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Diagnosis Serological of Toxoplasmosis using Recombinants Antigens

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Abstract

Toxoplasma gondii, the causative agent of the toxoplasmosis is an obligate intracellular parasite that affects humans, domestic and wild animals. The transmission of this parasitosis can occur by consuming contaminated food, by the accidental ingestion of oocysts in the environment or by transfer of the pathogen from the mother to the fetus. The life cycle presents three morphological stages: 1) tachyzoite, infectious form that quickly multiply in any cell of the intermediate host and non-intestinal epithelial cells of the definitive host; 2) bradyzoite, located in the tissue cysts of intermediate host and 3) sporozoite stage present in the environment. Serodiagnosis is critical in two risk groups: 1) patients with deficiencies in their immune system (immunocompromised) which due to its delicate state of health are very fragile and can die by the complications originated by toxoplasmosis, 2) pregnant women, in which can ocurr transmission of the parasite to the fetus and subsequently cause an abortion or severe neonatal malformations. The serodiagnosis of the congenital disease presents serious drawbacks, due to the absence of anti-*T. gondii* IgM antibodies in a large proportion of infected children. Native antigens obtained from extracts of the parasite (*Toxoplasma* lysate antigen (TLA)) generally have little specificity, because they are a complex mixture that causes cross-reactions. Advances in Molecular Biology have allowed to optimize the diagnosis through the obtaining of recombinant antigens (RA), which are a very useful tool, with many advantages: high sensitivity and specificity, production in large quantities at relatively low cost, their handling is safe for the researcher, can be purified to homogeneity through the use of affinity tags that allows its easy and rapid purification in few steps, are among the many benefits that such proteins exhibit for providing a safe and optimal diagnostic. Another biotechnological advance very used is the synthesis of the chimeras, designed through the fusion of several RA with excellent properties. The combination of immunoreactive epitopes from proteins of different stages of the life cycle of *T. gondii* is a spectacular strategy to overcoming the of antigenic complexity of the parasite, which allows the production of a much more immunodominant protein than the use of proteins separately and the obtaining of promising results for the acquisition of the ideal antigens to detect toxoplasmosis. In order to discriminate between persons in the acute and chronic phase of infection have been developed tests of avidity of IgG, very relevant in the case of pregnant women, which enable to administer chemotherapy in a timely and effective manner and, therefore, protect the fetus from the irreversible damage caused by the parasite. This review analyzes the different routes followed by the serodiagnosis in the course of many years, from its beginning to the present, with its difficulties and its great progress, seeking to achieve excellence in the detection of antibodies against *T. gondii* through the use of the recombinant DNA technology, with the aim of improving the living conditions of the people who suffer from this disease, which is of fundamental importance because of the large number of cases that are scattered all over the world.

Keywords: *T. gondii*; serodiagnosis; recombinants antigens; *Toxoplasma* IgG avidity assay; ELISA

Introduction

T. gondii is a protozoan Apicomplexa, ethiologic agent of toxoplasmosis, one of the infections most widely distributed in the world. During its life´s cycle presents three morphological stages: 1) tachyzoites are infectious forms of acute phase, which multiply rapidly in any cell of the intermediate host and in non-intestinal epithelial cells of the definitive host; they are divided into the parasitophorous vacuole (PV) by asexual reproduction. This form is oval, with a size of 3 µm × 6 µm, with one pointed end and the other rounded. Ultrastructurally, consists of various organelles and inclusion bodies including a external pellicle, apical and polar rings, conoid, rhoptries, micronemes, micropore, mitochondrion, subpellicular microtubules, Golgi complex, ribosomes, rough and smooth endoplasmic reticululum, nucleus, dense granules, nucleus and nucleolus, 2) bradyzoites are the form of slow replication of the parasite, during chronic toxoplasmosis it is observed in microscopic conglomerates surrounded by a wall called cysts, located in the infected muscle and brain tissue. The bradyzoites are thinner than the tachyzoites, with a nucleus located towards the posterior end. It has more electrodense rhoptries, less dense granules and more abundant micronemes. The bradyzoites are contained in cysts, which helps to protect the parasite from immune responses and chemotherapeutic treatments and 3) oocysts are ovoid structures formed by a protective layer that prevents mechanical damage and chemicals, which favors its survival during long periods in the environment [1]. The sexual cycle starts when the feline ingests oocysts or tissues that are infected with cysts of bradyzoite; these events occur in the intestine and culminate in the release of oocysts in cat´s feces. Matured oocysts are very infectious and can survive in extreme environments for a long time, even years. During the asexual reproduction, an infected animal is contaminated with oocysts, which release sporozoites and transform into tachyzoites, spread throughout the host organism (acute phase) to form bradyzoites that cause tissue

