



Efficiency of Immunoblot While Determination of Antibodies to *Treponema Pallidum* in Cerebrospinal Fluid

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Abstract

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BACKGROUND: A decrease in the incidence of syphilis has been observed in the world and in Ukraine in recent years. At the same time, an increase in cases of neurosyphilis is recorded. Diagnosis of neurosyphilis is quite difficult and based on the correct interpretation of the complex of various diagnostic tests.

OBJECTIVES: The paper is aimed to determine diagnostic potential of treponemal tests (TTs) and evaluate effectiveness of *Treponema pallidum* immunoblot (TPI) while research on cerebrospinal fluid (CSF) in differential diagnosis of neurosyphilis.

MATERIALS AND METHODS: The research object was CSF of patients with late forms of syphilis. The regulated serological and immunological methods in accordance with current guidelines and orders of the Ministry of Healthcare of Ukraine were used for laboratory diagnosis of neurosyphilis: Enzyme immunoassays (EIA), fluorescent treponemal antibody (FTA), *T. pallidum* hemagglutination assay (TPHA), and TPI.

RESULTS: Effectiveness of TTs in the diagnosis of neurosyphilis while research on 23 samples of CSF was carried out by the following methods: EIA (Immunoglobulin [Ig]M + IgG), FTA, and TPHA. The above-mentioned TTs used in serological diagnosis of CSF do not always meet the problem of confirming neurosyphilis diagnosis. According to these investigations, both positive and false positive results were obtained. In order to verify the diagnosis, a study on positive and false positive samples of CSF by TPI method was carried out. Positive results were obtained in 13 samples with the established duration of the disease.

CONCLUSIONS: TPI is an optimal treponemal immunological method of examination of CSF to diagnose neurosyphilis with a high degree of reliability. The use of TPI while research on CSF makes it possible to verify the diagnosis of neurosyphilis by differentiated detection of antibodies to the most immunogenic antigens of *T. pallidum* eliminating the subjective factor of the reaction and simplifies diagnostic procedure.

Introduction

In recent years, epidemiological situation in Ukraine and abroad is characterized by increase in the number of cases of neurosyphilis. The long-term consequences of syphilis epidemic in the 1990s resulted in the formation of new cases of neurosyphilis due to latent forms of syphilitic infection that tend to increase these days. Untimely detection of lesions of nervous system can contribute to development of late forms of neurosyphilis necessitating improvement of existing approaches to the diagnosis of neurosyphilis [1], [2], [3], [4].

To diagnose neurosyphilis, cerebrospinal fluid (CSF) research methods have been developed and used including clinical researches (determination of protein content, cytosis) that are important but do not confirm lesion specificity, so great importance is attached to immunological researches with non-treponemal and treponemal tests (TTs) [2], [5], [6].

Non-TTs (NTTs) are aimed at detecting antibodies to cardiolipin, phospholipids, cell component,

and mitochondrial bacteria membrane. TTs detect antibodies directed specifically against antigens, components of *Treponema pallidum* bacteria. Standard NTTs for research on CSF in patients with syphilis is the venereal disease research laboratory (VDRL) test and the rapid plasma reagin (RPR) test of rapid plasma reagents which specificity and sensitivity is 99–99.3% and 55–75%, respectively. In Ukraine, for research on CSF while syphilis diagnosis is used microprecipitation reaction which diagnostic efficiency does not differ from the above indicators VDRL, RPR [5], [7], [8].

Due to the low sensitivity of NTTs, TTs are widely used in while research on CSF. These include fluorescent treponemal antibody (FTA) reaction, *T. pallidum* hemagglutination assay (TPHA) reaction, enzyme immunoassays (EIA), and immunoblot method [2], [6].

FTA reaction is used as FTA-abs with adsorption and FTA with pure CSF. According to some authors, FTA sensitivity while research on CSF is 82–94%. In general, importance of this reaction in neurosyphilis diagnosis is highly valued. Positive FTA results with CSF

cannot necessarily be associated with neurosyphilis. In some cases, the reaction positivity can be due to the penetration of serum antitreponemal antibodies during lumbar punctures or while increasing permeability of the blood-brain barrier. Negative FTA results with CSF exclude the neurosyphilis diagnosis [5], [6], [9].

Another TT for CSF is the passive hemagglutination reaction (TPHA) with determination of antibodies to a recombinant analogue of the *T. pallidum* membrane protein A (TmpA) and 17 kDa *T. pallidum* protein. Researches by many authors have established possibility of using TPHA in the neurosyphilis diagnosis, in particular while research on CSF [2], [5], [6].

