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Preliminary evaluation of a new kit for differentiation of *Mycobacterium tuberculosis* complex species using Speed-Oligo MTBC

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We present the first evaluation of a novel molecular assay, the Speed-Oligo *Mycobacterium tuberculosis* complex (SO-MTBC), which is based on PCR combined with a dipstick for the differentiation of *M. tuberculosis* complex (MTBC) members. The results of this assay were compared with findings obtained using the Genotype MTBC assay. In this study, 189 strains of MTBC isolates from 2011 to 2014 were evaluated to determine the MTBC species. Most (174, 92 %) of the strains were identified as *M. tuberculosis sensu stricto*, 7 (3.7 %) as *Mycobacterium bovis*, 5 (2.6 %) as *M. bovis* bacillus Calmette–Guérin, 2 (1.1 %) as *Mycobacterium africanum* and 1 (0.5 %) as *Mycobacterium caprae*; no strains belonged to *Mycobacterium microti* and *Mycobacterium canettii* subsp. The concordance κ coefficient obtained was 0.96 with the results of the Genotype MTBC assay. SO-MTBC may represent a fast and easy-to-use alternative for differentiating among MTBC subspecies in laboratories with standard equipment.

INTRODUCTION

Tuberculosis (TB) is a major global health problem, with approximately 9 million new cases and 1.5 million deaths reported in 2013 (World Health Organization, 2014). The disease is caused by members of the *Mycobacterium tuberculosis* complex (MTBC), which consists of a group of closely related *Mycobacterium* spp., including *M. tuberculosis sensu stricto*, *Mycobacterium bovis*, *M. bovis* bacillus Calmette–Guérin (BCG), *Mycobacterium africanum* and four other rarely isolated members: *Mycobacterium microti*, *Mycobacterium canettii*, *Mycobacterium caprae* and *Mycobacterium pinnipedii* (Goodfellow *et al.*, 2012).

Abbreviations: BCG, bacillus Calmette–Guérin; LJ, Lowenstein–Jensen; MTBC, *Mycobacterium tuberculosis* complex; NTM, non-tuberculosis mycobacteria; RD, region of difference; SO-MTBC, Speed-Oligo *Mycobacterium tuberculosis* complex; TB, tuberculosis.

Species of this complex are the primary cause of TB in humans and also infect wild and domesticated animals. MTBC subspecies differ in their range of hosts, geographic distribution, drug resistance and pathogenicity (Wayne & Kubica, 1986). However, DNA–DNA hybridization, multilocus enzyme electrophoresis and sequencing of 16S rRNA genes and the 16S–23S rRNA internal transcribed spacer have demonstrated that MTBC subspecies are closely interrelated (Kasai *et al.*, 2000; Niemann *et al.*, 2000). Rapid and reliable identification of these subspecies is critical to guide public health and primary healthcare decisions.

The differentiation of MTBC subspecies according to their phenotypic characteristics (e.g. laboratory tests such as niacin, urease, pyrazinamide, cyclosporine, cycloserine, thiophen-2-carboxylic acid, aerobic/microaerophilic) is labour intensive, time consuming and dependent on their sufficient growth; furthermore, not all laboratories can perform

these tests in a routine manner (Okazaki *et al.*, 2005; Prodinger *et al.*, 2005).

Different DNA-based methods have been developed to overcome these shortcomings, including spoligotyping, PCR *gyrB*–RFLP analysis and PCR-based genomic detection assay (Kasai *et al.*, 2000; Liébana *et al.*, 1996; Parsons *et al.*, 2002). These techniques have proven useful for the discrimination of MTBC subspecies but are typically only available in specialized molecular laboratories rather than in routine diagnostic laboratories. Other commercial kits include the Genotype MTBC assay (Hain Lifescience), based on the detection of single-nucleotide polymorphisms of *gyrB* and on the presence or absence of region of difference (RD) 1 (Richter *et al.*, 2003, 2004).

The present study was designed to evaluate the performance of a novel oligo-chromatographic assay, Speed-Oligo-*Mycobacterium tuberculosis* complex (SO-MTBC; Vircell SL), in the direct molecular detection of MTBC subspecies in strains isolated from respiratory and extrarespiratory clinical samples. The SO-MTBC assay differentiates among the seven MTBC subspecies and may represent a fast and easy-to-use alternative for differentiating among them in laboratories with standard equipment (thermocycler and thermoblock). The study objective was to compare the results of this assay with findings obtained using the Genotype MTBC assay, the only currently available commercial kit.

