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Comparison among three methods for mycobacteria identification

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Abstract

Objective. To compare three methods: Biochemical tests, high-performance liquid chromatography (HPLC) and polymerase chain reaction-restriction fragments length polymorphism (PCR-RFLP), for the identification of mycobacteria, and to perform a cost-benefit analysis to define an optimum identification algorithm. **Material and methods**. One-hundred-and-seven mycobacteria isolates were identified by the three methods at Instituto de Diagnóstico y Referencia Epidemiológicos, between February of 1999 and January of 2000 and the results were compared with those of a reference laboratory using the Q-Cochran statistical test. **Results**. PCR-RFLP was the most rapid and specific procedure but also the most expensive; biochemical tests excelled for identification of *Mycobacterium tuberculosis,* but were lengthy and expensive for other mycobacteria; HPLC ranked in the middle for price, speed and specificity. **Conclusions**. Considering the expected proportion of *M. tuberculosis*, the following algorithm was proposed: Initially, biochemical tests should be performed; if the results indicate a non-tuberculous mycobacteria, the isolate should be analyzed with HPLC; if results are unclear, the isolate should be analyzed using PCR-RFLP. Isolates showing a previously undescribed PCR-RFLP pattern should be characterized by DNA sequencing.

Key words: *Mycobacterium*/diagnosis; diagnosis, laboratory; chromatography, high-performance liquid/diagnostic use; Mexico

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Resumen

Objetivo. Comparar tres métodos: pruebas bioquímicas, cromatografía líquida de alta resolución (HPLC, por sus siglas en inglés) y reacción en cadena de la polimerasa-polimorfismo del tamaño de fragmentos de restricción (PCR-RFLP) para identificar micobacterias a nivel especie, analizando costo-beneficio y proponiendo un algoritmo de identificación. **Material y métodos**. Entre febrero de 1999 y enero de 2000, en los laboratorios del Instituto de Diagnóstico y Referencia Epidemiológicos se tipificaron 107 aislados de micobacterias y los resultados se compararon con los obtenidos en un laboratorio de referencia utilizando la prueba estadística Q de Cochran. **Resultados**. Se encontró que el PCR-RFLP fue el método más específico y rápido pero también el más caro. Las pruebas bioquímicas fueron confiables para la identificación de *Mycobacterium tuberculosis*, pero lentas e inespecíficas para otras micobacterias. El HPLC estuvo en un nivel medio tomando en cuenta costo, tiempo y especificidad. **Conclusiones**. Considerando la proporción esperada de *M. tuberculosis*, se propone el siguiente algoritmo: si las pruebas bioquímicas indican una micobacteria no tuberculosa, el aislado será analizado por HPLC; si la identificación no es clara, el aislado será analizado usando PCR-RFLP. Si el aislado no pertenece a un patrón descrito, se identificará por secuenciación de ADN.

Palabras clave: *Mycobacterium*/diagnóstico; diagnóstico de laboratorio; cromatografía líquida de alta resolución/uso diagnóstico; México

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The diseases produced by species of the genus *Mycobacterium* are important causes of morbidity and mortality in the world; they have increased due to HIV infections, with the involvement mainly of *M. tuberculosis* and *M. avium* complexes.[1] The identification of mycobacteria to the species level is important because of the clinical significance; some species are pathogenic while others are not. Knowledge of species is also critical in order to provide adequate patient management because specific antimycobacterial drugs are required against different pathogenic mycobacteria species.[2]

The conventional methods for the identification of mycobacteria, currently used at Instituto de Diagnóstico y Referencia Epidemiológicos (InDRE) in Mexico City, are based on culture and biochemical tests, they require several weeks for adequate growth, and sometimes, accurate identification is not possible.[3] Difficulties such as lack of adequate reproducibility, the variability of phenotypes, and the fact that phenotype information is limited to common species, may lead to ambiguous or erroneous results.[2] Alternative techniques have been established, such as thin-layer chromatography, gas-liquid chromatography, high-performance liquid chromatography (HPLC), and molecular techniques based on hybridization, amplification, or sequencing of nucleic acids, but in developing countries they are generally limited to research laboratories.[3]

This study was designed to compare (from economic and operational points of view), mycobacteria biochemical tests with two new techniques, HPLC and PCR: HPLC separates mycolic acids extracted from bacterial cell wall, that are derivatized to their p-bromophenacyl esters as a function of their chain length, unsaturated acids, and functional groups, thus generating a distinctive pattern for each species of mycobacteria.[4,5] The polymerase chain reaction (PCR)-based method amplifies a fragment of the gene that codes for the 65-KDa heat-shock protein (*hsp65*), followed by restriction fragments length polymorphism (RFLP) analysis, using the restriction enzymes *Hae*III and *Bst*EII.[3,6]

The objective of the study was to implement a new algorithm for processing mycobacterial strains that arrive at this Institute, to offer a more efficient service.