EIA method is one of the most common TTs while neurosyphilis diagnosis. Diagnostic efficiency of EIA method while research on CSF is 92–100% depending on the neurosyphilis form [5], [6].

In recent years, immunoblotting that is one of the most modern methods of diagnosing syphilis has been actively used to differentiate treponemal serological reactions. *T. pallidum* immunoblot (TPI) is a highly specific and highly sensitive reference method for diagnosis of patients with positive or indeterminate test results including EIA reaction, TPHA reaction, and FTA reaction. TPI lies in its high informativeness and reliability of results. According to the references, sensitivity and specificity of the test are very high 99.6–100% and 99.0–99.5%. It is achieved through the separation of proteins, glyco- and lipoproteins, and maximum specificity of detectable immune sera or monoclonal antibodies [1], [2], [9].

TPI is a modification of classical and linear EIA. The method is intended for the analysis of serum or plasma of human blood for availability to *T. pallidum* immunoglobulins (Igs) and determines antibody spectrum (AB) to several antigens; it also evaluates contribution to the overall AB reaction of different specificity. The analysis can be performed both in an automatic enzyme-linked immunosorbent analyzer and by manual staging with subsequent recording on a spectrophotometer (scanner) with electrophoretic ally separated *T. pallidum* antigens [1], [2].

Recombinant proteins are usually used in EIA systems that allow to achieve high sensitivity and specificity and to avoid false positive results. For TPI production recombinant analogs of the most immunogenic *T. pallidum* lipoproteins TpN15, TpN17, TpN41, TpN47, and one synthetic peptide – TmpA p45 is used in diagnostic test systems. The list of antigens can differ in test kits from different manufacturers. The test result is considered positive if the test sample contains specific antibodies up to two or more antigens. The analysis result is considered negative if the optical density of the test sample is negative for all tested *T. pallidum* antigens. The test result is considered indeterminate if the test sample contains specific antibodies up to one *T. pallidum* antigen [2], [9].

This method can be used both to verify the syphilis diagnosis and as a criterion for the disease curability. The method is easily reproducible, relatively easy to perform and interpret the results. In one reaction, binding of antibodies to several antigens can be detected, each of which can be accurately identified. Thus, TPI has a number of advantages over other methods of detecting antibodies which results depend on standardization, sensitivity, substrate quality, instability, or insolubility of certain antigens.

The aim of this research is to determine diagnostic capabilities of TTs and evaluate effectiveness of TPI (TPI DIA-Trep-different) while research on CSF in differential diagnosis of neurosyphilis.

Materials and Methods

Diagnosis of neurosyphilis is quite difficult and based on the correct interpretation of the complex of various diagnostic tests. If clinical evidence of neurologic involvement is observed (e.g., cognitive dysfunction, motor or sensory deficits, cranial nerve palsies, symptoms or signs of meningitis or stroke), a CSF examination should be performed before treatment.

The research object was CSF of patients with late forms of syphilis. The regulated serological and immunological methods in accordance with current guidelines and orders of the Ministry of Healthcare of Ukraine were used for laboratory diagnosis of neurosyphilis: A combination of NTTs –RPR (“DIAPROF-med”) and TTs – EIA (“DIAPROF-med-EIA”), FTA absorption test (FTA) (“DIAPROF-med-FTA”), *T. pallidum* particle agglutination (TPPA) (TPHA) (“Biolik-TPHA”), TPI (“DIA-Trep-different”) [10].

NTTs: The RPR test is a NTT that uses antigen containing cardiolipin, which flocculates on reaction with IgM and IgG antibodies. Apparently, *T. pallidum* infection results in binding of host lipids to the *Treponema*, converting inert lipids into immunogens *in vivo* [10].

TTs. The TPPA is based on agglutination with the treponemal antigen. The TPHA test is a microhemagglutination assay for IgM and IgG antibodies. The FTA uses a fixed *T. pallidum* to bind IgM and IgG antibodies.

EIA detects IgG antitreponemal antibodies using a microtiter plate of wells with wild-type *T. pallidum* antigens.

TPI (subsequent EIA), used recombinant *T. pallidum* antigens instead of wild-type antigens. TPI test that used recombinant TpN15, TpN17, TpN41, TpN47, and had higher sensitivity than an EIA that used wild-type antigens (99% vs. 91.4%; $p < 0.01$) [10].

All tests were performed according to the manufacturer's instructions and were subject to quality assurance programs.

