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MOLECULAR GENETIC AND TRADITIONAL METHODS FOR DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX (DISCREPANCY ANALYSIS)*

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In the past six and half years, 862 different clinical samples [sputum, bronchoalveolar lavage, thorax puncture, cerebrospinal fluid and skin samples] were tested by Gen-probe amplified *Mycobacterium tuberculosis* direct test (MTD) or ligase chain reaction (LCR) or polymerase chain reaction (PCR). 239 parallel clinical samples were cultivated, and some samples were stained with Ziehl-Neelsen staining. 1-4 samples were tested per patient. 29 (12.13%) samples were positive and 177 (74.05%) samples were negative with both cultivation and molecular genetic methods. 2 (0.83%) samples were positive only on cultivation, and 31 (12.97%) samples were positive only with the molecular diagnostic methods. The differences are undoubtedly explained by the sensitivity of the molecular diagnostic methods.

Keywords: tuberculosis, MTD, LCR, PCR, discrepancy analysis

Introduction

Microbiological diagnostics has been reformed during the past 15 years by the introduction of molecular biological methods. Microbial pathogens, bacteria, viruses and fungi can now be detected within some hours with molecular diagnostic techniques. This was not possible with traditional methods, or only after a long culturing time, as in case of *Mycobacterium tuberculosis (M. tuberculosis).*

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The methods for the diagnosis of the *M. tuberculosis* complex have not progressed for decades because of the long cultivation time. The increases in AIDS opportunistic mycobacterial infections and the number of multiresistant strains have made it urgent to elaborate rapid diagnostic methods to detect the *M. tuberculosis* complex [1]. The development of amplification methods was a very important step forward in this field.

New radiometric liquid cultivation methods [BACTEC], and new rapid diagnostic methods have been introduced in recent years, e.g. chromatographic methods [HPLC and GLC], nucleic acid hybridization and amplification methods for diagnosis of the *M. tuberculosis* complex [2–5].

There are three different traditional levels of *Mycobacterium* diagnostics in Hungary: direct detection of the bacterium by acid-fast Ziehl-Neelsen staining, cultivation of the bacterium in regional centers, and identification and antibiotic-sensitivity testing methods in the national center.

The conventional cultivation method, which is regarded as the "gold standard" [Löwenstein-Jensen] and Gen-probe amplified *Mycobacterium tuberculosis* direct test (MTD) or ligase chain reaction (LCR) or polymerase chain reaction (PCR) were compared in our laboratories [6]. The members of the *M. tuberculosis* complex are *M. tuberculosis, M. bovis BCG, M. bovis, M. africanum* and *M. microti,* all of which are detected by MTD, while all except *M. bovis* BCG are detected by LCR and PCR.

Materials and methods

The clinical samples were digested and decontaminated by the NALC-NaOH method. The decontaminated samples were cultivated at 37°C for 6 or more weeks, and examined weekly.

The MTD method utilizes a proprietary isothermal enzymatic amplification of target rRNA via DNA intermediates. The detection of amplified product is achieved by using an acridine ester-labeled DNA probe [7]. The major processes of *M. tuberculosis* MTD are as follows: sonication breaks the mycobacterial cell wall, liberating rRNA, which is added to the amplification mix. This mix contains the nucleotides, the primers and enzymes. In the second step, the primers anneal to target RNA sequences. The reverse transcriptase and nucleoside triphosphates initiate the DNA synthesis. Subsequently, the reverse transcriptase converts cDNA:RNA duplex into template for RNA synthesis, and the transcriptase makes many RNA copies. The chemiluminescent-labeled hybridized DNA/RNA complexes were detected in a Leader 50 luminometer.

The LCR is an amplification method [8]. The four oligonucleotide probes recognize and hybridize to a specific target sequence within the chromosomal DNA of

the *M. tuberculosis* complex. The oligonucleotides are designed to be complementary to the target sequence so that in the presence of target, the probes will bind adjacent to one another. They can then be enzymatically joined to form the amplification product, which subsequently serves as an additional target sequence during further rounds of amplification. After sufficient numbers of target amplification product have accumulated, they can be detected by microparticle enzyme immunoassay (MEIA) that is measured by the MEIA optical assembly.

The Amplicor PCR assay includes three major steps: PCR target amplification, hybridization of the amplified product to a specific nucleic acid probe and colorimetric detection. Genus-specific primers located in a highly conserved region of 16S rRNA *Mycobacterium* gene are used to amplify a 584-bp sequence. Carry-over contamination in the Amplicor PCR test is prevented by incorporating dUTP [9-11]. The automatized Cobas Amplicor system was used for examination of clinical samples.

Results

862 different clinical samples [sputum, bronchoalveolar lavage (BAL), thorax puncture, cerebrospinal fluid (CSF) and skin] were tested by the MTD, LCR or PCR and cultivation methods, and some samples were subjected to Ziehl-Neelsen staining. 39 (4.52%) sputum samples, 27 (3.13%) BAL samples, 9 (1.04%) thorax puncture samples, and l2 (1.39%) other samples were positive among the 862 samples (Table I).

Of the 459 clinical samples from males 66 (7.65%) were positive. Of the 403 clinical samples from females 21 (2.44%) were positive (Table II).

