Molecular-based mycobacterial identification in a clinical laboratory setting: a comparison of two methods

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ABSTRACT

Many mycobacterial species are pathogenic to humans, with infection occurring worldwide. Infection with *Mycobacterium tuberculosis* is a well-described global phenomenon, but other mycobacterial species are increasingly shown to be the cause of both pulmonary and extrapulmonary infection and are managed differently from *M. tuberculosis* infection. Rapid and accurate differentiation of mycobacterial species is, therefore, critical to guide timely and appropriate therapeutic and public health management. This study evaluates two commercially available DNA strip assays, the Genotype Common Mycobacteria (CM) assay (Hain Lifescience, Nehren, Germany) and the Speed-oligo Mycobacteria assay (Vircell, Spain) for their usefulness in a clinical laboratory setting. Both assays were evaluated on 71 clinical mycobacterial isolates, previously identified using Gen-Probe AccuProbe and through a UK mycobacteriology reference laboratory, as well as 29 non-mycobacterial isolates. Concordant results were obtained for 98% of isolates using both assays. The sensitivity was 97% (95% confidence interval [CI]: 93.3–100%) for the CM assay and 98.6% (95% CI: 95.9–100%) for the Speed-oligo assay. Overall, both assays proved to be useful tools for rapid and sensitive mycobacterial species identification, although interpretation of results was easier with the CM assay. Finally, results were available within one day, compared to current identification times which range between seven days and four weeks.

KEY WORDS: Genotyping techniques.
Mycobacterium infections, nontuberculous.

Introduction

The *Mycobacterium* genus includes *Mycobacterium tuberculosis* complex (MTBC), which includes *M. tuberculosis* and *M. bovis*. There are many other diverse species referred to collectively as 'Mycobacteria other than tuberculosis' (MOTT), which includes the *Mycobacterium avium* complex (MAC). The incidence of infection with MTBC in Ireland is relatively high compared to many other European countries, being ranked joint 11th highest of 30 EU countries in 2009.[1]

Mycobacteria other than tuberculosis are ubiquitous in the environment, being found in soil, food, water and animals, and their capacity to cause disease in humans has been recognised since the 1960s.[2] At present, more than 130 species are known and infections due to MOTT represent an increasing proportion of all mycobacterial disease, especially in developed countries, causing pulmonary, lymph node, joint and soft tissue infections.[3]

Immunocompromised patients are more susceptible to these non-mycobacterial infections, and infections with MAC are well described in patients with advanced human immunodeficiency virus (HIV) disease. Cystic fibrosis patients are also at risk of infection with various MOTT species, and, in particular, *M. abscessus* infection has been reported in these patients.[4] Proper and expedient species identification is crucial for immunocompromised patients, guiding effective treatment, appropriate infection control measures, and helping the accurate collection of epidemiological data.

Currently, the Department of Clinical Microbiology at Cork University Hospital (CUH) processes samples submitted for diagnosis of mycobacterial infection using conventional culture methods and the Gen-Probe AccuProbe *Mycobacterium* system (GenProbe, San Diego, USA), which is used to confirm identification of MTBC and MAC. This test can identify mycobacteria strains that have been grown in culture within 2–3 h as MTBC, MAC, or neither. However, the main disadvantage of this assay is its inability to identify MTBC and MAC isolates in more detail, as well as its inability to characterise MOTT species.

The aim of the current study is to compare the efficacy and cost-effectiveness of the Genotype Common Mycobacteria (CM) assay, the Genotype MTBC assay and the Speed-oligo Mycobacteria assay for the identification of MTBC and MOTT isolates to species level, compared to the current method in use at CUH.

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Materials and methods

Mycobacterial isolates

Seventy-one fully identified clinical mycobacterial isolates and 29 non-mycobacterial isolates were included in this study. The 71 positive samples were identified previously by

means of current phenotypic methods (auramine and confirmatory Ziehl Neelsen staining methods, and automated Bactec/Alert 3D culture system) and by utilising the Gen-Probe AccuProbe system to identify MTBC and MAC. All further mycobacterial species identification for MOTT was performed previously by a UK reference laboratory (HPA National Mycobacterium Reference Laboratory, London).

The 29 non-mycobacterial isolates consisted of samples that remained negative for the duration of incubation, as well as samples that flagged falsely positive due to contamination with non-mycobacterial organisms such as streptococci and staphylococci. All these liquid cultures (n=100) were provided without knowledge of strain identity for the purpose of this study.

One hundred isolates were tested using the Vircell Speed-oligo Mycobacteria assay. Initially it was planned that all 100 isolates would be tested using the Genotype CM assay; however, due to cost limitations during the study, it was necessary to use either the CM assay or the MTBC assay (without indication of the identity of the species). Sixty-seven isolates were tested blindly with the Genotype CM assay, and the remaining 33 isolates were tested using the Genotype MTBC assay. Both assays were performed according to the manufacturers' instructions, without prior knowledge of the identity of the isolates.

