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Toxoplasma gondii in Meat for Human Consumption – A Brief Review of the Most Described Strategies for Its Detection and Quantification

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Abstract

Toxoplasmosis is a parasitic zoonotic disease widely distributed worldwide and is caused by the intracellular parasite *Toxoplasma gondii*. The definitive host of *T. gondii* is the domestic cat and the entire cat family, in which the sexual stages of the parasite develop. *T. gondii* can also infect a wide range of intermediate hosts, affecting most warm-blooded animals including humans. In humans, toxoplasmosis is usually asymptomatic in healthy individuals, but can develop lymphadenopathy and nonspecific symptomatology or even be fatal in infants with congenital toxoplasmosis and in immunocompromised patients. Transmission to humans is mainly through food, especially by eating undercooked meat or meat contaminated with tissue cysts. This has led to various public health organizations worldwide monitoring programs on *T. gondii* in animals intended for human consumption, especially in meat samples. One of the techniques employed in the laboratory is that based on the polymerase chain reaction and some of its variants, which have proven to be valuable tools for the detection of *T. gondii* in tissues for human consumption and many other types of biological samples. The development of different strategies for the molecular detection of *T. gondii* has led to the identification and quantification methodologies varying widely among laboratories. Therefore, this chapter reviews the main methods of extraction, purification, detection and quantification of *T. gondii* DNA in tissue samples from different species destined for human consumption.

Keywords: *T. gondii*, meat, DNA quantification, parasite load, zoonosis

1. Introduction

Toxoplasmosis is a zoonotic disease widely distributed throughout the world and is caused by the intracellular parasite *Toxoplasma gondii*. The definitive host of the parasite is the domestic cat and the entire cat family, in which the parasite reproduces sexually. *T. gondii* can also infect a wide range of intermediate hosts, affecting most warm-blooded animals [1].

In humans, toxoplasmosis is usually asymptomatic in healthy individuals, but can develop lymphadenopathy and nonspecific symptomatology or even be fatal in infants with congenital toxoplasmosis (CT) and in immunocompromised patients (such as people with AIDS), in persons with problems in bone marrow and recipient patients of transplanted organs) [2].

Toxoplasmosis transmission to humans is mainly through food and from exposure to different stages of *T. gondii*, particularly by ingestion of sporulated oocysts found in the environment and contaminated water and food, or by ingestion of tissue cysts or tachyzoites contained in meat and products derived from the meat of various animals, [1,3,4].

The consumption of undercooked, raw or cured meat is a major mode of transmission of *Toxoplasmosis* to humans, especially in cases of CT, which has been attributed to 30% to 60% of cases of infection during pregnancy [5]. This has led to various public health organizations worldwide monitoring programs introduced on *T. gondii* in animals intended for human consumption, mainly in meat samples [6].

2. Occurrence of toxoplasmosis

T. gondii is a parasite that is widely distributed around the world, showing a higher incidence in tropical areas and a decrease when the latitude increases [7]. The estimated prevalence in the human population varies widely in different geographical areas, between different cities within the same city and between different ethnic groups even when they live in the same area [6,8]. In humans, toxoplasmosis is considered the third leading cause of death among food-borne diseases [7].

As well in humans, *T. gondii* is widely distributed worldwide. Parasite presence has been described in several animal species (wild, companion and production animals). However, food-producing animals may represent a real risk for transmission of the disease to humans, either directly or through farming [9]. Several serological assays have been performed in free-ranging chickens, because the information provided is usually used as an index of environmental contamination with oocysts [10]. Prevalence rates in these animals in Central and South America ranges from 40 to 60% [11],and other reports from India describe prevalence rates from 20 to 40% [12,13]; in Egypt there are rates reported around 40% [10,14] and in China, 30.36% [15]. Pigs and sheep are also commonly infected. Pigs are very susceptible to become experimentally infected and show high prevalence rates in some regions of the United States, as well in warm and tropical countries [16–21]. Prevalence rates of *T. gondii* in sheep are also widely distributed. For example in the United States, there has been described prevalence rate

in range of 27 to 73 %; Uruguay, Argentina, Chile, Mexico and the United Kingdom showed prevalence rates of around 30%, but there are some countries with higher rates such as Ivory Coast, France and Turkey, where prevalence rates of 68, 89 and 95.7%, respectively, have been reported [22]. Cattle and buffaloes are not considered as probably sources of significant infection [22] as cows are relatively resistant to experimental infection [24].

In general, the occurrence infection by *T. gondii* in animals varies depending on weather conditions, geographical area, type of species, either productive or wild, as well as the age of the animals, the type of farming system (extensive, semi-extensive, intensive), the management and the existence of health programs [22,25,26]. Also, there have been described many factors that have an important impact on foodborne transmission of *T. gondii* to humans, such as the type of management and production of livestock, hygienic standards of slaughterhouses, food processing and technology, the density of cats or wild felines in the area and climate change, which may influence the sporulation of oocysts in the environment (i.e., temperature, humidity, wind) [6].

