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Assessment of *T. pallidum* recombinant proteins Tp0163 and Tp0971 as antigens for the diagnosis of syphilis by enzyme-linked immunosorbent assay

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Background. Genomic sequencing of *T. pallidum* and the application of bioinformatics and immunoproteomics resulted in many recombinant proteins of *T. pallidum* that have been analyzed for their diagnostic value, and some of them are used as diagnostic antigens in commercial tests. However, in practice, there are often significant difficulties in the diagnosis of asymptomatic forms of syphilis, especially of the late forms, when the sensitivity of serological methods may differ significantly. Therefore, the search for more sensitive and specific antigens for serological diagnosis of syphilis is ongoing.
Aim of study. Assessment of the potential of *T. pallidum* recombinant proteins Tp0163 and Tp0971 as candidate antigens for serum IgG determination in syphilis patients by enzyme-linked immunosorbent assay (ELISA).
Methods. *T. pallidum* recombinant proteins Tp0163 and Tp0971 were produced by Cusabio (China). Serum samples from subjects with a confirmed diagnosis of primary, secondary, early latent and late latent syphilis, as well as serum samples from healthy subjects as controls were used for the tests with indirect ELISA. To characterize the antibody concentration, a positivity coefficient equal to the ratio of the absorbance obtained for each sample to the critical absorbance was determined. The positivity coefficient was used to determine the difference between groups of syphilis patients and healthy individuals using the Mann-Whitney test for unpaired samples, and to assess the clinical informativeness of laboratory test. The diagnostic efficacy of ELISA was assessed by calculating the values in groups of patients with established diagnosis of syphilis and in a group of healthy individuals in compliance with the standard assessment of the diagnostic informativeness of laboratory tests.
Results. The overall diagnostic efficacy of the studied recombinant proteins for the detection of IgG class antibodies by ELISA is 65.4 and 66.7 % for Tp0163 and Tp0971, respectively, demonstrating high specificity and positive predictive value of the test. The sensitivity of ELISA to the studied forms of syphilis does not exceed 68.8 %.
Conclusion. In order to improve treponema-specific diagnosis of syphilis, we believe it is important to continue working with these antigens to determine the efficacy of ELISA to detect IgG, IgM, or IgM/IgG antibody ratios with more clinical serum samples from subjects with syphilis, subjects without clinical and laboratory signs of syphilis, and healthy individuals.

Keywords: ***Treponema pallidum*, recombinant proteins, Tp0163, Tp0971, enzyme-linked immunosorbent assay**
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Оценка рекомбинантных белков *T. pallidum* Tp0163 и Tp0971 как антигенов для диагностики сифилиса методом иммуноферментного анализа

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Обоснование. С расшифровкой генома *T. pallidum* и применением биоинформатики и иммунопротеомики появилось множество рекомбинантных белков *T. pallidum*, которые были проанализированы с точки зрения их диагностической ценности, и некоторые из них используются в качестве диагностических антигенов в коммерческих тестах. Однако на практике нередко возникают существенные затруднения при диагностике асимптомных форм сифилиса, особенно поздних, когда чувствительность серологических методов может значительно различаться. Поэтому поиск более чувствительных и специфических антигенов для серологической диагностики сифилиса продолжается.
Цель исследования. Оценка потенциала рекомбинантных белков *T. pallidum* Tp0163 и Tp0971 в качестве кандидатных антигенов для определения IgG в сыворотке больных сифилисом методом иммуноферментного анализа (ИФА).
Методы. Рекомбинантные белки *T. pallidum* Tp0163 и Tp0971 произведены фирмой Cusabio (Китай). Для исследования методом непрямого ИФА использовали образцы сыворотки крови пациентов с подтвержденным диагнозом первичного, вторичного, раннего скрытого и позднего скрытого сифилиса, а также образцы сыворотки крови от здоровых лиц как группы контроля. Для характеристики концентрации антител определяли коэффициент позитивности, равный отношению оптической плотности, полученной для каждого образца, к критической оптической плотности. Коэффициент позитивности использовали для определения различия между группами больных сифилисом и здоровых лиц по критерию Манна–Уитни для непарных выборок, а также для оценки клинической информативности лабораторного теста. Оценку диагностической эффективности ИФА проводили путем расчета показателей в группах больных с установленным диагнозом сифилиса и в группе здоровых лиц в соответствии со стандартом оценки диагностической информативности лабораторных тестов.
Результаты. Общая диагностическая эффективность исследованных рекомбинантных белков для определения антител класса IgG методом ИФА составляет 65,4 и 66,7% соответственно для Tp0163 и Tp0971, демонстрируя при этом высокую специфичность и положительную предсказательную ценность исследования. Чувствительность ИФА в отношении изученных форм сифилиса не превышает 68,8%.
Заключение. С целью совершенствования трепонема-специфической диагностики сифилиса мы считаем важным продолжить работу с данными антигенами по определению эффективности ИФА для выявления IgG, IgM или соотношения антител IgM/IgG с большим количеством клинических образцов сывороток от пациентов с сифилисом, пациентов без клинико-лабораторных признаков сифилиса и здоровых лиц.