Research Results

It is known that when positive serological reactions to syphilis are detected in the serum of patients with late forms of syphilis, diagnosis of neurosyphilis can be established only in availability of treponemal antibodies in CSF. All 23 patients with late forms of syphilis and suspected possible etiological role of syphilitic infection in the development of neurological symptoms were subject of research on CSF for availability of specific antibodies to *T. pallidum*. All CSF samples were examined by non-treponemal and TTs, as shown in Figure 1.

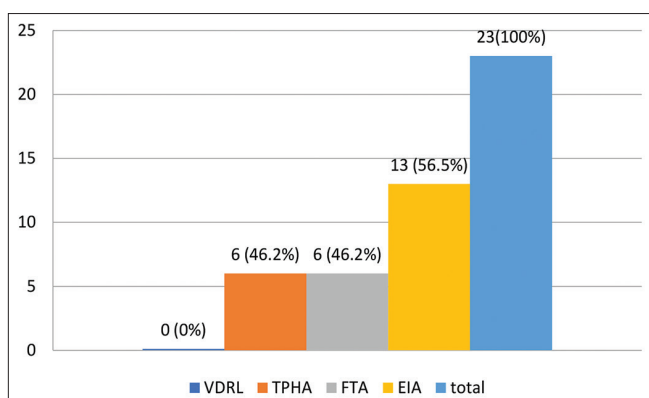


Figure 1: Results of the research on cerebrospinal fluid through non-treponemal tests and treponemal tests

In 23 (100%) samples of CSF while performing RPR (VDRL analog) test, non-specific antibodies to cardiolipin antigen were not detected that indicated the lack of active process. While research through TTs there was found that in 13 (56.5%) cases by EIA method antibodies to Igs of IgG + IgM *T. pallidum* classes were detected. In one case, the indicator was questionable. While further testing positive EIA samples of CSF TTs tests for TPHA and FTA results were positive (4+) in 6 (46.2%) samples, in 7 (53.8%) samples were weakly positive (2+). Detection frequency of antibodies to *T. pallidum* in CSF was higher through EIA than through TPHA, FTA and was 56.5–46.2%, respectively. False positive results without availability of neurological clinical infection manifestations are not sufficient indicators for the diagnosis of neurosyphilis, so we verified research results obtained through EIA, TPHA, and FTA using TPI to individual *T. pallidum* proteins.

Determination of Igs differentiated into separate *T. pallidum* proteins through TPI is a highly specific reference method for cases with positive, false-positive, or indeterminate results, including by TPHA, FTA, EIA tests. This method allows to detect antibodies

to corresponding antigens of *T. pallidum* depending on their molecular mass (TpN15, TpN17, TpN41, and TpN47). In one reaction, we found the binding of antibodies to several antigens, each of which was accurately characterized, shown in Figure 2.

No sample	TpN17	TpN15	TpN41	TpN47	combined
1	+	+	-	+	2 (15.4%)
2	+	+	-	+	
3	+	+	-	-	4 (30.8%)
4	+	+	-	-	
5	+	+	-	-	
6	+	+	-	-	
7	+	-	-	-	7 (53.8%)
8	+	-	-	-	
9	+	-	-	-	
10	+	-	-	-	
11	+	-	-	-	
12	+	-	-	-	
13	+	-	-	-	
B %	100%	46.2%	0%	15.4%	
+	found				up to 3 proteins
-	not found				up to 2 proteins
					up to 1 protein

Figure 2: Detection of immunoglobulins to *Treponema pallidum* proteins in cerebrospinal fluid samples by enzyme immunoassays

While performing TPI with 13 samples of CSF, all 100% of the samples were positive for TpN17 protein that coincided with EIA results and indicated syphilitic infection. Igs to TpN15 protein in 6 (46.2%) samples and TpN47 protein in 2 (15.4%) samples were detected. Simultaneously, the samples revealed combinations of Igs up to three proteins TpN15, TpN17, TpN47– in 2 (15.4%) cases; up to two proteins TpN15, TpN17– in 4 (30.8%) cases, respectively; to protein TpN41– there were no positive results in any case, shown in Figure 2.

Based on obtained data, according to the results of TPI in 6 (46.2%) samples of CSF detected Igs up to two or more proteins TpN17, TpN15, and TpN47 that confidently indicated neuroinfection. In 7 (53.8%) cases, Igs were detected only up to one protein – TpN17 that indicated availability of syphilitic infection with an early duration of infection. Thus, TTs, EIA, FTA, and TPHA used in the serological diagnosis of CSF do not always meet the task while diagnosing neurosyphilis with process activity.

