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The comparison between two methods for typing of nontuberculous mycobacteria: high pressure liquid chromatography and molecular assay GenoType Mycobacterium CM/AS

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Abstract

Introduction: The GenoType Mycobacterium CM and the GenoType Mycobacterium AS (HAIN Lifescience, Germany) were evaluated for the ability to differentiate mycobacterial species of clinical isolates. Serial use of the both assays is aimed to identify 38 different molecular patterns, of which 24 patterns can be assigned to single species, 10 patterns are allocated to two or more *Mycobacterium* species, and 4 patterns correspond to *Mycobacterium* species and gram-positive bacteria with a high G + C content. The analysis of mycolic acids by high pressure liquid chromatography (HPLC) was the reference method.

Material and methods: A set of 127 nontuberculous mycobacterial isolates on Loewenstein-Jensen media, derived from different patients between 1999 and 2007, was analyzed. The strains were primary classified by HPLC following the diagnostic procedure, and retyped by GenoType Mycobacterium CM/AS.

Results: In total, results obtained by both methods were interpretable for 113 strains. Concordant results were obtained for 105 (93%) mycobacterial strains. One out of 8 inconcordantly classified strains, which was classified as *M. abscessus*/ *M. chelonae* by HPLC, displayed a pattern of *M. tuberculosis complex* by a molecular method. Eleven clinical strains were differentiated only by one of used methods, either by HPLC (6 strains) or by GenoType CM/ AS (5 strains). Three strains were not classified at all.

Conclusions: Our results show that the GenoType Mycobacterium CM/AS system represents a useful tool to identify mycobacterial clinical isolates. The molecular system is as rapid and reliable as the HPLC, but much easier to perform and more friendly for the environment.

Key words: GenoType Mycobacterium CM/AS, high pressure liquid chromatography (HPLC), nontuberculous mycobacteria (NTM)
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Introduction

Over the past decade, along with the development of molecular techniques, we have witnessed considerable progress in the taxonomy of mycobacteria. The genus *Mycobacterium* currently includes 148 species and 11 subspecies, numbers that continue to grow. A list of already described taxa,

updated on an ongoing basis, can be accessed online [1]. Of these, *M. leprae* and *M. tuberculosis* are obligatory pathogens, while the remainder, called non-tuberculous mycobacteria (NTM), are saprophytes commonly found in the environment. However, about a third of NTM species are capable of causing opportunistic infections, most commonly associated with immune system dysfunction.

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Factors predisposing to NTM infections include immunosuppressive treatment, old age, past illnesses or co-morbidities. In the case of pulmonary manifestation of mycobacteriosis, these may include tuberculosis and chronic obstructive pulmonary disease, while in the case of mycobacterial skin infection, these may include skin or mucosal damage [2–4]. However, the commonest clinical manifestation of mycobacteriosis is chronic lung disease [4–7]. In Poland, the species most commonly isolated from samples collected from patients with pulmonary mycobacteriosis include: *M. kansasii*, *M. avium*/*M. intracellulare* and *M. xenopi*. In contrast to tuberculosis, mycobacteriosis is not transmitted to healthy individuals and therefore is not subject to obligatory reporting, with the result that epidemiological data are only rough estimates and usually concern specific regions of the country or just a single NTM species. It should be borne in mind that mycobacteriosis is a rare disease, although it has become more common than one might have expected a decade or two ago. Based on our experience, we believe that the pulmonary diseases in which NTM are the causative factor may account for over a dozen percent of all mycobacterial infections [8]. This is consistent with data reported from other parts of the world [9–12]. There has been a noticeable rise in the number of cases of mycobacteriosis in developed countries, and this has been paralleled by a fall in the incidence of tuberculosis. It is difficult to establish whether this is associated with increased numbers of people predisposed for NTM infections, or other it is instead related to progress in the techniques of differentiating mycobacteria, something which is naturally faster in developed countries [11]. Due to the necessity of differentiating aetiologically significant NTM from accidental contaminations of clinical samples, a microbiological confirmation of mycobacteriosis in symptomatic patients in whom tuberculosis has been ruled out consists of positive cultures for NTM of the same species on two separate occasions [4]. This sometimes requires testing of more than three clinical samples from a patient, which is a standard in the diagnosis of tuberculosis [8]. However, the fundamental diagnostic difficulties in the case of mycobacteriosis result from the fact that, based on the clinical manifestations, it is impossible to differentiate it from tuberculosis, and microbiological methods, even the most sensitive ones (molecular methods), do not detect infection with *M. tuberculosis* in all the patients affected by tuberculosis. It happens that in asymptomatic patients presumed to have tuberculosis without microbiological con-

firmation, antituberculous treatment is initiated. It is only when this treatment proves unsuccessful that the search for aetiological factors other than *M. tuberculosis* is prompted

