Chapter 7

Effective Diagnostic Marker for Serodiagnosis of *Toxoplasma gondii* Infection: New Developments and Perspectives

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Abstract

Toxoplasmosis is a prevalent parasitic infection caused by an obligate intracellular parasite *Toxoplasma gondii*. Various methods have been established in the laboratory diagnosis of toxoplasmosis. Among these methods, serological tests are common and provide satisfactory results. However, producing reliable reagents and standard antigen remains difficult and expensive. Replacing native antigens in all current diagnostic kits with standard and reliable reagents are speculated to achieve more sensitive and specific detection that can significantly improve the assay performance. This review provides updated data on toxoplasmosis serodiagnosis. It focuses on the recent trends of producing reliable and standard antigens that have been used in the serological tests of toxoplasmosis, as well as the future direction in this field.

Keywords: ELISA, serodiagnosis, multiepitope peptide, recombinant antigen, sensitivity, specificity, toxoplasmosis

1. Introduction

Toxoplasmosis is one of the most prevalent parasitic infections caused by an obligate intracellular parasite *Toxoplasma gondii.* This parasite can infect almost all warm-blooded animals including humans. The effect of *T. gondii* on public health and animal production is significantly evident worldwide. Therefore, it became one of the well-studied parasites because of its medical and veterinary importance. Although the prevalence rate of toxoplasmosis is

approximately one-third of the world's human population, *T. gondii* infection in immunocompetent individuals is usually asymptomatic, even though some patients may experience fever and other non-specific clinical signs [1]. In immunocompromised patients, this disease may be serious or even fatal; furthermore, primary invasion during pregnancy may endanger the life of the fetus as well as the infected mothers [1, 2]. Accurate diagnosis of the acquired infection by highly sensitive and specific methods is crucial for proper management of animal and human toxoplasmosis and represents the key step in the prevention and treatment of the disease [3].

Various methods have been established in the laboratory diagnosis of toxoplasmosis. Among these methods, serological tests are common and provide satisfactory results. However, producing reliable reagents remains laborious and expensive. Acquiring a specific and effective reagent that can be used in the serodiagnosis is necessary. In the present review, we provide updated data on toxoplasmosis serodiagnosis. The review focuses on the recent trends of producing reliable and standard antigens that have been used in the serological tests of toxoplasmosis, as well as the future direction in this field.

2. Serodiagnosis

Various direct and indirect detection methods have been established to detect *T. gondii* infectious agent or anti-*Toxoplasma* antibodies [4]. In particular, different sets of detection methods have also been developed and evaluated to achieve accurate diagnosis [5]. There are several diagnostic approaches which are applicable to detect the parasitic agents include: histological identification, isolation of the organism in tissue culture [4], and recovery of the parasite DNA by the polymerase chain reaction (PCR) [6, 7] or by a combination of these techniques, whereas the serodiagnostic tests are mainly designated to detect the different classes of antibodies or antigens [8].

Despite the various methods of toxoplasmosis investigation, the routine laboratory diagnosis in both humans and animals depends mainly on conducting various serological investigations to detect specific anti-*Toxoplasma* antibodies in serum samples [5, 9]. It remains the primary approach to achieve satisfactory results [10]. The production and appearance of each antibody isotype are correlated with the immune response after the infection, thus, the determination whether the host has got *Toxoplasma* infection or not can be achieved simply by monitoring the immune response especially humoral immunity. The disease symptoms are non-specific and not enough to characterize accurate diagnosis because of clinical signs mimicry between toxoplasmosis and several other infections [5, 11]. Many serological tests have been designed to measure different types of antibody, which show unique increases and decreases during or after infection [12]. All immunoglobulin isotypes have been used successfully in *T. gondii* serodiagnosis, including IgG, IgM IgA, and IgE [10]. Specific *T. gondii* IgM is considered as early and sensitive diagnostic marker that correlates with the occurrences of acute toxoplasmosis. Particularly, IgM can be detected in serum only 1 week following the infection.

