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Research Article

Standardization of Polymerase Chain Reaction for Molecular Detection of the B1 Gene of *Toxoplasma gondii* in Meat Products

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Received: December 07, 2018; **Published:** January 21, 2019

Abstract

The transmission of *Toxoplasma gondii* in Costa Rica indicates that the ingestion of undercooked meat, as well as some sausages, are an important infection mechanism. The purpose of this work was to standardize endpoint polymerase chain reaction (PCR), for the molecular detection of the *B1* gene of *T. gondii*, in meat products. For this intention, DNA extraction was standardized with two commercial protocols, without (protocol I) or with silica column (protocol II). The quality of the DNA was evaluated based on its spectrophotometric relationship 260/280 and integrity by electrophoresis. The tachyzoites of the RH strain of *T. gondii* were used as biological material alone or inoculated in beef, chicken or pork. The sensitivity and specificity of the endpoint PCR were standardized with the primers for the first round of the nested PCR, for gene *B1* amplification. A better quality and less degraded DNA was obtained when it was extracted with protocol II. The PCR detected one femtogram of DNA and one tachyzoite of *T. gondii* alone or 10 thousand tachyzoites in the presence of meat, used as extraction matrix. The PCR was 100% specific. With the standardized conditions, PCR obtained was as or more sensitive than the nested PCR reported in the literature. Standardized PCR can be used as a molecular tool for the detection of *T. gondii* DNA in meat products for human consumption.

Keywords: *Toxoplasma gondii; Molecular Detection; Meat; Gene B1; PCR Standardization*

Abbreviations

PCR: Polymerase Chain Reaction; *T. gondii: Toxoplasma gondii*; ng/rx: ng/reaction; tach./mL: tachyzoites/mL

Introduction

Toxoplasmosis, caused by the organism, *Toxoplasma gondii,* Apicomplexa [1] is an infection for human and animals where produce abortion and other symptoms that generates important economics losses and public health problems [2,3]. In human beings, although this disease is usually present as a subclinic infection, in special low immune conditions, ocular lesions, encephalitis besides abortion, can be observed [1,4]. This parasitosis is found worldwide showing a seroprevalence between 10 to 92%, depending of economic, social and cultural factors [5] in each country. The reports in Costa Rica indicate seroprevalences of 61,4% [6], 76% [7] and 58% [8].

Ingestion of raw or slightly raw meat, as well as water and other foods contaminated with cat feces [9,10], are the principal sources of Toxoplasma infection; sausage ingestion is also important [11]. Recent studies, feeding mice with meat or sausage [12], showed a decrease of presence of *T. gondii* in this foods (4%) as compared with similar analysis done by Madrigal., et al. [13] and Chaves., et al. [14] whom found a positivity of 40 or 8%, respectively.

On the other hand, parasite molecular detection, introduce specific techniques where the use of experimental animals and finding of the parasite, or the immune response [15] are not necessary. And detection by PCR is simple, sensitive, specific, consistent an cheaper [16], if compared with the biologic *in vitro* and *in vivo* models, that can last 10 days to several weeks [17]. Of course, sensibility and specificity of this method depends on the genetic material extraction, sample preparation, chosen sequence and parameters of amplification reaction [18].

T. gondii molecular detection previously described by Burg., *et al.* [19], was based on a nested PCR that detect the parasite with the gene *B1* identification; this gene has been frequently utilized for this detection [1,17]; because it has 35 copies within the genome of the parasite, the sensitivity of the test is better [20].

Implementation of molecular methods to detect *T. gondii* DNA in meat products has been performed in several countries [21-25], but there are not similar studies in Costa Rica.

Aim of the Study

The aim of the present study was standardize a PCR for the molecular detection from the *B1* gene of *Toxoplasma gondii* in meat products.

Materials and Methods

DNA source

Genetic material was obtained from *T. gondii* tachyzoites (RH strain, ATCC 50174 D) collected from a peritoneal exudate of white mice (Strain CD-1, *Mus musculus* Swiss), previously infected and maintained in the appropriate conditions, according to the national and international laws for animal care [26-28].

DNA extraction from tachyzoites (protocol A)

Two commercial protocols were used to obtain the DNA from *T. gondii* tachyzoites: the Wizard Genomic ADN Purification Kit (Promega, USA) without DNA purification column (protocol IA) and NucleoSpin (Machery-Nagel, USA) with that column (protocol IIA) Briefly,

to isolate the material for both protocols, 1 ml of mouse peritoneal exudate with *T. gondii* tachyzoites was washed with 1 ml of saline phosphate buffer (Gibco) and centrifuged (11.000 x g for 5 minutes). The supernatant was removed, the pellet resuspended and the DNA was extracted according to the corresponding commercial protocols, adapted to the procedures utilized for DNA extraction from culture cells. This material was separated in 20 µL aliquots and maintained at -20°C.