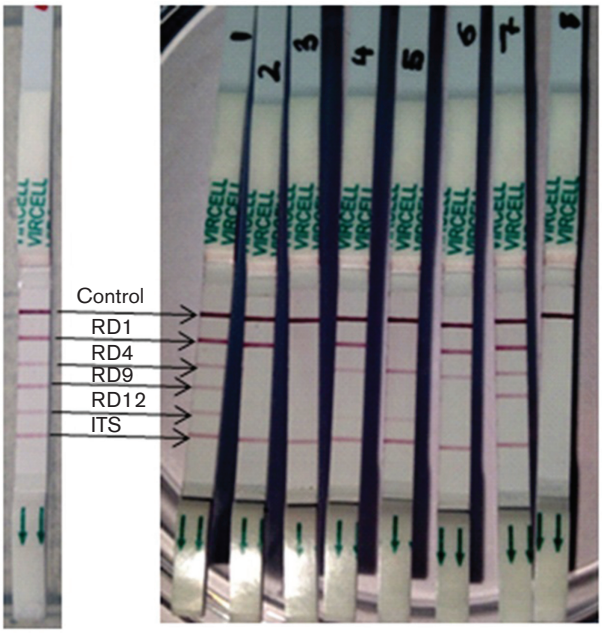

Fig. 1. SO-MTBC hybridization results for each MTBC species. 1, *M. tuberculosis*; 2, *M. bovis*; 3, *M. bovis* BCG; 4, *M. africanum*; 5, *M. microti**; 6, *M. caprae*; 7, *M. canettii**; 8, negative control. *Results obtained with collection strains: *M. microti* NC08710 and *M. canettii* NC 0743.

METHODS

The study included 264 clinical isolates obtained by our laboratory from patients with suspected mycobacterium infection between January 2011 and December 2014: 189 isolates belonging to MTBC; 73 isolates of non-TB mycobacteria (NMT) [*Mycobacterium chelonae* (7), *Mycobacterium gordonae* (7), *Mycobacterium fortuitum* (7), *Mycobacterium avium-intracellulare* (7), *Mycobacterium intracellulare* (7), *Mycobacterium abscessus* (7), *Mycobacterium avium* (5), *Mycobacterium xenopi* (5), *Mycobacterium lentiflavum* (4), *Mycobacterium mageritense* (4), *Mycobacterium scrofulaceum* (4), *Mycobacterium senegalense* (2), *Mycobacterium marinum* (2), *Mycobacterium kansasii* (2), *Mycobacterium porcinum* (1), *Mycobacterium septicum* (1) and *Mycobacterium gastri* (1)] and 2 isolates of bacteria related to genus mycobacterium (*Nocardia* spp. and *Rhodococcus* spp.). All strains were stored at −80 °C after their isolation from liquid and/or solid media [Mycobacteria Growth Indicator Tube (MGIT) and/or Lowenstein–Jensen (LJ)].

***M. microti* NC08710 and *M. canettii* NC0743 collection strains.** The confirmation of MTBC membership was performed with the BD MGIT TBc identification test (Becton-Dickinson Company) for isolates recovered in MGIT tubes and with the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) technique (Bruker Daltonics) or Genotype Mycobacterium CM/AS assay (Hain Lifescience) for those recovered in LJ. These last two techniques were also used for the identification of NMT, while standard laboratory procedures were used to identify *Rhodococcus* spp. and *Nocardia* spp.

Growing strains and nucleic acid extraction. Strains were thawed, and 50 µl was inoculated into a fresh MGIT tube, which was used, in positive cases, for DNA extraction after centrifugation at 12 000 r.p.m. for 15 min and removal of the supernatant. The pellet was resuspended in 150 ml of sample solution (Vircell) and incubated at 95 °C for 1 h. It was then centrifuged at 12 000 r.p.m. for 5 min, and the supernatant (DNA eluate) was collected in a new vial.

SO-MTBC assay. The SO-MTBC assay is a PCR-based method coupled to a dipstick device. The PCR mix (supplied in lyophilized format) contains PCR reagents and specific oligo pairs for mycobacterial amplification of the seven MTBC subspecies. The multiplex PCR was performed in a Labcycler thermocycler (Sensoquest) under the following conditions : denaturation for 1 min at 95 °C, followed by 40 cycles of 20 s at 92 °C, 45 s at 55 °C and 45 s at 72 °C, followed by an elongation step of 2 min at 72 °C. A PCR product encoding RDs (RD1, RD4, RD9 and RD12) was amplified to differentiate the MTBC subspecies. A non-competitive internal control was simultaneously amplified. Another line internal transcribed spacer that is present in all members of MTBC was included to differentiate MTBC subspecies from other *Mycobacterium* spp.