Ключевые слова: ***Treponema pallidum*; рекомбинантные белки; Tp0163; Tp0971; иммуноферментный анализ**
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Background

Serological methods hold a leading position in the laboratory diagnostics of syphilitic infection. In recent years, treponemal tests using recombinant immunodominant antigens of *T. pallidum* such as Tp15 (Tp0171), Tp17 (Tp0435) and Tp47 (Tp0574) and TmpA (Tp44.5, Tp0768) have become the platform of choice for performing enzyme immunoassays in laboratories. The method of enzyme-linked immunosorbent assay (ELISA) using recombinant antigens is widely used in the diagnosis of all forms of syphilis due to its high sensitivity and specificity, as well as the ability to detect the disease in the absence of clinical manifestations, which is relevant for the latent form of syphilis. Automated performance and objective interpretation of the results are also the advantages of the method, and its limitations are inability to differentiate various disease forms and low informativeness for the treatment efficacy assessment [1]. The search for additional sensitive and specific antigens may expand the abilities of serological diagnostics of syphilis.

The development of methods of functional genomics and proteomics and, on their basis, the technology of recombinant proteins production opened the possibility for large-scale detection of such antigens. A detailed review of the groups of proteins that exhibit immunogenic properties during the course of syphilitic infection was performed by M.A. McGill et al. [2] and M.B. Brinkman et al. [3]. Based on the works of these researchers and our own bioinformatic analyses results, the prospect of diagnostic use of a number of *T. pallidum* proteins, which immunogenicity in relation to human serum was confirmed by both proteomic platforms [4], was studied in the Federal State Budgetary Institution State Research Center for Dermatovenerology and Cosmetology of the MoH of Russia (SRCDC) in 2012. Six proteins were selected for which genetic expression systems were created *de novo* at the SRCDC. After purification by affinity chromatography, the obtained recombinant proteins were used as antigens for the determination of specific IgG in the serum of patients with different forms of syphilis by ELISA and immunofluorescence assay [5–9]. The data obtained characterized new antigens as promising for the diagnosis of syphilitic infection.

The search for *T. pallidum* antigens that could serve as markers of syphilis stage, or be suitable for response to treatment monitoring, or be used as recombinant vaccines is still ongoing [10–12].

In the present work, we continued to investigate candidate antigens for syphilis diagnosis selected by bioinformatic analysis of two proteomic platforms [4] and evaluated the prospects of using *T. pallidum* recombinant proteins Tp0163 and Tp0971 as antigens in the examination of syphilis patients' serum by ELISA.

Tp0163 (TroA) is a periplasmatic protein in the composition of ATP-binding transport complex. The work by M.V. Brinkman et al. shows a 2-fold increase over background values in the tests of antibodies to this antigen in the serum of patients with early latent syphilis, whereas no significant increase was described in the manifest forms [3]. Meanwhile, the screening by M.A. McGill et al. showed seroreactivity values of 3+ for serum samples of patients with primary, secondary, early and late latent syphilis [2].

Tp0971 is a periplasmatic lipoprotein believed to be attached to the inner membrane of *T. pallidum* [13, 14]. The work by Brinkman et al. showed a 4-fold increase over background values of the level of antibodies to this antigen in the group of patients with primary syphilis and an 8-fold increase in the group of early latent syphilis compared to control samples of healthy individuals [3]. The screening by M.A. McGill et al. showed seroreactivity values of 1+ for serum samples of patients with late latent syphilis and 2+ for primary, secondary, early latent syphilis [2].

More recent works have shown the induction of production of high levels of specific IgG antibodies in rabbits in response to the administration of recombinant Tp0971 [11] as well as serum reactivity to recombinant Tp0163 in subjects with primary syphilis [12].

The study aim was to assess the potential of *T. pallidum* recombinant proteins Tp0163 and Tp0971 as candidate antigens for IgG determination in the serum of patients with primary, early latent, late latent and secondary syphilis by ELISA.

