Chapter 6

The Laboratory Diagnosis in *Toxoplasma* Infection

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Abstract

The diagnosis of toxoplasmosis is of great importance due to the damage caused by this parasite in immunosuppressed people or in pregnant women, the diagnosis of an active toxoplasmosis represents a sign to initiate a pharmacological treatment immediately. The diagnosis of *Toxoplasma* can be performed with direct methods through intraperitoneal inoculation of serum or cerebrospinal fluid, in susceptible mice evaluating the survival and detection of tachyzoites of biological samples. Indirect methods detecting the IgM and IgG isotypes against *Toxoplasma* have been the tools mostly used and had leaded to discriminate between an active and acute, from a chronic toxoplasmosis. Molecular methods actually *Toxoplasma*-DNA identification by molecular biology tests like the polymerase chain reaction (PCR) allow the direct detection of the parasite. Polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLPs) have been used to identify three strain linages (type I, II and III). Recently, a high-resolution melting method was described to determine the genotype of the infection by *Toxoplasma gondii* directly from biological samples.

Keywords: *Toxoplasma* infection, diagnosis, toxoplasmosis, methods

1. Introduction

The diagnosis of toxoplasmosis represents a very important decision to whether to initiate or not an anti-parasitic treatment. The immunological methods that detect the IgM and IgG isotypes against *Toxoplasma* have been the tools mostly used and had leaded to discriminate between acute and chronic toxoplasmosis. In immunosuppressed people or in pregnant women, the diagnosis of an active toxoplasmosis represents a sign to initiate a pharmacological treatment immediately.

Several methodologies have been applied historically, including the isolation of the parasite in biological specimens and the xenodiagnostic evaluation through intraperitoneal inoculation

of serum or cerebrospinal fluid, in susceptible mice evaluating the survival and detection of tachyzoites in cerebrospinal fluid. Serologic diagnosis is frequently performed for the detection in serum of IgM and IgG anti-*Toxoplasma* antibodies, and recently *Toxoplasma*-DNA identification by molecular biology tests is the polymerase chain reaction (PCR) that allows the detection of just 10 or less parasites in the reaction volume.

2. Direct methods

2.1. Isolation of the parasite or bioassay

The diagnosis of toxoplasmosis could be performed by the inoculation of suspicious blood samples, cerebrospinal fluid, lymph node or other corporal fluids or tissues, in the peritoneal cavity of the immunosuppressed mice or in a susceptible strain like BALB/c. The presence of tachyzoites in the peritoneal fluid of the mice is analysed by phase contrast microscopy from 6 to 10 days after inoculation. The parasite is observed in its characteristic half-moon fixing the specimen with methanol and stain with Wright, Giemsa or May-Grunwald and observed by a microscope. The parasites present sometimes a granular membrane, dyed blue cytoplasm and a red nucleus at the center of the cytoplasm.

In cases of new-borns with toxoplasmosis, inoculation of mice with blood of the child obtained during the first week of birth reaches 75% of positive results; however, if the sampling is delayed by 1 month, the positive results are reduced to 52%. A drawback of this method is the need of specialized personal because of the risk of contamination by the handling of *Toxoplasma*, as well as the lapse of time to get the results [1–5].

2.2. Cellular culture

Cellular culture is more utilized nowadays for the diagnosis and isolation of parasite *in vitro* [5, 6], the parasite has been isolated from lung [5, 6] and cerebrospinal fluid from patients with acquired immunodeficiency syndrome (AIDS) [7]. Diagnosis by cellular cultures has also been utilized in ocular toxoplasmosis [8, 9]. Cellular cultures are also used for research purposes, like the study of host-parasite interaction, identification of factors involved on innate resistance, molecular and genetic characterization of *Toxoplasma* strains [10] and for the evaluation of candidate molecules on vaccine and treatment development [11].

The cell culture to identify tachyzoites from biopsy or corporal fluid of infected patients, leads to the destruction of the cellular monolayer and is related with the initial amount of tachyzoites in the biological sample. The sensitivity of parasite detection by cell culture from intraocular liquid (aqueous and vitreous humor) is 91%, compared with antibodies detection by ELISA with 67%.