Material and methods

This was a masked study, conducted between February 1999 and January 2000 at Instituto de Diagnóstico y Referencia Epidemiológicos in Mexico City. A total of 107 isolates were analyzed, 76 of which had been previously identified as non-tuberculous mycobacteria, and 31 as *M. tuberculosis* complex isolates.

Sample size was calculated for a confidence interval of 95% ($p<0.05$). Strains were selected and analyzed in a double-masked fashion. Four subcultures for each strain were obtained independently to perform biochemical tests, HPLC, PCR-RFLP, and one external control test (Texas Department of Health, Austin TX, USA); the identification algorithm consisted firstly of HPLC and then, if any strain was difficult to identify, genetic probes and biochemical tests were used.

Ultraviolet-detection HPLC was performed as described by the U.S. Department of Health and Human Services.[7] A newer reference version that has now been published,[8] was not available when this study was conducted.

For PCR-RFLP, DNA was extracted as described by van Soolingen.[9] Basically, bacterial cells were lysed with lysozyme, proteinase K and SDS, followed by CTAB/NaCl and chloroform extraction; DNA was then precipitated with isopropanol, washed twice with ethanol, and resuspended in 20 µl of sterile distilled water. The PCR procedure was partially based on Telenti *et al.*[6] Primers Tb11 (5′ –ACC AAC GAT GGT GTG TCC AT) and Tb12 (5′ –CTT GTC GAA CCG CAT ACC CT) were used to amplify a 439-bp fragment of the *hsp65* gene; reaction conditions included a pre-incubation time of 2 min at 94 ºC, followed by 40 two-step cycles of 30 sec at 94 ºC, and 2 min at 67 ºC. PCR products were electrophoresed in 2% agarose gels and digested separately by restriction endonucleases *Bst*EII and *Hae*III. Restriction products were analyzed on 12% polyacrilamide gels using pBR322/*Hae*III as the molecular weight standard. Restriction patterns were compared with the algorithm reported by Devallois *et al*.[3]

Biochemical analysis began by determining growth rate and pigment production for each isolate. To find the optimal temperature for growth, each strain was incubated at several temperatures (25, 37, and 42 ºC). Three biochemical tests; niacin, nitrate reduction, and catalase production at 68 ºC, were performed. Niacin and nitrate-positive isolates and 68 ºC catalasenegative isolates were reported as *M. tuberculosis*. Niacin-negative, 68 ºC catalase-positive and/or nitrate-negative isolates were further characterized with other biochemical tests like iron uptake, 5% NaCl tolerance, Tween hydrolysis, 1mM and 3mM aryl sulfatase, potassium tellurite reduction, and urease.[10-12]

Result data were entered into a database, to calculate the sensitivity for each method, and concordance percentages between methods and with the reference laboratory. Finally, a time and resources analysis was

made for each method. Statistical analysis was performed using the WinstatTM statistical package; Q-Cochran's test was used to obtain *p* values of $p<0.05$, $p<0.01$ and $p<0.001$.

Results

One-hundred-and-seven *Mycobacterium* isolates from our institutional collection were identified to the species level. Thirty-one belonged to the *M. tuberculosis* complex and the remaining were non-tuberculous mycobacteria.

The fastest method was the PCR-RFLP; results from HPLC and biochemical testing required 5 and 15 additional days, respectively. The differences were mainly due to the larger amount of cells required for ultraviolet-detection HPLC and to the large number of biochemical tests necessary for the identification of non-tuberculous mycobacteria.

Identification results with the three methods compared with the reference laboratory are summarized in Table I. Basically, for the identification of *M. tuberculosis* complex, the three methods were adequate. PCR-RFLP was more sensitive, since it correctly identified all 31 *M. tuberculosis* isolates; HPLC identified 28 isolates, and the biochemical tests identified 26. For the non-tuberculous mycobacteria, PCR-RFLP was also more sensitive; especially remarkable was that this technique identified 20 from 24 *M. avium* complex isolates, while the HPLC and the biochemical tests only identified 18 and 5 isolates, respectively. This was partially due to the observation of 6 unusual HPLC patterns (Table II) and 19 pigmented isolates that also showed an altered growth rate.

Specificity was calculated for each species, considering as negatives all the isolates not belonging to that particular species and as false negatives, the isolates erroneously identified as belonging to the species.

