The usefulness of IS*6110* nested polymerase chain reaction in neurotuberculosis diagnosis before and after empirical treatment starting

Utilitatea tehnicii IS*6110 nested polymerase chain reaction* în diagnosticul neurotuberculozei înainte şi după începerea tratamentului empiric

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Abstract

Objectives*. Neurological forms of tuberculosis require prompt diagnosis and urgent therapy. Due to the slow bacteriological diagnosis, most suspected neurotuberculosis patients are urgently treated only on clinical and cerebrospinal fluid (CSF) non-specifically features. The study's aim was to compare the rapid IS6110 Nested-PCR assay with classical CSF criteria of neurotuberculosis diagnosis regardless of therapy starting. **Materials and Methods***. 91 CSF samples obtained before and during the first 30 days of antituberculosis treatment from 16 neurotuberculosis patients and 15 controls were evaluated. The CSF samples were analysed using IS6110 Nested-PCR method by comparison with cytological-biochemical and bacteriological CSF criteria of diagnosis. **Results.***The sensitivity (Sv) of CSF Nested-PCR before antituberculosis therapy was 86.66% with a specificity (Sp) of 87.5% by comparison with 33% Sv and 100% Sp for Lowenstein culture, 6% Sv and 100% Sp for Ziehl Neelsen smear 80% Sv and 87.5% Sp for cytochemical CSF criteria. Nested PCR remained positive after 21 days of therapy at 86.66% patients and up to 30 days at 60% of patients. **Conclusions.***IS6110 Nested-PCR detection of M. tuberculosis in CSF samples was rapid and accurate in tuberculous meningitis, tuberculoma, cerebral miliaria and tuberculous arahnoiditis, before treatment starting and during the first 21 days of antituberculosis therapy. **Importance.***The CSF samples were analysed in rare and difficult to diagnose forms of NTB with classic and Nested-PCR methods of diagnosis. IS6110 Nested-PCR method with a Sv of 86.66% improved the diagnosis of high gravity forms of neurotuberculosis even after treatment starting.*

Key words*: IS6110 Nested-PCR, neurotuberculosis, treatment*

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Rezumat

Obiectiv. Formele neurologice de tuberculoză reprezintă urgenţe de diagnostic şi terapie. Diagnosticul bacteriologic fiind lent, cei mai mulţi pacienţi suspecţi de neurotuberculoză încep tratamentul urgent, pe baza unor modificări necaracteristice, clinice şi ale lichidului cefalorahidian (LCR). Studiul de faţă compară tehnica rapidă IS6110-Nested PCR cu cea clasică bazată pe criteriile LCR, în diagnosticul neurotuberculozei chiar după începerea tratamentului. Material şi metodă. 91 probe LCR obtinuţe de la 16 pacienţi cu neurotuberculoză şi 15 martori au fost evaluate înainte şi în timpul primelor 30 zile de tratament antituberculos. Probele LCR au fost analizate folosind IS6110-Nested PCR, prin comparaţie cu criteriile citochimice şi bacteriologice de diagnostic ale LCR. Rezultate. Înainte de începerea tratamentului antituberculos, metoda Nested PCR a avut o sensibilitate (Sb) în LCR de 86.66% cu specificitate (Sp) 87.5%, comparativ cu cultura pe mediul Lowenstein Sb 33% şi Sp 100%, frotiul Ziehl Neelsen Sb şi Sp 100 % şi criteriile citochimice ale LCR Sb 80% şi Sp 87.5%. Metoda Nested PCR a ramas pozitivă după 21 zile de tratament la 86.66% pacienţi şi până la 30 zile la 60% dintre pacienţi. Concluzii. Detecţia M. tuberculosis în LCR folosind IS6110 Nested-PCR, în forme de meningită tuberculoasă, tuberculom, miliaria cerebrală şi arahnoidita tuberculoasă, a fost rapidă şi precisă înainte de tratament şi în timpul primelor 21 zile de tratament antituberculos. Importanţa. S-au folosit metode clasice şi Nested-PCR pentru a analiza LCR în forme de neurotuberculoză rare şi dificil de diagnosticat. Metoda IS6110 Nested–PCR avand o Sb de 86.66% a îmbunătăţit diagnosticul în neurotuberculoza gravă chiar şi după începerea tratamentului antituberculos.