3. Clinical signs in animals and humans

3.1. Toxoplasmosis in humans

The clinical spectrum of the disease varies widely and depends primarily on the immune status of the host and *Toxoplasma* lineage to which a person was exposed. In humans, toxoplasmosis may manifest basically in five ways: asymptomatic, acute infection, congenital, ocular and the immunocompromised patient. In immunocompetent persons toxoplasmosis is usually asymptomatic or develops mild symptoms that go unnoticed in 80–90% of cases [27]. The usual clinical manifestations, trend to be laterocervical lymphadenopathy which is often treated, less frequently supraclavicular nodes, occipital, inguinal, mediastinal lymph node or any other chain are affected; patients with lymphadenopathy also have other symptoms such as fever, malaise, arthralgia, asthenia, night sweats, sore throat or maculopapular rash, no itching, which usually spares the palms and soles. The clinical picture of acute toxoplasmosis is usually benign and self-limiting; in <1 month, progression to the severe form is rare in immunocompetent hosts [27–30]. In immunocompromised subjects, acute toxoplasmosis usually occurs widely where the main sites of spread are the central nervous system, eyes, heart, liver and lungs, with preference in the tissues where the immune response is limited, causing injuries. In immunocompromised patients, such as AIDS patients with very low CD4 counts, patients under immunosuppression to prevent or treat transplant rejection and fetuses, a reactivation of an earlier infection, rather than a newly acquired one, is common. In these individuals the parasite can induce besides encephalitis and retinochoroiditis, carditis, pneumonia, and meningitis, among other manifestations [31]. Ocular toxoplasmosis is produced in most cases by breaking tissue cysts that are congenitally acquired; however, it can occur in acquired infections too. It manifests as uveitis or retinochoroiditis with exudate and decreased vision; the lesion can be observed by fundus evaluation. The ocular presentation is commonly characterized by necrotic lesions destroying retinal architecture, sometimes engaging and

choroid (retinochoroiditis) [28,30]. CT is the most significant complication of active infection. When the infection is acquired during pregnancy, either by reactivation of latent-phase bradyzoites or by a primary infection, the parasites migrate to the placenta and from there to the fetus. The severity of the disease depends on the stage of pregnancy when the parasites cross the placenta. At the beginning of pregnancy, fetal infection is rare, but when it happens, it triggers severe injury or death of the fetus. As the pregnancy progresses, congenital infection is most common, but the damage tends to be lower [29,32]. When the disease occurs in utero, there may be consequences observed at birth. Most congenital infections are asymptomatic, although in some cases the child is born with acute illness, where cases of hydrocephalus, eye damage and visceral can be found. In other cases, the disease develops after birth or become dormant for a long time [29,32].

3.2. Toxoplasmosis in animals

Natural infection in non-pregnant animals usually elapses without symptoms, but primary infection during pregnancy can cause embryonic death, abortion, birth weak or clinically normal but infected animals. Globally, *T. gondii* is the cause of 11–14% of the abortions that occur in sheep and goats [33]. In cattle, by contrast, *T. gondii* infection is not considered a common cause of abortion and presented asymptomatic. *T. gondii* in dogs is considered an opportunistic pathogen and infection is usually subclinical, but under certain conditions clinical signs are present, predominantly respiratory and neuromuscular manifestations (Dubey, 2010). Cats even as definitive hosts usually enrolled asymptomatic infection, even during removal of oocysts; however, sometimes clinical signs are present, primarily associated with respiratory type interstitial pneumonia, dyspnea, lethargy and anorexia, ocular signs (uveitis, retinochoroiditis) or neuromuscular signs [34]. In cats it has also been described as intrauterine infection; thus, infected animals generally have more severe signs: encephalitis, hepatitis, ascites, respiratory signs and perinatal death or weaning [35]. In the pigs, the disease usually attends in subclinical and can be seen in some cases of weak animals born or stillborn; in adult animals weight loss, anorexia and fever have been observed, which usually disappear by the third week after infection regardless of variant of *T. gondii*. Pigs are considered an important source of infection for humans because of the high parasite loads encountered in their tissues compared to other productive animals [22,35].

4. Economic impact of toxoplasmosis

The real economic impact of toxoplasmosis is difficult to estimate, because in most immuno-competent individuals, the infection goes unnoticed or has claimed clinical presentation to other diseases [22,29]. However, it is estimated that the economic impact should be very high due to the loss of one or more days of work in mild cases, treatment and care needs, sick children, especially those with mental retardation and blindness, loss of quality lifestyle and the costs of hospitalization in severe cases and the cost of monitoring pregnant women and treatment during pregnancy who are *T. gondii* positive [26,36,37].

In United States,, despite the low incidence, the economic impact of CT is high due to the severity of the infection, associated complications, treatment and social costs. CT costs have been estimated as $ 1.26 million per case and were mainly attributed to drug costs, annual losses of productivity, special education and health care costs [38]. On the other hand, in the United States, some 3,000 babies are born every year with CT and the annual cost of treating this disease is between US$ 31 and US$ 40 million [39]. The total economic impact of CT just in the United States has been estimated as over $ 7.7 billion per year, which makes it the second most important infection to humans after foodborne salmonellosis [40]. In the United Kingdom, the annual economic impact is estimated $ 12 million [26].

In most productive animals, toxoplasmosis occurs asymptomatically; however, in animal production, toxoplasmosis is considered as an economically important disease of livestock, especially sheep and goats, where it can cause early embryonic death and resorption, fetal death and mummification, abortion, stillbirth and neonatal death [41].

Regarding economic impact of infection with *T. gondii* in productive species, just in the United Kingdom, economic losses were estimated to be between 15 and 20%, from scanning to sale, but on some farms losses can reach over 30% [42]. This is often a result of a disease outbreak of *T. gondii* abortion. Other authors had mentioned that cost of *Toxoplasma* in the UK flock was estimated to be £12 million [43]. These estimates included loss of production, cost of treatment, control and monitoring but did not include the costs associated with human health. As a matter of fact, the economic losses caused by *T. gondii* infection in sheep are difficult to estimate because the disease occurs sporadically. Moreover, only a small number of the lambs aborted are subjected to diagnosis [43]. In addition, the material sent for diagnosis, besides being potentially inadequate, might also be examined erroneously and, finally, serological testing lacks specificity [45].

In pigs, the infection with *T. gondii* also use to be asymptomatic, but in some cases, the active infection or parasitemia reactivation could impact over reproductive parameters (abortions or weak stillborns), mainly when is present in concomitance with other viral agents such as parvovirus [46] or circovirus.

5. Molecular biology techniques for *T. gondii* detection

The diagnosis of toxoplasmosis is usually based on the detection of antibodies by ELISA serology, agglutination assays or other methods such as Western blot immuno- or Sabin-Feldman [47] staining. However, there are times when serological or with any of the aforementioned detection methods are not possible; in these cases the techniques of molecular biology have been helpful in the diagnosis of *T. gondii*, especially the PCR technique, which has proved to be a valuable tool [2,48].

