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Evaluation of GenoType NTM-DR Assay for Identification of *Mycobacterium chimaera*

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ABSTRACT Identification of species within the *Mycobacterium avium* complex (MAC) is difficult, and most current diagnostic laboratory tests cannot distinguish between species included in the complex. Differentiation of species within the MAC is important, as *Mycobacterium chimaera* has recently emerged as a major cause of invasive cardiovascular infections following open heart surgery. A new commercial diagnostic assay, GenoType NTM-DR ver. 1.0, is intended to differentiate between three species within the MAC, namely, *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium chimaera*. In this study, we investigated an archival collection of 173 MAC isolates using 16S rRNA and 16S-23S internal transcribed spacer (ITS) gene sequencing, and GenoType NTM-DR was evaluated for identifying *M. chimaera* and other species belonging to the MAC. Species identification of 157/173 (91%) isolates with the GenoType NTM-DR assay was in agreement with 16S rRNA and 16S-23S ITS gene sequencing results. Misidentification occurred with 16 isolates which belonged to four species included in the MAC that are rarely encountered in clinical specimens. Despite some limitations of this assay, GenoType NTM-DR had 100% specificity for identifying *M. chimaera*. This novel assay will enable diagnostic laboratories to differentiate species belonging to the *Mycobacterium avium* complex and to accurately identify *M. chimaera*. It can produce rapid results and is also more cost efficient than gene sequencing methods.

KEYWORDS GenoType NTM-DR, *Mycobacterium avium* complex, *Mycobacterium chimaera*, genotypic identification

Mycobacterium chimaera is a slow-growing nontuberculous mycobacterium recently recognized as a novel species within the *Mycobacterium avium* complex (MAC) (1). Similarly to *Mycobacterium avium* and *Mycobacterium intracellulare*, *M. chimaera* is associated with infections primarily in immunosuppressed patients and in those with an underlying lung disease (2–4). However, identifying *M. chimaera* as the cause of these infections is difficult, as it is often misidentified as *M. intracellulare* using current phenotypic and molecular tests (5). Many commercial assays such as GenoType Mycobacterium CM (Hain Lifescience, Nehren, Germany), AccuProbe MAC culture identification test (Genprobe, United States), and Inno LiPA Mycobacteria (Immunogenetics, Ghent, Belgium) cannot distinguish between these two closely related species (6).

The importance of accurate identification of *M. chimaera* is highlighted by its recent emergence as a major cause of life-threatening cardiovascular infections following open heart or vascular surgery (5, 7–11). Recent outbreaks of invasive *M. chimaera* infections following cardiac surgery have been reported in several countries in Europe and in the United States (10–13). Investigations in Switzerland have demonstrated an airborne transmission route of *M. chimaera* infection, whereby aerosols from contaminated water in heater-cooler units are transmitted to the patient during open heart surgery (12). Following these reports, many mycobacteriology reference laboratories

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TABLE 1 Comparison of gene sequencing methods and GenoType NTM-DR for the identification of 173 *Mycobacterium avium* complex species

No. of strains	Gene sequencing result		GenoType NTM-DR result	
	16S rRNA	16S-23S ITS	Band patterns[a]	Identification
29	*M. avium*	*M. avium*	CC, UC, SP1	*M. avium*
49	*M. intracellulare*	*M. intracellulare*	CC, UC, SP2	*M. intracellulare*
79	*M. chimaera*	*M. chimaera*	CC, UC, SP2, SP3	*M. chimaera*
2	MAC[b]	*M. arosiense*	CC, UC, SP2	*M. intracellulare*
3	MAC	*M. timonense*	CC, UC, SP2	*M. intracellulare*
5	MAC	*M. bouchdurhonense*	CC, UC, SP2	*M. intracellulare*
6	MAC	*M. marseillense*	CC, UC, SP2	*M. intracellulare*

[a]A faint band was produced in the universal control for all the isolates tested, and only bands with intensities stronger than the universal control were considered. CC, conjugate control probe; UC, universal control probe; SP1 to SP3, species-specific probes.
[b]MAC, *Mycobacterium avium* complex.

are assisting national investigations in identification of clinical and environmental isolates of *M. chimaera* (12, 14).