PCR products were denatured by heating in the thermocycler at 95 °C for 1 min. PCR products were detected by using the dipstick according to the kit instructions. Briefly, 10 µl of denatured PCR product was diluted in 35 µl running solution and placed in a thermoblock set at 55 °C. Amplification products were hybridized on a dipstick using five specific probes bound to colloidal gold and five probes immobilized in the membrane. When placed in contact with the dipstick, the product flows into the strip and reacts first with the gold-bound probes. The PCR product–gold probe complex then reaches the specific test lines within the nitrocellulose, where a second hybridization takes place. The final reading was visually accomplished after 15 min of incubation. Reactivity was confirmed by visualization of a red line. Results were interpreted by identifying a specific band pattern for each subspecies identified in the test (Fig. 1). The entire process took around 90 min.

Test results were performed by two observers who were blinded to previous results.

Genotype *Mycobacterium* CM/AS and Genotype MTBC assays. DNA was extracted with the GenoLyse (Hain Lifescience) method in three steps: centrifugation of material, addition of lysis buffer to the pellet and incubation for 5 min at 95 °C and addition of neutralization buffer (30 min).

Amplification was done with 35 µl of primer nucleotide mix and amplification buffer containing 2.5 mM $MgCl_2$, 1.25 U of HotStarTaq polymerase (Qiagen) (not provided with the kit) and 5 µl of DNA in a final volume of 50 µl. The amplification protocol consisted of 15 min of denaturation at 95 °C, followed by 10 cycles comprising 30 s at 95 °C and 120 s at 58 °C, an additional 20 cycles comprising 25 s at 95 °C, 40 s at 53 °C and 40 s at 70 °C and a final extension at 70 °C for 8 min.

Hybridization and detection were carried out in an AutoLipa automated washing and shaking device (Siemens Healthcare). Twenty microlitres of the amplification products were mixed with 20 µl of denaturing reagent (provided with the kit) for 5 min in separate troughs of a plastic well. After it was placed in the device, 1 ml of hybridization buffer was automatically added, followed by a stop to put the membrane strips into each trough. The hybridization procedure was performed at 45 °C for 30 min, followed by two washing steps. For colorimetric detection of hybridized amplicons, streptavidin-conjugated alkaline phosphatase and the appropriate substrate were added at room temperature in the automated device. After the final washing step, the strips were air-dried. A template sheet showing the positions of the lines and the interpretation table were used for the interpretation of the test results.

MALDI-TOF MS technique. The strains were processed for the MALDI-TOF assay at 1–3 days after the culture resulted positive. Samples were prepared according to the manufacturer's instructions (Bruker Daltonics) using the protocol recommended at the time of the application. From mycobacteria isolates grown in solid media, several colonies were harvested and suspended in 300 µl of HPLC grade water. In the case of mycobacteria grown in liquid medium, 1.2 ml from the culture was centrifuged at 13 000 r.p.m. for 15 min. The supernatant was discarded, and 300 µl of HPLC grade H_2O was added.

The culture was inactivated for 30 min at 100 °C. Afterwards, 900 µl of absolute ethanol was added and centrifuged at 13 000 r.p.m. for 2 min. The supernatant was removed. The residual ethanol was evaporated at room temperature. According to the pellet obtained, 100–200 mg of zirconia/silica beads was added along with 10–50 µl of acetonitrile. Samples were vigorously shaken at maximum speed for 1 min. Formic acid 70 % (v/v) was added. The samples were shaken using the Minilys for 5 s and centrifuged at 13 000 r.p.m. for 2 min (Bruker Daltonics Inc., 2013, 2014).

One microlitre of the final supernatant was spotted onto an MSP 96-spot plate (Bruker Daltonics, and 1 µl of the MALDI-TOF matrix HCCA solution (Bruker Daltonics) was added and left to dry. The spectra acquisition was performed with a Microflex™ mass spectrometer (Bruker Daltonics), using the FlexControl™ software. The analysis was carried out in automatic mode.

Discrepancy between SO-MTBC and Genotype MTBC. Any isolate showing a discrepant or abnormal result was analysed by *hsp65* sequencing. If the nucleotide at the position corresponding to 631 of the homologous *hsp65* of *M. tuberculosis* H37Rv was cytosine, the isolate was considered to be *M. tuberculosis sensu stricto* (Huard *et al.*, 2003; Ueyama *et al.*, 2014). Isolates with discrepancies were sent to the national reference microbiology centre (Institute Carlos III, Madrid, Spain), which uses a published research protocol for MTBC subspeciation (Herrera-León *et al.*, 2009).

Statistical analysis. The concordance of the results obtained by the two methods was evaluated by calculating the concordance κ coefficient by reference to the following values of strength of concordance: κ<0.20, poor; κ 0.21–0.40, weak; κ 0.41–0.60, moderate; κ 0.61–0.80, good; κ 0.81–1.00, very good.