Vircell Speed-oligo Mycobacteria assay

Testing of the isolates and interpretation of the results was conducted by one of the authors who was unaware of the results of previous work on the isolates (N. O'D). For DNA extraction, 1 mL of each liquid bacterial culture was concentrated by centrifugation at 12,000 x***g*** for 15 min and resuspended with 150 µL Vircell Sample Solution. The bacterial suspensions were then incubated for 60 min at 95˚C. Following centrifugation for 5 min at 12,000 x***g***, 10 µL supernatant was used directly for polymerase chain reaction (PCR) analysis or frozen at –20˚C for future use.

For PCR amplification, the lyophilised PCR mix (provided in the kit) was resuspended with 150 µL Vircell PCR Mix Reconstitution Solution. Then, 10 µL sample was added to 15 µL PCR mix in PCR tubes in a DNA-free pre-amplification area and the amplification procedure (1 min at 92˚C, 40 cycles of 92˚C for 20 sec, 55˚C for 30 sec, 72˚C for 30 sec, and a final extension of 72˚C for 1 min) was conducted. The PCR products were then denatured by heating for 1 min at 95˚C and immediately cooled on ice for no more than 2 min. Detection of PCR products was performed using the dipstick. Denatured PCR product (5 µL) was diluted with 40 µL preheated (55˚C) running solution in a 1.5 mL tube. Nitrocellulose strips were inserted (one per sample) and incubated for 5 min. The strips were then removed and interpreted immediately. A valid result was indicated by the presence of a PCR amplification control line (PCRCL) and a product control line (PCL).

A generic *Mycobacterium* genus band (test line [TL] 7) and six species-specific mycobacterial identification test lines (TL1–6) are included in each test strip: TL1 *M. chelonae*/*M. abscessus* complex, TL2 *Mycobacterium gordonae*, TL3 *M. kansasii*/*gastri*, TL4 *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. microti*, *M. africanum*), TL5 *M. avium*/*scrofulaceum*/*intracellulare* complex, TL6 *Mycobacterium fortuitum*. Results were interpreted by identification of specific bands for each of the mycobacterial species. The strips were then fixed on their prenumbered positions on a results sheet.

Genotype CM and Genotype MTBC assays

The Genotype CM assay generates a banding pattern on membrane strips coated with specific probes for the following 14 mycobacterial species: *M. avium* subspecies, *M. chelonae*, *M. abscessus*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. scrofulaceum*, *M. interjectum*, *M. kansasii*, *M. malmoense*, *M. peregrinum*, *M. marinum*, *M. ulcerans*, *M. tuberculosis* complex and *M. xenopi*, and a genus control specific for a member of the genus *Mycobacterium*. The Genotype MTBC assay allows further differentiation of the MTBC, identifying the following species of this complex: *M. tuberculosis*, *M. africanum*, *M. microti*, *M. bovis* ssp. *bovis*, *M. bovis* ssp. *caprae* and Bacillus Calmette-Guérin (BCG).

The following procedure was applied for each assay. Briefly, 1 mL each liquid bacterial culture was concentrated by centrifugation at 10,000 x***g*** for 15 min and resuspended with 300 µL molecular grade water. The suspension was then incubated at 95˚C for 20 min, followed by sonication at 60˚C for 15 min. Following centrifugation for 5 min at 10,000 x***g***, 5 µL supernatant was used for the assay or frozen at –20˚C for use later.

A master mix was prepared according to instructions. A 5 µL sample was added to 45 µL PCR master mix in PCR tubes in a DNA-free pre-amplification area and the amplification procedure (15 min at 95˚C, 10 cycles of 95˚C for 30 sec and 58˚C for 2 min, 20 cycles of 95˚C for 25 sec, 53˚C for 40 sec and 70˚C for 40 sec, and a final extension at 70˚C for 8 min) was conducted. Hybridisation and detection were performed using an automated hybridisation GT-Blot 48 instrument (Hain).

Conjugate concentrate and substrate concentrate were diluted (1 in 100) with conjugate buffer and substrate buffer, respectively. The following reagents were then placed into their respective positions on the instrument: hybridisation buffer, stringent wash solution, rinse solution, diluted conjugate, distilled water and diluted substrate. The hybridisation buffer and stringent wash solutions were preheated for 1 h at 37˚C. Furthermore, a preheating phase of 15 min for these reagents occurs on the instrument.