To date, accurate identification of *M. chimaera* has required complex molecular techniques based on sequencing the 16S rRNA gene and/or the 16S-23S internal transcribed spacer (ITS) region (1, 5). GenoType NTM-DR ver. 1.0 (Hain Lifescience, Nehren, Germany) is a new commercial assay based on reverse hybridization of a PCR product to a nitrocellulose strip containing immobilized probes which are specific for species belonging to the MAC, as well as to the *Mycobacterium abscessus* complex. The GenoType NTM-DR ver. 1.0 assay targets the 23S rRNA gene region for the identification of species belonging to the MAC, whereas the *erm* gene is used to differentiate between members of the *M. abscessus* complex. The binding of the amplified product to a specific probe results in the development of a colored band on the strip which allows the species to be identified by its banding pattern.

The aim of this study was to identify *M. chimaera* isolates among MAC isolates in a retrospective collection that were previously identified to the species level with the GenoType Mycobacterium CM assay. In this study, gene sequencing methods were used to reidentify MAC species and the specificity of the GenoType NTM-DR assay for identifying species belonging to the MAC was determined.

RESULTS

Sequencing of both 16S rRNA and ITS genes reidentified 79/144 (55%) of our *M. intracellulare* isolates as *M. chimaera*. All nonclinical isolates were also identified as *M. chimaera* by sequencing the two gene regions. However, only 49/144 (34%) of *M. intracellulare* identifications by 16S rRNA and ITS gene sequencing were in concordance with the original identifications that were made with the GenoType Mycobacterium CM assay. On the other hand, ITS gene sequencing identified 16/144 (11%) as other species, including *Mycobacterium arosiense* ($n = 2$), *Mycobacterium timonense* ($n = 3$), *Mycobacterium bouchedurhonense* ($n = 5$), and *Mycobacterium marseillense* ($n = 6$). All 29 *M. avium* isolates previously identified by the GenoType Mycobacterium CM assay were also in 100% agreement with 16S rRNA and ITS gene sequencing results. Following this, the 173 MAC isolates were tested with the GenoType NTM-DR assay.

The GenoType NTM-DR assay correctly identified 157/173 (91%) of MAC isolates to the species level, while misidentification occurred in 16 cases (Table 1). Results for all 79 *M. chimaera*, 49 *M. intracellulare*, and 29 *M. avium* isolates were in 100% agreement with 16S rRNA and ITS gene sequencing results. Misidentification of isolates occurred with 2 *M. arosiense* strains, 3 *M. timonense* strains, 5 *M. bouchedurhonense* strains, and 6 *M. marseillense* strains which were identified only by ITS gene sequencing. Although the GenoType NTM-DR assay does not contain specific probes for these uncommon species, the hybridization patterns identified them as *M. intracellulare*. The sensitivity and specificity of the GenoType NTM-DR assay for the identification of *M. chimaera* were 100%.