Table 1. Results of 189 clinical MTBC isolates with SO-MTBC and Genotype MTBC

	No. of strains (%)	
	SO-MTBC	Genotype MTBC
M. tuberculosis	174 (92)	175 (92.6)
M. bovis	7 (3.7)	7 (3.7)
M. bovis BCG	5 (2.6)	5 (2.6)
M. africanum	2 (1)	1 (0.5)
M. caprae	1 (0.5)	1 (0.5)

RESULTS

Of the 189 clinical MTBC isolates analysed with the SO-MTBC assay, 174 (92 %) were identified as *M. tuberculosis sensu stricto*, 7 (3.7 %) as *M. bovis*, 5 (2.6 %) as *M. bovis* BCG, 2 (1.1 %) as *M. africanum* and 1 (0.5 %) as *M. caprae*. No strains belonging to *M. microtii* and *M. canettii* subsp. were identified. Fig. 1 depicts the dipstick after 5 min incubation, showing each of the possible outcomes. A concordance κ coefficient of 0.96 was obtained for the concordance with the findings of the reference Genotype MTBC method, with one strain identified as *M. africanum* by the SO-MTBC but as *M. tuberculosis* by Genotype MTBC (see Table 1). Sequencing of *hsp65* and the reference centre study both identified the strain as *M. tuberculosis sensu stricto*.

A specificity for identifying the MTBC of 100 % was obtained for the SO-MTBC assay, which detected hybridization for the MTBC subspecies alone and not for NTM or related bacteria (*Rhodococcus* sp. and *Nocardia* subsp.).

DISCUSSION

The elevated incidence of TB worldwide has been accompanied by the emergence of drug-resistant MTBC strains (World Health Organization, 2014), and global partnerships have been encouraged to improve the diagnosis of TB and its appropriate treatment to prevent further global spread of the disease.

Accurate diagnostic tests are essential to support clinical decisions on the most appropriate drug regimens for mycobacterium infections. Novel, rapid and accessible tests are required in both developed and developing countries. In this study, we present a rapid, simple and affordable test for MTBC subspeciation that can be used in almost any laboratory with basic equipment. The differentiation of MTBC subspecies is challenging because they share 99.9 % genetic identity (Gordon *et al.*, 1999; Brosch *et al.*, 2002), but it is

essential for a correct therapeutic approach for the identification of *M. bovis* BCG in possible complications after its therapeutic utilization (e.g. treatment of urothelial bladder cancer) and for the rapid detection (e.g. providing information relating to contact and source tracking) and control of outbreaks (e.g. *M. bovis* remains an important cause of zoonotic TB worldwide and it should not be overlooked in a clinical setting).

Many authors have designed in-house techniques to differentiate some species of this complex according to the presence of genetic deletions in the genomes of MTBC compared to *M. tuberculosis* H37Rv genome or the presence of specific genetic polymorphisms in different targets (g*yrB*, *kat G*, *hsp65*) or by using techniques such as RFLP-PCR of *hsp65* or *gyrB* or spoligotyping (Bouakaze *et al.*, 2010; Devallois *et al.*, 1997; Halse *et al.*, 2011; Reddington *et al.*, 2012; Somoskovi *et al.*, 2008).

The only commercially available MTBC test is Genotype MTBC (Hain Lifescience), which differentiates among *M. tuberculosis*, *M. canettii*, *M. africanum*, *M. microti*, *M. bovis*, *M. bovis* BCG and *M. caprae* subsp. using PCR and reverse hybridization, with the genetic targets being polymorphisms in the *gyr*B gene and RD1 for the identification. The procedure takes around 4 h to perform. The SO-MTBC assay differentiates the seven MTBC subspecies according to the RD level and offers the following benefits: it differentiates between *M. tuberculosis* and *M. canettii* and MTBC subspecies in a rapid manner (about 90 min) and does not require sophisticated equipment. The SO-MTBC price will be around 10 euros for determination. Its lower price compared to the price of Genotype MTBC (around 40 euros for determination) represents another advantage of this new identification system.

SO-MTBC results correlated well with the results of the Genotype MTBC (κ=0.96). The discrepancy was related to a case of *M. tuberculosis*, which was identified as *M. africanum* in the SO-MTBC assay. These are very similar subspecies. In fact, *M. africanum* was previously separated into types I and II, and it was only recently decided to include *M. africanum* type II in *M. tuberculosis* spp. (de Jong *et al.*, 2010; Mostowy *et al.*, 2004), and it is recognized that some lineages of *M. africanum* type I are very difficult to distinguish from *M. tuberculosis* (Köser *et al.*, 2011). The main limitations of the SO-MTBC assay are that its automation is not possible and that separate laboratory areas must be used for the strain preparation, target amplification and amplicon detection.

In conclusion, the SO-MTBC is an easy-to-use alternative for differentiating members of the MTBC (difference among the seven subspecies) in any microbiological laboratory (thermocycler and thermoblock) and offers a short total turnaround time.

The kit described and evaluated in this work will soon be commercially available.

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