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However, it may remain in the serum for several months or years [8]. Thus, the interpretation of the serology results sometimes becomes more difficult. Furthermore, the presence of IgM antibodies in the maternal circulation even 18 months after invasion influence the accuracy of this diagnostic approach because determining whether an antibody is from active or previous infection is crucial during gestation [13]. If an antibody is from a previous infection, no consequences for the fetus normally occur. If the infection occurs during pregnancy, the clinician should decide on administering anti-parasitic treatment to avoid disease complication in the unborn child [5, 11]. Recent studies have shown that the use of IgM alone for the establishment of acute toxoplasmosis is insufficient [8].

Detection of IgG antibodies in patients may aid diagnosis. IgG antibodies can be detected within 1–2 weeks following infectionacquisition, and normally peak within 1–2 months, and decline at various rates, but usually persist lifelong at residual titers [5, 11]. High levels of this antibody indicate previous infection. Therefore, measuring of IgG antibody is a common diagnostic marker that helps clinicians to decide whether a patient has chronic infection or not. However, this antibody still has difficulty in distinguishing previous infection from a recent infection. Consequently, an auxiliary test based on the IgG avidity has been established to differentiate acute from chronic infection in an asymptomatic patient [5, 11]. In recent years, precise dating and infection have been proven to be obtained by using IgE and IgA. However, they produced during the first weeks of infection, and disappear early [9]. There are various serological procedures have been established for the detection of anti *T. gondii* antibodies; these include Sabin-Feldman dye test (SFDT), indirect fluorescent assay (IFA), agglutination tests, and enzyme-linked immunosorbent assays (ELISAs), or a combination of these methods are also required for determining recent and previous infections [9, 10, 12].

3. Sabin-Feldman dye test (SFDT)

It was a greatest advancement in the field of toxoplasmosis diagnosis when Sabin and Feldman described the dye test as a novel diagnostic test 60 years ago [14]. Though SFDT is the first assay developed for the laboratory investigation of *T. gondii* infection [10], but still considered as "gold standard" with high sensitivity and specificity [15]. The principle of the assay is based on incubating live tachyzoites with patient serum and complement. Subsequently, if the serum contains specific antibodies against *T. gondii*, it will immediately coat the parasite and the tachyzoite will be lysed by the complement system and fail to stain with dye methylene blue. The number of unstained (dead) and stained (live) tachyzoites will be counted and used to establish the end-point titer [10, 15, 16]. In comparison with other diagnostic tests, SFDT has the potential to detect both IgM and IgG. On the other hands, the assay has certain limitations. For example, application of live parasite is considered a biohazard; thus, it can be only applied in a few laboratories. The antibody titers also do not give strong clues to determine whether the infection is acute or chronic [15, 16].

4. Indirect fluorescent assay (IFA)

IFA is one of the most simple, sensitive, safe, and widely used assays in the usual detection of anti-*Toxoplasma* antibodies [17]. It has been established as an alternative diagnostic method in response to overcome the biohazard of using live tachyzoites in SFDT [10]. Diluted serum specimens are incubated with killed *Toxoplasma* tachyzoites fixed on a glass slide to allow specific antigen-antibody interaction [8]. The interaction will then detected by addition of fluorescent-labeled anti-human IgG or IgM antibodies, and the result is viewed under a fluorescence microscope [18]. Although IFA is relatively inexpensive, fluorescence microscope is required, and the individual differences in result reading are considered as one of the test limitation. Furthermore, false-positive results may occur in case the sera contain rheumatoid factors or antinuclear antibodies [10]. Nevertheless, high levels of *T. gondii*-specific IgG in some acute acquired toxoplasmosis patients may interfere with the IgM and cause false negative results [19].

5. Agglutination tests

Different agglutination tests, including direct agglutination test (DAT), indirect hemagglutination test (IHAT), and latex agglutination test (LAT), are applied in toxoplasmosis serology [9, 12, 20]. Development of DAT assists tremendously in identifying of anti-*T. gondii* antibody in humans and animal sera [12]. Fulton achieved this test in 1965; the important features of this test are the absence of secondary antibody and no special equipment needed. The principle of DAT starting with coating of microtiter plates with formalinized *Toxoplasma* tachyzoites. Diluted patient sera will then added. Accordingly, the agglutination will occur if anti-*Toxoplasma* antibodies are present in the tested sera. However, precipitated tachyzoites will be noticed at the bottom of the wells if the sample is negative [21]. The test is very simple and has proved to be very sensitive and inexpensive, although a large quantity of antigen is required. Despite that DAT is only used for detection of IgG antibodies, non-specific agglutination can be induced by occurrences of IgM antibodies [12, 20].

