Use of the AMPLIFIED™ *Mycobacterium tuberculosis* direct test for the diagnosis of tuberculosis

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استخدام الاختبار المباشر المضخَّم للمتفطرات السلية في تشخيص السل

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الخلاصة: أجرى الباحثون تحليلاً مقارناً لثلاثة اختبارات تقليدية للسل في مقابل طريقة المسبار الدَّنَوي لدى مرضى السل في مركز إحالة في اليونان. ففي عام 2004 حلَّل الباحثون 2961 عينة مأخوذة من 2234 مريضاً بالطرق التالية: التلوين بصيغة تسيل نيلسون، والزرع في مستنبت لوفنشتين جنسن، وأنابيب مؤشرات النمو للمتفطّرات BACTEC والاختبار المباشر المضخَّم للمتفطرات السُلِّية بالمسبار الجيني (اختبار مباشر معدَّل)؛ وقد شخَّص الباحثون 136 مريضاً بالسل يتلقّون المعالجة المضادة له، منهم 133 مريضاً (98٪) إيجابيون للاختبار المباشر المعدَّل المضخَّم، و112 مريضاً (82٪) كشفهم الباحثون بطريقة أنابيب مؤشرات النمو للمتفطرات، و102 مريضاً (75٪) كشفهم الباحثون بالزرع في وسط لوفنشتين جنسن، و75 مريضاً (55٪) كشفهم الباحثون بالتلوين بصبغة تسيل – نيلسون. ثم إن استخدام الاختبار المباشر المعدَّل يعطي نتائج إيجابية تصبح جاهزة خلال ساعات في حين يتطلَّب غيره أياماً أو أسابيع.

ABSTRACT A comparative analysis was made of 3 conventional tests for tuberculosis (TB) versus a DNA probe technique among suspected TB patients at a reference centre in Greece. During 2004, we tested 2961 biological specimens from 2234 patients with the following methods: Ziehl–Neelsen staining, Löwenstein–Jensen culture, BACTEC mycobacteria growth indicator tubes (MGIT) and the Gen-Probe AMPLIFIED™ ***Mycobacterium tuberculosis*** direct test (MTD). Of a total of 136 TB patients diagnosed and under anti-TB treatment, 133 of them (98%) were positive by amplified MTD. There were 112 TB (82%) detected by the MGIT method, 102 (75%) by Löwenstein–Jensen culture and 75 (55%) by Ziehl–Neelsen staining. Using MTD the positive result is ready within hours compared with days or weeks.

Utilisation du test *AMPLIFIED™ Mycobacterium tuberculosis Direct Test* dans le diagnostic de la tuberculose

RÉSUMÉ Une analyse comparative entre trois tests classiques de dépistage de la tuberculose et une technique utilisant une sonde à ADN a été réalisée sur des cas suspects de tuberculose dans un centre de référence en Grèce. Pendant l'année 2004, nous avons effectué des tests sur 2961 échantillons biologiques provenant de 2234 patients à l'aide des méthodes suivantes : la coloration de Ziehl-Neelsen, la culture sur milieu de Löwenstein-Jensen, les tubes MGIT (***Mycobacteria Growth Indicator Tubes***) de BACTEC et le test AMPLIFIED™ ***Mycobacterium tuberculosis direct test*** (AMTD) de Gen-Probe. Sur un total de 136 patients tuberculeux diagnostiqués et sous traitement antituberculeux, 133 (98 %) étaient positifs au test AMTD. Cent douze cas de tuberculose (82 %) ont été détectés grâce à la méthode MGIT, 102 (75 %) à la culture sur milieu de Löwenstein-Jensen et 75 (55 %) à la coloration Ziehl-Neelsen. Avec le test AMTD, les résultats positifs sont prêts en quelques heures et non plus en plusieurs jours ou semaines.

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Received: 20/12/05; accepted: 13/06/06

Introduction

Tuberculosis (TB) like no other disease has taken its toll of human life over the millennia and has spread worldwide [*1*]. It is estimated that 2 million people each year die from TB and nearly one-third of the world's population is infected with the bacterium that causes TB. Left untreated, a person who develops active TB will infect an average of 10 to 15 other people every year. TB is curable only if it is diagnosed quickly and appropriately with medication. The laboratory diagnostic tests used for detecting *Mycobacterium tuberculosis* disease are summarized in Table 1.