cysts. When a susceptible host eats undercooked meat contaminated with these cysts, the bradyzoites invade the intestinal epithelium and differentiate into the tachyzoite stage to complete the asexual reproduction. If the new host is a cat, the bradyzoites differentiate into sexual stages and thus ends the life cycle of this pathogen [2]. The tachyzoites multiply rapidly during the acute phase of toxoplasmosis, invade the host cells through a process of active penetration [3] and are transformed into ovoid structures surrounded by a PV, which is a replicative niche formed by the plasma membrane of the host cells [4]. The formation of vacuoles induced by these pathogens is directed by organelles called micronemes, rhoptries and dense granules; this process involves the interconnectivity between the host endoplasmic reticulum (hER) and PV, then the migration of molecules occurs from the microorganism to the cytoplasm of the host cell [5]. This type of mechanism allows infecting almost any kind of nucleated cell and the establishing a long-term chronic disease in all warm-blooded animals, including humans.

Toxoplasmosis in the majority of the immunocompetent patients is asymptomatic and, for this reason, it is not diagnosed or being a part of the epidemiological records of the affected countries, i.e. the health statistics are underestimated. Humans usually acquire the infection by oral transmission due to contamination with oocysts from cat feces, ingested with raw or undercooked meat, vegetables, and fruits poorly washed or water; it can also occur by congenital transmission through transfer of tachyzoites to the fetus, through the placenta [6]. Taking into account the serious pathological effects in people at high risk, it is essential to have effective systems of serological diagnosis to detect the presence of this disease in its early stages, which favors the administration of the chemotherapy treatment that leads to the removal of these microorganisms in a timely and effective manner [7].

This review shows part of the evolution of the serological diagnosis of toxoplasmosis using recombinant antigens over many years of research. The history beginning with the detection of antibodies called anti-*T. gondii* through the use of extracts of parasites and their innumerable disadvantages, the development of RA together with different strategies that favor the increase of the sensitivity and specificity among other advantages, the design of chimera systems that improve the quality of the tests, up to the trials of avidity of IgG that allow to determine the stage of the disease, a very important test in infected pregnant women to carry out a proper clinical management of the newborn that will improve their health conditions. Of course, are still developing new research and biotechnological advances in the search of excellent antigens to detect toxoplasmosis in all the cases of infected patients, independently of the phase of the disease, strain, geographical area of origin of the affected individual or immune status of the patient, that is to say, that allows an extremely efficient and safe diagnosis.

Serodiagnosis De Toxoplasmosis

The most commonly used proteins in serological tests for detecting toxoplasmosis are TLA obtained from tachyzoites of RH strain propagated *in vivo* (intraperitoneal inoculation in mice) or *in vitro* (cell cultures) [8,9]. These extracts of parasites are complex mixture difficult to standardize, which provide results that are not reproducible due to the lack of a unified methodology for their elaboration, and for this reason, there are differences in the experiments realized by various research centers; they have a high cost because it need a large amount of biological material and reagents for the production of small portions of proteins, they require a great investment of time for their preparation; using this antigens it is impossible to the early recognition of infection or the precise distinction between the phases of this disease. In addition, the use of this antigens can cause problems of specificity (false positives) [10,11], since it is formed by a set of molecules that generate cross reactions, for example, with rheumatoid factors or antinuclear antibodies, this problem also can occur due to antigenic preparations contaminated with reagents from culture media; added to this fact, they are heterogeneous mixtures that contain lipids, carbohydrates and other proteins that are shared by other microorganisms [12,13]. They also generate sensitivity problems (false negatives), by the use of specific antigens of the morphological stage or strain of the microorganism; in the immunocompromised patients, these disadvantages appear due to a minimal production of anti-*T. gondii* antibodies [12].

The propagation of *Toxoplasma* with the purpose of getting native antigens is a very expensive and laborious procedure, because it requires an animal facilities; specialists in its handling, since they are infectious potentially samples; besides the great investment for the maintenance of mouses, since they survive only 4 to 5 days after the inoculation and it requires a lot of time for their development; in this procedure the tachyzoites are contaminated with mouse peritoneal cells, which causes problems of specificity [10]. Although the maintenance of tachyzoites in the cell culture is more economical than its production in mice, it is still a very expensive method because it implies its growth in complex culture media; it requires a great investment of time and represents a high risk of contamination for the experimenter.