In the IHAT test, red blood cells (RBCs) are sensitized with *T. gondii* soluble antigen. Subsequently, sensitized cells will then incubated with patient sera. Accordingly, the RBCs will agglutinate if the sera contain anti-*T. gondii* antibodies. Furthermore, IHAT is also simple to perform and inexpensive and can be used for both humans and animals [8]. In the LAT, tachyzoite particle is fixed to latex beads. When the beads are incubated with patient sera containing specific IgG antibody, visible agglutination reaction will occur [22]. The disadvantage of this assay is the low sensitivity and specificity, particularly in immunocompromised patients; moreover, non-specific IgM can generate false-positive result [9, 20].

5.1. Enzyme-linked immunosorbent assay (ELISA)

ELISA is highly sensitive and specific analytical assay for quantitative detection of antibodies or virtually all types of antigenically active molecule. Application of ELISA in the diagnosis of toxoplasmosis has been established since 1976 [23]. Since developed up to now, it has been one of the most common biochemical techniques used in research and clinical laboratories, including detection of anti *T. gondii* antibodies [8, 24]. In this assay, a microtiter plate is coated with antigens, and then diluted patients sera are applied. If the serum contains anti-*Toxoplasma* antibodies, it will bind directly to coated antigen, and the presence of *Toxoplasma*-specific antibody will detect by using anti-human enzyme-conjugate (secondary antibody). Any unbound reagents are removed in the washing steps. Finally, the substrate is added, and subsequently, color reaction develops (which is relatively correlated with the quantity of the antibody). Interpretation of the ELISA results normally depends on the assessment of color change spectrophotometrically [25].

ELISA can easily determine a positive or negative sample by correlating the optical density of the serum with the control after a threshold value is established [25]. In general, the advantages of ELISA test are as follows: it shows high sensitivity which allows quantitative and semi-quantitative antibody measurements, automatically adopted and is inexpensive [26]. Furthermore, the assay is simple and easy to carry out and can be used to investigate a large number of serum samples in a short period of time [4]. Moreover, it can be used to detect both IgG and IgM antibodies [25]. The disadvantage of the ELISA includes standardization of used antigens [26]. A photometer is also necessary to assess the results, otherwise, it is difficult to distinguish between negative reaction and weak positive reaction by visual examination, and this may increase the cost of the test [25]. Generally, detection of anti-*T. gondii* IgM antibody possesses a high degree of accuracy according to the reports; however, it might sometimes give false-positive result [27]. It is most probably due to the presence of rheumatoid factor in serum, and on the other hands, it is common to get false-negative results due to specific IgG competitive inhibition [28].

5.2. Production of reliable and standard antigen for serological diagnosis

Establishing of *T. gondii* serology is an essential diagnostic tool, which gives satisfactory results. However, producing specific and standard antigens is a real challenge. Currently, most commercial serological kits use crude *Toxoplasma* antigens prepared through mouse passages or cell culture systems [29, 30]. The soluble native antigen of *T. gondii* parasite has been applied as a diagnostic marker in various seroepidemiological studies [4], a researcher used crude native antigen of type I and II, for example [1]. Crude *T. gondii* tachyzoite antigen applied in an indirect ELISA yielded sensitivity and specificity of 95.75 and 85.11%, respectively [31]. A significant variation between laboratories exists in the process of producing native antigen. Furthermore, antigen contamination with non-parasitic materials is also possible because of the lack of standard method for antigen purification [32]. Moreover, the use of live pathogens in antigen preparation process needs extra care because of biological hazards

generated by the parasite [33]. Therefore, serological tests that use tachyzoite crude antigens seem difficult to standardize [32]. To overcome this limitation and improve the serodiagnostic tests, recombinant antigen may be used as an alternative diagnostic marker, and it could replace the native antigens [34].