Cuvinte cheie: IS6110 Nested-PCR, neurotuberculoză, tratament

Introduction

Neurotuberculosis (NTB) is a granulomatous infection of the Central Nervous System (CNS) caused by *Mycobacterium tuberculosis* (*M. tbc*) infection. Tuberculous meningitis (TM) represents the most frequent manifestation and the major cause of NTB mortality (1). The other severe manifestations of NTB such as tuberculoma, cerebral miliary or arachnoiditis are quite rare.

NTB is one of the most devastating forms of tuberculosis (TB) and a well known diagnostic challenge. In developing countries, NTB is a common neurological disorder accounting for 10% of all the TB patients (2). Many NTB atypical pictures escaping diagnosis and increasing the mortality have been described, especially on immunosuppressed patients. The high NTB mortality estimated of 80% is still a result of missed diagnoses and delayed treatment (3). As a matter of fact, the diagnostic failure due to the lack of a rapid, sensitive and specific diagnostic test led to the evolution of NTB as one of the most dangerous and difficult to recognize form of TB.

Thus the TM "gold standard" diagnosis is still bacteriologic confirmation of cerebrospinal fluid (CSF) mycobacteria either on Lowenstein medium culture, a slow method (6-8 weeks) with a low sensitivity (Sv) of only 15%-50% or evidence of acid fast bacilli on CSF Ziehl-Neelsen stain, a rapid but insensitive method (4). Moreover, in cases such as tuberculoma, cerebral miliaria and other localized cerebral TB lesions, the values of CSF undergo unspecific modifications. Therefore a bioptic invasive method is highly required to confirm the TB etiology. Unfortunately the TB diagnosis should never be ruled out, if the bacteriologic CSF or bioptic samples results proves negative (5, 6).

In the absence of confirmed bacteriologic NTB diagnosis and since precocious treatment is crucial for the final outcome, more than 50% of NTB suspected patients promptly receive a long course of empiric antituberculosis treatment based on the clinical picture and CSF characteristics like CSF lymphocytosis, high CSF proteins and low CSF glucose level (7). As NTB manifestations like tuberculoma or cerebral miliaria have no CSF distinct characteristics,

the diagnosis must be strengthened by other indirect evidences of TB (other concurrent TB lesions, suggestive cerebral neuroimaging aspects) (8,9) or a good response to the empiric antituberculosis therapy. Starting the empiric anti TB treatment, although urgent to the survival, impedes unfortunately any subsequently bacteriologic investigations. Therefore an initially doubtful and never confirmed NTB diagnosis is frequently followed by a long unnecessary antituberculosis therapy (10).

Throughout the past decades, many tests have been advocated to support the NTB diagnosis, including adenosine deaminase assay, enzyme-linked immunosorbent assay (11), dot immunobinding assay (12) and high-pressure liquid chromatography (13). After 1990, many studies emphasized the polymerase chain reaction (PCR) assay as a suitable and reliable method for the detection of *M. tbc* in clinical samples, including CSF (14-16); some of them suggested the possibility to detect *M. tbc* into the CSF even after 14-29 days of antituberculosis treatment (17-20). Previous researches reported that all *M. tbc* strains have at least one copy but usually 6 to 15 copies of Insertion sequence (IS) *6110* elements in their genome. Because most *M. tbc* complex strains contain multiple copies of the IS *6110*, this element has frequently been used as the main target for PCR amplification.

While many studies have demonstrated an improving diagnostic NTB sensitivity using PCR methods, other studies highlighted the low sensitivities associated with PCR assays (21). Molecular methods have been only sporadically assessed in rare NTB manifestations such as tuberculoma, arahnoiditis or cerebral miliary TB (21). Some studies reported a better sensitivity (Sv) and specificity (Sp) of Nested PCR (N-PCR) assay targeting the multi-copy IS *6110* insertion element compared to other conventional single-step PCR assay in TM (18, 22) or other clinical forms of NTB (23,24).

The aim of our study was to assess the usefulness of IS *6110* N-PCR method to detect CSF *M tbc* before and after the start of antituberculosis therapy, in different forms of NTB (TM, tuberculoma, TB arachnoiditis, cerebral miliary TB).

Materials and methods

A total of 91 CSF specimens have been collected from 31 adult patients with suspected meningitis, who agreed to enter the study. The specimens were collected from September 2007 to January 2009 from the patients hospitalized into the National Institute of Infectious Diseases "Matei Bals", Bucharest, Romania.