The serological diagnosis of toxoplasmosis is based on the detection of IgG and IgM antibodies. IgM antibodies appear after a week of infection (acute phase). Toxoplasmic IgG appears after 2 weeks of infection and peaks at 3 months. It then remains at a plateau level for 6 months and after 1 year starts to slowly decrease to lower levels until the end of infected subject's life due to the persistence of latent cysts in immune-privileged organs [14]. However, the presence of IgM does not indicate recent infection, because in some affected individuals is detectable for long periods of time after the acute phase [15]. The use of native antigens presents multiple disadvantages, for this reason, investigations have continued in order to achieve the obtention of better antigens. Some laboratories around the world have carried out numerous studies thanks to advances in Molecular Biology techniques that have allowed the development of high quality AR, some of these proteins have very relevant properties, since they allow to detect toxoplasmosis in its initial stages, very important because if the infection occurs during the gestation stage, the parasite can infect the fetus and cause inflammatory lesions, which trigger neurological and ophthalmological diseases [16]. Discrimination between the acute phase and the chronic phase of toxoplasmosis is essential, since the detection of the initial stage of the disease during pregnancy allows early care of the mother and the fetus and thus avoids the serious problems associated with this pathology.

Diagnosis with Recombinant Antigens

Toxoplasmosis is asymptomatic in immunocompetent individuals, but can cause serious problems in two risk groups: a) immunosuppressed patients, such as people with HIV/AIDS transplanted and individuals with malignant tumors, who have impressive invasions by T. *gondii* that can cause death [17-19], (b) *Toxoplasma*-infected pregnant women can transmit this pathogen to the fetus and cause severe neonatal pathologies, such as mental

retardation, hydrocephalus, retinochoroiditis, intracranial calcification, microcephaly, hydrocephalus, mental retardation, or even result in the spontaneous abortion and death [20,21]. These cases of risk require a fast, accurate diagnosis, in order to administer the appropriate treatment without loss of time.

The need to improve the quality of the diagnostic has led to the development of numerous investigations that use as a tool the recombinant DNA technology, which allows get great proteins with the following characteristics: 1) it provides well-defined antigens, selected with sequences of aminoacids very immunogenic to guarantee the recognition of all the patients with toxoplasmosis, 2) have been achieved develop tests with mixures of RA in order to improve the sensitivity of diagnosis, 3) the purification can be standardized [22], 4) easy of purify to homogeneity because they are produced with labels of affinity (for example, His-Tag), 5) obtained in large quantities due to the use of systems of overexpression of proteins, 6) the manipulation presents no risk, as they are not infectious for the researcher, 7) these proteins can be used as markers of the acute or chronic phase of the disease, 8) are sensitive and specific, depending on the sequences used and 9) their production has a relatively low cost.

Criteria for Choice of Antigen Sequences

The technology of phage display of *T. gondii* cDNA libraries is a powerful application for the obtaining of antigen sequences from a complex mixture, makes it possible to exploit affinity selection, by incubation of specific sera from patients with toxoplasmosis [23] with collections de bacteriophages that express portions of proteins of the *Toxoplasma* on their capside and that contain the corresponding genetic information. The bacteriophages that specifically bind the antibodies present in the serum are easily recovered, remaining bound (by the antibodies themselves) to a solid support (e.g. magnetic beads); the non-specific ones, by contrast, are washed away. Direct screening, i.e. the analysis of the ability of single phage clones to bind the antibodies of a given serum, is done only at a later stage, when the complexity of the library (i.e. the different number of sequences) is substantially reduced, precisely as a result of the selection. Lambda display is a useful system for expressing tachyzoite cDNA libraries of *T. gondii*, and several investigations have demonstrated efficient visualization of a variety of protein domains [24]. The use of tachyzoite phage display library together with a well characterized panel of human sera reactive with *T. gondii*, have allowed to isolate several positive clones, to then carry out their respective sequence analysis. The displaying *T. gondii* antigen fragments can been further characterized, which leads to the identification of a human immunodominant epitope of the antibody-mediated response.

Beghetto et al. developed the methodology lambda-display of tachyzoite cDNA libraries to identify a human immunodominant epitopes as follows below [25]:

Construction of cDNA fragments display library

T. gondii mRNA was purified from tachyzoite cells (RH strain) as previously described [26]. Double-strand cDNA was synthesised from poly(A)+RNA by using the SMART cDNA Construction Kit (Clontech). Total cDNA (10 mg) was randomly fragmented using 1.5 ng of DNaseI (Sigma Aldrich) for 20 min at 15°C. cDNA fragments were then purified by using the Qiaquick PCR Purification Kit (Qiagen). 30-Protruding termini of 3 mg of cDNA fragments were recessed by using 9 units of T4 DNA polymerase (New England Biolabs) for 60 min at 15°C, in the presence of 0.2 mM dNTP. cDNA fragments were then purified by phenol extraction and ethanol precipitation using standard methods. Synthetic adapters bringing SpeI or Not I restriction sites were added to cDNA ends by incubating 0.5 mg of cDNA fragments with 20-fold molar excess of adapters, in the presence of T4 DNA ligase (New England Biolabs). A mixture of six different adapters was used, obtained by annealing the following oligonucleotide pairs: K185 50-CTAGTCGTGCTGGCCAGC-30 and K186 50-GCTGGCCAGCACGA-30; K187 50-CTAGTCGTGCTGGCCAGCT-30 and K188 50-AGCTGGCCAGCACGA-30; K189 50-CTAGTCGTGCTGGCCAGCTG-30 and K190 50-CAGCTGGCCAGCACGA-30; K191 50-TCTGGTGGCGGTAGC-30 and K192 50-GGCCGCTACCGCCACCAGA-30; K193 50-TTCTGGTGGCGGTAGC-30 and K194 50-GGCCGCTACCGCCACCAGAA-30; K195 50-TTTCTGGTGGCGGTAGC-30 and K196 50-GGCCGCTACCGCCACCAGAAA-30. Fragments having a molecular size between 300 and 1,000 bp were isolated by electrophoresis on 2% agarose gel electrophoresis and purified by using the Qiaquick Gel Extraction Kit (Qiagen). Size-selected cDNA fragments were cloned into Spe I-Not I digested lKM4 vector (Beghetto et al.). The cDNA-fragments lambda library was finally obtained by using the Ready-To-Go Lambda Packaging Kit (Amersham Pharmacia Biotech), yielding 107 independent clones [26]. Average size of cDNA inserts of 50 random clones, analysed by PCR (see below), was around 300 bp.

Sera samples

Sixty sera from 39 women who contracted primary toxoplasmosis in gestation were anonymously analysed at different time intervals from primary infection (sera collected within 1-24 months after acquisition of infection infection). Three sera from three infants with congenital symptomatic toxoplasmosis were also analysed (age 1-6 months). Diagnosis of primary toxoplasmosis in gestation was based on documented seroconversion: time of infection was established on the basis of the last negative and the first positive control, and levels of specific immunoglobulins (IgG, IgM, IgA and IgG avidity) achieved. All the samples were analysed with ELFA IgG and IgM tests (ELFA bioMerieux), IgA assay (Platelia IgA, Pasteur) and IgG avidity test (IgG avidity, bioMerieux). IgG levels ranged from 3,000 to 30 IU/ml.

Affinity selection

Magnetic beads (100 ml), linked to Protein G (Dynabeads Protein-G, Dynal), were used for each affinity selection. Beads were washed twice with coating buffer (Naphosphate buffer 0.1 M, pH 8) and subsequently incubated with 10 ml of human serum in 0.1 ml of coating buffer for 40 min at room temperature. After being washed with coating buffer, beads were incubated with 1 ml of blocking solution (5% non-fat dry milk in PBS, 0.25% Triton X-100, 10 mM $MgSO_4$) for 1 h at room temperature. 5×10^{10} pfu (plaque forming units) of recombinant lambda phages, diluted in 1 ml of blocking solution, were then added to the beads. The mixture of phages and beads was incubated for 3-4 h at room temperature with rotation. Beads were then extensively washed with washing buffer (1% Triton X-100, 10 mM $MgSO_4$ in PBS) and then added to 1.2 ml of *E. coli* cells [27] (BB4 strain) BB4 cells were grown in LB medium supplemented with 0.2% maltose and 10 mM $MgSO_4$, up to O.D.600=1.0, then centrifuged and resuspended in half volume of SM buffer. BB4 cells were directly incubated with the phage particles adsorbed to the beads

for 20 min at room temperature. Top-Agar medium (15 ml) was added to 0.6 ml of BB4-infected cells and subsequently adsorbed to LB plates. Plates were then incubated at 37ºC until plaques were clearly visible (12-16 h). Finally, recombinant phages were recovered from bacterial plaques using standard procedures. Several rounds of affinity selection were done using the same method and the immunoreactivity of phage population was monitored, after each round, by phage-ELISA [26].