It seems that from the laboratory point of view, failure to diagnose mycobacteriosis can be equally blamed on the arbitrary designation of NTM isolates without identification of the species as environmental contaminants, and on the lack of techniques of performing typing. We believe that an important step forward in the diagnosis of mycobacterioses would be the dissemination of rapid and low-cost molecular methods for differentiating mycobacterial species.

Until recently, the reference method in mycobacterial typing, also used in Poland, was the analysis of mycolic acids by high performance liquid chromatography (HPLC) [13–15]. The set of mycolic acids, or more accurately their elution profile in HPLC, differentiates many, but not all, mycobacterial species. It seems that, although excellent, this technique is becoming obsolete because of the equipment-related costs and the environmental burden of organic solvents.

The current gold standard in experimental research is 16S rDNA sequencing [16], although this is not a method available for routine diagnostics. At the same time, molecular assays, certified for clinical purposes, are being introduced. One of the proposals includes the following EU-certified, mutually complementary assays: GenoType Mycobacterium CM and GenoType Mycobacterium AS (HAIN Lifescience, Germany).

The GenoType Mycobacterium CM (Common Mycobacteria) system offers 15 molecular patterns differentiating between the species: *M. avium*, *M. chelonae*/*M. immunogenum*, *M. abscessus*/*M. immunogenum*, *M. fortuitum* 1, *M. fortuitum* 2/*M. mageritense*, *M. gordonae*, *M. intracellulare*, *M. scrofulaceum*/*M. paraffinicum*/*M. parascrofulaceum*, *M. interjectum*, *M. kansasii*, *M. malmoense*/*M. haemophilum*/*M. palustre*, *M. marinum*/*M. ulcerans*, *M. tuberculosis complex*, *M. peregrinum*/*M. alvei*/*M. septicum* and *M. xenopi*. The GenoType Mycobacterium AS (Additional Species) offers 19 molecular patterns differentiating between the species: *M. simiae*, *M. mucogenicum*, *M. goodii*, *M. celatum*, *M. smegmatis*, *M. genavense*/*M. triplex*, *M. lentiflavum*, *M. heckeshornense*, *M. szulgai*/*M. intermedium*, *M. phlei*, *M. haemophilum*, *M. kansasii* (4 subtypes), *M. ulcerans*, *M. gastri*, *M. asiaticum*, *M. shimoidei*. In addition, each system offers the possibility of obtaining a molecular pattern for *Mycobacterium* sp. (Genus Control).

The aim of our study was to compare the precision in identifying mycobacterial species by the molecular system GenoType Mycobacterium CM/AS (HAIN Lifescience, Germany) versus mycolic acid analysis by HPLC used in routine diagnostics.

Material and methods

Test strains

This was a retrospective study that included NTM strains isolated from clinical materials from 127 patients between 1999 and 2007 at a teaching hospital of the Medical University of Warsaw. The strains were cultured from 64 sputum samples, 38 bronchial lavage fluid samples, 19 bronchoalveolar lavage fluid samples, 2 pleural effusion samples and 4 other clinical samples. The clinical samples were liquefied and decontaminated using soda lye with N-acetylcysteine and sodium citrate (final concentration: 2% NaOH, 0.5% NAC, 1.3% $C_6H_5O_7Na_3$) [17]. The samples were then concentrated and cultured on the Lowenstein-Jensen (L-J) medium. We qualified for the study only acid-fast bacterial strains by Ziehl-Nielsen stain whose elution pattern by HPLC confirmed *Mycobacterium* sp. and differed from *M. tuberculosis complex*.

Species typing by HPLC

The isolated mycobacterial strains were typed in accordance with the Centres for Disease Control and Prevention (CDC) guidelines [18] analysing mycolic acids by HPLC, which is the method routinely used in the microbiological diagnostics of tuberculosis and mycobacterioses at the laboratory of the Medical University of Warsaw [14, 15].