Currently different strategies for molecular diagnosis of *T. gondii* have been developed; this has meant that various methodologies are reported (both for the extraction of nucleic acids and for the detection of different amplification targets) and brings about a large variation

between results from different laboratories [49]. So the aim of this chapter is to review the main methods of extraction, purification, detection and quantification of DNA from *T. gondii* in different species intended for human consumption.

5.1. DNA extraction methods

The extraction and purification of nucleic acids is the first stage of most molecular biological studies; extraction methods allow to obtain nucleic acids from various sources and then perform specific analysis by polymerase chain reaction (PCR) or its variants [49]. The quality and purity of the nucleic acids are two of the most important elements of such analysis since contaminants can interfere by inhibiting the amplification process in which PCR [50] rests. This makes clear that the importance of the process of sample preparation and DNA extraction methodology used, to have significant impact on the sensitivity of the test [50,51].

To extract the nucleic acids of the biological material, samples must be homogenized, causing cell lysis, proteins be removed by incubation with a protease and finally nucleic acids should be separated from other cellular components [50]. The ideal lysis procedure, which usually consists of a balancing techniques, must be strong enough to break the complex starting material (a fabric, for example), but gentle enough to preserve the nucleic acids. The lysis process is generally performed by physical or chemical processes, which break the bonds between the cells to facilitate interaction with lysis solutions that help release the genetic material [50, 51]. Among the conventional methods for extracting genetic material from T. *gondii* in tissue include the following procedures previous to DNA extraction.

5.1.1. Mechanical homogenization with liquid nitrogen

This process involves macerating the sample with liquid nitrogen using a mortar to obtain a fine powder. The nitrogen immediately can freeze the sample to prevent crystals formation, thus avoiding the breakdown of cell structures and the start of DNA degradation process by the action of DNase [51].

5.1.2. Chemical homogenization

In the chemical homogenization, the samples are maintained in solution at high temperatures in the presence of proteases, detergents and chaotropic agents to break the bonds between the cells or can even pierce the cell membrane. In fibrous tissue it is recommended that you cut into small pieces to facilitate their decomposition. Before starting this type of homogenization it is necessary to have information about the right amount of tissue; for rapid and complete homogenization, it is necessary to ensure the recovery of DNA and prevent degradation [51].

5.1.3. Pepsin digestion

Pepsin digestion is a method developed by Jacos et al. in 1960 and is modified by Dubey (1998) for retrieving from *T. gondii* from 50 g of muscle tissue (Dubey, 2010). The method is based on the digestion process by which the parasite is released from tissue cysts to invade its host. In this method, the homogenization of muscle tissue occurs by digestion with porcine pepsin

solution, HCl, NaCl and distilled water at 37°C for 1 hour. Subsequently, a series of centrifugations allow the parasites to concentrate and be more likely to find their genetic material when purification is performed. At the end of centrifugation the product has to be buffered with bicarbonate to prevent degradation of the nucleic acids contained in the homogenate [22].

Pepsin requires acidic pH for activation; it breaks the bonds between tyrosine and phenylalanine partially degraded proteins. Pepsin polypeptides of different sizes and some amino acids are obtained without degrading completely, so this procedure is commonly followed by the enzymatic action of another protease [52].

5.1.4. Proteinase K

Proteinase K is most often used because it is the widest-spectrum (degrades all proteins) protease and it is often used with buffers containing SDS and EDTA [50]. Proteinase K is a protease obtained from the fungus saprophyte *Tritirachium album* and is particularly suitable for digestion in a short time. Proteinase k acts on the carboxyl group of amino acids and is highly stable in a wide range of temperatures and pH values, having its greatest activity at elevated temperatures [53].

5.2. DNA purification methods

The DNA purification methods can be classified into two major branches: traditional protocols and by commercial kits [51].

5.2.1. Conventional protocols

They were developed in the 1950s; organic solvents used to separate proteins and DNA, once suspended in the aqueous phase by ethanol precipitation isolate [51]. In the case of *T. gondii*, more purification methodology used in the traditional methods is performed by phenol/chloroform.

5.2.2. Phenol/chloroform

The phenol/chloroform purification is a method of liquid-liquid organic extraction consisting of separate mixtures of molecules, which is based on the difference in solubility of individual molecules in two different liquids [52,53]. The nucleic acid extraction with phenol/chloroform involves adding equal volumes of phenol/chloroform aqueous cell lysate or tissue homogenate, mixing the two phases and allowing to separate by centrifugation (Alejos et al., 2014). The phenol/chloroform method ensures the separation of liquids in two phases (organic and aqueous lower than), because chloroform is miscible with phenol due to its higher density (1.47 g/cm3) phenol [54,55].