For *M. tuberculosis* the specificity was 98.7% for two methods, PCR-RFLP and biochemical tests, with only one error in 76 samples. HPLC specificity was initially only 83.1% because *M. terrae* and *M. avium* complexes isolates showed different patterns from those found in the CDC manual,[7] and only a limited number of *M. terrae* complex reference patterns were available at our Institute at the time of the study. Additional *M. terrae* and *M. avium* complexes reference patterns were obtained and compared with the isolates that were mistakenly identified as *M. tuberculosis*. It was then possible to clearly differentiate all of *M. terrae* and *M. avium* complexes isolates from *M. tuberculosis*. After correcting for these isolates, specificity of HPLC was 100%.

Table I

SENSITIVITY OF THE THREE METHODS FOR SPECIES IDENTIFICATION COMPARED WITH THE REFERENCE LABORATORY

Species	Reference	Method					
		PCR-RFLP		HPLC		BT	
	n	*n*	%	*n*	%	*n*	%
Mycobacterium tuberculosis complex	31	31	100	28	90	26*	84
Mycobacterium fortuitum	19	18	95	17	90	13*	68
Mycobacterium avium complex	24	20*	83	18*	75	5§	21
Mycobacterium gordonae	14	11	79	6‡	43	8*	57
Mycobacterium terrae complex	10	3	30	0	0	3	30
Mycobacterium simiae	5	5	100	3	60	0	0
Mycobacterium flavescens	2	0	0	0	0	1	50
Mycobacterium asiaticum	1	1	100	1	100	0	0
Mycobacterium marinum	1	1	100	0	100	0	0

Q-Cochran test, * $p<0.05$, ‡ $p<0.01$, § $p<0.001$

Table II

NUMBER AND PERCENTAGE OF STRAINS WITH ATYPICAL PATTERNS

Species	Refernce	PCR-RFLP		HPLC		BT	
	n	*n*	%	*n*	%	*n*	%
Mycobacterium tuberculosis complex	31	0	0	3	10	0	0
Mycobacterium avium complex	24	0	0	6	38	19	80
Mycobacterium fortuitum	19	0	0	2	11	6	32
Mycobacterium gordonae	14	3	21	8	62	6	43
Mycobacterium terrae complex	10	7	70	10	100	7	70

Statistical analysis showed significant differences ($p<0.05$) among the three tests for the identification of *M. tuberculosis* and *M. avium* complexes, but not for *M. fortuitum* and *M. gordonae*. Statistical significance and differences between each test and the reference test are shown in Table I.

Cost analysis of the three methods is shown in Table III. In this analysis we separated the biochemical procedures for *M. tuberculosis* from those required for the identification of non-tuberculous mycobacteria. The least expensive method for *M. tuberculosis* identification was the biochemical test; it was also the fastest, since it required only one day. For non-tuberculous

Table III
RESOURCES CONSUMED FOR EACH IDENTIFICATION METHOD OF MYCOBACTERIA TO SPECIES LEVEL

Method	*Time (days)*	*Workers*	*Samples per day*	*Salary per sample (USD)*	*Reagents (USD)*	*Material (USD)*	*Cost per sample (USD)*
BT (*M. tuberculosis*)	1	1	20	1.7	1.3	1.0	4.0
BT (NTM)	30-40	2	20	3.4	3.05	3.65	10.1
HPLC	1	1	5	6.8	3.7	2.3	12.8
PCR-RFLP	2	2	20	3.4	3.6	5.9	13.0

BT: biochemical tests
NTM: non-tuberculous mycobacteria
HPLC: high performance liquid chromatography
PCR-RFLP: polymerase chain reaction-restriction fragments length polymorphism

mycobacteria, biochemical tests were inefficient, costly, and required more than 30-40 days. HPLC was as fast as the biochemical tests for *M. tuberculosis* identification, with the advantage that it can identify other mycobacteria at the same time, but at a higher price. Finally PCR-RFLP was the most expensive procedure, and required one more day than the other methods; but like HPLC, PCR-RFLP can also immediately identify non-tuberculous mycobacteria. Furthermore, it was also the most accurate method, as shown in Tables I and II.