Conventional culture methodologies can detect TB growth as early as 1 week, but may take up to 8 weeks [*2–8*]. Comparatively, the *M. tuberculosis* direct (MTD) test provides detection of *M. tuberculosis* complex rRNA within 2.5 to 3.5 hours after beginning the test procedure. The need for a quick diagnosis and a quick start of anti-TB drug therapy lead our laboratory to the use of the amplified MTD genetic technique (San Diego, California, Gen-Probe) for the diagnosis and laboratory follow up of suspected TB patients [*9*]. The Gen-Probe amplified MTD test utilizes transcription-mediated amplification (TMA) and the hybridization protection assay (HPA) to qualitatively detect *M. tuberculosis* complex ribosomal ribonucleic acid (rRNA). The MTD test will detect rRNA from both cultivable and noncultivable organisms [*10*].

This paper reports a comparative analysis of 3 conventional tests for TB versus amplified MTD among suspected TB patients at a TB reference centre in Greece where the Gen-Probe method has been improved to achieve better specificity and sensitivity by titration of the method with positive and negative controls every day and where there is follow-up of patients before and after anti-TB therapy for more than 6 months.

Methods

Background to the study

The study was conducted during 2004 at the TB reference centre of northern Greece—the pneumonological clinic laboratory of G. Papanikolaou Hospital, Aristotle University of Thessaloniki. Our reference centre covers mostly the population of northern Greece (approximately 2 500 000 people). Samples from both Greek nationals and immigrant adults are tested. During 2004 a total of 7023 suspected TB patients were examined at the centre, two-thirds of whom were hospitalized in the pneumonological and the medical pathology department while one-third were referred to our laboratory as outpatients from pneumonologists. The majority of the patients suffered from fever, night sweating, weakening, body weight loss, cough (dry or mucopurulent expectoration) and haemoptysis with bloody sputum or large haemoptysis. Some of these patients were asymptomatic but had chest X-ray findings compatible with TB infection (nodules or patchy shadows).

Data collection

We tested 2961 biological specimens from 2234 patients with the Gen-Probe amplified MTD and with 3 conventional methods: Ziehl–Neelsen staining, Löwenstein–Jensen culture and BACTEC mycobacteria growth indicator tubes (MGIT**) (**Becton, Dickinson and Company, Franklin Lakes, New Jersey). Table 2 shows the site of the 2961 biological specimens. Samples from the remaining 4789 patients were examined with the 3 conventional methods only.

The criteria for the clinical diagnosis of pulmonary TB were as follows [*11*]. Smear-positive cases: at least 2 positive smears, or 1 positive smear and radiographic abnormalities compatible with pulmonary TB, or 1 positive smear and 1 positive culture. Smear-negative cases: at least 3 negative

Table 1 **Advantages and disadvantage of different laboratory tests for tuberculosis**

Laboratory method	Description	Advantages	Disadvantages
Ziehl–Neelsen staining of clinical specimen and culture material	Microscopic examination for acid-resistant mycobacteria	Low cost, can be applied in non-specialized laboratories	Needs 3 samples, low sensitivity
Fluorescent auramine staining	Similar to Ziehl–Neelsen staining but fuchsin is replaced by auramine	Rapidity of reading	Requires costly equipment, constant electricity supply and trained technicians
Löwenstein–Jensen culture	Solid egg-enriched medium	Each live bacillus forms colonies on culture	Requires at least 3 weeks of incubation for the colonies to be visible to the naked eye
Culture on solid agar-based medium (Middlebrook 7 H10 and 7 H11)	Solid synthetic transparent materials	Useful for studying the colonial morphology	Costly
Culture on liquid medium (Bactec MGIT 960 system)	Modified Middlebrook 7H9 broth with fluorescent indicator and an antibiotic mixture	Bacilli can be detected in 8–14 days	Easily contaminated
Polymerase chain reaction	Genome amplification technique using specific probes to identify the different mycobacteria	Allows the amplification of the minimum DNA molecule that exists in clinical specimen. Result available within 24 to 48 hours	Costly equipment
Amplified mycobacterium tuberculosis direct test (Gen-Probe)	Target-amplified nucleic acid probe test for the in vitro diagnostic detection tuberculosis complex rRNA	Rapid test (3.5 hours result), high specificity and sensitivity of mycobacterium	Costly equipment, special training

References: [2–8***].***

smears and 1 or more positive cultures, or at least 2 series of negative smears from samples taken at least 2 weeks apart, with persisting radiographic abnormalities compatible with active TB, not improved with treatment using broad-spectrum antibiotics for at least 1 week.