Nucleic acids are soluble in the upper aqueous phase because of their negatively charged phosphate backbone, while proteins and lipids are separated in the organic phase [55]. Phenol causes precipitation of proteins and polymers (including carbohydrates) that are contained in the interface between the two phases (often as a white supernatant); in the case of lipids, these
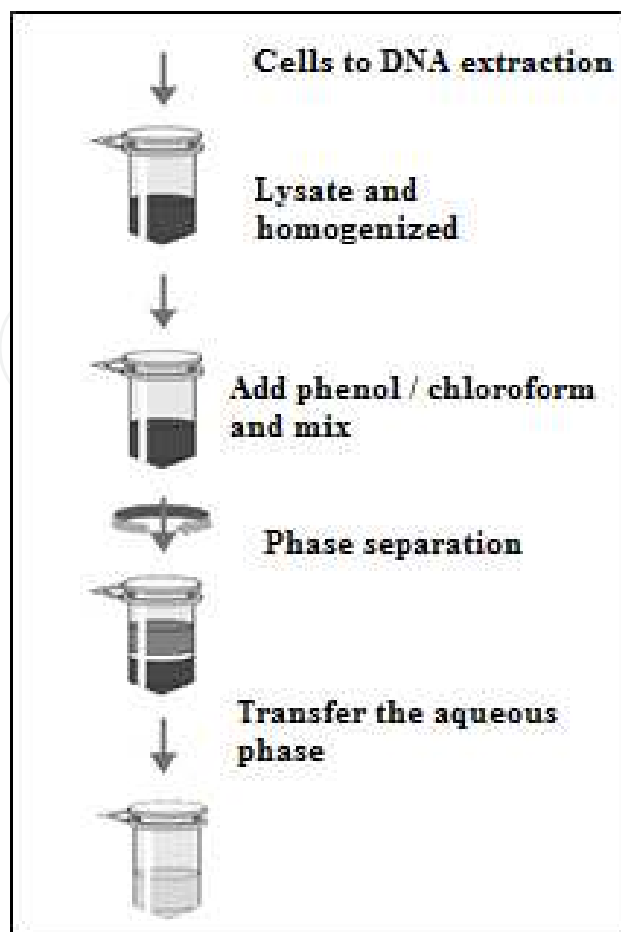
Figure 1. DNA purification with phenol/chloroform [55].

are dissolved in the lower organic phase. The separation between the aqueous and organic phase by centrifugation allows isolation of the DNA in the aqueous phase (Karp, 2009; Soma, 2010). Subsequently the DNA is recovered from the aqueous phase with ethanol and is insoluble, causing centrifugation to precipitate it [51].

5.3. DNA extraction using commercial kits

From the 1990s were introduced to market commercial purification kits; these kits commonly used membranes or inorganic matrices to which the DNA will bind to specific conditions (Karp, 2009). Often these arrays are stacked into small columns in centrifuge tubes so that the binding steps, washing and elution can be performed efficiently by applying a centrifugal force. Some of the advantages of using commercial kits are to increase efficiency of DNA recovery, to obtain inhibitor-free extract and to decrease the time spent for purification {51,54]

The purification procedure with commercial kits can be summarized in three steps: 1) homogenization of tissues to facilitate the selective attachment of DNA to the matrix; 2) washing to remove DNA contaminants and 3) recovering the DNA from the matrix using an eluting buffer [51].

5.3.1. Silica matrix

This method is based in selectively adsorption/desorption of nucleic acids and it has proven more efficient for the recovery of pure DNA beside biological samples (i.e., blood, tissue). Silica extraction methods produce increased DNA yield while efficiently removing PCR inhibitors; those protocols usually include a small-scale silica-based spin column. The selective adsorption/desorption occurs when, by the action of ethanol, the DNA loses its humectant layer, exposing its phosphate groups and thereby facilitating the adsorption of the molecule to the positively charged membrane. Lipids and proteins are not related to the membrane and are removed using the wash solution and a cycle of centrifugation, while the genetic material remained is bound to the matrix [51].

5.3.2. Magnetic beads

Extraction methodology using magnetic beads or a magnet that attracts magnet to separate the beads from solutions in which they are suspended is applicable. In this case, the lysis buffer solution at acidic pH, which allows to positively charge the beads, favoring DNA binding [51,53], is added.

The methodology for the extraction and purification of DNA from *T. gondii* in tissue with magnetic beads was described by Opsteegh et al. [56]. Tissue homogenization is carried out in a bag filter Stomacher400 containing a lysis buffer (100 mM Tris HCl pH 8.0, 5 mM EDTA pH 8.0, 0.2% SDS, 200mMNaCl and 40mg/L Proteinase k), by reaction using 2.5 mL of lysis buffer per gram of sample for 2 minutes at high speed. The homogenate is placed in incubation at 55°C overnight and, after the incubation, the extract obtained is homogenized again for 1 minute; 50 mL of the homogenate is transferred to a 50-mL tube and is centrifuged at 3500 g × 45 min; 12 mL of the supernatant (crude extract) is collected for use in the purification of the genetic material of *T. gondii*.

The first step in the purification process based on magnetic beads is the removal of free biotin in the sample because this method is based on the target binding sequence labeled with biotin to the magnetic beads that are coated with streptavidin (protein high affinity for biotin). To remove free biotin from the sample streptavidin sepharose should be added to allow the biotin precipitation and to form a pellet by centrifuging and 10 mL of the supernatant are finally used for purification process [56,57].

To mark the sequences of interest with biotin is needed the addition of specific primers that are marked with this molecule [57]. The bonding occurs during hybridization, so it is first necessary to denature the sample nucleic acids by heating at 95°C for 15 min and then lower the temperature to allow hybridization; if the proposed protocol is by Opsteegh et al. [56]. the hybridization temperature is 55°C for 45 min. Once labeled primers are hybridized to the target sequence, we proceed to introduce the magnetic beads in the sample and proceed to incubate at room temperature for 60 minutes to allow binding sequences labeled with magnetic beads. They are separated from the beads using a magnet and then the DNA of the beads was recovered by washing with buffer B & W (Binding & Washing) provided in the kit extraction