2.3. Histology

The detection of tachyzoites in histological sections from suspicious biopsies indicates an acute infection; on the contrary, the detection of tissue cysts that contain bradyzoites in histological

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samples only confirms a chronic toxoplasmosis infection. The presence of cysts in the placenta, foetus or new-born indicates a congenital infection, according to the number of cysts in histological sections, can be suggested as an active infection and needs immediate treatment. In biopsy or autopsy material, the encysted tachyzoites and bradyzoites are usually demonstrable with wright, hematoxylin and eosin dyes, where the bradyzoites contain glycogen vacuoles. The size of the cyst is important in histological sections of the brain (**Figure 1**). There are other stain techniques like Schiff-periodic acid, specific immunohistochemistry stained with inmunoperoxidase that are equally efficient. These procedures have great diagnosis value but are limited due to the difficulty to grow parasites *in vivo* as well as the identification of tachyzoites by histochemistry methods [3, 5, 8].

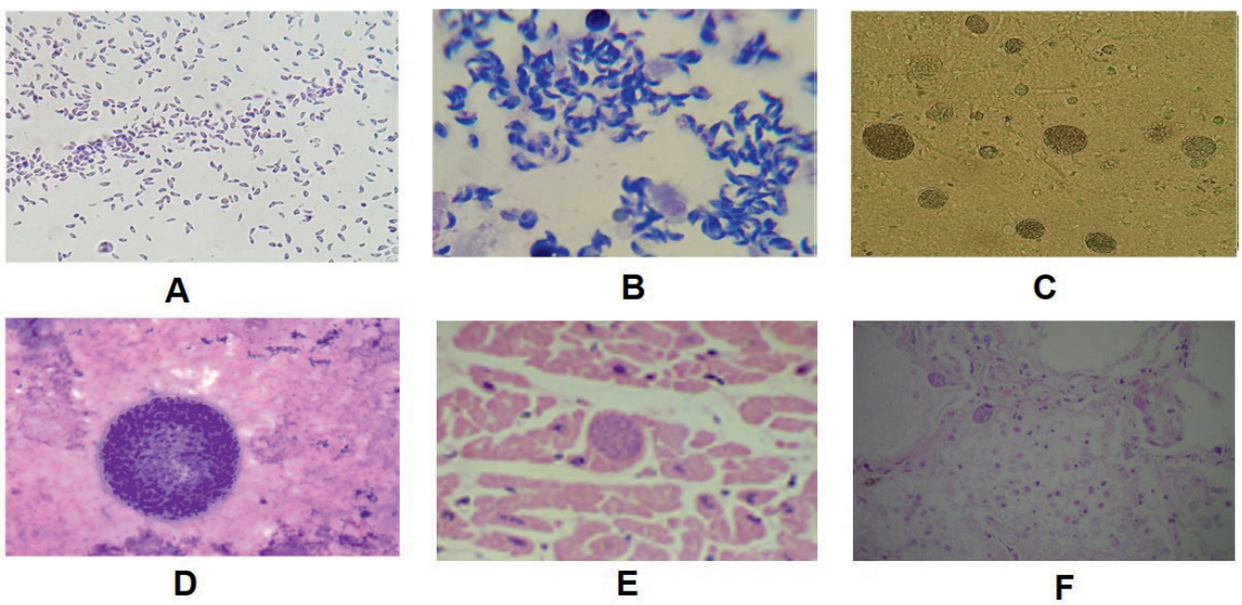

Figure 1. (A) Tachyzoites from mice peritoneal exudate, Diff-Quick stain, 100×. (B) Tachyzoites from mice peritoneal exudate, alkaline metilen blue, 10×. (C) Multiply tisular cysts en mouse brain, without stain, 20×. (D) Tisular cyst in mouse brain, Diff-Quick stain, 40×. (E) Histological section of the necropsy from patient (400×) cyst of *Toxoplasma gondii* in the sarcoplasm of a cardiac muscle fiber. (F) Histological section of the necropsy from patient (400×) H&E stain, in which some cysts of *Toxoplasma* are observed in the alveolar epithelium, necrosis in the adjacent cells, detritus, some inflammatory cells and proteinaceous material H&E stain. (A–D) Courtesy of Gustavo Salas Lais. Student of Doctoral Science in Biomedicine and Molecular Biotechnology, ENCB-IPN.Mexico. (E and F) Courtesy of Dr. Ramón Franco Topete: Medical Pathologist, Department of Microbiology and Pathology CUCS and the New Civil Hospital of Guadalajara, Mexico.