Each strip was placed into separate wells within the tray, which was fixed into the instrument. The programme started after 20 µL denaturation solution (provided with the kit) was mixed with 20 µL amplification products for 5 min. Automatically, 1.5 mL prewarmed hybridisation buffer was then added. The hybridisation procedure was performed at 45˚C for 30 min, followed by two washing steps. For colorimetric detection of hybridised amplicons, streptavidin conjugated with alkaline phosphatase and substrate buffer was added. After final washing steps, the strips were removed and interpreted. A valid result was indicated by the presence of a conjugate control and a universal control.

Results

Overall, 98% of isolates correlated with the standard methods of identification used routinely at CUH (Table 1). One *M. tuberculosis* isolate was not identified by the Speed-oligo Mycobacteria assay, producing bands at TL4 and TL5, and thus failed to distinguish *M. tuberculosis* from

Table 1. Comparison of the Vircell Speed-oligo Mycobacteria assay and the Hain Genotype Common Mycobacteria (CM) and Genotype *Mycobacterium tuberculosis* complex (MTBC) assays for the identification of mycobacterial species.

Mycobacterium Species	Number of strains	Speed-oligo identification (*n*=100)	Genotype CM identification (*n*=67)	Genotype MTBC identification (*n*=33)
M. tuberculosis	45	*M. tuberculosis* complex (*n*=44) Not determined (*n*=1)	*M. tuberculosis* complex (*n*=34)	*M. tuberculosis* (*n*=11)
M. szulgai	1	*Mycobacterium* genus (*n*=1)	Gram positive, high G+C content (*n*=1)	NA
M. bovis[*]	2	*M. tuberculosis* complex (*n*=2)	*M. tuberculosis* complex (*n*=2)	NA
MAC	15	*M. avium/scrofulaceum/ intracellulare* complex (*n*=15)	*M. avium* spp. (*n*=9) *M. intracellulare* (*n*=6)	NA
M. abscessus	4	*M. chelonae/ abscessus* complex (*n*=4)	*M. abscessus* (*n*=4)	NA
M. fortuitum	1	*M. fortuitum* (*n*=1)	*M. fortuitum* (*n*=1)	NA
M. kansasii	2	*M. kansasii* (*n*=2)	*M. kansasii* (*n*=2)	NA
M. scrofulaceum	1	*M. avium/scrofulaceum/ intracellulare* complex (*n*=1)	Not determined (*n*=1)	NA
Non-mycobacterial isolates[†]	29	Negative (*n*=29)	Negative (*n*=7)	Negative (*n*=22)
Total tested	100	100	67	33
Sensitivity	–	98.6%	97.%	100%

[*]*M. bovis* starins were blindly assayed by the Genotype CM assay, and not by the Genotype MTBC assay.

[†]Sputum samples processed for TB which were negative on the Bactec/Alert culture system and were blindly assayed by the Genotype MTBC assay.

NA: not applicable

M. avium/scrofulaceum/intracellulare complex. *M. szulgai* is not included in the specific Speed-oligo probes and was thus not identifiable by this assay.

Ninety-seven percent (65/67) of isolates tested using the Genotype CM were identified appropriately. In one case, *M. szulgai* was not recognised as a member of the genus *Mycobacterium* but as a Gram-positive bacterium with a high G+C content. A specific pattern was not produced by the assay because it does not have a probe specific to *Mycobacterium* in its nitrocellulose test strip. The other non-identified isolate, *M. scrofulaceum*, generated a mixed banding pattern on the nitrocellulose test strip; the presence of *M. malmoense*, *M intracellulare* or *M. scrofulaceum*, or a mixture thereof, could not be ruled out for this specimen. This discrepancy was resolved by a phenotypic feature (i.e., pigment production). The sample produced pigmentation in the light and dark, indicating a scotochromogen, namely *M. scrofulaceum*.

Among the 33 isolates tested using the Genotype MTBC assay, all 11 isolates of *M. tuberculosis* were identified correctly.

Discussion

Diagnostic services for mycobacterial infections are among the slowest to generate results in a clinical microbiology laboratory. Mycobacteria are, for the most part, notoriously slow growing, with a mean detection time in one study of 24 days for *M. tuberculosis*, which results in a prolonged turnaround time.[5] Identification of *Mycobacterium* species is difficult when using the traditional phenotypic microbiological methods as these are frequently time-consuming and inconclusive. This inherent delay is problematic as treatment varies depending on the species isolated.[6]

Cork University Hospital is an acute hospital, and also acts as a referral centre for other hospital laboratories in the south of Ireland; however, reliance on an external reference laboratory for mycobacterial species identification increases turnaround times. Providing an on-site molecular mycobacterial species identification system at CUH would yield same-day results after growth on culture, thereby assisting prompt implementation of appropriate patient management, infection control and public health protocols.