Analysis of recombinant lambda phage clones

Phage clones were isolated from reactive phage pools by immunoscreening [26]. Phage particles from a single plaque were diluted in SM buffer (300 ml). Chloroform was added (50 ml), and the samples left at 37°C for 20 min. After centrifugation, supernatant was collected and used to amplify the cDNA of the recombinant phage inserts by PCR. Phage supernatant (2 ml) was used as template, and oligonucleotides K47 (50-GGGCACTCGACCGGAATTATCG-30) and K85 (50-GGGTAAAGGTTTCTTTGCTCG-30) as specific primers, mapping respectively at N- and C-terminus of cDNA inserts. Reaction was performed for 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, for 25 cycles. PCR products were then purified by using the Qiaquick PCR purification Kit (Qiagen). Recombinant cDNA inserts were sequenced from the purified PCR products through standard methods. Single recombinant phage-clones were amplified by BB4 cells infection and tested by phage-ELISA as previously described [26].

Recombinant fusion proteins

cDNA inserts of phage clones tx-cl4.11, tx-cl15.11, txcl1.11, tx-cl8.0, tx-cl1.16 tx-cl9.18 and tx-cl4.18 were subcloned into bacterial expression vector pGEX-SN, while the insert of phage tx cl7.11 was cloned into vector pKM4-6H. pGEX-SN vector allows the expression of cDNA inserts as fusion products at the carboxyl terminus of glutathione S transferase protein (GST). pGEX-SN was obtained by inserting a synthetic DNA linker bringing BamHI, Spe I, Not I, and Eco RI restriction sites into pGEX-3X vector [28]. The synthetic linker was obtained by annealing the oligonucleotides K108 (50-GATCCTTACTAGTTTTAGTAGCGGCCGCGGG-30) and K109 (50 -AATTCCCGCGGCCGCTACTAAAACTAGTAAG-30). The linker was finally cloned into pGEX-3X vector previously digested with Spe I and Not I endonucleases. pKM4-6H is an expression vector that allows the production of cDNA inserts from the display library as fusion products at the amino terminus of soluble lambda protein D. In this vector the viral protein D has been modified by the addition of six histidine residues at the carboxyl terminus. cDNA inserts were amplified from phage supernatant by PCR, subsequently digested with Spe I/Not I endonucleases and then ligated into pGEX-SN or pKM4 vectors, digested by Spe I and Not I, using standard procedures. Competent E. coli cells (AD202 strain) were transformed with the recombinant plasmids and single clones isolated. To induce the expression of fusion proteins, transformed cells were grown in LB medium to O.D.600=0.5. IPTG was then added to (up to 0.4 mM) and the culture incubated at 37°C for 3 h. Cells were then centrifuged and stored at -80°C. GST fusion proteins were purified from bacterial cytoplasm as previously described [26]. The purification of the D fusion protein (clone tx-cl7.11) was performed by one step of affinity chromatography using Ni-NTA beads (Qiagen). The ELISA on GST fusion product was performed by coating Maxisorp-multiwells plates (Nunc) overnight at 4°C with recombinant proteins at a concentration of 5 mg/ml in coating buffer (50 mM $NaHCO_3$ buffer, pH 9.6). D fusion protein (D-cl7.11) was coated at a concentration of 2.5 mg/ml. After incubation overnight at 4°C, plates were blocked 1 h at 37°C using blocking solution (5% non-fat dry milk, 0.05% Tween-20 in PBS) and subsequently incubated for 1 h at 37°C with human sera diluted 1:200 in blocking solution. Plates were then washed and anti-human-IgG HRP-conjugate (1 mg/ml; Sigma) diluted 1:10,000 in blocking solution was added to each well. Finally, enzymatic activity was revealed by incubating the plates with chromogenic substrate tetramethylbenzidine (TMB; Sigma Aldrich). The results were recorded as the difference between the absorbance at 450 and 620 nm, detected by an automated ELISA reader (Labsystem Multiskan). To characterise the immunological properties of the selected *T. gondii* protein fragments, were examined the reactivity of the corresponding recombinant phage clones with the immunoglobulins of infected individuals. In particular, the recombinant lambda clones were analysed by using sera samples coming from three infants with congenital toxoplasmosis and 25 pregnant women who were infected by *T. gondii* during gestation.

Once completed the database search, all selected phage clones matched the sequence of *T. gondii GRA1, GRA2, GRA3, GRA7, GRA8, MIC3, MIC5* and *SAG1* genes. Sequence analysis revealed that several clones coming from distinct affinity selections contained the same cDNA insert. At the end of the affinity selection procedure fourteen different recombinant phage clones were chosen for further analysis.