6. Recombinant antigens

Application of the recombinant antigens in serodiagnostic kits reduces the native antigen production time and significantly reduces the antigen production cost. Furthermore, better test standardization is achieved when recombinant antigen is used [35]. Moreover, the particular antigenicity of specific antigen can be easily investigated, and with the ability to combine more than one antigen to evaluate their diagnostic value, definitely this will facilitate the standardization of diagnostic assays [29]. If the synthesis of any selected antigens during the parasite life cycle is related to acute or chronic phase of infection, application of this particular antigen as discriminating tool leads to significant improvement in *Toxoplasma* diagnosis [35]. The use of recombinant DNA technology to obtain identical antigenic protein helps in overcoming the biohazard problem related to native antigens production and is greatly beneficial to reduce time, cost and labor consumption. Interestingly, one of the most advantageous of using recombinant antigen in the development of diagnostic tests is that the potential to determine precise protein composition, with reduced non-parasitic material. In addition, more than one antigen can be combined in a single test [32].

Recently, several *T. gondii* antigenic genes have been successfully cloned and expressed using various expression systems. Many reports indicate the successful use of recombinant antigen in toxoplasmosis serodiagnostic tests [33]. The antigens that have been extensively involved in the progress of *T. godii* diagnosis improvement include the surface antigens (SAGs) [36], dense granule antigens (GRAs) [36], microneme antigens (MICs) [37], and rhoptry antigens (ROPS) [33]. More details about the application of *T. gondii* recombinant antigens in the diagnostic studies are illustrated in **Table 1**. Most previous and current studies approved that recombinant protein technology significantly improves the serodiagnosis of *T. gondii* infection. Moreover, the uses of recombinant antigen allow not only the detection of anti-*T. gondii* antibodies but also differentiation between recently acquired and previous infections, resulting in reduced medical complication risk of the disease in immunocompromised patients, especially pregnant women [32]. The potential ability of specific *T. gondii* recombinant antigen to identify the clinical phases of the disease was extensively studied, in the attempts to discriminate acute from chronic infection. The performance of recombinant antigen seems to be sensitive enough and promising in differentiating acute versus chronic infection [35, 38]. Although there is no clear definition of either chronic or acute infection, identification of recently acquired infection normally relies on the detection of specific IgM in patients serum; however, IgM might persist for a long time after infection. Thus, the results may not be precise to indicate recent infection, unless the serum is subjected to additional method such as the IgG avidity, definitely this will improve the accuracy of identification of the infection occurrence. Performance of IgG avidity assay by using *T. gondii* recombinant antigens may be useful for identification of toxoplasmosis phases.

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Antigen	Diagnostic test	Sensitivity %	Specificity %	Reference
SAG1	ELISA (IgG)	100	100	[39]
	Rapid diagnostic test (RDT)	100	99.4	[40]
SAG2	ELISA (IgG)	100	89.4	[41]
SAG3	ELISA (IgG)	95.4	91.2	[41]
GRA1	ELISA (IgG)	100	100	[42]
GRA2	Western blot	100	90	[43]
GRA5	Western blot	100	46.8	[38]
GRA7	ELISA (IgG)	93.2	94.0	
ROP1	ELISA (IgG)	100	100	[44]
ROP8	Western blot	90	94	[33]

Table 1. Recombinant antigens of *T. gondii* as serodiagnostic markers.

7. Epitope-based antigen

The epitope or antigenic determinant is an antigenic part of a protein that possesses the potential ability to be recognized by T and B cells receptors or the antibodies binding sites [45]. The epitope is classified into two categories based on their primary structure: linear (continuous) epitope if the amino acids sequences in the epitope are continuous or conformational (discontinuous) epitope which composed from discontinuous sections of amino acid [46]. Attempts to achieve high sensitive and specific diagnostic assay usually assume that replacing the native antigen in all current diagnostic kits by standard and reliable reagents can significantly increase the assay performance [30]. In recent years, it has been demonstrated that the use of peptide-based antigen can meet the demand to standardize the serodiagostic test of toxoplasmosis and increase the sensitivity and specificity of these assays. Furthermore, the ability to distinguish between previous and recently acquired infection can be also achieved [47].