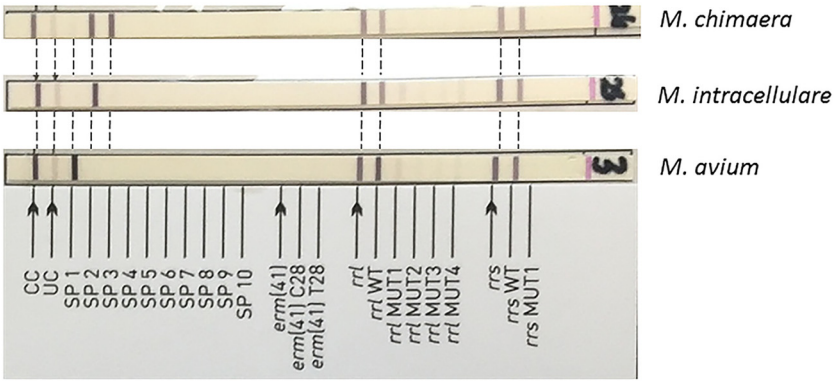

FIG 1 GenoType NTM-DR test strips of *M. chimaera*, *M. intracellulare*, and *M. avium*. All 79 *M. chimaera*, 49 *M. intracellulare*, and 29 *M. avium* isolates produced the correct species banding pattern. However, the universal control (UC) band were very faint in all the isolates tested and only bands with intensities stronger than those seen with the UC were considered. No antibiotic resistance mutation bands were present in the three test strips shown at the top of the figure.

DISCUSSION

In this study, GenoType NTM-DR was evaluated for the identification of *M. chimaera* and other *Mycobacterium* species belonging to the MAC. The novelty of this assay is that it allows differentiation of *M. intracellulare* from *M. chimaera*. With the use of 16S rRNA and ITS gene sequencing, 79 *M. chimaera* isolates were identified from a collection of 144 isolates which were previously identified as *M. intracellulare*. The ITS sequences obtained showed 100% identity with that of *M. chimaera* FI-0169T (1). A similar retrospective Belgian study found that 63% of clinical *M. chimaera* isolates had been incorrectly identified with the GenoType Mycobacterium CM assay (15). Results of recent whole-genome sequencing of respiratory *M. chimaera* isolates also support the use of 16S rRNA and ITS gene sequencing to distinguish *M. intracellulare* and *M. chimaera*, as there is an even greater divergence between these two species at the genomic level (16).

All MAC isolates identified by 16S rRNA and ITS gene sequencing were tested with the GenoType NTM-DR assay. Of 173 isolates, 157 developed the expected banding pattern and 16 were incorrectly identified. A conjugate control band was produced on the strips of all tested isolates, indicating the efficiency of the conjugate binding and substrate reaction for the production of a colored band on the strip. However, faint bands were produced in the universal control (UC) for all the isolates tested (Fig. 1). This may have been due to the inefficient amplification of DNA which was displayed when confirmation of PCR product was performed. All *M. chimaera* isolates developed strong species-specific (SP) bands at SP probe 2 (SP2) and SP3 only. The interpretation of banding patterns present which represent more than one species must be carefully performed, and only patterns showing strong species-specific bands should be taken into account. This limitation has also been noted with the previous line probe assay GenoType Mycobacterium CM and the AS assay (Hain Lifescience, Nehren, Germany) (17). In our study, some *M. chimaera* isolates also produced a weak band at SP1, which might have been interpreted as a mixture of *M. avium* and *M. chimaera*. However, these were not considered true species-specific bands, as only bands that were much stronger than that seen with the UC were taken into account. Similarly, all *M. intracellulare* isolates tested produced a strong species-specific band at SP2 only and very few *M. intracellulare* isolates produced a faint band at SP3. However, these bands were always very faint or fainter than that seen with the UC and were similarly disregarded. Likewise, all *M. avium* isolates produced a strong SP1 band only, resulting in correct identification. From our study, we would propose that only those bands with intensities that are stronger than that seen with the UC should be considered.

The MAC also comprises other species which are often misidentified as *M. intracel-*

lulare with the GenoType Mycobacterium CM assay (18). We observed several other MAC species, including *M. arosiense*, *M. marseillense*, *M. timonense*, and *M. bouchedurhonense*, which were misidentified as *M. intracellulare* with the GenoType Mycobacterium CM assay (19). Likewise, GenoType NTM-DR misidentifies these species also, as they cross-react with the *M. intracellulare* species probes present on the strips. However, those other species are isolated from clinical samples infrequently. Similarly, misidentification of *M. arosiense* as *M. intracellulare* with the line probe assays Accuprobe, Inno LiPa Mycobacteria, and GenoType Mycobacterium CM has been previously reported (18, 20). However, there is a lack of published information on these less frequently encountered species and further research is needed to determine their clinical significance and relationship to *M. intracellulare*. Another limitation to our study is that other MAC species such as *Mycobacterium vulneris* and *Mycobacterium colombiense* were not tested with the GenoType NTM-DR assay, and we cannot be sure whether they would react with the probes present on the strip. However, these species are rarely reported and we did not identify these species in our collection of isolates referred between 2007 and 2016.