1–4 samples were tested per patient. 239 (27.73%) of 862 amplified and cultivated samples were compared. 29 samples (12.13%) were positive and 177 (74.05%) samples were negative by both the cultivation and the amplified methods. 2 (0.83%) samples were positive only on cultivation, and 31 samples (12.97%) were positive only with the amplified methods. The parallels of amplified positive samples were negative on cultivation in a higher percentage, whereas the clinical symptoms and results of the amplified methods generally correlated. Comparative results on 26 patients are given in Table III.

The different cultivation and amplification results are explained in Table IV. The differences in the first four cases are undoubtedly explained by the sensitivity of the amplification methods. For patients 5, 24 and 25 different samples were processed for the culture and molecular genetic methods. For patient 6, carcinoma was diagnosed on the basis of the clinical, X-ray and CT examinations.

Table I

Distribution of results on clinical samples for detection of M. tuberculosis *complex between 01 July 1994 and 31 December 2000*

	Sputum N (%)	BAL N (%)	Thorax puncture N (%)	Others N (%)	Total N (%)
Positive	39	27	9	12	87
	(4.52)	(3.13)	(1.04)	(1.39)	(10.09)
Negative	201	156	51	367	775
	(23.32)	(18.09)	(5.92)	(42.58)	(89.91)
Total	240	183	60	379	862
	(27.84)	(21.23)	(6.96)	(43.97)	(100.0)

Table II

Distribution of clinical samples by sex between 01 July 1994 and 31 December 2000

	1994 N (%)	1995 N (%)	1996 N (%)	1997 N (%)	1998 N (%)	1999 N (%)	2000 N (%)	Total N (%)
Male positive	20	17	13	6	4	0	6	66
	(2.32)	(1.97)	(1.51)	(0.69)	(0.46)	(0.00)	(0.69)	(7.65)
Female positive	1	6	2	2	4	3	3	21
	(0.12)	(0.69)	(0.23)	(0.23)	(0.46)	(0.35)	(0.35)	(2.44)
Male negative	16	70	77	63	48	48	71	393
	(1.86)	(8.12)	(8.93)	(7.31)	(5.57)	(5.57)	(8.24)	(45.59)
Female negative	5	27	64	61	73	67	85	382
	(0.58)	(3.13)	(7.43)	(7.08)	(8.47)	(7.77)	(9.86)	(44.32)
Total	42	120	156	132	129	118	165	862
	(4.87)	(13.92)	(18.10)	(15.31)	(14.97)	(13.69)	(19.14)	(100.0)

After lobectomy, the morphological results confirmed this diagnosis. For patient 7, on the basis of the X-ray and molecular genetic results antituberculotic treatment was started in spite of a negative culture result. This homeless and alcoholic patient was released after 6 months of anti-TB treatment, with X-ray regression. For patients 8 and 9, the culture became positive after 10 weeks (Table IV).

Table III

Discrepancies in detection of Mycobacterium tuberculosis *complex (N 25)*

Patients	Samples (N)	Cultivation positive (N)	Molecular genetic positive (N)
1	4	1	3
2	4	1	4
3	2	1	2
4	3	1	3
5	1	0	1
6	2	0	1
7	1	0	1
8	2	0	2
9	1	0	1
10	2	1	2
11	2	0	2
12	2	0	1
13	1	0	1
14	3	0	3
15	1	0	1
16	1	0	1
17	1	0	1
18	1	0	1
19	1	0	1
20	1	0	1
21	1	0	1
22	1	0	1
23	1	0	1
24	1	1	0
25	1	1	0

Discussion

The conventional cultivation methods are time-consuming. The cultivation, identification and antituberculotic drug sensitivity testing requires weeks or months in some cases.

The amplification methods require only some hours after decontamination. Detection of the *M. tuberculosis* complex by MTD involves the detection of living bacteria in clinical samples. The procedure is suitable for detection of the *M. tuberculosis* complex in immune complexes and white blood cells too.

The cultivation method is not indispensable because of drug sensitivity testing. The costs of the amplification methods are apparently high, but the benefit is really much higher, considering the advantages of the rapid diagnosis: the beginning of immediate therapy, the prevention of further mycobacterial infections and the lower hospital hotel costs [12-14]. In consequence of their sensitivity, molecular diagnostic methods are able to amplify the nucleic acid of 1-2 bacteria, even if the patient has been treated with antibiotics in the meantime [15].

Table IV

Explanation of the different culture and molecular genetic results

Cases	Explanation
1-4, 10-23	The differences are undoubtedly explained by the sensitivity of the molecular diagnostic methods.
5, 24-25	Different samples were processed for cultivation and molecular genetic methods
6	Carcinoma was diagnosed on the basis of clinical, X-ray and CT examination. After lobectomy, the morphological results confirmed the diagnosis.
7	On the basis of clinical, X-ray and molecular genetic results, antituberculotic treatment was started in spite of a negative culture result. This homeless and alcoholic patient was released after 6 months of anti-TB treatment, with X-ray regression.
8, 9	The culture became positive after 10 weeks.

The development of amplification methods was a very important step forward in this field. The application of rapid diagnostic methods in mycobacteriology was emphasized by several unsolved problems. The introduction of amplification methods (MTD, PCR, LCR, and NASBA) has opened up new dimensions in mycobacterial diagnostic methods.

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