membrane. With SPEED-OLIGO Mycobacteria, the efficiency of hybridization could be reduced since some denatured single-strand DNA will ultimately reanneal to from double-stranded DNA. In contrast, Q Gene Mycobacteria is based on the hybridization of a single-stranded DNA tag added to the PCR products with a probe comprising a sequence that is complementary to the DNA tag, which is immobilized on a membrane; with this, the denaturation of PCR products is unnecessary. In fact, Q Gene Mycobacteria has less steps and more efficiency compared to SPEED-OLIGO Mycobacteria. As a result, Q Gene Mycobacteria developed more distinct color compared to SPEED-OLIGO Mycobacteria.

This study had some limitations. First, we tested only 16 species of clinical isolates, and thus, additional research might be needed with not only the type strains but also rare clinical isolates that are genetically close to the target species of the kit, such as *M. arosiense*, *M. chimaera, M colombiense*, *M. marseillense*, *M. vulneris*, and *M. yongonense*. However, we could not test clinical isolates of these species because we did not isolate them until 2016. Second, fewer mycobacterial species can be identified by Q Gene Mycobacteria compared to that with other commercial kits. Recently, infections caused by NTM have been increasing in industrialized countries; especially, *Mycobacteroides abscessus* is often isolated as a causative agent of respiratory infection (Namkoong et al., 2016; Novosad et al., 2016). It is thus believed that the addition of a *M. abscessus*-specific probe to this Q Gene Mycobacteria assay will be useful for the diagnosis of respiratory NTM infections.

In conclusion, Q Gene Mycobacteria allowed for the accurate differentiation of five targeted species from others, with the exception of *M. paragordonae*. As it was simple, rapid, and feasible without special instruments, it could be appropriate for use in ordinary clinical laboratories.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.105657.

Funding

This study was supported by research grants from Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan.

Declaration of interest

Dr. Hiroyuki Hata and Ms. Akiko Kawai are employees of Kyokuto Pharmaceutical Industrial Co., Ltd. Other authors have no conflicts of interest to declare.

Acknowledgments

We thank Miroku Medical Laboratory Co., Ltd., Bio Medical Laboratories, Inc., LSI Medience Co., SRL Inc., National Hospital Organization Tokyo National Hospital, Kawasaki Municipal Ida Hospital, Fukujuji Hospital, and Kobe Institute of Health for providing various clinical isolates.

References

Ben Salah, I., Cayrou, C., Raoult, D., Drancourt, M., 2009. *Mycobacterium marseillense* sp. nov., *Mycobacterium timonense* sp. nov. and *Mycobacterium bouchedurhonense* sp. nov., members of the *Mycobacterium avium* complex. Int. J. Syst. Evol. Microbiol. 59,