Patients

All 31 patients (16 male and 15 female, mean ages of 38 years, range 18-55 years) were evaluated for history, clinical examination and laboratory analysis, including CSF cell counts, biochemistry, bacteriology, serology, radiology (chest radiography, cerebral or spinal magnetic resonance examination -MR) and also CSF N-PCR method. 15 patients were diagnosed with NTB (TM 11 patients, TB arachnoiditis 1 patient, tuberculoma 1 patient, cerebral miliary 2 patients) and 16 with nonTB meningitis (controls). Ten of the control patients were confirmed with bacterial etiology (1 with *L. monocytogenes*, 5 with *N. meningitides*, and 4 with *S. pneumoniae*), 5 patients with viral meningoencephalitis and 1 patient with fungal (*Cryptococcus neoformans)* meningitis. Five out of the 31 patients (4 with non TB meningitis and one patient with tuberculoma) were HIV infected with a mean of CD4 cell count of 204/µl (range: 140-350/µl).

Criteria used for the TM diagnosis and TB arachnoiditis included fever, headache and neck stiffness for more than a 14-30 days, signs of meningeal irritation, suggestive CSF cytological and biochemical findings such as CSF pleocytosis (300 - 870 leucocytes/µl), CSF lymphocytosis (60%-85% lymphocytes), hypoglycorrhachia (CSF/blood glucose ratio ≤ 0.5) and elevated CSF proteins (150 mg/dl - 370

mg/dl). Clinical manifestations and CSF modifications were associated with other documented TB lesions (9 cases with pulmonary TB lesions, 3 patients with TB adenoiditis, 1 patient with TB orchitis) or specific features on MR examination (the patient with TB arachnoiditis). Five patients (4 with TM and 1 with TB arachnoiditis) were also bacteriologicaly confirmed (positive Lowenstein CSF culture and/or positive CSF Ziehl Neelsen staining).

Criteria used for the other CNS tuberculosis lesions were: chronic headache, seizures, MR evidence of cerebral lesions, histological criteria (cerebral follicles with central caseous necrosis on bioptic samples), other documented TB lesions (adenoiditis or pulmonary TB lesions) and a good clinical response to the antituberculosis treatment. All these patients had non-specific CSF cytological and biochemical modifications and negative Lowenstein culture or Ziehl Neelsen stain. *Criteria used for the* diagnosis *of non NTB control patients* were based on CSF analysis (cytological, biochemical and bacteriological criteria), hemoculture or specific serologic tests, which confirmed the nonTB etiology.

The treatment of NTB patients

The NTB patients have been treated with standard antituberculosis regimen including daily oral isoniazid (5mg/kg/day), rifampin (10 mg/kg/day), pyrazinamide (15 mg/kg/day) and ethambutol (20 mg/kg/day); prednisone 1 mg/kg/day was associated in the first 21 days of therapy.

Samples

A total of 91 CSF samples (75 samples from NTB patients and 16 samples from non NTB patients) were collected after patients' admission. Five samples of CSF have been collected and analyzed for each of the 15 NTB patients: one CSF sample before antituberculosis treatment (day 0) and 4 CSF samples on the 7th, 14th, 21st, 30th day during antituberculosis therapy. Each of the 16 non NTB patients was sampled on admission, once.

CSF Analysis

Each CSF sample (about 6-8 ml/per sample) from NTB patients and non TB meningitis patients (controls) was analyzed as follows:

-about 2 ml of CSF was sent to cytological analysis (CSF cell counts) and biochemistry (CSF proteins and glucose) to be performed by standard methods (25);

-about 5 ml of CSF was sent to smear staining (Gram and Ziehl Neelsen staining), culture (standard and Lowenstein culture) and IS*6110* N-PCR method. N-PCR was performed on the same CSF specimens obtained for smears and culture examinations.

CSF N-PCR method

An IS*6110* N-PCR method using the EUROCLONE kit (Euroclone Diagnostica Molecolare- Euroclone Sp A, via Lombardia 12, 27,010 Silex, Italy) amplifying IS *6110* in the *M. tbc* genome was used to analyze CSF. This sequence is a repetitive element exclusively found in the genome of the *M. tbc* complex and is usually present 6 to 15 copies (up to 30-40 copies) per bacterium. This kit allows getting to a detection limit of 100 Mycobacterium cells by PCR in 1ml of sample.