Methods

Study design

A pilot, uncontrolled, observational study was conducted with a control group of serum samples from healthy donors (n = 20) and 58 serum samples from subjects with a confirmed diagnosis of syphilis of the following forms: primary (n = 15), secondary (n = 8), early latent (n = 16), and late latent (n = 19).

Acceptance criteria

Inclusion criteria: subjects of both sexes 18 years of age and older diagnosed with primary, secondary, early latent and late latent syphilis. The control group consisted of practically healthy individuals of both sexes 18 years of age and older.

Exclusion criteria: pregnancy, syphilis of other forms.

Study conditions

The diagnosis was established by a dermatovenereologist of the SRCDC in compliance with the clinical recommendations "Syphilis" (2020) based on the medical history data, clinical signs and serological test results. Blood sampling was performed at the clinical laboratory of the SRCDC, and serological tests (ELISA) were performed at the same institution in the Department of Laboratory Diagnostics of Sexually Transmitted Infections and Dermatoses.

Serum obtained from subjects from the central region of the Russian Federation who addressed the SRCDC between September 2023 and May 2024 was studied.

Study description

Indirect IgG ELISA was used. *T. pallidum* recombinant proteins of Tp0163 and Tp0971 were produced by Cusabio (China).

Recombinant antigens Tp0163 and Tp0971 were individually diluted in 0.05 M carbonate bicarbonate buffer (pH 9.6) to 1 μg/mL concentration and added into 96-well microplates (ServiceBio) at amount of 100 μL per well, then incubated overnight at room temperature. The microplates were rinsed once with phosphate saline buffer solution containing 0.05 % of tween 20 (FSSt) and blocked with 3 % skim milk powder solution (with 5 % sucrose) in FSSt at amount of 150 μL per well for 1.5 h at room temperature.

After removal of the blocking buffer and drying, 100 μL each of the test serum samples at a dilution of 1:100 **(**in FSSt with 1 % skim milk powder) were added to the plates and incubated at 37 °C for 30 min. The plates were rinsed three times with FSSt to remove unbound antibodies. 100 μL

HRP-conjugated goat anti-human IgG antibodies (Merck Millipore) were added to each well and incubated at 37 °C for 35 min. To detect immunocomplexes, the plates were developed by adding 100 µL of tetramethylbenzidine-H_2O_2 substrate per each well for 15–20 min with incubation at 37 °C in the dark. The reaction was stopped by adding 100 µL of 0.2 M sulfuric acid.

Outcome reporting methods

The absorbance (A) of immunoenzyme reaction products in the plate wells was measured at 450 nm using Multiskan FC ELISA analyzer (Thermo Scientific, USA). For results interpretation the A threshold level (A_{thresh}) was defined as the average A of serum samples from healthy individuals.

A_{thresh} was used to determine positivity coefficient (PC):

$$CP = A_{sample}/A_{thresh},$$

where A_{sample} is A of any test serum sample.

Further, CP values exceeding 4, the recommended value for our test system, were considered positive.

Statistical analysis

Quantitative characteristics of the CP were tested for normality of distribution using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Integroup differences were evaluated using Fisher's test for single-factor analysis of variance. In pairwise comparison of averages, Student's test for independent groups was used. Significance level complied with $p < 0.05$.

Serum samples from subjects with a confirmed diagnosis of syphilis classified as positive in ELISA were designated as true positive (TP); those classified as negative in ELISA were designated as false negative (FN); classified as positive in ELISA were classified as false positive (FP); serum samples from healthy donors classified as negative in ELISA were classified as true negative (TN).

The diagnostic value of recombinant antigens Tp0163 and Tp0971 of *T. pallidum* was analyzed in compliance with GOST (All-Union State Standard) R 53022.3-2008 "Clinical Laboratory Technologies. Requirements for Quality of Clinical Laboratory Tests. Part 3. Rules for the Clinical Informativeness of Laboratory Tests Assessment" [15] using the CP of the test serum.

Diagnostic indices were calculated using the following formulas:

$$\text{Sensitivity} = TP / (TP + FN) \times 100\ \%, \quad (1)$$

$$\text{Specificity} = TN / (TN + FP) \times 100\ \%, \quad (2)$$

$$\text{Positive predictive value} = TP / (TP + FP) \times 100\ \%, \quad (3)$$

$$\text{Negative predictive value} = TN / (TN + FN) \times 100\ \%, \quad (4)$$

$$\text{Diagnostic efficacy} = (TP + TN) / (TP + FN + TN + FP) \times 100\ \%, \quad (5)$$

where TP, TN are true positive and true negative results, respectively; FN, FP are false negative and false positive results, respectively.

