Original Article

Evaluation of a genetic probe (Gen-Probe Accuprobe® system) in comparison to traditional methods for identifying members of the Mycobacterium tuberculosis complex*

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Background: The appearance of tuberculosis/human immunodeficiency virus co-infection and the growing number of diseases caused by nontuberculous mycobacteria, as well as the confusion that these can cause in relation to emerging multidrug-resistant strains, require more accurate and rapid laboratory results, not only in the isolation of strains but also in their identification.

Objective: A comparative study evaluating a new tool of molecular identification, which uses a genetic probe based on the 16S rDNA sequence of the Mycobacterium tuberculosis gene (Gen-Probe Accuprobe® Gen Probe, Inc.), and the classic methodology.

Method: Fifty-five Mycobacterium strains, isolated from the sputum of patients treated at a tuberculosis reference clinic, were selected for study. Subcultures were performed in three tubes: one submitted to genetic identification, one analyzed through classical tests (production and accumulation of niacin; growth in the Lowenstein Jensen medium with the inhibitor agents p-nitrobenzoic acid and thiophene-2-carboxylic acid hydrazide added), and one held in reserve.

Results: The probe identified 51 cases as belonging to the M. tuberculosis complex (one associated with M. kansasii) and the other 4 as nontuberculous mycobacteria, later identified as M. kansasii (3) and M. avium (1). Using traditional methods, 47 samples were identified as belonging to the M. tuberculosis complex, 4 were classified as fitting the profile of nontuberculous mycobacteria (in agreement with the genetic probe results), and 4 were unidentified, 1 of which presented the exact characteristics that 2 mycobacterium species have in common.

Conclusion: The benefits of the molecular biology technique justify its implementation and routine use, in combination with classical methods, in a high-traffic clinic where complex cases of tuberculosis are treated.

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INTRODUCTION

The pandemic caused by the human immunodeficiency virus has modified tuberculosis (TB) epidemiology, complicated TB diagnosis and made it difficult to treat and control TB in several regions of the world[1-3]. This combination has also produced an increase in the number of diseases caused by nontuberculous mycobacteria (NTM), which causes confusion in diagnosis, especially in relation to the differential diagnoses between these diseases and those caused by multidrug-resistant strains[4]. In first world countries, which possess state-of-the-art technology, the reemergence of TB and the study of the problems above led to the development of automated laboratory processes as well as of molecular biology techniques for the diagnosis of TB that not only isolate and identify but also determine the resistance profile of the mycobacteria more accurately and more rapidly than do traditional methods.

The introduction of these new methods in countries with higher TB prevalence, but more limited financial resources, is dependent on the capacity to invest and make decisions based on studies that evaluate the comparative applicability of these new technologies in relation to the existing ones as well as on the cost-benefit ratios involved[5].

This was the reason for the present study, carried out at a TB outpatient referral clinic (Clemente Ferreira Institute) in the city of São Paulo. At this clinic, the annual numbers of cases of multidrug-resistant TB (so defined when resistant to rifampin, isoniazid and one more typically prescribed drug) and of TB/human immunodeficiency virus co-infection are estimated at 60 to 70 and 30 to 35, respectively[6].

The present study aims to evaluate the applicability and efficiency of an advanced diagnostic tool for identifying the Mycobacterium tuberculosis complex, a genetic probe (Accuprobe® System; Gen-Probe, San Diego, CA, USA), in comparison to the traditional method of biochemical identification of production and accumulation of niacin and the method of growth in cultures containing inhibitor agents such as p-nitrobenzoic acid (PNBA) and thiophene-2-carboxylic acid hydrazide (TCH), which are routinely used in the laboratory of the Clemente Ferreira Institute.

METHODS

In order to standardize the comparative tests, strains isolated from identified patient sputum samples were selected from among those available at the Clemente Ferreira Institute laboratory between October and December of 2001. From among the cultures with the highest number of colonies in Löwenstein-Jensen medium, strains were chosen on the basis of viability, and subcultures were performed. According to patient charts, all HIV test results were negative. The samples were collected from patients who had never undergone treatment and from patients under treatment control (this datum was reported in the cases in which there was discordance among the methods used and in the cases in which the presence of NTM was determined). Since procedures were carried out using the subculture of the first isolate, previous use of tuberculostatic drugs did not interfere in the results. The genetic probe can only be employed using culture in liquid or solid medium, or following methods of gene amplification, according to the kit guidelines (kit directions for use available at http://www.genprobe.com). The procedures for collection of subcultures, the decontamination of the samples and the laboratory techniques for culture in Löwenstein-Jensen medium were performed in accordance with the guidelines established in the Manual de Bacteriologia da Tuberculose (Guidebook for Tuberculosis Bacteriology)[6].