The Speed-oligo Mycoabacteria assay is a relatively new assay for mycobacterial species identification and few published data are available for this method. Overall, 98/100 (98%) of the isolates tested with this assay showed concordant results. This identification method unexpectedly failed to identify an isolate of *M. tuberculosis*, and, by producing a mixed banding pattern on the test strip, was unable to distinguish *M. tuberculosis* from *M. avium/ scrofulaceum/intracellulare* complex. In contrast, the Genotype CM assay successfully identified this isolate as *M. tuberculosis* complex. A fundamental difference between *M. tuberculosis* and other mycobacteria is the lack of disease transmission between humans in the latter group, In addition, as the treatment regime for *M. tuberculosis* differs from that given for other mycobacterial infections, this misidentification could lead to inappropriate therapy. Species identification is critical for regulating chemotherapy.[7]

The Speed-oligo system demonstrated high sensitivity, and results were available within three hours; however, the interpretation of the bands proved more difficult. According to the manufacturer's instructions, interpretation of results must be performed immediately after the strips have been

removed from the heating block, and they must not be left to dry as this can cause weak additional bands to form in negative samples. In the present study, the manufacturer's instructions were followed exactly; however, weak background bands also occurred with positive *Mycobacterium* strains.

In a previous study,[8] the Speed-oligo system produced a 1.2% false-positive rate, with three MOTT isolates misidentified (*M. marinum* and two *M. peregrinum* isolates). This study concluded that the main drawback with the Speed-oligo assay is the small spectrum of mycobacterial species it can identify, but concluded that this dipstick assay is rapid and easy to perform in a laboratory. In addition, the study reported weak bands in mycobacterial species.

The Genotype CM assay showed 98% concordance with CUH's current algorithm using GenProbe and a reference laboratory. Discrepant results occurred in two cases using the CM assay. The first was due to a limitation of the CM assay, as it does not include a specific probe for *M. szulgai*. This test was valid, generating a conjugate and universal control but failed to generate the genus control.

In Japan, four pulmonary cases caused by *M. szulgai* were reported in 2008,[9] and an untreated *M. szulgai* pulmonary infection was described in 2010 in the USA.[10] These studies appear to indicate that this organism is a potential pathogen, even though it accounts for less than 1% of all MOTT infections.[11] Similarly, Richter *et al*. reported failure of the Genotype CM assay to specify a number of strains as mycobacterial species, only identifying them as Gram-positive bacteria with a high G+C content.[12] Therefore, indication by this assay of Gram-positive bacteria with a high G+C content does not rule out the presence of mycobacteria. *M. szulgai* has a similar clinical presentation to *M. tuberculosis*, causing pulmonary infection. However, treatment regimens differ and therefore identification of this rare pathogen is important in order to permit correct patient management.[9]

The other disadvantage of the Genotype CM assay was failure to identify *M. scrofulaceum*. This specimen generated a non-specific banding pattern that might have indicated *M. scrofulaceum*, *M. intracellulare* or *M. malmoense*. These species are closely related, sharing many similar genetic, ecologic, biochemical and cultural characteristics, as well as similar antibiotic susceptibility patterns. All three species can cause pulmonary disease, but treatment regimens differ depending on susceptibility to antimicrobials.[13]

Pulmonary infection with *M. abscessus* is described increasingly in patients with cystic fibrosis. The present study included four *M. abscessus* isolates which were identified correctly by the Genotype CM assay but were identified as *M. chelonae/M. abscessus* complex using the Speed-oligo Mycobacteria assay. *M. abscessus* is potentially more virulent than any other MOTT in cystic fibrosis patients.[14] The presence of these species in cystic fibrosis patients has consequences for potential lung transplantation, and *M. abscessus* has been cited as a disqualifying factor for this intervention.[15] The inability of the Speed-oligo Mycobacteria assay to differentiate these two species would therefore prove problematic in this situation.

A time and cost analysis was also made between current validated methods and the two proposed methods. There is a very significant time saving, overall up to four weeks if the services of the external reference laboratory are required. Such a reduction in turnaround time would be beneficial to patient management, allowing prompt, optimal drug therapy. Of the isolates sent by CUH to the reference laboratory in 2010, all could have been identified to species level using the Genotype CM assay. Current transportation of these isolates is costly because of stringent European Union packaging regulations, which require containment and the services of a courier.

The Genotype CM assay and the MTBC assays are more expensive than the Speed-oligo Mycoabcteria assay and the AccuProbe assay. However, this increase in cost must be weighed against the ability of the Genotype CM assay to identify 14 of the most relevant mycobacterial species. The Genotype CM assay was found to be more suitable for the identification of mycobacteria isolated in the CUH laboratory because of its wider strain selection, less stringent reaction conditions, cost-effectiveness when compared to the algorithm currently in use, and the easier interpretation of results. □

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