and finally the DNA recovered is resuspended in distilled water. Magnetic capture process is presented in Figure 2.
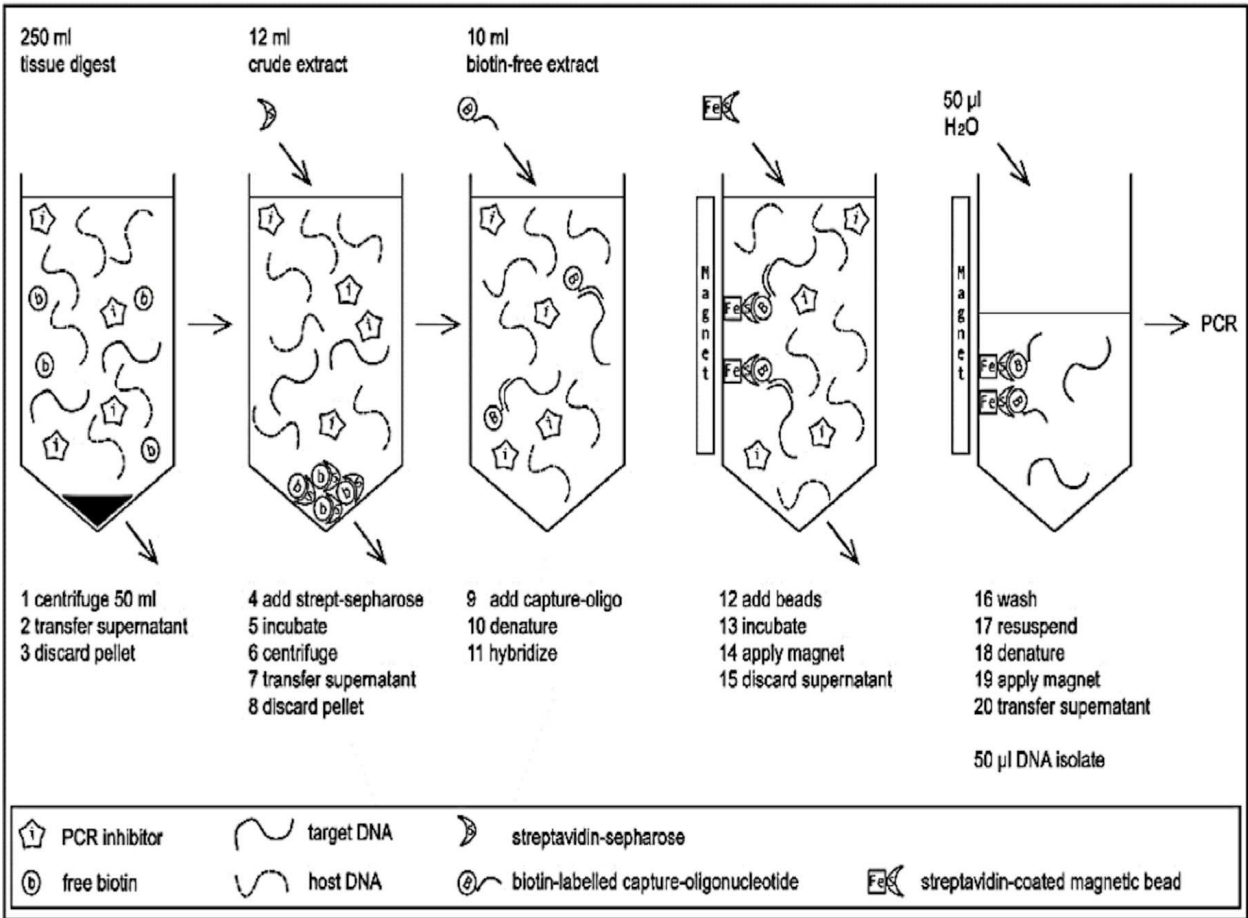

Figure 2. DNA purification from tissue employing magnetic beads [56].

Today there are also cases that implement business purification methods with organic solvents. Usually these methods are based on the phenol/chloroform by adding guanidine or any other part that improves the extraction and purification of nucleic acids. Examples of these traditional methods are converted to commercial kits extraction and extraction with TRIzol® DNAzol® [58,59]. These commercial kits based home methods are usually cheaper than commercial kits based on inorganic matrices, although purification performance is very similar in some cases, there is a risk of contamination of samples with phenol and inhibition of amplification in the PCR process [60]. Another important factor to consider is security, since this type of extraction involves the use of corrosive and irritant substances and therefore requires some experience to handle; accidental contact with the reagents that can burn skin and vapors inhalation could cause damage to the respiratory system, in both cases, medical assistance is required. Table 1 presents the main commercial kits used for the extraction and purification of DNA from *T. gondii*.

Commercial kit	Fundamental	Column material	Amount of recovered DNA	Reference
Universal Genomic DNA Extraction Kit (TaKaRa, USA)	Absorption/ desorption	Silica	10μg (2–30 mg of tissue)	[61].
Easy-DNA®Kit (Invitrogen)	Organic extraction	N/A	150μg (50 mg of tissue) 5–10 ng/μL blood	[62].
High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim Germany)	Absorption/ desorption	Glass fiber filter	3–9 μg (200–300 μL blood) 25–50 mg of tissue (variable amount recovered depending the tissue tested)	[6]. [63].
QIAamp DNA Mini Kit (QIAGEN, Valencia, CA)	Absorption/ desorption	Silica	4–12μg (200μL blood) 25–45μg (25mg of cardiac tissue)	[21,47,64–68].
TRIzol® LS Reagent (Invitrogen, Paisley, the United Kingdom) (Fenol-Cloroformo)	Organic liquid-liquid extraction	N/A	2–3μg (for each mg of sample)	[69].
Phenol-Chloroform	Organic liquid-liquid extraction	N/A	---	[70–76]
NucleoSpin Tissue Kit (Macherey Nagel, Czech Republic)	absorption/ desorption	Silica	20–35μg (25mg of tissue)	[77].
Biotin labeled capture oligonucleotides and Streptavidin labeled magnetic beads (Invitrogen)	magnetic capture	Magnetic beads	Recovered DNA from 5–100pb	[5,47,56]
NucliSens easy MAG system (BIOMERIEUX)	magnetic capture	Magnetic Silica	---	[49,78,79]
Genomic Prep Blood DNA isolation kit (Amersham Pharmacia Biotech, the United Kingdom)	absorption/ desorption	Silica	Recovered DNA from 100–200 kb	
DNAzol® (Life technologies)	organic extraction	N/A	3–5μg/mg from tissue	[80].
Qiaquick y PCR purification kit (QIAGEN, Valencia)	absorption/ desorption	Silica	10 μg DNA recovered from 100pb–10kb	[81,82]
DNeasy Blood & Tissue Kit (QIAGEN)	absorption/ desorption	Silica	10–30ug (25mg of tissue)	[83,84]

Table 1. Leading commercial kits used for DNA extraction and purification from different tissues for *T. gondii* detection

6. Methods for molecular detection of *T. gondii* in meat for consumption

The presence of *T. gondii* in biological samples can be diagnosed by molecular techniques aimed at detecting its genetic material [74,85] (Switaj et al., 2005; Garcia et al., 2006). A specific fragment of the parasite genome can be amplified by PCR to visualize on a polyacrylamide or agarose gel, to stain, to sequence directly in real time. The sensitivity and specificity of PCR-based techniques rely on an appropriate method of purification of genetic material from the samples, the characteristics of the DNA sequences chosen for amplification and the parameters of the amplification reaction [50,85]. Various amplification methods for *T. gondii* will be explained below.