Molecular typing

Species typing at the molecular level was performed with the GenoType Mycobacterium CM/AS systems (HAIN Lifescience, Germany), in which the product of multiplex PCR (polymerase chain reaction) hybridize with specific oligonucleotide probes on a nitrocellulose strip. The system does not involve isolation of mycobacterial DNA, which should be isolated by any technique used for this type of isolation. The manufacturer of the kits does not provide DNA polymerase either.

In our study, we obtained the DNA matrix using the AMPLICOR Respiratory Specimen Preparation Kit (Roche Diagnostics, USA) and continued amplification as described previously [19]. Hybridisation and detection were carried out in an automated system (TwinCubator, HAIN Lifescience, Germany) according to the manufacturer's instructions briefly presented here: 20 μ l of the am-

plification product was incubated for 5 minutes at room temperature with 20 μ l of denaturation reagent (all the reagents and strips are available in the kit). Then 1 ml of hybridisation buffer at 37°C was added, and after the nitrocellulose strip with the probes were placed, hybridisation was carried out for 30 minutes at 45°C. After washing in 1 ml of Stringent Wash Solution at 45°C for 15 minutes, followed by 1 ml of Rinse Solution (RIN) at room temperature for 1 minute, the strips were incubated for 30 minutes at room temperature with 1 ml of alkaline phosphatase conjugated with streptavidin, diluted with an appropriate buffer. The strip was washed three times at room temperature: twice in 1 ml of RIN for 1 minute, and once in 1 ml of water for 1 minute. The washed strip was incubated in 1 ml of the substrate at room temperature, in the darkness, for between three and 20 minutes, until the CC (Conjugate Control) band was stained. The colour reaction was inhibited by washing twice the strip with water. After drying the strip, the pattern of stained bands consistent with specific DNA probes was assessed by comparing it with the pattern provided by the manufacturer. Assessment was only carried out with respect to strips in which the control bands had stained.

Results

For 113 mycobacterial strains out of the 127 clinical isolates tested, reliable results were obtained using both the GenoType Mycobacterium CM/AS molecular system and HPLC. In 105 (93%) cases, the results for both typing methods were consistent. This applied to 35 isolates of *M. kansasii*, 22 isolates of *M. xenopi*, 16 isolates of *M. avium*/*M. intracellulare*, 11 isolates of *M. goodii*, 10 isolates of *M. fortuitum*, 10 isolates of *M. abscessus*/*M. chelonae* and one mixed isolate, namely *M. fortuitum* + *M. kansasii*. The molecular method allowed us to increase the precision of typing of 16 strains identified by HPLC as *M. avium*/*M. intracellulare*, 15 isolates of which turned out to be *M. avium*, and one *M. intracellulare*. Similarly in the case of ten isolates of *M. abscessus*/*M. chelonae* (HPLC), there were nine strains of *M. chelonae* and one strain of *M. abscessus* (GenoType Mycobacterium CM) (Table 1). Inconsistent typing results were obtained in the case of eight strains (7%) (Table 2).

In addition to the aforementioned 113 strains, there were also strains successfully identified by only one of the two methods (6 strains by HPLC and five strains by the molecular method) (Table 3), plus three clinical isolates consistently qualified as NTM but whose identification was unsuccessful by either method.

Table 1. Mycobacterial strains concordant when typed by both methods

Number of strains	HPLC	GenoType Mycobacterium CM/AS
35	<i>M. kansasii</i>	<i>M. kansasii</i>
22	<i>M. xenopi</i>	<i>M. xenopi</i>
16	<i>M. avium</i> / <i>M. intracellulare</i>	15 <i>M. avium</i> 1 <i>M. intracellulare</i>
11	<i>M. gordonae</i>	<i>M. gordonae</i>
10	<i>M. fortuitum</i>	<i>M. fortuitum</i>
10	<i>M. abscessus</i> / <i>M. chelonae</i>	9 <i>M. chelonae</i> 1 <i>M. abscessus</i>
1	<i>M. fortuitum</i> + <i>M. kansasii</i>	<i>M. fortuitum</i> + <i>M. kansasii</i>

Table 2. Mycobacterial strains non-concordant when typed by HPLC and GenoType Mycobacterium CM/AS