The knowledge of the biology of *T. gondii* has been used on the way to obtaining RA. This microorganism is characterized by an apical complex with secretory organelles, micronemes, rhoptries and dense granules that are involved in host cell invasion [23,29]. Several genes for these proteins have been cloned and expressed to produce RA, such as surface antigens (SAG), rhoptries (ROP) and dense granules (GRA). These proteins are present in the tachyzoite and bradyzoite forms of *T. gondii* [24,30]. The tachyzoites of *T. gondii* possess a set of 8-12 ROP [25,31], ROP2, ROP4 and ROP7 are the best protein members that have been identified within the ROP family and are highly specific for detecting antibodies against this parasite. ROP2 and ROP4 are expressed in bradyzoites and sporozoites [26,32].

Over the years, many research groups have conducted evaluations of sera with several RAs, trying to obtain antigens with excellent properties. Macre et al. has evaluated ELISA-ROP2 to detect anti-*T. gondii* antibodies in pregnant women with acute toxoplasmosis in Brazil [33]. This test shows a sensitivity of 87% and a specificity of 88%. Serodiagnosis in the gestation period is essential because of early detection it allows prophylactic measures to prevent the baby from suffering from the complications associated with this infection. The detection of anti-*T. gondii* antibodies in pregnant women at the Arba Minch Hospital, Ethiopia, was carried out by Yohanes et al. [34]. To detect anti-*Toxoplasma* IgM and IgG antibodies, the ELISA kit (Human Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany) was used. The seroprevalence was 79.3%, distributed as follows: 175 (75.43%) seropositive IgG, 9 (3.9%) seropositive IgM and 2 of the 9 pregnant women tested positive for both IgG and IgM. In general, 9 (3.9%) pregnant women tested positive for IgM antibodies. The existence of IgM in pregnancy indicates an acute infection and a potential probability of infection for the fetus, so it is necessary to perform an effective chemotherapeutic treatment as quickly as possible. Tests carried out by Pietkiewicz et al. used SAG1, GRA1 and GRA7 to determine its usefulness in the serodiagnosis of human toxoplasmosis. These proteins were obtained from expression systems in *Escherichia coli* and were purified with a high degree of

homogeneity to guarantee the quality of this ELISA assay [35]. The choice of these proteins is due to their very particular characteristics such as: SAG1 is a highly immunogenic and antigenic surface antigen, involved in the activation of a potent immune response in tachyzoites during the acute phase of toxoplasmosis [36]; GRA1 is expressed in all forms of the parasite, predominantly in sporulated oocysts [37] and in the parasitophores vacuoles of tachyzoites [38]; GRA7 is expressed by all the infectious forms of *Toxoplasma* [33], which is very important to ensure a good sensitivity in the serodiagnosis and is located in the vacuolar parasitophore membrane, it is secreted in the late intra-vacuolar phase. It has been suggested that dense granule antigens stimulate humoral immunity in the host [39], it is presumed that these proteins have a role in the interactions between the parasite and the host.

The diagnosis of toxoplasmosis with a single RA can lead to the appearance of false negative results, due to the use of specific antigens of the strain or morphological stage of *T. gondii*. For these reasons, some researchers have opted for the use of mixtures of RAs to increase the sensitivity of the test, particularly when evaluating sera samples with low antibody titers against *T. gondii* [40-44]. Subsequent studies aimed at improving the quality of diagnostic tests, have been proposed to design chimera antigens, which consist of the fusion of several immunoreactive regions of high quality recombinants proteins of *T. gondii*. An example of the methodology employed for production of chimeras has been made by Hajissa et al. [45]. This experience was carried out as follows: the complete amino acid sequences of the SAG1, GRA2 and GRA7 proteins were obtained from the GenBank database. The immunodominant epitopes expressed within these antigens are identified with bioinformatics software especially designed for it function, in this case was used on ABCpred online prediction server [46]. Three epitopes for each antigen were chosen with high levels of antigenicity and immunogenicity and then they were assembled to facilitate the design of the complementary oligonucleotides. Ultimately, taking into account the DNA sequence of the epitopes, a 456 bp gene (USM.TOXO1) is synthesized using VNTI computer program software; said gene was then cloned into a pET-32a expression vector. The recombinant plasmid is transformed into *E. coli* BL21 (DE3) plysS competent cells and then performing DNA sequencing and confirming that everything is in order, proceed to express the chimera of interest.

A series of experiments carried out by Holec-Gasior et al., have designed a chimera with MIC1 (residues 25-182), MAG1 (residues 30 to 222) and SAG1 (residues 30 to 222) denominated MIC1-MAG1-SAG1 (MMS) [47]. The mixture of the three individuals antigens (M), MIC1-MAG1 (MM) and MMS was analyzed against 270 sera cataloged in four study groups: group I, 47 sera from individuals with acute toxoplasmosis; group II, 19 sera from individuals with postacute toxoplasmosis; group III, 96 sera from patients with chronic toxoplasmosis; group IV, 108 serum samples from seronegative individuals. The sensitivities were 98.1% for MMS, 90.7% M and 81.5% for MM; this confirms that properly constructed chimeric antigen containing several different immunodominant regions is better than the mixture of three proteins individuals and can replace the preparation of TLA to obtain an optimal serodiagnosis for human toxoplasmosis.