Discussion

Results obtained in this work showed that the basic methodology for mycobacteria identification, including colonial morphology, pigmentation, growth rate, and enzymatic tests, was fast and reliable for the identification of *M. tuberculosis* isolates, requiring three tests performed in a single day from a well-grown homogeneous culture. However, a non-tuberculous mycobacteria strain implied a lengthy process that required up to nine different tests and took approximately 6 to 8 weeks. After finishing this long and time-consuming procedure, we often found that the results were not absolute, because sometimes intra-species variation in enzymatic activity and phenotypic characteristics were observed. In the present work, these variations made particularly difficult the identification of species belonging to the *M. terrae* and *M. avium* complexes. Wallace *et al*[13] reported that 80% of *M. avium* complex strains recovered from patients with HIV show pigmentation. This feature was not considered for *M. avium* complex identification at our laboratory, leading to an erroneous assignation of species. Another source of uncertainty was the reported heterogeneity for *M. terrae* complex, that led to the low sensitivity observed for those isolates with the three methods. Interestingly, the three strains belonging to *M. terrae* complex identified by PCR-RFLP were *M. nonchromogenicum*, which has been shown to be pathogenic to humans.[14,15]

With the use of an adequate collection of reference patterns, identification by HPLC was reliable. The required time to obtain a result from a pure culture was 1 to 2 days. Nowadays, this technique is well standardized and we have now patterns allowing the identification of up to 20 species.[8] In our study, HPLC took more time than PCR-RFLP, partially because there were too many strains to analyze simultaneously, but also because ultraviolet-based HPLC needs more cells than the PCR procedure and therefore more time was required to obtain the cell mass. Faster identification by HPLC could be possible by developing more sensitive fluorescence detection of mycolic acid esters.[16]

The problem with identification of *M. avium* and *M. terrae* complexes emphasized the need for adequate HPLC reference patterns.

The PCR-RFLP was the most precise of the three methods and at least as fast as the HPLC; moreover, it can identify up to 34 *Mycobacterium* species[3] and can also identify several *Nocardia* and other aerobic actinomycetes.[17,18] In fact, as described above, this method was the first to finish the identification of all the isolates, because it allows the technicians to process more isolates on a daily basis.

Another important aspect was the cost of the techniques. In a direct comparison, we found that the biochemical tests used for *M. tuberculosis* identification were the least expensive, followed by HPLC and biochemical tests for non-tuberculous mycobacteria, while PCR-RFLP was the most expensive. However, we also have to consider that a faster and more accurate identification of mycobacteria allows a faster recovery of the patients and important savings in drugs; since cur-

rent tuberculosis treatment takes about 6 months, costs around $100 USD in Mexico, and is useless when the infection is caused by non-tuberculous mycobacteria.

We think that more efficient identification of mycobacteria could be achieved by a combination of the three methods in an optimized algorithm (Figure 1), considering cost-benefit and time-saving criteria. This algorithm will be used at our Institute following the publication of this study: When the primary culture is obtained, the three basic biochemical tests to identify *M. tuberculosis* are performed. When these tests suggest a non-tuberculous mycobacteria, the isolate is then analyzed by HPLC, which allows the identification of most species at a relatively low cost. If an unusual HPLC pattern is found or there is not enough growth for a clear identification, the isolate is analyzed by PCR-RFLP, which allows the identification of a wider variety of species with a smaller sample. When a sample with clinical or epidemiological characteristics suggesting non-tuberculous mycobacteria arrives at our Institute, the sample will be analyzed directly by PCR-RFLP to obtain faster results. If the PCR-RFLP is unable to give an unequivocal result, sequencing of 16S RNA and the *hsp*65 gene will be performed for a final identification or for its inclusion in the data banks if new species are encountered. Finally, the importance of other methods like the hybridization tests used in the reference laboratory at the Texas Department of Health should be considered. These tests have the advantage of being highly specific, but the disadvantage that a specific test for each species is required. These tests are not recommended for the screening of a large number of unknown isolates, but are the best available to confirm an uncertain identification performed by other methods, or when the identification reaches a point from which only a limited number of species can be assigned.

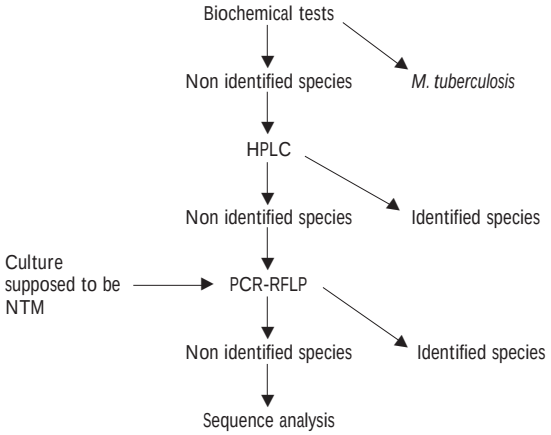

FIGURE 1. OPTIMIZED ALGORITHM FOR IDENTIFICATION OF MYCOBACTERIA TO THE SPECIES LEVEL

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