Subcultures were performed in three tubes: one submitted to genetic identification using the probe based on the 16S rDNA gene sequence of the M. tuberculosis complex; one analyzed through classic phenotype tests (niacin production, niacin accumulation and growth in Löwenstein-Jensen medium augmented with the inhibitors p-nitrobenzoic acid and thiophene-2-carboxylic acid hydrazide); and one held in reserve in order to repeat, if necessary, the tests that presented discordances and identify the NTM through the use of probes. The Löwenstein-Jensen medium - augmented or not with the inhibitor agents - was created in the Clemente Ferreira Institute laboratory in accordance with the guidelines established in the Manual de Bacteriologia da Tuberculose do Ministério da Saúde (Health Ministry Guidebook for Tuberculosis Bacteriology)[6].

Tests with an HV37Ra ATCC strain of M. tuberculosis, an ATCC strain of M. bovis and an ATCC strain of M. smegmatis were used as controls. As required, the interval between subculture and the performance of all tests did not exceed 30 days. Tests with discordant results were repeated in order to exclude the possibility of any error in the use of the technique, and all of them confirmed the results obtained.

The identification tests using the genetic probe were performed using the sample from the second

tube of the isolate subculture. In accordance with the recommendation for the M. tuberculosis complex kit, the sample was collected with the aid of a disposable platinum loop. Since the stipulated times and temperatures are critical factors in the technique, they were strictly adhered to.

In order to apply the technique, a considerable number of colonies (approximately three loops) were collected in each test, and the sample was exposed to a lytic process using specific reagents that are available in the kit for the extraction of genetic material. The sample was homogenized together with lytic reagents and placed into a device called sonicator (a sound wave emitter) for fifteen minutes in order to perform the extraction. At the end of this period, the technician carefully removed the sample from the sonicator, avoiding any agitation, and exposed it to a temperature of 95°C ± 5°C for ten minutes. The aim of the whole process described above is to extract nucleic acids. After this extraction, 100 µl of the lytic solution were hybridized using the probe (which is already marked with ester of acridine and is affixed to the internal surface of a specific tube in the kit). This hybridization is performed using a thermoblock at a temperature ranging from 59.5°C to 61°C for fifteen minutes.

Through the use of a specific selection reagent (300 µl) and homogenization, the material that had not been hybridized was eliminated from the reaction (as was the case for samples that tested negative by the probe). This selection was performed using a thermoblock at a temperature ranging from 59.5°C to 61°C for ten minutes (in the case of the probe for the M. tuberculosis complex, the specific length of time stipulated in the kit is strictly observed).

The sample was allowed to settle for 5 min, after which the reading and interpretation were performed at room temperature using a device known as a luminometer. The reading is expressed as relative light units (RLU) and interpreted as follows: 20,000 RLU = negative; from 20,000 to 29,999 RLU = inconclusive; above 30,000 = positive.

For identification of NTM, a similar procedure was carried out using the samples of the tube held in reserve.

For the tests of identification through growth in the Löwenstein-Jensen medium augmented with inhibitors, we seeded 0.1 ml of a 10-3 dilution of the colonies (that obtained using the 1.0 McFarland standard) into three other tubes containing the same medium: the first augmented with PNBA (500 µg/ml); the second augmented with TCH (2 µg/ml); and the third with no inhibitors.

Niacin production and accumulation by the isolate was demonstrated using a reagent strip (TB niacin test strips, BBL taxo®; Becton Dickinson, Cockeysville, MD, USA). The method was applied in accordance with the kit guidelines.

The niacin production and accumulation test was carried out on the same day that the subcultures were read and the other tests were carried out, that is, within 30 days (3- to 4-week cultures, with at least 50 colonies, in accordance with the requirements of the technique). The medium was torn using a disposable loop, 1.5 ml of sterile distilled water were added, and it was inclined so that the liquid covered the surface for 15 to 20 min. At the end of this period, 0.6 ml of this liquid were withdrawn and placed into a sterile screw cap tube. A BBL reagent strip was placed into this tube with the aid of a pair of forceps. The exam was defined as positive if the water turned yellow and negative if it did not.