3. Immunodiagnostics'

3.1. Sabin and Feldman test

The Sabin and Feldman reaction, also known as the dye test [12], is considered as golden standard by detection of *Toxoplasma*-specific antibodies. The patient's serum is mixed with a suspension of live tachyzoites, and then methylene blue dye is added. If the patient's serum has anti-*Toxoplasma* antibodies, by the effect of complement factors, the parasites are lysed losing the property of capturing the dye, and as a result they are observed without colour under the

microscope (positive result for toxoplasmosis). When there are no anti-*Toxoplasma* antibodies in the assayed serum, the parasites remain with their cellular layer intact and thereby capture the dye showing a blue colour under the microscope (negative result to toxoplasmosis). The problem with this test is the use of live tachyzoites, a potential risk to laboratory staff conducting the test. Also, it requires specialized infrastructure for the maintenance of the parasites, so only research and reference centres perform this diagnostic tool [12]. Interpretation of results shows that the parasites dyed blue (negative) and more than 50% non-dyed (positive) parasites are reported with the highest serum dilution with the positive result, and the values that reflect infection with the parasite are >1:16 IU.

3.2. Hemagglutination

The hemagglutination assay was first described by Jacobs L. in 1957. This test is based on the capacity of anti-*Toxoplasma gondii* antibodies to agglutinate sensitive erythrocytes masked with cytoplasmic *Toxoplasma* antigens [13, 14]. This test is useful, rapid and highly sensitive ideal to be used in association with indirect immunofluorescence [15]. Also, it could interfere in the diagnosis of congenital toxoplasmosis, as the identified antibodies are IgG and so on. Interpretation of the test: positive if agglutination is present and negative if the agglutination is inhibited. The result is considered the lecture at the higher dilution. Afterwards Yamamoto in 1991, standardized a test for the determination of IgM antibodies, with a sensibility of 98% and specificity of 95% [16].

3.3. Modified agglutination test (MAT)

For this assay, the tachyzoites are fixed with formaldehyde in microtiter plates in the form of U plates with diluted serum added. In positive serum samples, a thin layer of agglutination is produced, while in negative samples compact sediment is formed due to precipitated tachyzoites at the bottom of the well. This assay was first described by Fulton and Turk [17], with low specificity and sensitivity, due to the union of normal IgM at the surface of the parasite and improved by the preparation of the antigen using a buffer with 2-mercaptoethanol, that eliminates the non-specific IgM. This assay detects IgG antibodies, without limitation of host species, but false negative results could occur during early stages of acute infection [17]. The specificity and sensitivity of MAT are similar to the Sabin and Feldman assay in most species. Also, MAT can be used in corporal fluids, being of much used in field work.

3.4. Indirect immunofluorescence (IFI)

This assay was described by Garin in 1967 [18], consists of fixation of tachyzoites on microscope slides. Upon contact with the antibodies present in the patient's serum, the antibody antigen reaction is carried out, which is visible with a second antibody labelled with fluorescein. The antigen-antibody reaction is evidenced by fluorescence microscope. In positive cases, a bright yellow greenish fluorescence is evident and a red colour without fluorescence is observed in negative cases. The colour of the fluorescence depends on the fluorochrome conjugated to the antibodies. The immunofluorescence is a test that shows high precision when it is adapted for specific antibodies. It is known that when the human is infected, it responds first with the production of IgM that disappears later. The detection of specific

IgM in the new-born shows difference in the antibodies transmitted from the mother to the product (IgG goes through placental barrier and IgM is not capable to do it). The detection of specific IgG contributes to the diagnosis most of the times; however, considerable titles of antibodies may persist for months or years [18].

3.5. Enzyme linked immunosorbent assay (ELISA)

The enzyme linked immunosorbent assay (ELISA) was described initially in 1971 by Engvall and Perlman, uses antibodies conjugated to an enzyme [19]. The antibody conserves its capacity of specific union to the antigen, while the enzyme is capable of a redox reaction, in which the substrate precipitates in a coloured product. In this system, the antigen or the antibody is absorbed to an insoluble solid phase (micro polystyrene plates). There are several variants of ELISA: direct, indirect, capture and competitive. All of them allow the determination of antigens in biological fluids except the indirect method that only detects antibodies.