Holec-Gasior et al. showed that the sensitivity of the IgG ELISA for the MIC1-MAG1 chimeric protein was almost as high as that for the *Toxoplasma* lysate antigen (TLA), 90.8% and 91.8%, respectively, whereas the sensitivities of IgG ELISAs for the mixture of two recombinant proteins (rMAG1 and rMIC1ex2) or for single recombinant antigens were definitely lower, 69.1% for the mixture, 75.5% for rMIC1ex2, and 60% for rMAG1 [48]. Thus, the MIC1-MAG1 recombinant chimeric antigen has the potential to replace the TLA in the IgG ELISA. Ferra et al. constructed two chimeras with SAG2, GRA1 and ROP1; with the same fragments of SAG2 and GRA1 changing the size of the ROP1 fragments. SAG2-GRA1-ROP1L (SGRL) (amino acids 85-396 of ROP1) and SAG2-GRA1-ROP1S (SGRS) (amino acids 85-250 of ROP1) [49]. The SGRL used in ELISA IgG format has a higher sensitivity (100%) than the mixture of the three antigens (99.4%) or the extract obtained from *Toxoplasma* lysate antigens (TLA) (97.1%). This indicates that anti-T IgG antibodies. gondii recognize more frequently the chimera with the sequence ROP1 major (SGRL); this because it presents a C-terminal region with many arginines, which is determinant in the binding to antibodies; furthermore, it is assumed that the C-terminal end of ROP1 has an immunodominant B-cell epitope. This new study indicates that a chimera is better than a mixture of RA; this construction must have different immunodominant regions, making an accurate evaluation of the size and sequences of the proteins to obtain successful results. This chimera is postulated as a candidate to be used in the serodiagnosis of toxoplasmosis. Some RAs are the molecular markers of the acute or chronic stage of the infection [50].

In the search for better antigens, comparative experiments of RA with TLA have been performed, as shown in Table 1. Here can see the sensitivity and specificity of TLA and RA against serum in different laboratories of the world. We show that there are discrepancies in the results obtained with purified TLA in different experimental studies, which indicates that there is a problem of inconsistency in the quality of the final product. The methods of producing these antigens may also vary significantly between laboratories. It is important to know that the antigen(s) obtained from tachyzoites may contain various nonparasitic materials from culture media and eukaryotic host cells. Serological tests based on tachyzoite antigenic extracts are thus difficult to standardize, and frequently provide insufficient specificity and results which are too inconsistent is, to say, sometimes there are no conclusive and reliable results, as specificities of 83.7 or 70%. In some cases it can be observed that recombinant antigens provide better results than TLA, for example, rMAG1-ELISA has 93.9% sensitivity and 98.3% specificity, values greater than those found using TLA with 87.8% sensitivity and 96,7% specificity. The use of purified recombinant proteins obtained through molecular biology is an alternative for the detection of serum antibodies and allow a better standardization of immunoassays. In addition, the use of the combination of recombinant antigens It can improve the sensitivity of an antibody-based assay.

In recent years, IgG avidity assays have been developed to discriminate between phases of toxoplasmosis. The trials conducted by Emelia et al. at the Universiti Kebangsaan Malaysia Medical Center (UKMMC) demonstrate the importance of the IgG avidity test as a tool to determine acute and chronic toxoplasmosis during pregnancy [51]. The IgM and IgG anti-*Toxoplasma* specific antibodies were evaluated, followed by the IgG avidity test in 281 serum samples. A seroprevalence of 35.2% was detected (anti-*Toxoplasma* IgG 33.5% and anti-*Toxoplasma* IgG and IgM antibodies 1.8%). Five (1.8%) samples of sera positive for IgM ELISA, 4 had high avidity antibodies, suggesting past infection, 1 with limit avidity index and 2 with low avidity were from IgM negative serum. The IgG avidity assay showed a specificity of 97.6%, which demonstrates its great effectiveness in determining whether a IgM positive serum sample corresponds to a recently acquired toxoplasmosis infection. The use of RA in IgG avidity trials offers a promising outlook, since it offers the possibility of better

distinguishing between acute and chronic toxoplasmosis. While avidity assays based on *T. gondii* lysates obtained from whole cells detect low or extreme avidity antibodies in many patients with the chronic phase of the disease [52], which means that they are discarded to carry out this kind of tests.