In these tests, the M. tuberculosis complex is considered present when there is sensitivity to PNBA, resistance to TCH and positivity for the accumulation of niacin.

All procedures were carried out in a biosafety laminar flow hood in accordance with the biosafety guidelines established in the Health Ministry Guidebook for Tuberculosis Bacteriology[6].

Finally, patient charts were reviewed in order to evaluate clinical aspects, as well as complementary tests that contributed to diagnosis and therapeutic evolution, comparing the findings to the results of the diagnostic tests under study.

The study was approved by the Ethics in Human Research Committee of the Instituto de Ciências Biomédicas da Universidade de São Paulo (University of São Paulo Institute of Biomedical Sciences). There was no conflict of interest since the present study was funded entirely by the Clemente Ferreira Institute.

RESULTS

Isolates from the sputum of 55 patients were tested. The genetic probe reading was positive for the M. tuberculosis complex in 51 of the samples (one of them associated with M. kansasii) and 4 classified as fitting the profile of NTM, being identified by specific probes (3 cases of M. kansasii and one case of M. avium). Using traditional methods, 47 samples were identified as belonging to the M. tuberculosis complex, 4 were classified as fitting the profile of NTM (in agreement with the genetic probe results), and 4 were unidentified, one of which being exactly the one that presented two mycobacterium species in the probe reading (Table 1).

TABLE 1

Comparative results obtained using the genetic probe and traditional methods for identification of M. tuberculosis, Clemente Ferreira Institute-SP, 2001

	GPAS	Traditional methods
M. tuberculosis complex	51	47
NTM	4	4 *
Unidentified	0	4
Total	**55**	**55**

* One of them presenting M. tuberculosis and M. kansasii concomitantly.
GPAS: Gen-Probe Accuprobe System; NTM: nontuberculous mycobacteria.

When evaluating the 4 samples that were unidentified using traditional methods and identified as strains of the M. tuberculosis complex using the probe, we observed that 1 presented discordance regarding niacin accumulation, 1 presented discordance regarding TCH, and 2 presented discordance regarding PNBA. In the 1 case identified as belonging to the M. tuberculosis complex in the probe reading and in the niacin accumulation test, there was growth in Löwenstein-Jensen medium augmented with PNBA. When the sample in question was tested using other probes, the M. kansasii species was identified, proving that it presented both species (Table 2).

All tests with discordant results were repeated in order to exclude the possibility of any error in the use of the technique, and all confirmed the results obtained.

In reviewing the charts of the 51 patients in whom the M. tuberculosis complex was identified by the probe, we found that all were considered TB cases with a compatible clinical and radiological diagnosis and most of them were cured as the treatment evolved, including the case that presented the two different species, without showing symptoms related to NTM during this evolution. A few were considered multi-drug resistant, in treatment and with favorable evolutions. The 4 patients identified as being infected with NTM strains were diagnosed as follows: 1 with multi-drug resistant TB (cured, colonized by the M. avium); 2 with mycobacteriosis caused by M. kansasii (cured through alternative treatment regimens); and 1 with pulmonary TB (cured, with no extensive sequelae or related symptoms), who was considered to have been colonized by the M. kansasii (Table 3).

DISCUSSION

For many years, sputum microscopy has been recommended as the basis and main tool for diagnosing TB[3,7]. Some use it almost exclusively as a means of screening for the disease in patients presenting respiratory symptoms. The current Ministério da Saúde Manual Técnico para o Controle da Tuberculose (Health Ministry Technical Guidebook for Tuberculosis Control)[8], published in 2002, states, as it has since its third edition (1988), that, in addition to those cases in which there is positivity in sputum microscopy and a positive culture, it is possible to define cases in which "the physician makes the diagnosis based on clinical and epidemiological data as well as on the results of complementary tests" as cases of TB. This has revived old and new opinions that bacteriology is equally as important as imaging diagnosis, roentgenography[9,10] and conventional chest X-rays[5,11] in the discovery of TB cases in risk groups.