The direct ELISA methods involve attachment of the antigen to the solid phase, followed by an enzyme-labelled antibody. This type of assay generally makes measurement of crude samples difficult, since contaminating antigens compete for plastic binding sites. Whereas, indirect ELISAs also involve attachment of the antigen to a solid phase, but in this case, the primary antibody is not marked. An enzyme-conjugated secondary antibody, directed at the first antibody, is then added. This format is used most often to detect specific antibodies in serum.

The captured ELISA assay involves attachment of a captured antibody to a solid phase support. Samples containing known or unknown antigens are then added in a matrix or buffer that will minimize attachment to the solid phase. An enzyme-labelled antibody is then added for detection.

The most used enzymes are horse radish peroxidase (HRP), alkaline phosphatase (FA) and the β-D-galactosidase (BG), the substrates for the HRP enzyme are hydrogen or urea peroxide, which on reducing make certain chromogens oxidized in the enzymatic reaction, developing a coloured product. The oxidized ortho-phenylene diamine remains soluble after the oxidation reaction and is the most used. The FA substrate p-nitrophenyl phosphate that produces colour when degraded by the enzyme at temperatures over 30°C, the 5 bromo-4cloro-3-indoilphosfate can be employed in ELISA in tube or in dot-ELISA. The ELISA method detects from 70 to 80% of cases in congenital infections, a rise in the titles of IgM that extends more over the first week is indicative of acute infection, since the average of duration of maternal IgM is from 3 to 5 days. Also, antibodies can be determined in serum and aqueous humour.

Interpretation of the test: If ELISA is qualitative, a development of colour is observed in seropositive samples. If the test is quantitative, the intensity of the developed colour is measured at a determined wavelength (nanometres) and the lecture of absorbance is proportional to the magnitude of the reaction antigen-antibody [20–22].

3.6. Recombinant antigens

At the final of the nineteenth century, several recombinant antigens of different sites of the parasites were analysed [23, 24]. The dense granule antigen—GRA1, GRA2, GRA4, GRA6,

GRA7 and GRA8, rhoptries protein—ROP1 and ROP2, the matrix protein—MAG1, protein micronemes—MIC2, MIC3, MIC4 and MIC5 or the surface antigen—SAG1 and SAG226, were evaluated as diagnostic markers in human beings or animals by ELISA to detect IgG and IgM specific antibodies [25]. The combinations of recombinant antigens have showed more sensibility and specificity for the use of only one antigen. For example, the combinations of SAG2A, GRA2, GRA4, ROP2, GRA8 and GRA7 are potentially useful to detect antibodies IgG in human beings with the infection recently acquired [26], ROP1, SAG1, GRA7, GRA8 and GRA6 are promising to detect specific IgM antibodies while GRA7 and GRA8 are used to detect IgA specific antibodies. A specific protein of sporozoites related with embryogenesis (TgERP) was identified in a study, it can react with specific antibodies of oocysts and can be used to detect an early infection by sporozoites implying the oocysts as infection source [27, 28].

3.7. Avidity test

The IgG avidity test was described by Hedman et al. [24]. This assay measures the avidity of the binding of specific antibodies to *T. gondii* antigen. The proteins present in the serum are denatured with a solution of urea. The avidity can be variable during the course of infection [29, 30]. In early stages of infection, the values of avidity are low and are increasing with the course of infection [24]. Therefore, the avidity test can distinguish between acute and chronic infection. The assay is performed by different serological procedures, such as ELISA. However, there are limitations for the IgG test in pregnant women, since these antibodies can persist for several months and the treatment of *T. gondii* can affect the results [31].

3.8. Western blot

Western blot is an immunoenzymatic technique, based on the binding of *Toxoplasma* antigens fixed on nitrocellulose paper with specific antibodies present in the serum of infected patients. First, the protein antigens must be separated by electrophoresis in polyacrylamide gel (PAGE-SDS), then transferred and immobilized in a solid phase (nitrocellulose layer). Nitrocellulose paper must be blocked or saturated with non-antigenic protein (bovine serum albumin) to prevent the unspecific binding of the antibodies. Then a second antibody conjugated with an enzyme (horseradish peroxidase or alkaline phosphatase) is added, this antibody binds to the Fc of the first antibody, when the substrate of the enzyme is added. The substrate is transformed to a product and visible specific bands in situ indicate the antigens of *T. gondii* recognized by specific antibodies [32].