Antigen	Sensitivity (%)	Specificity (%)	Author
H4/GST H11/GST	81.3	100	Johnson et al. [53]
TLA (Abbott)	96.9	83.7	
TLA (Mercia)	100	95.3	
TLA (Sorin)	93.8	95.3	
TLA (FMC)	96.9	100	
GRA1	81.0	95.4	Wang et al. [54]
GRA7	91.0	97.7	
TLA	88.1	96.8	
SAG2-GRA1-ROP1L	100	100	Ferra et al. [49]
TLA	98.9	100	
rSAG1	93.3	90.0	Orozco [55]
rGRA7	86.7	80.0	
rROP1	33.3	50.0	
TLA (an-ELISA)	93.3	70.0	
rMAG1-ELISA	93.9	98.3	Zhuo et al. [56]
TLA	87.8	96.7	

Table 1: Comparison of the sensitivity and specificity of TLA with several recombinant antigens.

Beghetto et al. demonstrated that MIC3 can be used as a molecular marker that discriminate on the basis of the results of avidity between sera from patients infected with *T. gondii* collected within 2 months after primary infection [57]. In testing conducted by Pfrepper et al. was built a proof of avidity in which recombinant RA7 were used, MAG1 and SAG1 [58]. The test strip of *Toxoplasma* IgG recom-Line (Mikrogen GmbH, Germany) has low avidity for GRA7 during the period of one month of infection, however, the low avidity for MAG1 and SAG1 is presented in a postinfeccion period of 3 months. In 2008, Sickinger et al. evaluated the ARCHITECT IgG Avidity test with SAG1 and GRA8, which recognized the 124 (100%) of acute-phase sera with low avidity (<4 months after infection), this assay showed excellent specificity and sensitivity for detection acute phase and past infection, can be used for evaluation of acute infection in pregnant women [59].

In addition, in 2010, Hossein et al. showed a better clinical utility for a IgG avidity assay based on recombinant GRA6 for the Euroimmun avidity test (Euroimmun, Lübeck, Germany) to the exclusion of the recent infection, which occurred less than 4 months before, in pregnant women [60]. The results of a selected mixture of recombinant proteins, namely, GRA7, SAG1 and GRA1, also published [35], which shows that the maturation of avidity of IgG against this recombinant mixture is different from the received against *Toxoplasma* lysate antigen (TLA). The fundamental importance of the IgG avidity tests is that they allow discriminating between the acute phase and the chronic phase of toxoplasmosis, particularities essentials in the diagnosis of infection in pregnant women, as it prevents serious illness at the fetus that could ultimately cause fatal effects.

Conclusions

Toxoplasmosis is widely disseminated globally; its distribution is so wide that one third of the world's population is infected. In immunocompetent individuals, it disease usually goes totally unnoticed, because it does not show distinctive clinical symptoms. Special attention should be paid to the presence of cases in risk groups, such as pregnant women, as there may be congenital transmission and the fetus may have serious complications, which may even cause death; in addition, in immunocompromised people, whose immune system is defective and they do not have the possibility of responding to their maximum capacity, such as, for example, people with HIV/AIDS, autoimmune diseases, malignant tumors, among others. In people who are in risk groups it is essential to carry out an efficient serological diagnosis in order to quickly administer chemotherapeutic compounds and improve their living conditions. The detection of the infection phase using serological diagnosis is difficult, due to the presence of long-lasting IgM or low avidity IgG in the sera. To solve these problems a variety of tests have been developed using recombinant proteins, alone or in combination; as well as chimeric proteins, products of the fusion of several recombinant antigens that have been demonstrated to be excellent in the detection of anti-*T. gondii* antibodies, this allows check that the use of rationally designed chimeras with the appropriate sequences is useful in the high quality

serological diagnosis for the detection of toxoplasmosis. It has even been detected that some proteins are molecular markers of the acute or chronic stage of the infection.

Accurate discrimination between acute and chronic phase of infection can be done by using tests of avidity of IgG, which allows the exclusion of acute toxoplasmosis in pregnant women with high titers of IgM for extended periods, as well as in other cases. The risk groups, such as neonates or immunocompromised patients. These would be the complementary tests of easy interpretation and increased security, to be used in conjunction with serological tests that use recombinant antigens of high quality, with high values of sensitivity and specificity, in the hospitals and laboratories of bioanalysis for the detection of the toxoplasmosis. The research studies continue in the search to improve the serodiagnosis of toxoplasmosis.

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