Ethical expert evaluation

The study was approved by the Local Independent Ethics Committee of the Federal State Budgetary Institution "State Research Center for Dermatovenerology and Cosmetology" (Protocol No. 2 dated February 28, 2023). Biological material for the study was obtained from subjects in compliance with written voluntary informed consent.

Results

Preliminary analysis of the possibility of diagnostic use of recombinant proteins Tp0163 and Tp0971 of *T. pallidum* for determination of IgG antibodies by ELISA was carried out with 58 serum samples from four groups of patients with the following forms of syphilis: primary syphilis (n = 15), secondary syphilis (n = 8), early latent syphilis (n = 16) and late latent syphilis (n = 19). The control group consisted of 20 serum samples from healthy individuals. The results of ELISA using both antigens are given in Table 1, and the diagnostic characteristics of recombinant antigens Tp0163 and Tp0971 are given in Table 2.

The studies have shown that the ELISA results for the determination of IgG class antibodies based on Tp0163 and Tp0971 are generally consistent with the clinical diagnosis. Significant variability of immune response is characteristic for both antigens in all studied groups of syphilis patients (see Table 1). Some amount of false negative results was obtained in each group of patients, while no false positive results were observed in the group of healthy individuals.

The differences in CP between the groups of healthy individuals and secondary syphilis patients were statistically insignificant with respect to both antigens. At the same time, the average CP values in the group of secondary syphilis patients were quite high and were 19.90-fold and 11.41-fold higher than the CP values in the group of healthy individuals for Tp0163 and Tp0971, respectively. In addition, the lowest amount of false negative results in the whole sample was reported in these groups — only one with Tp0163. Nevertheless, large scatter of values with a small sample leveled out the above differences.

For patients diagnosed with primary, early latent, and late latent syphilis, the CP values are statistically significantly higher than those for the control group. The diagnostic sensitivity of IgG determination in this case for primary, early latent and late latent syphilis is 40; 62.5; 31.6 % with Tp0163 and 66.7; 68.8; 15.8 % with Tp0971, respectively.

The overall diagnostic efficacy of the studied recombinant proteins for the detection of IgG antibodies is characterized by low values — 65.4 and 66.7 % for Tp0163 and Tp0971, respectively, at the same time demonstrating high specificity and positive predictive value of the test (100 %). In our study, both antigens showed maximum sensitivity with respect to secondary syphilis, but due to the small sample size and significant variation in CP values, the results were statistically insignificant. The sensitivity of ELISA to other forms of syphilis does not exceed 68.8 %.

Discussion

With the elucidation of *T. pallidum* genome and the use of bioinformatic analysis, many *T. pallidum* recombinant proteins such as Tp15, Tp17, TmpA, Tp47, Tp0453, Tp0319, Tp1038, Tp0277, Tp0684, Tp0965 have been evaluated for their diagnostic value [5, 6, 8, 9, 12, 14]. Some of them — Tp15, Tp17, TmpA, Tp47 — are used as diagnostic antigens in commercial tests. To date, however, the diagnosis of syphilis is based on the combination of several assays, and there is no single commonly accepted serological test. Thus, the search for sensitive and specific antigens for serological diagnosis of syphilis remains an important task.

Table 1. Values of positivity coefficients in healthy individuals and in groups of patients and the level of statistical significance of differences between groups by the Mann–Whitney test
Таблица 1. Значения коэффициентов позитивности у здоровых лиц и в группах больных и уровень статистической значимости различий между группами по критерию Манна–Уитни

Group of subjects	Range	Average	*p*
	Tp0163		
Healthy (*n* = 20)	0,5–1,7	0,90	
Primary syphilis (*n* = 15)	0,6–19,2	5,15	$p_{1,2} < 0,05$
Secondary syphilis (*n* = 8)	2,7–27,7	19,96	$p_{1,3} > 0,05$*
Early latent syphilis (*n* = 16)	0,8–32,0	10,17	$p_{1,4} < 0,05$
Late latent syphilis (*n* = 19)	1,2–12,5	4,34	$p_{1,5} < 0,05$
	Tp0971		
Healthy (*n* = 20)	0,3–1,8	1,18	
Primary syphilis (*n* = 15)	1,7–19,9	6,54	$p_{7,8} < 0,05$
Secondary syphilis (*n* = 8)	4,5–15,9	11,41	$p_{7,9} > 0,05$*
Early latent syphilis (*n* = 16)	0,9–19,3	7,62	$p_{7,10} < 0,05$
Late latent syphilis (*n* = 19)	0,7–8,4	2,38	$p_{7,11} < 0,05$

Note. * — differences are statistically insignificant.
Примечание. * — различия статистически незначимы.