2803–2808.
Bru, D., Martin-Laurent, F., Philippot, L., 2008. Quantification of the detrimental effect of a single primer-template mismatch by real-time PCR using the 16S rRNA gene as an example. Appl. Environ. Microbiol. 74, 1660–1663.
Brunello, F., Ligozzi, M., Cristelli, E., Bonora, S., Tortoli, E., Fontana, R., et al., 2001. Identification of 54 mycobacterial species by PCR-restriction fragment length polymorphism analysis of the hsp65. Gene 39, 2799–2806.
Cheung, C.Y., Cheng, N.H.Y., Ting, W.M., Chak, W.L., 2017. *Mycobacterium paragordonae*: a rare cause of peritonitis in a peritoneal dialysis patient. Clin. Nephrol. 88, 371–372.
Chikamatsu, K., Igarashi, Y., Aono, A., Yamada, H., Takaki, A., Mitarai, S., 2016. Specificity evaluation of TRCReady® MTB and TRCReady® MAC for identifying *Mycobacterium tuberculosis* complex, *Mycobacterium avium* and *Mycobacterium intracellulare*. Kekkaku 91, 623–629.
Fukano, H., Yoshida, M., Kazumi, Y., Fujiwara, N., Katayama, K., Ogura, Y., et al., 2018. *Mycobacterium shigaense* sp. nov., a slow-growing, scotochromogenic species, is a member of the *Mycobacterium simiae* complex. Int. J. Syst. Evol. Microbiol. 2437–2442.
Henkle, E., Hedberg, K., Schafer, S., Novosad, S., Winthrop, K.L., 2015. Population-based incidence of pulmonary nontuberculous mycobacterial disease in Oregon 2007 to 2012. Ann. Am. Thorac. Soc. 12, 642–647.
Ikegame, S., Sakoda, Y., Fujino, N., Taguchi, K., Kawasaki, M., Kajiki, A., 2012. Clinical evaluation of COBAS TaqMan PCR for the detection of *Mycobacterium tuberculosis* and *M. avium* complex. Tuberc. Res. Treat. 2012, 1–5.
Iwamoto, T., Saito, H., 2006. Comparative study of two typing methods, *hsp65* PRA and ITS sequencing, revealed a possible evolutionary link between *Mycobacterium kansasii* type I and II isolates. FEMS Microbiol. Lett. 254, 129–133.
Kim, B.J., Lee, S.H., Lyu, M.A., Kim, S.J., Bai, G.H., Chae, G.T., et al., 1999. Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (*rpoB*). J. Clin. Microbiol. 37, 1714–1720.
Kim, B.J., Hong, S.H., Kook, Y.H., Kim, B.J., 2014. *Mycobacterium paragordonae* sp. nov., a slowly growing, scotochromogenic species closely related to *Mycobacterium gordonae*. Int. J. Syst. Evol. Microbiol. 64, 39–45.
Lucke, K., Hombach, M., Friedel, U., Ritter, C., Böttger, E.C., 2012. Automated quantitative drug susceptibility testing of non-tuberculous mycobacteria using MGIT 960/EpiCenter TB eXiST. J. Antimicrob. Chemother. 67, 154–158.
Mok, S., Rogers, T.R., Fitzgibbon, M., 2017. Evaluation of GenoType NTM–DR assay for identification of *Mycobacterium chimaera*. J. Clin. Microbiol. 55, 1821–1826.
Moreno, E., Miller, E., Miller, E., Totty, H., Deol, P., 2018. A novel liquid media mycobacteria extraction method for MALDI-TOF MS identification using VITEK® MS. J. Microbiol. Methods 144, 128–133.
Morimoto, K., Hasegawa, N., Izumi, K., Namkoong, H., Uchimura, K., Yoshiyama, T., et al., 2017. A laboratory-based analysis of nontuberculous mycobacterial lung disease in Japan from 2012 to 2013. Ann. Am. Thorac. Soc. 14, 49–56.
Murcia, M.I., Tortoli, E., Menendez, M.C., Palenque, E., Garcia, M.J., 2006. *Mycobacterium colombiense* sp. nov., a novel member of the *Mycobacterium avium* complex and description of MAC-X as a new ITS genetic variant. Int. J. Syst. Evol. Microbiol. 56, 2049–2054.
Nagai, S., Miyamoto, S., Ino, K., Tajimi, S., Nishi, H., Tomono, J., 2016. Easy detection of multiple *Alexandrium* species using DNA chromatography chip. Harmful Algae 51, 97–106.
Namkoong, H., Kurashima, A., Morimoto, K., Hoshino, Y., Hasegawa, N., Ato, M., et al., 2016. Epidemiology of pulmonary nontuberculous mycobacterial disease, Japan1. Emerg. Infect. Dis. 22, 1116–1117.
Novosad, S.A., Beekmann, S.E., Polgreen, P.M., Mackey, K., Winthrop, K.L., 2016. Treatment of *Mycobacterium abscessus* infection. Emerg. Infect. Dis. 22, 511–514.
Ramis, I.B., Cnockaert, M., Von Groll, A., Mathys, V., Simon, A., Tortoli, E., et al., 2015. Evaluation of the speed-oligo mycobacteria assay for the identification of non-tuberculous mycobacteria. J. Med. Microbiol. 64, 283–287.
Richter, E., Niemann, S., 1999. Ru S. identification of *Mycobacterium kansasii* by using a DNA probe (AccuProbe) and molecular techniques. J. Clin. Microbiol. 37, 964–970.
Rodriguez-Temporal, D., Perez-Risco, D., Struzka, E.A., Mas, M., Alcaidea, F., 2018. Evaluation of two protein extraction protocols based on freezing and mechanical disruption for identifying nontuberculous mycobacteria by matrix-assisted laser desorption ionization–time of flight mass spectrometry from liquid and solid cultures. J. Clin. Microbiol. 56 (pii: e01548-17).
Russo, C., Tortoli, E., Menichella, D., 2006. Evaluation of the new GenoType Mycobacterium assay for identification of mycobacterial species. J. Clin. Microbiol. 44, 334–339.
Scorzolini, L., Mengoni, F., Mastroianni, C.M., Baldan, R., Cirillo, D.M., De Giusti, M., et al., 2016. Pseudo-outbreak of *Mycobacterium gordonae* in a teaching hospital: importance of strictly following decontamination procedures and emerging issues concerning sterilization. New Microbiol. 39, 35–44.
Shah, N.M., Davidson, J.A., Anderson, L.F., Lalor, M.K., Kim, J., Thomas, H.L., et al., 2016. Pulmonary *Mycobacterium avium-intracellulare* is the main driver of the rise in non-tuberculous mycobacteria incidence in England, Wales and Northern Ireland, 2007-2012. BMC Infect. Dis. 16, 1–6.
Shojaei, H., Daley, C., Gitti, Z., Hashemi, A., Heidarieh, P., Moore, E.R.B., et al., 2013. *Mycobacterium iranicum* sp. nov., a rapidly growing scotochromogenic species isolated from clinical specimens on three different continents. Int. J. Syst. Evol. Microbiol. 63, 1383–1389.
Springer, B., Stockman, L., Teschner, K., Roberts, G.D., Böttger, E.C., 1996. Two-laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods. J. Clin. Microbiol. 34, 296–303.
Talbot, E.A., Williams, D.L., Frothingham, R., 1997. PCR identification of *Mycobacterium bovis* BCG. J. Clin. Microbiol. 35, 566–569.
Tomita, M., Yoshida, S., Tuyuguchi, K., Suzuki, K., Okada, M., Hayashi, S., 2014. Genetic analysis reveals misidentification of *Mycobacterium lentiflavum* as *Mycobacterium intracellulare* by the COBAS TaqMan MAI test. Kekkaku 89, 703–709.
Tortoli, E., Rindi, L., Garcia, M.J., Chiaradonna, P., Dei, R., Garzelli, C., et al., 2004. Proposal to elevate the genetic variant MAC-A, included in the *Mycobacterium avium* complex, to species rank as *Mycobacterium chimaera* sp. nov. Int. J. Syst. Evol. Microbiol. 54, 1277–1285.
WHO, 2018. Global Tuberculosis Report 2018. World Health Organization, Genova, Switzerland. http://apps.who.int/iris/bitstream/handle/10665/274453/9789241565646-eng.pdf.
Yamamuro, T., Miyamoto, S., Kitamura, M., Muro, T., Iwata, Y.T., Segawa, H., et al., 2018. Development of simple and accurate detection systems for *Cannabis sativa* using DNA chromatography. Forensic Sci. Int. 291, 68–75.