Laboratory methods

Standard methods were used for Ziehl–Neelsen staining [*12*], Löwenstein–Jensen culture [*13*] and MGIT [*14*].

Ziehl–Neelsen staining

The smear was stained with carbol fuchsin, destained with sulfuric acid and alcohol then restained with methylene blue. The stained smear was examined for acid-fast bacilli (AFB) using a binocular microscope with an immersion lens (× 100). The criteria for 1+ positive were 10–99 AFB per 100 immersion fields; for 3+ positive were > 10 AFB per field.

Table 2 **Distribution of the 2961 biological specimens by site**

Site	No. of specimens
Pulmonary samples	
Sputum	709
Bronchial aspirates	239
Pleuritic fluid	169
Bronchoalveolar lavage	203
Bronchial washings	654
Total	1974
Non-pulmonary samples	
Whole blood	480
Urine	357
Ascitic fluid	78
Arthritic fluid	72
Total	987

Löwenstein–Jensen culture

After decontamination, specimens were centrifuged and the sediment neutralized using a mild acid before adding Löwenstein–Jensen medium. The inoculated tubes were incubated at 37 °C for 4–12 weeks. The culture medium was inspected for large, rounded, buff-coloured, "cauliflower-like" colonies visible to the naked eye which indicate a positive result. The criteria for 1+ positive were < 10 colonies; for 3+ positive were > 100 colonies

BACTEC MGIT

A fluorescent compound was embedded in silicone on the bottom of round-bottom tubes. Tubes entered into the BACTEC MGIT 960 system were continuously incubated at 37 ºC and monitored every 60 min for increasing fluorescence. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth from actively respiring microorganisms. A positive tube contains approximately 10^5–10^6 colony forming units per millilitre (CFU/mL). Culture vials which remained negative for a minimum of 42 days (up to 56 days) were removed from the instrument as negatives.

Gen-Probe amplified MTD test

The Gen-Probe MTD test is a 2-part test in which amplification and detection take place in a single tube. Initially, nucleic acids are released from mycobacterial cells by sonication. Heat is used to denature the nucleic acids and disrupt the secondary structure of the rRNA. The Gen-Probe TMA method, using a constant 42 °C temperature, then amplifies a specific mycobacterial rRNA target by transcription of DNA intermediates, resulting in multiple copies of mycobacterial RNA amplicon. *M. tuberculosis* complex-specific sequences are then detected in the RNA amplicon using the Gen-Probe HPA method [*15*]. The *M. tuberculosis* hybridization reagent contains a single-stranded DNA probe with a chemiluminescent label. This probe is complementary to *M. tuberculosis* complex-specific sequences. When stable RNA:DNA hybrids are formed between the probe and the specific sequences, a hybridized probe is selected and measured in a luminometer.

Our methods have been described before [*16*]. We observed that the manufacturer's amplification cell negative control was not steady, which encouraged us to modify the interpretation of the results using a control that was calculated on a daily basis and was relevant to the biological specimens that were to be examined, under the same conditions daily.

Specimens were collected in sterile plastic containers, and stored at 2 °C to 8 °C for no more than 4 days (generally less than 24 hours) prior to processing. Specimens that are grossly bloody should not be tested with the MTD test. Sediments were decontamination using NALC-NaOH and then centrifuged at > 3000 × g. Specimens and controls were pipetted into lysing tubes,

buffer was added and after sonication for 15 minutes the lysates were ready for amplification.

Amplification tubes were prepared and 50 µL reconstituted *M. tuberculosis* amplification reagent and 200 µL mycobacterium oil reagent were added and 25 µL of lysate was transferred to the bottom of the amplification tubes. Tubes were incubated at 95 °C for 15 minutes in a dry heat bath. The enzyme reagent was reconstituted and 25 µL enzyme mix added to each amplification tube and incubated at 42 °C for 30 minutes.

Hybridization was done at room temperature with reconstitute lyophilized mycobacterium hybridization buffer, vortexed until clear, followed by 100 µL of reconstituted hybridization reagent, vortexed 3 times until the reaction mixture was uniformly yellow. This was incubated at 60 °C for 15 minutes in a dry heat bath.