6.1. Conventional PCR (endpoint)

The PCR is a molecular biology technique developed by Dr. Kary B. Mullis in 1985 [54,86]. The impact of his discovery was such that Dr. Mullis received the Nobel Prize for Chemistry in 1993 (Welch, 2012). PCR is a technique "*in vitro*" used to amplify enzymatically a specific region of DNA located between two regions of DNA whose sequence is known [86]. PCR simulates what happens during cell replication taking advantage of features of the chemical structure and semiconservative DNA replication [50].

DNA is a polymer formed from two complementary strands anti-parallel, each chain consists of nucleotide units which in turn are comprised of a nitrogenous base (adenine = A, guanine = G, cytokine = C and thymine = T) attached to sugar deoxyribose and a triphosphate group [50,87]. To replicate the DNA separates its two complementary strands, serving each mold or template for the "de novo synthesis" of its complementary strand, the specificity of pairing of the nitrogenated bases (TA, CG) to obtain two identical DNA molecules, each consisting of an original and a new chain. The enzyme that performs this process is called DNA polymerase [54,87]. PCR synthesis of new DNA strands is performed by mixing: containing DNA or fragments to be amplified; polymerase; primers (DNA fragment of 15 to 30 nucleotides flanking the region to be amplified and to provide the free 3 'end to initiate transcription); deoxynucleotides (dNTPs); magnesium chloride (MgCl2) or other cofactor necessary to work polymerase [86]. Generally, the PCR begins with denaturation or separation of the double helix of DNA by heating the sample at a temperature between 94 and 96°C to break the hydrogen bonds that bind them, so as each string is a template for synthesis A new complementary strand of DNA [88].

Once separated the chains of DNA primers (initiators or primers) are aligned in complementary-specific sites of the single strands in the region to be amplified; for this to happen it is necessary to lower the temperature between 40 and 60°C, allowing binding (hybridization or alignment) of the primers. Finally, a new strand is synthesized in the 5′ to 3′ for which the temperature is increased, generally at 72°C, which is the optimum temperature to work Taq polymerase [89]. These three stages — 1) denaturation, 2) hybridization and 3) elongation of DNA — are repeated successively in each new cycle and amplified the region of interest of the two complementary strands [86] simultaneously. The essential equipment for the process to take place is the thermal cycler, which has a heating pad where each reaction is placed and

where temperature changes are accurate and can be pre-programmed in three stages by several cycles [50, 86].

Detecting the PCR product is usually accomplished by electrophoresis. Separation matrices (agarose, polyacrylamide) at various concentrations are used depending on the size of the amplification product and the resolution desired. The posterior viewing can be done with ethidium bromide with a UV lamp, silver staining, fluorescence or radioactivity light. The sizes of the PCR products are determined by comparing them with markers containing DNA fragments of known size, which are run in a gel with PCR products.

6.2. Nested PCR

Nested PCR (nested) consists of two successive processes of amplification, using the product of the first amplification as template for the second [2]. In the second amplification primers used should be different from the first amplification and are targeted to amplify a smaller fraction contained within the product of the first PCR [90]. This methodology increases both the sensitivity and specificity of the test. Furthermore, the risk of contamination increases significantly due to the increased amount of amplification products and work steps involved [91]. In this type of test validation it is always recommended by both negative and positive controls, ensuring that the positive controls are highly diluted to avoid contamination of the samples [90].

6.3. PCR-LAMP

This is a variant of PCR developed for parasites of the phylum Apicomplexa, among which is *T. gondii*. The amplification method LAMP (Loop-mediated isothermal amplification) is based on a displacement auto cyclic reaction chain using a set of four oligonucleotides (primers) which recognize six sequences within the genomic DNA target region and form a loop structured amplicon, the polymerase that performs this function is Bst polymerase having activity displacement [92,93].

6.4. Real-time PCR (qPCR)

Real-time PCR is a technique used to quantify specific nucleic acid sequences in a sample of interest. The assay is based on generating a fluorescent signal that is directly proportional to the amount of target DNA. Real-time PCR is able to monitor the fluorescence emission that occurs during the reaction progress, so is said to be in real time [94]. Among the major fluorophores used for determination of *T. gondii* load are as follows:

6.4.1. SYBR Green™

It is an intercalator that binds to dsDNA resulting fluorescence increased with increasing the amount of PCR product. An important aspect to consider is that the SYBR Green™ can also join primer dimers and nonspecific amplification products, resulting in an overestimation of the concentration of target DNA. The detection of DNA of *T. gondii* using SYBR Green it could be useful mainly during experimental infections. It has been used for monitoring parasitemia

loads in animal models, however for clinical diagnosis or monitoring any other natural infection (i.e., in animals for consumption) the performance it is not enough sensitive to detect the presence of low amount of the target in the sample.

6.4.2. TaqMan™

TaqMan probes are hydrolysis probes which allow increase the specificity of quantitative PCR. They have attached a reporter (a fluorophore) and a quencher. When both (fluorophore and the quencher) are in proximity, the reporter emits no signal, however, when the probe hybridizes to the sequence of interest during PCR, the endonuclease activity of Taq polymerase to short photochromic other reporter probe, allowing emission of a fluorescent signal. The reporter fluorescent signal is cumulative in each of the subsequent cycles [79].