Western blot has the advantage to identify specific antigens recognized by IgG, IgM and IgA antibodies from serum and cerebrospinal fluid of patients with chronic and acute toxoplasmosis, specially in immunocompromised patients with low titers of antibodies. In immunocompetent patients this method has been used to identify parasite proteins as markers for toxoplasmosis diagnosis (**Figure 2**) [33]. Also, diagnosis of congenital toxoplasmosis from colostrum samples by Western blot is very useful, since IgA antibodies recognize a greater

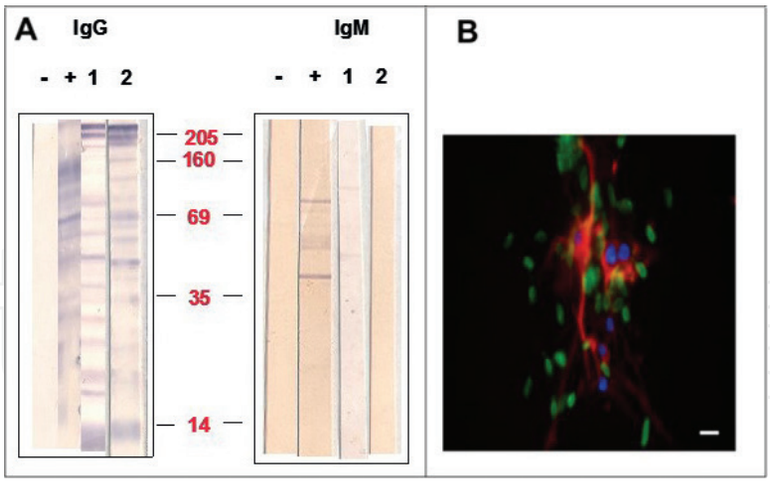

Figure 2. Recognition pattern of antibodies IgG and IgM by Western blot. The antibodies IgG and IgM were detected in samples of serum from a patient with acute infection. Negative control (−), positive control (+), samples 1 and 2 correspond to the bands of antigen *T. gondii* revealed by specific antibodies type IgG and IgM.

number of antigens [34]. This method has also been used with other corporal fluids such as saliva [35].

3.9. Immunocytochemistry and immunohistochemistry

The immunocytochemistry involves detection of epitopes expressed by the antigens of *Toxoplasma* within a tissue sample using a 'primary antibody' capable of binding those epitopes with high specificity. After the epitope-antibody binding event, a 'secondary antibody' capable of binding the primary antibody with high specificity is added. The secondary antibody is coupled to a reporter molecule and after the antibody-antibody binding event, a chemical substrate is added which reacts with the reporter molecule to produce a coloured precipitate at the site of the whole epitope-antibody complex (**Figure 2B**). The immunohistochemistry has the same base as that of the immunocytochemistry, the difference is that this one is realized on tissue slides. Studies have been done for a neuropathological test of cerebral tissue in patients with infection by the virus of human immunodeficiency VHI/AIDS, to evaluate the diffuse and spread brain multifocal lesions [36]. With immunohistochemistry there have been realized studies in different tissues to demonstrate *T. gondii*, such as ocular toxoplasmosis [37–39].

4. Molecular techniques

4.1. Generalities of the genome of *T. gondii*

Molecular biology has permitted the advance in molecular knowledge of *T. gondii*. Its genome contains approximately 65 megabases (MB), distributed in 14 chromosomes which varies from 1.9 to 7.4 MB. It contains 52.3% of GC and codifies for almost 7988 proteins and contains

296 small non-coding genes (Ensemble protists): *T. gondii:* [40, 41]. The complete genome sequence of GT1, ME49 and VEG strains classifies the *Toxoplasma* in three types I, II and III, respectively. Till date, the analysis of the restriction fragment length polymorphisms (RFLPs) with more than 900 strains of the parasite permits its classification in 140 genotypes grouped in 12 main groups. Similar results were obtained by the sequencing of introns of a smaller number of samples and also using microsatellite markers. In spite of these results, it is necessary to sequence more complete genomes for a better precision in the clarification of the linages (information taken in the page of Institute J, Craig Venter) [42].