GenoType NTM-DR also contains probes for detecting genes conferring antibiotic resistance to macrolides and aminoglycosides. Resistance to these classes of antibiotics is encoded by the *rrl* and *rrs* genes, respectively, and they have been reported previously in the *Mycobacterium abscessus* complex (21, 22). Among those studied in our collection of MAC isolates, only two *M. intracellulare* isolates developed a corresponding mutation band for macrolide resistance (*rrl* MUT2; mutation A2058G) or aminoglycoside resistance (*rrs* MUT1; mutation A1408G). Retrospective analysis confirmed that these two isolates were phenotypically resistant to clarithromycin (MIC, >64 μg/ml) or resistant to amikacin (MIC not available). Resistance to macrolides and aminoglycosides was not detected in our collection of *M. chimaera* and *M. avium* isolates with this assay (data not shown). Further research is needed to evaluate the performance of this assay for characterizing antibiotic resistance genes.

In conclusion, the GenoType NTM-DR assay allows for rapid and specific detection of *M. chimaera* isolates and is in good agreement with 16S rRNA and ITS gene sequencing. Despite some of the limitations of this novel assay, it allows *M. chimaera* to be distinguished from *M. intracellulare*, with 100% specificity. GenoType NTM-DR is suitable for use in diagnostic laboratories as it is simple, cost-effective, and more accessible than gene sequencing methods. The GenoType NTM-DR kit contains 96 test strips, and the assay can be performed without the need to refer samples to other laboratories for gene sequencing. This assay will be an invaluable test in addition to the GenoType Mycobacterium CM assay for the identification of *M. chimaera*, as the differentiation of MAC species is becoming more important.

MATERIALS AND METHODS

MAC isolate collection. All 173 MAC isolates had been referred to the Irish Mycobacteria Reference Laboratory between 2007 and 2016. Of these isolates, 162 were previously recovered from clinical specimens from 119 patients and 11 isolates were recovered from water samples taken from heater-cooler units referred by Irish cardiothoracic surgery centers. The collection of MAC isolates consisted of 144 *M. intracellulare* and 29 *M. avium* isolates which were subcultured from frozen pure cultures at −80°C in a Bactec MGIT 960 liquid culture system (Becton Dickinson and Company, USA). Species identification had been performed with the GenoType Mycobacterium CM assay (Hain Lifescience, Nehren, Germany) as part of routine diagnostics. Further identification methods were not in place to identify *M. chimaera* at the time. A reference strain of *M. chimaera* DSM 44623 was also cultured in a Bactec MGIT 960 liquid culture system and was included as a positive control for 16S rRNA and ITS gene sequencing and also for the GenoType NTM-DR assay (1).

16S rRNA and ITS gene sequencing. Identification of MAC isolates to the species level was performed using 16S rRNA and ITS gene sequencing. Mycobacterial DNA was extracted from liquid cultures using a GenoLyse (Hain Lifescience, Nehren, Germany) extraction kit according to the manufacturer's instructions, and the 16S rRNA gene was amplified using Seq1 forward and Seq2 reverse primers as previously described (23). Similarly, the ITS region was amplified using Sp1 forward and Sp2 reverse primers (5, 24). PCR amplification was performed on a Veriti 96-well thermal cycler (Applied Biosystems, USA), and subsequent PCR products were sequenced using 244 and 259 primers for 16S rRNA PCR products as previously described (23, 25). ITS gene products were sequenced with the same primers as those used for DNA amplification. Raw sequence data were analyzed using DNAStar Lasergene