7. Determination of parasite load in meat by qPCR

7.1. Absolute quantification

Absolute quantitation is a quantization strategy based on comparison of the test samples against a standard curve created from a template of known concentration (Sivaganesan et al., 2010). This template of known concentration is used to make serial dilutions and generate a curve from the CT values (threshold cycle) obtained for each concentration. The curve can interpolate directly the CT values of the test samples and get your concentration by the equation $Q = 10^{\left(\frac{CT - b}{m}\right)}$ where: Q = amount shown, CT = amplification cycle, b = insertion axis "and" m = gradient of the line. In traditional protocols creating absolute quantification standard curve in each qPCR experiment conducted it suggested, however, can also be employed master calibration curve generated from multiple experiments qPCR [95]. The absolute quantification method is conceptually easy, however, requires a reliable source as a template of known concentration.

7.2. Relative quantification

Such quantification measures changes in the basal state of a gene of interest versus constant gene expression that acts as a control. The difference lies in the absolute quantification that are not part of a known amount of DNA, but an endogenous housekeeping gene control or reference "housekeeping". Because the absolute amount of internal standard is unknown, can be determined only relative changes of the gene of interest with reference to the endogenous gene. Some of the most commonly used reference genes include glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, hypoxanthine guanine phosphoribosyl transferase (HPRT) and 18S ribosomal RNA.

The advantage of using mass units and normalizing the experimental design is that it is conceptually simple, but requires accurate quantitation of starting material to be used as a normalizer. There are some methods and models to determine the amount of DNA that are based on comparison of the CT values using the efficiency of PCR reaction as a correction

factor. However, there is also a model that does not require the reaction efficiency of accessing a correction factor, assuming 100% efficiency in the PCR reaction in real time, both study gene as reference gene, this Method 2 is delta-delta CT (2-$\Delta\Delta$CT) (Vinuesa, 2009; Aguilera et al, 2014.). Method 2-$\Delta\Delta$CT expresses the ratio obtained from the relationship between the CT values of the sample and control values CT. Assay validation is made by serial dilution problem for both the endogenous gene to gene. ΔCt values (CT gen – CT endogenous) are obtained. These are plotted on the "y" axis versus the logarithm of the concentration in each of the dilutions in the "x" axis. The slope of the line should be less than or equal to 0.1 so that the method is valid [69]. Table 2 shows the most commonly used primers for the detection by different variants of PCR and also are listed the ones used to estimate the parasite load of *T. gondii* in tissues.

Amplification primers target		Sequence 5′–3′	Amplicon size	Reference
PCR endpoint				
529pb Repeat Element (*RE*)	Toxo4	CGCTGCAGGGAGGAAGACGAAAGTTG	529pb	[62,74,75]
	Toxo5	CGCTGCAGACACAGTGCATCTCGGATT		
Gen B1	Tg1	AAAAATGTGGGAATGAAAGAG	469pb	[3,21,64]
	Tg2	ACGAATCAACGGAACTGTAAT		
Gen B1	F	AGCCTCTCTCTTCAAGCAGCGTA	300pb	[71,83]
	R	TCGGAGAGAGAAGTTCGTCGCAT		
Nested PCR				
Gen B1	S1	TGTTCTGTCCTATCGCAACG	580 pb	[49]
	AS1	ACGGATGCAGTTCCTTTCTG	530pb	
	S2	TCTTCCCAGACGTGGATTTC		
	AS2	CTCGACAATACGCTGCTTGA		
Gen GRA6	GRA6-F1x	ATTTGTGTTTCCGAGCAGGT	546pb	[76]
	GRA6-R1	GCACCTTCGCTTGTGGTT	351pb	
	GRA6-F1	TTTCCGAGCAGGTGACCT		
	GRA6-R1x	TCGCCGAAGAGTTGACATAG		
Gen B1	B1F1	TCAAGCAGCGTATTGTCGAG	194pb	[96]
	B1R1	CCGCAGCGACTTCTATCTCT		
	B1F2	GGAACTGCATCCGTTCATGAG		
	B1R2	TCTTTAAAGCGTTCGTGGTC		
529 pb *RE*	REF1	TGACTCGGGCCCAGCTGCGT-3′	164pb	[93,96]
	RER1	CTCCTCCCTTCGTCCAAGCCTCC-3′		
	REF2	AGGGACAGAAGTCGAAGGGG-3′		
	RER2	GCAGCCAAGCCGGAAACATC-3′		
PCR LAMP				
529bp *RE*	F3	ACGAGAGTCGGAGAGGGA	202pb	[93, 96]
	B3	TGGATTCCTCTCCTACCCCT		
	FIP (F1 c-F2)	GGATCGCATTCCGGTGTCTCTTAAGATGTTTCCGGCT		
	BIP (B1c-B2)	TGGC		