4.2. Molecular diagnosis by the polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) consists in making a repetitive replication *in vitro* of specific DNA sequences. Amplifying or copying several times a fragment of DNA, the analytical sensibility of the test increases proportionally. The amplified product is analysed by electrophoresis in agarose or polyacrylamide gel. This technique is useful in the detection of the parasite in serum samples, peripheral blood mono-nuclear cells (PBMC), urine, placenta, amniotic and spinal brain liquid. The sensibility and specificity of PCR depends on the technique used in the extraction of DNA, the primers and the parameters of the amplification reaction [43, 44]. The most conserved genes of *Toxoplasma* are B1 with 35 copies in the genome of the parasite, followed by the repetitive region REP of 529 base pairs (bp) with an approach of 300 copies in the genome; these genes are the most selected for the PCR test as diagnostic tool. Other sequences of unique copy, which are used in investigation laboratories mainly for genotyping purposes are SAG1, SAG2, SAG3, SAG4 and GRA4 genes [45]. The polymerase chain reaction (PCR) is a very valuable diagnosis method in acute infections, when the parasite crosses placental barrier and infects the foetus or on infected patients with AIDS and other immunosuppression or reactivation of the parasite [46–48]. Unfortunately, it is difficult to have a confidentially valor in the PCR assays. For a pre-natal diagnosis, the reported sensibility goes from 64 to 97.4% with a predictive negative value of 87.7–99.7%, respectively; in case of patients with AIDS and toxoplasmosis, the reported sensibility goes from 13 to 87.5% [49]. With the detection of the sequence REP 529-bp, a sensibility of 100% has been reported in amniotic fluids [48]. The sequence REP 529-bp is having an increase in the utility for the diagnosis of *T. gondii* by PCR since it demonstrates greater sensibility, possibly the high number of repetitions in the parasites sequence increases the possibilities of detection. The variations in the results are due to different time of infection in the patients, type of sample, storage conditions that affect sensibility. Also, actually there is no agreement in the primers that should be used in the PCR, one of the regions which is more conserved is gen B1 [47], still there exists some polymorphisms and the strains of *Toxoplasma* in clinical samples are not the same genotype, these factors can affect the parasite detection. It requires large number of studies which compare gen B1 and the sequence REP 529-bp to meet and agreement in the selection of primers. Another factor that can affect the results is the variability reported in the techniques used for DNA extraction.

In patients with AIDS, the detection of *T. gondii* by PCR in cerebrospinal liquid has shown different sensibilities, reaching to 87% of sensibility when the sample is taken soon enough

preferably during the first 3 days and a maximum of 7 days after starting the specific treatment [48].

The polymerase chain reaction (PCR) can be in the future, undoubtedly a successful tool in the diagnosis of toxoplasmosis, when a satisfactory correlation exists between the patient's clinical profile and the presence of *T. gondii* DNA aspects which still have to be studied and standardized. Also, in countries with low development and still in many with high development, it has not been implemented for daily use due to its high cost.

4.3. PCR in real time

Unlike the conventional PCR, the real-time PCR using fluorescence probes measures the product of amplification in each cycle and can be quantified with the use of known concentration standards. Real-time PCR has been successfully used to detect the *T. gondii* DNA in human blood, cerebrospinal fluid, aqueous humour, amniotic liquid and other samples [49–51]. Also, it is used to evaluate the progression of the toxoplasmosis and the efficiency of the treatment, since it can estimate the intensity of the infection by *T. gondii*. The PCR assay in real time with REP 529-bp sequence is considered as the technique with greater efficiency for the diagnosis of toxoplasmosis in comparison with the detection of gen B1.