Pre-extraction sample treatment. The CSF samples were processed with a standard procedure of decontamination using N-acetyl-L-cysteine-sodium hydroxide (31). Approximately 200 µl of decontaminated pellet were used for DNA isolation.

The DNA extraction method. Extraction was carried out with the EUROCLONE kit, Mycobacterium DNA Gene Releaser (EM-R058050) according to indications, as follows: 200µl of decontaminated sample were transferred into a sterile microtube (contained in the kit) then inactivated at 80^0C for 30 minutes. 500µl of sterile water was added, mixed well, centrifuged at 12000 rpm for 15 minutes and supernatant was discarded. Thereafter 50µl of solution A were added to the pellet and vortexed for 1 minute; 50µl of solution B were added and vortexed for 1 minute. The mixed solu-

tion was centrifuged at 12000 rpm for 15 minutes and 10µl of the supernatant containing the mycobacterial DNA was aspirate and used for genic amplification.

The N-PCR method. 10 µl of extracted DNA were dispensed in a tube of 0.2 ml with readymade master–mix (EUROCLONE MO-LEC- Master Mix e Oligo mix Ready to Use, *Mycobacterium tuberculosis* detection kit EBR00103) containing the external primers, dNTPs, amplification buffer and Taq polymerase. A negative control containing all the PCR ingredients except the DNA template was included for amplification program. DNA extracted from the *M. tbc* strains cultured on Lowenstein-Jensen medium in the laboratory served as template for the positive control.

The first amplification program was as follows: 95°C - 8 minutes (1 cycle) and 94°C - 30 seconds, 65°C - 30 seconds, 72°C - 30 seconds (30 cycles). Before the second amplification program 80 µl of solution containing the internal primers were added to each reaction tube. The second amplification program was as follows: 95°C - 8 minutes (1 cycle); 93°C - 30 seconds, 50°C - 30 seconds, 72°C - 30 seconds (30 cycles). The detection was achieved by agarose gel 1% electrophoresis. In positive tested sample, a DNA band of 200 bp must appear in the run gel.

The PCR method was considered positive when a specific sequence of DNA was visualized by agarose gel electrophoresis.

Results

A total of 91 CSF samples from the 31 hospitalized patients were collected and analyzed. 31 samples were collected before treatment from all the patients and 60 samples were collected during the first 30 days of antituberculosis therapy from NTB patients only. CSF bacteriological, cytological, biochemical and N-PCR results were compared.

A) The results of CSF examination before treatment

Bacteriological CSF results. *M. tbc* CSF detection was positive in 5 of the 15 patients with NTB. Thus, *M tbc* was isolated on Lowenstein culture at one patient with TB arachnoiditis and 4 patients with TM. Only one patient with TM had both positive Ziehl Neelsen smear and Lowenstein culture. All the 16 non NTB patients had negative Ziehl Neelsen stain and Lowenstein culture. The Sv of CSF *M tbc* detection was 33.33 % for Lowenstein culture and 6.66% for Ziehl Neelsen smear. The Sp was 100% for both culture and smear. The positive predictive value (PPV) was 100% for both Lowenstein and Ziehl Neelsen stain; the negative predictive value (NPV) was 61.53 % for Lowenstein culture and 53.33% for Ziehl Neelsen stain.

Cytological and biochemical CSF results. Twelve out of the 15 NTB patients had CSF cyto-chemical characteristic features suggesting TB etiology, (e.g. CSF lymphocytosis, increased proteins and decreased glucose). The patient with tuberculoma and those with cerebral miliary TB had only non-specifically increased CSF proteins. Two of the 16 non NTB controls, one with Listeria and one with fungal meningitis, both HIV positive, had the same CSF modifications as TM patients.

The Sv of CSF cytochemical features suggesting TB diagnosis was 80%, the Sp was 87.5%, the PPV 85.71% and the NPV 84.21%.