Table 2. Diagnostic parameters of the recombinant proteins Tp0163 and Tp0971 as antigens for syphilis diagnosis by the method of enzyme immunoassay
Таблица 2. Диагностические параметры рекомбинантных белков Tp0163 и Tp0971 как антигенов для определения антител класса IgG при сифилисе методом иммуноферментного анализа

Diagnostic characteristics	Tp0163	Tp0971
General (*n* = 58), %:		
Sensitivity	53,4	66,7
Specificity	100,0	100,0
Positive predictive value	100,0	100,0
Negative predictive value	42,6	43,5
Diagnostic efficacy	65,4	66,7
Sensitivity (%) in determination of:		
Primary syphilis (*n* = 15)	40,0	66,7
Secondary syphilis (*n* = 8)	87,5	100,0
Early latent syphilis (*n* = 16)	62,5	68,8
Late latent syphilis (*n* = 19)	31,6	15,8

Note. Parameters were determined in accordance with GOST R 53022.3-2008.
Примечание. Параметры определялись в соответствии с ГОСТ Р 53022.3-2008.

The proteins Tp0163 and Tp0971 are localized on the cytoplasmic membrane of *T. pallidum* on the periplasmic side and, as shown in studies of the *T. pallidum* [2, 3] proteome, have high immunogenicity. They were also selected as candidate antigens for syphilis diagnosis as a result of bioinformatic analysis of two proteomic platforms [4]. The prospectivity of the study of their diagnostic properties was confirmed by the works by M.B. Brinkman et al. [3] and M.A. McGill et al. [2], and later - by the studies by X. Zhang et al. [11] and A.M. Haynes et al. [12].

Our study confirms the immunogenicity of recombinant proteins Tp0163 and Tp0971. Both antigens induce a reliably significant increase in specific IgG antibodies in the serum of patients with early latent syphilis. The specificity and positive predictive value of the tests with both antigens were 100 %. The diagnostic efficacy of

ELISA in syphilis has been shown to vary depending on antigens used to detect anti-*T. pallidum* antibodies [16] and on infection stage [17, 18]. In addition, ELISA based on isolated recombinant antigens is less sensitive than that using antigen kits [19, 20]. Moderate diagnostic efficacy observed in our experiment for Tp0163 and Tp0971 when determining IgG antibodies (65.4 % and 66.7 % for Tp0163 and Tp0971, respectively) suggests that each protein itself may not be an effective candidate for diagnosis, however, we believe that the testing performance established in our experiment could be improved by additional detection of IgM class antibodies, or IgM/IgG antibody ratio, or by inclusion of the test proteins in kits of several *T. pallidum* antigens.

The study is designed to include further investigation of diagnostic value of the recombinant proteins Tp0163 and Tp0971 alone or as part of kits with other *T. pallidum* antigens with a large number of serum samples from patients with various forms of syphilis as well as probable differentiation of latent forms of syphilis with false-positive reactions for syphilis. In addition, it is planned to investigate the dynamics of IgG antibody profile or IgM/IgG ratio in the serum of patients with syphilis, particularly that late latent, before and after specific therapy in order to assess the ability to use these antigens to develop serological criteria of therapy efficacy.

The search for *T. pallidum* antibodies specific for certain stages of syphilis or characterized by alternative expression patterns in different forms of the disease has been actively developed in the recent decades due to the expansion of proteomic platforms [10–12] and the ability to obtain recombinant analogues of hard-to-reach treponema proteins. The results obtained to date are scarce and contradictory [16, 19, 21–25], including those for supposedly well-studied antigens Tp15 (Tp0171), Tp17 (Tp0435) and Tp47 (Tp0574) and TmpA (Tp44,5, Tp0768) [19, 22]. The results of such studies are preliminary and help to see the prospect of further work with certain antigens as well as to improve the understanding of immune response development in syphilis.

Conclusion

T. pallidum recombinant proteins Tp0163 and Tp0971 retain prospects as novel antigens for serological diagnostics of syphilis. Further studies should encompass a wider range of syphilis cases and determine the potential of Tp0163 and Tp0971 in combination with other treponemal antigens to both diagnose syphilis and monitor the efficacy of specific treatment.

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