Traditional bacteriology remains valid in regions with limited resources, where the number of cases is also low. However, in large urban centers, where TB incidence and prevalence are actually increasing, diagnosis cannot be made based on sputum microscopy alone. This is especially true in referral clinics, where the most complicated and difficult to diagnose cases are concentrated. In addition, such facilities use

TABLE 2

Discordant samples in the comparison of the results obtained using the genetic probe and traditional methods for identifying M. tuberculosis, Clemente Ferreira Institute-SP, 2001

		Traditional methods			
Sample	GPAS	LJ PNBA	LJ TCH	NAC	Discordance
3853-CFI*	Mtb(+)	S	S	(+)	LJ TCH
3837-CFI*	Mtb(+)	R	R	(+)	LJ PNBA
4204-CFI*	Mtb(+)	S	R	(-)	B3
4316-CFI*	**Mtb(+)**	**R**	**R**	**(+)**	**LJ PNBA****

GPAS: Gen-Probe Accuprobe System; Mtb: Mycobacterium tuberculosis complex; LJ PNBA: Löwenstein-Jensen medium augmented with p-nitrobenzoic acid; LJ TCH: Löwenstein-Jensen medium augmented with thiophene-2-carboxylic acid hydrazide; B3: niacin; S: sensitive; R: resistant. *Number of the sample of the Clemente Ferreira Institute laboratory; **M.tuberculosis concomitant with M.kansasii.

TABLE 3

Comparison of clinical profile and the results obtained using genetic and traditional methods for identifying M. tuberculosis, Clemente Ferreira Institute-SP, 2001

	GPAS	Traditional methods	Clinical profile
Identified as belonging to the Mtb complex	51	47	TB diagnosis*
Unidentified	0	4	
NTM	4	4	MDR-TB cure-coloniz. M. avium
			Mycobacteriosis- M. kansasii
			Mycobacteriosis- M. kansasii
			TB cure-coloniz. M. kansasii

GPAS: Gen-Probe Accuprobe System; Mtb: Mycobacterium tuberculosis; NTM: nontuberculous mycobacteria; TB: tuberculosis; MDR-TB: multidrug-resistant tuberculosis; coloniz.: colonization. *One of the unidentified cases presenting M. tuberculosis and M. kansasii concomitantly.

differentiated therapeutic procedures (whether due to the resistance profile, adverse effects of treatment, risk associations or other problems). Therefore, it is necessary that more reliable and rapid laboratory resources, which can meet the demand, be used.

It can be said that, in Brazil, there is a distinct tendency to accept, as well as an evident need to use, modern methods for diagnosing TB. These methods have been rapidly and progressively introduced in universities, referral centers and private clinics. A policy for the implementation of more complex resources in public health clinics must reconcile needs with feasibility. As recommended by Kritski et al.[5] in their guidelines for the approach to TB at the Universidade Federal do Rio de Janeiro (Rio de Janeiro Federal University), It is necessary to evaluate the efficacy, the cost-benefit ratio and the adaptability of the new technologies to the treatment demands, taking into consideration the availability of local investment.

This was the motivation for the present study, which compares the genetic probe, an advanced diagnostic technology for identifying the M. tuberculosis complex, to the methods routinely used in referral clinics.

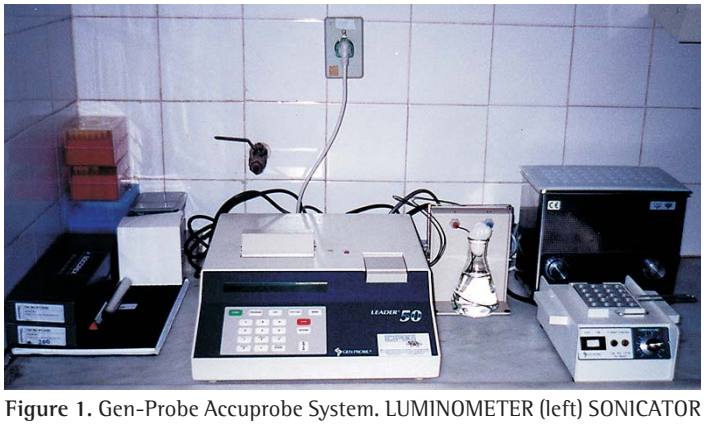

Figure 1. Gen-Probe Accuprobe System. LUMINOMETER (left) SONICATOR (back right)-THERMOBLOCK (front right)

Since the expectation that the probe to be tested would, due to its nature, produce much more accurate results than those obtained using the classic methods, making it impossible to determine its predictive values and use them as a basis, an initial problem was the lack of a gold standard. In addition, the initial idea of carrying out a more detailed study of the cost-benefit ratio was abandoned due to the fact that the device and the materials are imported, and it is difficult to estimate the costs of implementation and reproducibility in view of exchange rate fluctuations.