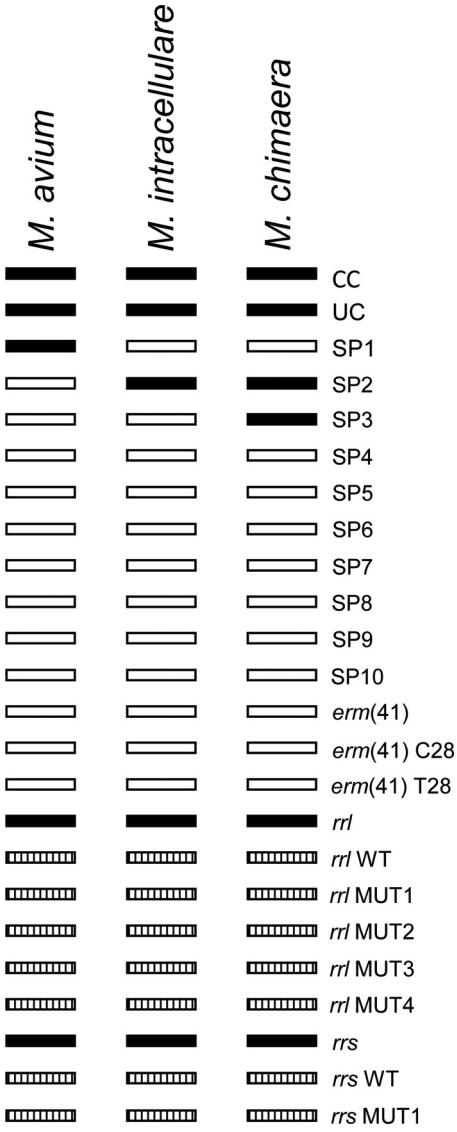

FIG 2 Interpretation chart for the GenoType NTM-DR assay. The specificities of the bands are as follows: CC, conjugate control probe; UC, universal control probe; SP1 to SP10, species-specific probes [specific probes for macrolide resistance mediated by *erm*(41) which are not applicable to members of the *Mycobacterium avium* complex, specific probes for the *rrl* gene and *rrs* gene, and mutation probes for possible detection of resistance to macrolides and aminoglycosides]. Filled boxes indicate complete staining, striped boxes indicate facultative staining, and blank boxes indicate no staining. WT, wild type.

software version 12.2, and aligned sequences were compared with sequences available on the National Center for Biotechnology Information (NCBI) website using the Basic Local Alignment Search Tool (BLAST).

GenoType NTM-DR assay. The GenoType NTM-DR ver. 1.0 assay was performed according to the manufacturer's instructions, and the same DNA extracts as those that had been used for gene sequencing were used for the hybridization assay. For amplification of DNA, a master mix containing 10 μl AM-A and 35 μl AM-B per sample was prepared, and 5 μl of DNA was added to each PCR tube (Hain Lifescience, Nehren, Germany). The amplification profile consisted of 15 min of denaturation at 95°C, followed by 10 cycles consisting of 30 s at 95°C and 2 min at 65°C, and an additional 20 cycles comprising 25 s at 95°C, 40 s at 50°C, and 40 s at 70°C, followed by a final extension for 8 min at 70°C. A positive-control isolate of *M. chimaera* DSM 44623 was also included for each assay.

Hybridization of amplified products was performed on an automated GT-Blot 48 instrument (Hain Lifescience, Nehren, Germany) after confirmation of amplified products on a 2% agarose gel. Hybridization buffer (1 ml) was added by the instrument to each strip followed by a stop to allow addition of membrane strips. Following the hybridization procedure, the strips were removed from the instrument and were allowed to dry. Each strip was labeled and pasted to an evaluation sheet. Results were then interpreted using a Genotype NTM-DR interpretation chart (Fig. 2).

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