IS6110 N- PCR results of M tbc CSF detection. Thirteen out of the 15 NTB patients had positive N-PCR CSF results as well as two of the 16 non NTB patients. N-PCR false negative results were observed in two patients with TM who also had CSF negative Ziehl Neelsen and Lowenstein culture results. N-PCR false positive results were recorded in two patients with bacterial meningitis (1 patient with *S. pneumoniae* meningitis, HIV positive and one with Listeria meningitis, also HIV positive). We point out that the patient with tuberculoma

Table 1. Comparative results of IS6110 Nested PCR method over bacteriological and cyto-chemical cerebrospinal fluid results in neurotuberculosis patients, before treatment

NTB form	Total patients	Positive results, by method			
		Nested-PCR	Lowenstein culture	Ziehl-Neelsen staining	Cytochemical features*
Tuberculous meningitis	11	9	4	1	11
Arachnoiditis	1	1	1	0	1
Tuberculoma	1	1	0	0	0
Cerebral miliary	2	2	0	0	0
NTB patients (total)	15	13	5	1	12

Table 2. IS*6110* Nested PCR, bacteriological and cytochemical cerebrospinal fluid comparative results of controls (non NTB patients)

Clinical form of disease	Total patients	Positive results, by method			
		Nested-PCR	Lowenstein culture	Ziehl-Neelsen staining	Cytochemical features*
Bacterial meningitis:					
-*L. monocytogenes*	1	1	0	0	1
-*N. meningitides*	5	0	0	0	0
-*S. pneumoniae*	4	1	0	0	0
Criptococcus meningitis	1	0	0	0	1
Mumps meningitis	1	0	0	0	0
Other viral meningitis	4	0	0	0	0
Non NTB patients (total)	16	2	0	0	0

* Patients with CSF predominant lymphocytosis, increased proteins and decreased glucose
CSF (cerebrospinal fluid), NTB (neurotuberculosis)

and those with cerebral miliary TB had positive CSF N-PCR results despite a negative Lowenstein culture, Ziehl Neelsen smear and non-specific CSF cyto-chemical modifications. They urgently received antituberculosis therapy; histological examination of the brain biopsy confirmed the tuberculoma diagnosis later.

The overall Sv of N-PCR method was 86.66% and the Sp 87.5%; the PPV was 86.66% and NPV 88.88%.

The bacteriological, cyto-chemical and N-PCR results obtained before treatment from all the 31 patients are summarized in *Table 1* (NTB patients) and *Table 2* (non TB patients). The comparative Sv, Sp, PPV and NPV of methods are shown in *Table 3*.

B. The results of CSF examination during antituberculosis treatment

Bacteriological results. All 5 bacteriologic positive NTB patients turned negative after the first 7 days of treatment.

CSF cytochemical results. CSF cytological and biochemical modifications remained characteristic in 80% of patients up to 14 days

Table 3. Comparative sensitivity, specificity, positive predictive value and negative predictive value between bacteriological, cytochemical and Nested PCR methods before antituberculosis treatment

Method	Sv (%)	Sp (%)	PPV (%)	NPV (%)
IS-*6110* N-PCR	86.66	87.5	86.66	88.88
Lowenstein culture	33.33	100	100	61.53
Ziehl Neelsen smear	6.66	100	100	53.33
CSF Cytochemical features*	80	87.5	85.71	84.21

Sv=sensitivity; Sp= specificity; PPV=positive predictive value; NPV=negative predictive value
* *Cerebrospinal fluid cytological and chemical TB characteristic modifications (predominant lymphocytosis, increased proteins and decreased glucose)*

Table 4. Results of *Mycobacterium tuberculosis* detection in cerebrospinal fluid by IS*6110* Nested PCR in neurotuberculosis patients before and after antituberculosis treatment starting

	IS *6110* Nested-PCR results (day of CSF sampling for Nested PCR)				
	0	**7**	**14**	**21**	**30**
1. Tuberculous meningitis	+	+	+	+	+
2. Tuberculous meningitis	+	+	+	+	+
3. Tuberculous meningitis	+	+	+	+	+
4. Tuberculous meningitis	+	+	+	+	+
5. Tuberculous meningitis	+	+	+	+	+
6. Tuberculous meningitis	+	+	+	+	+
7. Tuberculous meningitis	+	+	+	+	+
8. Tuberculous meningitis	+	+	+	+	+
9. Tuberculous meningitis	+	+	+	+	-
10. Tuberculous meningitis	-	-	-	-	-
11. Tuberculous meningitis	-	-	-	-	-
12. Tuberculoma (HIV positive)	+	+	-	-	-
13. Cerebral miliary TB	+	+	+	+	-
14. Cerebral miliary TB	+	+	+	-	-
15. TB Arahnoiditis	+	+	+	+	+
Total number of positive patients	13	13	12	11	9

of treatment. Nevertheless, unspecific CSF lymphocytosis and increased proteins persisted in all patients even after 30 days of therapy. However, patients with tuberculoma and cerebral miliary TB showed only unspecific CSF increased proteins.