Number of strains	HPLC	GenoType Mycobacterium CM/AS
2	NTM	Other than <i>Mycobacterium</i> sp.
1	<i>M. interjectum</i>	<i>M. avium</i>
1	<i>M. interjectum</i>	<i>M. xenopi</i>
1	<i>M. scrofulaceum</i>	<i>M. gordonae</i>
1	<i>M. xenopi</i>	<i>M. xenopi</i> + <i>M. intracellulare</i> + <i>M. scrofulaceum</i>
1	<i>M. gordonae</i>	<i>M. gordonae</i> + <i>M. xenopi</i>
1	<i>M. abscessus</i> / <i>M. chelonae</i>	<i>M. tuberculosis</i> complex

Table 3. Mycobacterial strains typed only by one of two used methods

Number of strains	HPLC	GenoType Mycobacterium CM/AS
1	<i>M. flavescens</i>	NTM
1	<i>M. neoaurum</i>	NTM
1	<i>M. nonchromogenicum</i>	NTM
1	<i>M. avium</i>	Polymerase inhibition
1	<i>M. fortuitum</i>	Polymerase inhibition
1	<i>M. kansasii</i>	Polymerase inhibition
1	NTM	<i>M. fortuitum</i> 2/ <i>M. mageritense</i>
1	NTM	<i>M. intermedium</i>
1	NTM	<i>M. lentiflavum</i>
1	NTM	<i>M. mucogenicum</i>
1	NTM	<i>M. smegmatis</i>

Discussion

In two cases, the strains identified as NTM by HPLC were identified as non-mycobacterial strains by genotyping. This may be explained by the presence of mycolic acids in the cell walls of microorganisms other than those belonging to the *Mycobacteria* genus, such as *Nocardia* [20], which

may sporadically grow on the L-J medium. The library of elution patterns only includes profiles for 29 mycobacterial species.

When *M. interjectum* was identified by HPLC, molecular typing yielded *M. avium* in one case, and *M. xenopi* in the other. Without genotyping based on a reference method, such as 16S rDNA sequen-

cing, these cases are not unequivocally justified. One can only suppose that the elution profile of mycolic acids by HPLC defined as characteristic of *M. interjectum* could in fact result from the presence of non-mycobacterial microorganisms dominating the culture, with concurrent presence of *M. avium* in the first and *M. xenopi* in the second instance.

The discrepancy in identifying *M. scrofulaceum* vs *M. gordonae* (by HPLC and molecular genotyping, respectively) may be explained by the diversity of elution profiles in HPLC. In the library of elution patterns, *M. gordonae* is characterised by one group of peaks. We demonstrated that within the *M. gordonae* species there was another subtype characterised by two groups of peaks [21], similarly to *M. scrofulaceum* [13], for which we did not have the elution patterns in our database. This is where we see the possibility of error in HPLC typing, and despite not having the results by another genotyping method, we are inclined to recognise the correctness of typing by the molecular method.

In two cases, HPLC analysis revealed an elution pattern for one species while the molecular analysis demonstrated the presence of genomes of this and two additional mycobacterial species (Table 2). This precision of molecular typing is the most important advantage of the GenoType Mycobacterium CM/AS system compared to HPLC. This important advantage of the assay has also been emphasised by other authors [22].

It is important that the GenoType assay offers the opportunity to detect *M. tuberculosis* in species-heterogenous in which fast-growing environmental mycobacteria predominate and mask the presence of slow-replicating *M. tuberculosis*. In our study, this happened in one case, confirmed by another molecular assay (AMPLICOR MTB, Roche Diagnostics, USA), in which the presence of *M. tuberculosis* was masked by fast-growing mycobacteria (*M. chelonae*/*M. abscessus*).

The GenoType Mycobacterium CM/AS system enables rapid and reliable species-specific identification of mycobacterial clinical isolates. The GenoType assay procedure is relatively simple, although it does require certain preparation in using molecular methods, because it does not involve DNA isolation, so that each laboratory should carry it out for itself, using one of the commonly accepted techniques. In contrast to the currently used HPLC method, the molecular system is environment-friendly.

Our results fully confirm the positive evaluation reached by other authors regarding the usefulness of the GenoType Mycobacterium CM/AS system for routine diagnostics [22–24]. This simple molecular method may speed up considerably the diagnosis of mycobacterial infections.

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