Theoretically, epitope or multiepitopes show numerous advantages compared with crude native antigen because they apply only the antibody-binding sites of the antigen, which definitely increase the accuracy of the test. The applied antigen composition in the serodiagnostic test will be known precisely, the ability to apply more than one identified epitope, easily standardization of the assays, application of only antigen epitope will reduce the biohazard problems in using live pathogens [30]. Nowadays, the use of a diagnostic marker that contains a high density of antibody binding site increases the chances of antibody detection in serum samples and provides the potential to acquire inexpensive diagnostic methods with a high degree of specificity and sensitivity. Therefore, a great possibility to improve diagnostic tests performance exists if multiepitope peptide is applied [48]. Application of multiepitopes antigen in diagnostic tests has been conducted successfully in several studies, employing multiepitope peptide in hepatitis C [49], influenza virus [50], leishmaniasis [51], leprosy

[52], *Trypanosoma cruzi* [53], leptospirosis, and *Mycobacterium tuberculosis* [54, 55] as well as *T. gondii* infection [30]. In all these studies, multiepitope proteins were proposed to enhance the test sensitivity. Identification of specific peptide epitope targeted by the host immunoglobulin allows better understanding of the immune response toward the parasite, as well as the development of accurate diagnostic tool, for development of diagnostic kit multiepitope antigen, appears as most promising antigens that can be used in routine toxoplasmosis screening [56].

Recently, bioinformatics tools are widely applied for epitope identification in protein analysis. Consequently, various epitope peptides are used to develop diagnostic antigen and epitope-based vaccines [47]. By using software-based prediction techniques, the SAG1 B cell epitope of *T. gondii* has been analyzed. Consequently, 11 selected peptides were successfully synthesized. The potential antigenicity of these peptides was evaluated by ELISA using pig sera collected from different periods of post infection. Four of 11 peptides showed high reactivity and were identified by all sera, whereas the rest of the peptides interacted with selected sera only [57]. When the same procedure was applied to *T. gondii* GRA1, 3 of 11 peptides were successfully recognized by all sera. The study suggested that these epitopes can activate the host immune system and generate constant immune response because its immunoreactivity was not affected when evaluated using sera collected from different time periods following the infection [47].

The involvement of GRA1 immunodominant B cell epitope in inducing antibody production by the human immune system was previously identified [58], whereas the immunodominant epitope on GRA1 was also located and accessed using pig sera. The result indicates high immunoreactivity as well as the capability of the peptide to improve the toxoplasmosis serodiagnostic tests [56]. The newly synthesized immunodominant epitopes of *T. gondii* antigens (SAG1, SAG2, SAG3, GRA5, GRA6, and P35) appear to be the most promising diagnostic reagents can be applied in routine toxoplasmosis screening The demonstrated multiepitope peptides can successfully replace the recombinant antigens for differentiating the recently acquired infection from the previous infection; thus, it can serve as an effective tool for human serodiagnosis [30].

The advantage of using epitope peptide in serodiagnostic tests can easily increase the assay sensitivity by applying different peptides. Alternatively, the use of multiepitope peptide that expresses a high density of conserved antigenic determinant can contribute in achieving a high degree of sensitivity and specificity [48, 53]. Multiepitope peptide has been evaluated to obtain specific and standard antigens for serodiagnosis of *T. gondii* infection. Findings showed promising results for developing more accurate assays with high degree of sensitivity and specificity and can differentiate between chronic and acute phases of infection [30, 46]. Multiepitope antigen expressed in *Escherichia coli* system provides the potential to replace the currently used crude antigen. This antigen can also contribute in developing accurate and inexpensive diagnostic assay with high degree of sensitivity and specificity. Therefore, recombinant multiepitope peptide appears as an attractive starting point for future development of *T. gondii* serodiagnosis and immunization [30, 48].

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8. Concluding remarks

The diagnosis of *T. gondii* infection is still a huge challenge to the medical knowledge and clinical acumen of physicians. Currently, the serological diagnosis plays a vital role in the identification of both human and animal toxoplasmosis. However, the insufficient accuracy of current diagnostic tests due to the lack of standardization in the production of the *T. gondii* whole-cell lysate necessitates the exploration of standard diagnostic reagents. Nowadays, it is speculated that application of recombinant multiepitope antigens in toxoplasmosis serodiagnostic methods would significantly improve the sensitivity and specificity of these methods. Accordingly, the increased usage of the bioinformatics and recombinant DNA technology will dramatically assist in designing and producing of novel epitope-based antigen, which will be crucial to acquire effective serodiagnostic methods for toxoplamosis.

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