IS6110 N-PCR results. Eleven patients with TM remained N-PCR positive during the first 21 days of specific therapy and 9 out of them even up to the 30th day of the treatment. The patients with tuberculoma and cerebral miliary TB remained N-PCR positive up to 14 days

Table 5. Positive results of bacteriological, cytochemical and IS*6110* Nested -PCR methods on cerebrospinal fluid in neurotuberculosis patients, before and after onset of antituberculosis treatment

Method of diagnosis	Day of CSF control				
	0	**7**	**14**	**21**	**30**
CSF IS*6110* Nested PCR	13	13	12	11	9
Lowenstein culture	5	0	0	0	0
CSF Ziehl Neelsen smear	1	0	0	0	0
CSF Cytochemic profile*	12	12	12	0	0

CSF (cerebrospinal fluid)

* CSF cytological and chemical TB characteristic modifications (predominant lymphocytosis, increased proteins and decreased glucose)

after starting the treatment. The results of N-PCR in NTB patients during the first 30 days of antituberculosis treatment are shown in the *Table 4*.

Overall bacteriological, cyto-chemical and N-PCR results of NTB patients before and after starting antituberculosis therapy are shown in *Table 5*.

Discussion

The rapid diagnosis of NTB remains elusive. Gold standard criteria of diagnosis based on the low Sv of CSF microscopic examination and time consuming Lowenstein culture are nevertheless disappointing. In this respect, 50% out of the TM suspected patients still receive antituberculosis therapy without bacteriologic confirmation. 70-80% out of tuberculoma suspected patients either require an extensive excision of tumoral lesions due to inconclusive bioptic diagnosis or start an empiric antituberculosis treatment. However the rapid starting of antituberculosis therapy is vital in order to reduce the high rate of mortality and complications of NTB (26, 27). Although the recommended standard therapy in NTB is of 12 months (27) the optimal regimen and treatment length are unknown and difficult to monitor. Unfortunately empiric treatment itself could be associated with severe drug toxicity. The high

gravity "paradoxical reaction" during antituberculosis treatment worsens the patients' outcome in about 30% of cases and is difficult to differentiate from other complications or clinical entities (drug resistance, NTB relapses or other neurological complications) (28). Therefore, although imperious to decrease mortality, empiric treatment of bacteriological unconfirmed NTB patients could be hazardous, difficult to control and questionable.

After 1995, many studies reported PCR as a reliable complementary method in TM diagnosis. The PCR methods in TM are time efficient and have a Sv of 50-90% with a Sp of 80-90%. Molecular methods may compensate the difficulty of bacteriologic diagnosis but their reliability depends on the specific primers used for DNA amplification, optimal DNA isolation and the PCR technique (29). The PCR method amplifying a 123 base-pair fragment of the IS*6110* sequence specific for *M. tbc* complex has been assessed by some authors proving a better Sv over traditional smear and culture methods in TM (22, 30). We assessed in our study the value of IS*6110* N-PCR method using EUROCLONE kit (Mycobacterium DNA Gene Releaser) to detect *M. tbc* in CSF samples, before and after starting antituberculosis treatment, in different NTB manifestations and controls.

Ninety one CSF samples from a total of 31 patients (15 patients with different clinical

forms of NTB and 16 with non NTB meningitis) were studied. The CSF samples of NTB patients were collected at admission and then weekly during antituberculosis treatment for 30 days. IS*6110* N-PCR CSF results were then compared with classic NTB diagnostic algorithms based on cyto-biochemical CSF features and bacteriological results. At admission (day 0), the bacteriological confirmation of TB etiology was very low: only 5 patients (33.33%) could be diagnosed bacteriologically. Thus, the Sv was 6.66 % for Ziehl Neelsen microscopy and did not exceed 33.33% for Lowenstein culture. CSF cyto-biochemical typical features (increased proteins, decreased glucose and lymphocytosis) suggested TB etiology in 80% of cases but also at 2 controls. All the localized forms of NTB showed non-specific CSF increased proteins only. IS*6110* N-PCR CSF positive results were observed in most NTB patients (Sv 86.66%) but also in two control patients (Sp 87.5%). IS*6110* N-PCR CSF positive results have been recorded despite an atypical CSF cytochemical profile and negative bacteriological results in patients with cerebral miliary TB and tuberculoma. These results suggested a CSF mycobacterial invasion in the absence of specific CSF inflammatory reaction. N-PCR *M tbc* detection was useful and non-invasive in patients with localised NTB lesions, allowing a quick diagnosis and precocious treatment.