Amplification primers target		Sequence 5'–3'	Amplicon size	Reference
		GACGACGCTTTCCTCGTGGTCAAGCCTCCGACTCTGTCT		
529bp RE	F3	CCACAGAAGGGACAGAAGTC	202pb	[96,97]
	B3	TCCGGTGTCTCTTTTTCCAC		
	FIP	TCCTCACCCTCGCCTTCATCTAGGACTACAGACGCGATGC		
	BIP	TGGTTGGGAAGCGACGAGAGTTCCAGGAAAAGCAG		
	LF	CCAAG		
	LB	TCCAAGACGGCTGGAGGAG		
		CGGAGAGGGAGAAGATGTTTCC		
Gen B1	BIP	TCGCAACGGAGTTCTTCCCAGTTTTGGCCTGATATTA	212 pb	[96]
	FIP	CGACGGAC		
	F3	TGACGCCTTTAGCACATCTGGT		
	B3	TTTTGATGCTCAAAGTCGACCGC		
		GGGAGCAAGAGTTGGGACTA		
		CAGACAGCGAACAGAACAGA		
qPCR				
Gen B1	SYBR Green™	BE-F: CTCCTTCGTCCGTCGTAATATC BE-R: TGGTGTACTGCGAAAATGAATC	451pb	[47]
SAG 1	SYBR Green™	F: CTGATGTCGTTCTTGCGATGTGGC R: GTGAAGTG-GTTCTCCGTCGGTGT	128pb	[61]
Gen B1	SYBR Green™	F: CGTCCGTCGTAATATCAG R: GACTTCATGGGACGATATG	98pb	[47]
529pb RE	TaqMan™	F: CACAGAAGGGACAGAAGT R: TCGCCTTCATCTACAGTC RE probe: (JOEᵉ)-CTCTCCTCCAAGACGGCTGG-(TAMRAᶠ)	94pb	[47,63,69]
Gen B1	TaqMan™	GENE_B1_TG-TX2F: CTAGTATCGTGCGGCAATGTG GENE_B1_TG-TX2R: GGCAGCGTCTCTTCCTCTTTT GENE_B1_TG-TX2M1: 6-FAM)-CCACCTCGCCTCTTGG-(NFQ-MGB)	62 pb	[79]
Gen B1	TaqMan™	BE-F: CCCCACAAGACGGCTGABE-R: TGGTGTACTGCGAAAATGAATC TaqMan probe: (6-FAM)-CATTTGCAAAACAGCGGCAGCGTCT-(DQ)	248pb	[47]
529pb RE	TaqMan™	TOX-9F: AGGAGAGATA TCAGGACTGT AG TOX-11R: GCGTCGTCTC GTCTAGATCG TOX-TP1: (6-FAM)-CCGGCTTGGC TGCTTTTCCT-(BHQ1)	524pb	[5,47,56,77]

Table 2. Amplification targets, primers and probes used in the diagnosis and quantification of *T. gondii* in meat.

Although there are several methods for the diagnosis of toxoplasmosis, the present work focused on the most used by researchers working with *T. gondii* in animal tissue samples. In this aspect the molecular, especially the PCR technique and its variants, has been a major technique for diagnosing the presence and in some cases abundance of the parasite in the tissues of the various productive species that are commonly consumed tool.

The sensitivity of PCR techniques is influenced by several factors, among which are tissue type, sample handling, the process employed in the extraction and purification of nucleic acids and the type of card selected amplification. All these factors cause variation sensitivity in these tests of about 20% to over 80% in some cases. Variations of the PCR most commonly used for the diagnosis of toxoplasmosis are conventional PCR, LAMP-PCR, nested PCR and real time PCR, considered as the most sensitive nested PCR and PCR techniques in real time, can detect the presence of nucleic acids of *T. gondii* from a single parasite in the sample.

The techniques of DNA extraction and purification significantly affect the development of PCR techniques; the presence of contaminants may inhibit the amplification process. Nowadays, there are different methodologies used for DNA extraction and also, there is a wide variety of commercial options available to perform the procedures, however, even almost all of them have been designed to obtain as much amount of DNA as possible from any specimen while minimizing manual handling and co-extraction of PCR inhibitors. Methodologies based in small-scale silica-based spin column, has proven more efficient for the recovery of pure DNA in comparison with phenol/chloroform extraction, because they have showed several disadvantages besides being unable to remove potential PCR inhibitors efficiently.

The sensitivity of PCR assays depends heavily on the number of copies of the target sequence; in the case of *T. gondii* although various cards amplification as is the case of SAG1, SAG2, SAG3 and GRA6 genes, most widely used are those which are directed to highly repetitive genes as gene B1 is repeated 35 times in the genome of *T. gondii* or repeated 529pb fraction, which is repeated 200 to 300 times in the parasite genome. Although the B1 gene is considered to be the gene of choice for the diagnosis, repeated fraction 529pb has begun to have a greater impact due to it being repeated more times increases the sensitivity of PCR testing. This trend toward the use of these cards amplification is observed in most variants of the PCR.

An important aspect for the selection of the PCR technique used in the diagnosis of toxoplasmosis has been the cost involved, the techniques of real-time PCR are usually much more expensive due to the use of probes and specialized equipment for reading the results, so this type of quantitative PCR are mainly used for research.

It is important to consider that molecular methods allow us to estimate whether the presence (when we use some variants as endpoint PCR or nested PCR) or quantity (by qPCR) parasite DNA in the evaluated sample. However, these findings do not allow for determination whether the parasites are viable and capable of producing infection.

Tissues that tend to have higher parasite loads are often brain, heart and spleen, but it is feasible to detect the presence of the parasite in other tissues intended for human consumption. Loading and distribution of parasites depends on biotype and density involved in the environment, as well as the animal's age and the type of production system. Due to this high variability, it is important to determine which tissues have higher loads and biotypes which are involved in order to avoid risks of transmission to consumers of meat from different regions and emphasize the importance of giving proper management to meat products (such as cooking or freeze) to reduce the risk of acquiring infection by eating them.

8. Conclusion and recommendation

Toxoplasmosis is a zoonotic disease transmitted by common foods; the occurrence of cysts of *T. gondii* in meat for human consumption represents a potential health risk, especially if the meat is consumed raw or cooked at lower temperatures of 67°C. In this sense the existence of accurate and reliable diagnostic tests is essential for the detection, monitoring and control of infections in intermediate hosts and to minimize the risk of infection in humans.

For the diagnosis of infection in tissue samples from naturally infected animals intended for consumption, it is desirable to use the technique Taqman qPCR probes since it is a more sensitive alternative to the same qPCR detection when used with SYBR Green dye. The format for performance this technique (qPCR with a Taqman probe) is versatile, allowing to evaluate both few samples (in a research environment for example) and in environments where it is required to test a greater number of specimens in a short time period (i.e., specialized laboratories for monitoring food safety), and increasingly, inputs for this procedure are more affordable.

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