The present study evaluated only relative yield and applicability of the genetic probe as compared to the traditional methods used at the Clemente Ferreira Institute laboratory.

Isolates from the most significant and prolific cultures were selected for subculture, thereby synchronizing the starting point for the methodologies being compared. The kit guidelines for the use of the new technology were strictly followed, and the routine methods were used in compliance with the guidelines established by the Brazilian Ministry of Health in the Guidebook for Tuberculosis Bacteriology[6].

The results met our expectations, confirming the superiority of the probe over the traditional methods. Using the probe, 55 isolates were diagnosed definitively, 51 of which were identified as belonging to the M. tuberculosis complex (100% yield). Of those 51 isolates, one was associated with M. kansasii and 4 were identified as NTM through the use of specific probes: 2 as mycobacteriosis (M. kansasii) and 2 as colonizations in cured TB patients (M. avium and M. kansasii). The classic tests routinely used did not allow us to arrive at a definite diagnosis for 4 isolates (92.7%) presenting discordant results. The clinical profile of the patients was consistent with the identification made through the use of the probe.

In cases identified as NTM, diagnosis could not be made solely through the use of the probe for M.

tuberculosis, and it was confirmed through the use of other specific probes, after the traditional phenotypic methodology indicated its presence. This fact indicates that the two methodologies should be used in concert, thereby fostering financial savings and the rationalization of the use of more advanced resources.

The probe may also be useful for defining the cure and controlling TB treatment, thereby preventing delays and unnecessary expenses. This point is illustrated by the cases in which NTMs were isolated in TB patients who were clinically cured and who would have continued to receive TB treatment if the isolates had not been identified as NTM.

Although the probe is much more expensive than the routine techniques, it is also more rapid. Results are obtained within approximately three hours, compared with 28 to 30 days for traditional articulated methods. The latter demand more staff time, incubators, glassware and, as at the Clemente Ferreira Institute, the preparation of all media. The probe laboratory protocol is very simple, using ready-made reagents and only three pieces of equipment (Figure 1).

The Accuprobe was launched in the United States in 1989 and was approved by the Food and Drug Administration. Its efficacy has been confirmed in various international studies, such as that carried out by Middleton et al.[12], in which the ability of the probe to detect the M. tuberculosis complex in a culture also containing the M. avium complex was investigated. The study suggested that the M. avium complex did not interfere with the detection of the M. tuberculosis complex and vice-versa when a specific probe for each one was used. Analyzing 134 clinical isolates, Lebrun et al.[13] found that the probe presented 100% specificity for the M. tuberculosis complex, the M. avium complex, M. gordonae and M. Kansasii; 100% sensitivity for the M. tuberculosis complex and M. gordonae; and 95.2% sensitivity for the M. avium complex. Through the analysis of 2727 isolates obtained from cultures performed in MB/BacT® (Organon Teknika, Durham, NC, USA), Badak et al.[14] confirmed that the probe presented 96.4% sensitivity and 100% specificity for the M. tuberculosis complex and 100% specificity and sensitivity for M. gordonae, considerably higher than the specificities and sensitivities obtained using standard biochemical identification tests.

In a nationwide consensus, the II Diretrizes Brasileiras para Tuberculose (Second Brazilian Guidelines on Tuberculosis Management), it was recommended that, since it is a simple method, is readily available in the market and has been validated, identification of the isolated mycobacterium species through the use of the probe be implemented in referral laboratories[15].

For the identification of the M. tuberculosis complex, the probe is highly specific, sensitive, rapid and effective. It also allows a prompt response for clinical intervention, thereby facilitating diagnosis. The failures resulting from the laboratory process involved in the classic methodology, as shown in the present study, indicate that molecular methods for diagnosing mycobacteria constitute fundamental methodologies, rather than alternative resources, for diagnosing TB. The benefits of the molecular biology technique justify its implementation and routine use, in combination with classic methods, in high-traffic clinics where complex cases of tuberculosis are treated.

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