In our study the overall Sv of N-PCR CSF *M tbc* detection (86.66 %) was superior to all other diagnostic methods but the Sp (87.5%) was inferior to the Lowenstein and Ziehl Neelsen Sp (100%). These results are comparable with previous studies of Seth (85% Sv) (15) or Lee (83% Sv) (51), inferior to Liu (90% Sv) (23), Scarpelinni (100% Sv) (18), Chedore (93.8% Sv) (31), but are superior to Chao Quan (75% Sv) (32), Lin (70% Sv) (33), Donald (63% Sv) (34), Miorner (54% Sv) (35); Kox (48% Sv) (36), Nguyen (33% Sv) (14) and some studies using commercially available kits such as MTD Gene-Probe and Roche AMPLICOR (16, 37).

IS*6110* N-PCR *M. tbc* detection during antituberculosis treatment was also studied. As other authors reported (38, 39), N-PCR CSF positive results in our study were observed up to 21 days after starting therapy in 86.66% patients and up to 30 days in 60% of patients. On the other hand, Ziehl Neelsen staining and Lowenstein culture rapidly became negative during the first week of therapy.

Our results have suggested the usefulness of IS*6110* N-PCR CSF *M tbc* detection in different forms of NTB diagnosis before and during antituberculosis therapy by comparison with bacteriological or unspecific CSF cytobiochemical criteria of diagnosis. The long persistence of CSF *M. tbc* detected with N-PCR method during anti TB therapy could also be used in antituberculosis therapy control. Other studies should also be performed in order to verify the usefulness of N-PCR in NTB diagnosis and treatment monitoring.

N-PCR assay limitations. In our study two patients with TM failed to be detected with IS*6110* N-PCR. As CSF typically lacks common inhibitors of PCR assays, a low number of mycobacteria or their poor lysis probably explains the false negative reactions in our study (40, 41). False negative reactions are also known to occur in rare cases of negative non-european IS*6110* strains (42). The disadvantage of PCR method remains the false positive results due to the cross contamination with amplified DNA product in the laboratory, a well-recognized problem in many laboratories. Although separated rooms and instruments were used at each PCR step in our study, two patients with bacterial meningitis were recorded as N-PCR positive; in these cases biochemical and cytological CSF results and the positive bacterial CSF culture clarified the diagnosis. The N-PCR interpretation in the clinical context prevents an incorrect diagnostics in most cases. More studies are required, especially in countries with a high incidence of TB, in order to assess:

- the high NTB mortality rate as a result

of incomplete and delayed diagnosis or treatment regarding the advantages of PCR based methods implementation;

- the rapid, sensitive, specific but high cost of a N-PCR method compared to the slow but inexpensive methods of diagnosis.

Conclusion

In our study IS *6110* N-PCR method using EUROCLONE kit to detect *M tbc,* proved superior for a rapid NTB diagnosis by comparison with classic methods of diagnosis. IS *6110* N-PCR method also proved a good potential of assisting the NTB diagnosis in different clinical forms including TM, tuberculoma, cerebral miliary TB and TB arachnoiditis. The long term positivity of IS *6110* N-PCR *M tbc* detection during antituberculosis therapy may be useful for the NTB confirmation regardless of empiric antituberculous treatment starting.

Ethical implications. The Hospital Scientific and Ethical Committee of National Institute of Infectious Diseases "Matei Bals", Bucharest has approved this study. The authors declare that the procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

Competing interests. The authors declare that they have no competing interests and no funding concerning the manuscript.

Abbreviations

CSF = Cerebrospinal fluid
HIV = Human immunodeficiency virus
M. tbc = Mycobacterium tuberculosis
MR = Magnetic resonance
N-PCR = Nested polymerase chain reaction
NPV = Negative predictive value
NTB = Neurotuberculosis
PCR = Polymerase chain reaction

PPV = Positive Predictive value
Sp = Specificity
Sv = Sensitivity
TB = tuberculosis
TM = Tuberculous meningitis

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