According to the results of our research, the TPI method of the domestic test system made it possible to verify the diagnosis in 6 (46.2%) out of 13 (56.5%) patients positive for the above-mentioned TTs for availability of Ig samples in CSF to some specific proteins of *T. pallidum* with a late duration of infection and in 7 (53.8%) patients with an indefinite duration of a disease.

Discussion

Invasion of the central nervous system by *T. pallidum* can occur at any stage of syphilis. Regarding the fact that *T. pallidum* is not cleared promptly, certain individuals may experience progression to neurosyphilis, which manifests as cognitive and

behavioral abnormalities, limb paralysis, and potentially fatal outcomes. Early identification or prevention of neurosyphilis is therefore crucial. CSF examination plays an important role in the diagnosis of neurosyphilis but lacks the gold standard.

The current laboratory recommendation for neurosyphilis diagnosis includes CSF analysis with NTTs such as VDRL or RPR, and with TTs such as FTA, TPHA, and EIA, alongside CSF cellularity and protein levels. However, there are important limitations, as CSF NTTs are not sensitive enough and do not eliminate the possibility of neurosyphilis in case of negative results [11]. On the other hand, CSF TTs are more specific but less sensitive, so they do not confirm the diagnosis but can exclude it. Finally, the hypercellularity and elevated protein levels can support the diagnosis in the presence of a negative non-treponemal CSF test and warrant empiric treatment.

Another challenge is regarding diagnosis in asymptomatic patients, investigated with usual tests for the hypothesis and diagnosis of neurosyphilis: FTA-ABS and RPR. Laboratory parameters (such as increased protein and leukocyte levels or even positive RPR) do not offer a significant statistical gain to confirm the disease, but, if not altered, they moderately reduce the individual's chance of having neurosyphilis [10].

Analysis of diagnostic specificity of TTs as a result of studies of 23 samples of CSF in patients with syphilis showed that the highest rates were obtained in treponemal immunological methods as EIA (56.5%) and TPI (56.5%), and slightly lower in TPHA (46.2%) and FTA (46.2%). The maximum indicator of diagnostic efficiency from TTs was noted in EIA and TPI (100% and 100%, respectively); in TPHA – 46.2% and FTA – 53.8%.

The TPI method showed the highest predictable value for positive and weakly positive results obtained by TPHA and FTA methods. As a result, in all 13 samples of CSF which were positive and weakly positive by TPI (regardless of the presence or absence of clinical manifestations of infection and the duration of infection), we detected antibodies to the protein TpN17 (100%), up to two and three proteins – TpN17, TpN15, and TpN47 (from 15.4% to 30.8% of cases). NTTs are less suitable to detect this pathology due to their low sensitivity. In our study, the RPR (VDRL) method was negative in all 23 samples of CSF.

Studies by modern authors have shown high efficiency of linear immunoblot in the study of CSF in patients with late forms of syphilis [12], [13], in patients with meningovascular neurosyphilis [10], which is also confirmed by our studies using the TPI with differential examination of antibodies to individual antigens of *T. pallidum*.

We set priorities for using of individual research methods in the diagnosis of neurosyphilis based on our results. Early examination of patients with neurosyphilis

is very relevant and important, especially among people who have previously suffered from syphilis. CSF testing performed on individuals suspected of having neurosyphilis, including patients with latent forms of syphilis and individuals who have experienced syphilis with positive immunological blood tests for *T. pallidum*.

TPI is an optimal treponemal immunological method of examination of CSF to diagnose neurosyphilis with a high degree of reliability (100% sensitivity and predicted value with positive and weakly positive results of EIA, TPHA, and FTA).

Our review has limitations that should not be ignored. To better determine the test performance characteristics of the CSF antibody tests, head-to-head studies of CSF nontreponemal (lipoidal antigen), and treponemal antibody tests would be conducted with larger samples and in more populations with well-characterized symptom status.

Conclusions

Thus, all the above indicates possibility and necessity of use in clinical practice in complex diagnostic situations, where adequate interpretation of laboratory results, compliance with the principle of comprehensive clinical and serological examination of patients with positive serological reactions to syphilis. The use of TPI while research on CSF makes it possible to verify the diagnosis of neurosyphilis by differentiated detection of antibodies to the most immunogenic antigens of *T. pallidum* eliminating the subjective factor of the reaction and simplifies diagnostic procedure.

Authors' Contributions

All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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