4.4. Loop mediated isothermal amplification (LAMP)

Loop mediated isothermal amplification (LAMP) is a technique of DNA amplification under isothermal conditions, which combines speed, efficiency and high specificity. The amplification of DNA proceeds from the repetition of two reactions of elongation in a structure that has loop form. It uses DNA polymerase with strand displacement activity and two inner primers and two outer primers. The inner primers contain sense and antisense sequences of DNA and initiate the LAMP reaction. The outer primers hybridize a few bases of the inner and initiate the DNA synthesis with strand displacement, forming a loop structure, which initiates a DNA synthesis by auto recognition. In this technique, 10 copies of DNA can be produced in less than one hour [52]. The final products are multiple loop DNA formed by alignment between inverted repeated sequences present in the same chain [53]. It only requires a water bath or thermo block to realize the amplification of DNA and the amplified products can be analysed by real time PCR equipment or by electrophoresis [54]. This technique is more sensitive than conventional PCR but low sensitive than real time PCR [53]. The LAMP assay has been evaluated identifying REP 529-bp, SAG1, SAG2 and B1 *T. gondii* sequences from PBMC [54]. The reported sensitivity was 0.1 tachyzoites and crossed reactivity with other parasites was not found. The detection of SAG2 showed the higher sensitivity (87.5%) followed by SAG-1 (80%) and B1 (80%). The specificity was 100% in all of them [55]. However, all techniques of nucleic acid amplification have a high sensitivity, for that reason a rigorous quality control is necessary to discard false positives.

4.5. Polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLPs)

The PCR-RFLP is based on the capacity of restriction endonucleases that recognize polymorphisms of only one nucleotide (SNPs). Polymerase chain reaction (PCR) products treated with restriction enzymes to generate polymorphic DNA fragments of variable length, that are visualized after electrophoresis in agarose or polyacrylamide gels with a band pattern [55]. In 1995, Howe and Sibley identified three main linages (type I, II and III) from 106 strains of *T. gondii* isolated from humans and animals by PCR-RFLP using six markers [56].

Since then, in different laboratories, several groups of different multi-locus markers have been selected, to characterize by PCR-RFLP *T. gondii* isolates from persons and animals [57, 58]. Currently, there has been developed a method for *T. gondii* classification into genotypes known as multiplex nested PCR-RFLPs (Mn-PCR-RFLPs). This method uses 10 genetic markers including the gens SAG1, SAG2, SAG3, Btub, GRA6, C22-8, c29-2, L358, PK1 and apico [59]. The Mn-PCR-RFLP has been applied broadly in the typing of positive clinically samples and has generated a big amount of data about the genetic diversity of the parasite [60, 61]. As in all techniques, where DNA amplification is performed, several negative controls must be included to have a strict quality control.

The microsatellites (MS) are short tandem repeats (STR) sequences of DNA (2 to 6 pb), that are widely distributed throughout the nuclear genomes of eukaryotes. They are highly polymorphic, the MS length varies depending the numbers of STR. The tandem repetitions in *T. gondii* are often simple, with only repetitions of two nucleotides, which occur from 2 to 20 times [62, 63]. Around 15 markers of MS have been used, including (tub2, W35, TgM-A, B18, B17; M33, IV.1, XI.1, M48, M102, N60, N82, AA, N61, N83) to determine the genotype of *T. gondii* in different laboratories [61, 62]. Ajzenberg et al. developed an easy method to genotype *T. gondii* in one multiplex PCR using 15 MS markers, in which eight MS markers (tub2, W35, TgM-A, B18, B17, M33, IV.1 and XI.1) could differentiate the types I, II and III from all the atypical genotypes, and the other seven markers (M48, M102, N60, N82, AA, N61, N83) could improve the resolution from the genetic differentiation of strictly related isolates in an haplo-group or clonal linage [62, 63]. The multiplex 15-MS assay is the best available tool to characterize *T. gondii* from genetically different or identical isolates during an outbreak, laboratory contamination and from mixed infections. The limitation of this assay is the request of an automatized sequencer with the 'gene scan' tool. The disadvantage is that samples with poor DNA can cause low intensity signals, generating non-specific PCR products [61, 62].

4.6. High-resolution melting (HRM)

In homogeny sample, the HRM method can analyse genetic variations, which allows to characterize polymorphisms based on the melting temperature generated by the DNA length and GC content [62]. On the base of only one SNP from gene B1 which is found in multiple copies, the HRM can classify correctly three different types of strains of *T. gondii* [63]. This assay was developed to determine the genotype of *T. gondii* directly from biological samples, with a better genotyping when use multiple copy genes in comparison to one copy genes, avoiding a cellular culture or bioassay.

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