Selection was done by adding 300 µL mycobacterium selection reagent to the tubes and vortexing until uniformly pink, incubating at 60 °C for 15 minutes in a dry heat bath, then cooling at room temperature for at least 5 minutes but not more than 1 hour.

The tubes were read in a luminometer (Gen-Probe Leader) with a 2 s read time. The results are expressed in relative light units (RLU). The cut-off value was set by the manufacturer: samples with values ≥ 30 000 RLU were considered positive to *M. tuberculosis* complex rRNA, and values of < 30 000 RLU were interpreted as negative.

Results

Of a total of 136 TB patients clinically diagnosed and under anti-TB treatment, 133 of them (98%) were positive by amplified MTD (130 were detected with RLU ≥ 30 000 and 3 were detected with RLU < 30 000). The 3 TB patients detected with RLU < 30 000 had values of 24 354, 27 977 and 29 351.

There were 112 TB patients (82%) detected by the MGIT method, 102 TB patients (75%) by Löwenstein–Jensen culture and 75 TB patients (55%) by Ziehl–Neelsen staining (Table 3).

Discussion

While the conventional methods for cultivation of *Mycobacteria* on solid media such as Löwenstein–Jensen and Middlebrook agars are relatively straightforward, they do require several weeks of incubation for the detection of organisms, and may lack sensitivity when used alone. For example, using the classic culture method (Löwenstein–Jensen) the positive result is ready in 3 weeks or more and the negative results ready after 2 months. The sensitivity of Löwenstein cultivation is 75%–80% [*17*]. The positive result is ready by Ziehl–Neelsen staining in 4 days, but the sensitivity of Ziehl–Neelsen is only 55%–65% [*17*]. MGIT supports and will detect the growth of a wide variety of *Mycobacterium* species, but requires a large number of tubes and requires 8–14 days.

Using amplified MTD the positive result is ready in 3.5 hours, the sensitivity is 96%–100% and the specificity 95%–100% [*17*]. The amplified MTD test is specific for, but does not differentiate among, members of the *M. tuberculosis* complex, i.e., *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. microti* and *M. canetti* [*18*]. However, *M. microti* infects only animals, *M. bovis* is uncommonly transmitted from infected animals to humans, and *M. africanum* causes pulmonary disease in humans in tropical Africa. *M. tuberculosis* is by far the most common member of the

Table 3 **Comparison of the results of 4 tests on 136 tuberculosis patients clinically diagnosed and under anti-TB treatment, by relative light unit (RLU) values**

Test/RLU values	No. (%) of patients positive	Criterion
Amplified MTD		
< 30 000	3	+
≥ 30 000	130	+
Total	133 (98)	
MGIT		
> 546 841–	6	< 28 days
> 1 300 500–	41	< 21 days
> 1 783 000–	38	< 14 days
> 2 578 000	27	< 7 days
Total	112 (82)	
Löwenstein–Jensen		
≥ 188 000–	27	+
≥ 440 000–	12	+
≥ 1 250 000–	15	+
≥ 2 508 000–	34	+
≥ 3 620 000	14	+
Total	102 (75)	
Ziehl–Neelsen		
≥ 805 000–	7	+
≥ 1 600 000–	18	+
≥ 2 440 000	50	+
Total	75 (55)	

MTD = Gen-Probe Mycobacterium tuberculosis direct test; MGIT = BACTEC mycobacteria growth indicator tubes test.

complex responsible for the human disease worldwide.

The DNA probe detection methods have been practised for years in research laboratories, but their use has recently been broadened to clinical laboratories. Advantages of the rapid diagnosis include the reduced costs of medical treatment. The medical treatment of a TB patient, according to the bibliography, costs €235 per day. Previously, suspected patients were hospitalized for at least 6 days at a cost of around €1408. Besides the economic side, there are social and psychological advantages. Rapid diagnosis means that the patient is not marked as having an infectious disease, a stigma that, even if the result is negative, may be indelible. Families, who naturally try to protect the other family members, may not accept having the suspected patient at home. Psychological stress that can arise in the patient while waiting for the result is another problem.

For optimum results, however, close cooperation between the clinician and the laboratory is needed to define those specimens which urgently have to be screened by amplified MTD, i.e. those cases in which the clinical usefulness of amplified MTD is justified.

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