Standardization of DNA extraction from meat products (protocol B)

200 mg of beef, chicken or pork placed in 1 ml of cold PBS was triturated in a sterile mortar and the DNA was extracted using the techniques already described. For practical purposes we identified the extraction with Kit de Wizard- Genomic (Promega) as the protocol IB, and when we used the Kit Nucleo-Spin (Machery -Nagel), as protocol IIB

Measurement of the quality of the extracted DNA and PCR analysis

This evaluation was done on basis of the relations of 260 nm and 280 nm absorbance (DNA/proteins), that can be found between 1.8 and 2.0 [29,30]. For this purpose we used the spectrophotometer UV-visible (PharmaSpec UV-1700, Shimadzu) and the DNA quantification was performed by fluorometry (Qubit, ThermoFisher, USA. DNA degradation was determined after an 1% agarose gel electrophoresis (SeaKem LE, Cambrex, USA) dissolved in TRIS-Acetate-EDTA and GelRed stained (Gel Stain, Biotium, Cat: 41003). Electrophoresis was executed at 100 volts for 30 minutes (Power Source FB1000, Fisher Scientific). (FastRuler™ Low Range DNA, Thermo Scientific). DNA and PCR amplifiers were observed in a UV Transilluminator (Slimline Series; Spectroline), the image was captured with a specific digitizer (Enduro TM GDS, Labnet international, Inc.) and the analysis was done with the software Total Lab 1D version 14.0.

PCR standardization for *T. gondii* B1 gene

Amplification of this gene was done according to the specifications of Burg., et al [19]. Briefly, the PCR was performed with ToxoN1 (5´-GGA ACT GCA TCC GTT CAT GAG- 3´) and ToxoC1 (5´-TCT TTA AAG CGT TCG TGG TC- 3´), which give rise to a product of 196 pb, after 40 amplification cycles of: 94ºC/1 min, 53ºC/1 min and 72ºC/1 min with previous treatment at 94°C/5 min and a final polymerization at 72°C/10 min. A final volume of 50 µL was adjusted with nuclease free water (Life Technologies, Cat.: AM9939, USA). According to the suggestions of commercial supplier Dream Taq Polymerase (Thermo Scientific Cat. #EP0702) at 1.25 U per reaction and deoxyribonucleotide triphosphate (dNTPs) at 0.2 nM final concentration (Sigma. Cat. #D7295), were utilized. To standardize the amplification of DNA obtained from tachyzoites, 10 to 50 ng were used besides primer differential concentrations between 0.5 and 1.0 µM.

Sensitivity and specificity of PCR

Sensitivity was qualify in two categories according to the minimal *T. gondii* DNA that gave rise to an expected amplification (sensitivity A) and the minimal number of tachyzoites detected by the PCR (sensitivity B). For the first one, the DNA was quantified and titled, obtained at least 1 femtogram (fg)/ml. For the second, 10^6 *T. gondii* peritoneal tachyzoites were prepared until obtaining one organism/ml. Each aliquot was centrifuged, the supernatant discarded and the DNA was extracted following the protocols already mentioned. The specificity was studied working with DNAs from several bacteria: (*Escherichia coli, Proteus vulgaris, Salmonella* spp.; *Staphylococcus aureus, Listeria* spp. and *Pseudomonas aeruginosa*), previously studied with VITEK 2 Compact (BioMeriex, España). The following ATCC parasites were also used: *Trypanosoma cruzi* (Cepa Y), *Plasmodium berghei* (Cepa NK65), *Toxoplasma gondii* (Cepa RH), and *Leishmania mexicana* (strain gently donated by Dr. Rodrigo Zeledon). In addition, we did an assay with kidney tissue of mice infected with *Trypanosoma duttoni* or *Plasmodium berghei*, inoculated or not *in vitro* with 105 *T. gondii* tachyzoites.

Results

DNA quality obtained from *T. gondii* tachyzoites

7.25 X 10^6 of these organisms, worked with the protocols IA and IIA, produced 43.1 ng/µL and 266 ng/µL of DNA respectively. After an electrophoresis analysis, we observed that the DNA obtained with the protocol IIA, was degraded (Figure 1) and there was a major DNA concentration probably for this degradation. Since in this assay, the temperature used was 58 and not 70°C, as recommended by the commercial kit, and therefore the proteinase K was not proteinase K was not inactivated, we concluded that the temperature was the factor for DNA degradation. To demonstrate this hypothesis, we extracted the DNA with a controlled temperature of 70°C, either with the proteinase K or not, using the protocol IIA. In this case, we obtained 43.7 and 61.2 ng/µL of DNA from tachyzoites, without any degradation, as observed in electrophoresis in agarose gel (Figure 2). In conclusion, it is possible to obtain DNA of good quality with both protocols and in the protocol IIA, it is independent of the proteinase K presence.

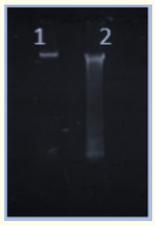

Figure 1: *Electrophoresis of the ADN. Line 1: extracted with Protocol I. Line 2: extracted with Protocol II*

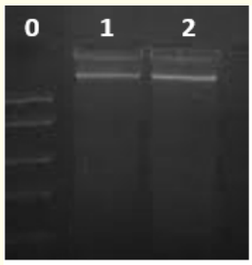

Figure 2: *Electrophoresis of DNA extracted with Protocol IIA. Lines: 0. Marker of 50 pb; 1. with Proteinase K; 2. without Proteinase K.*

Primers concentration

We utilized the protocol II for the DNA extraction from tachyzoites testing 10, 1.0 and 0.1 ng/reaction. An amplifier of 196 pb (Figure 3) was obtained with similar results either for a final concentration of primers of 5 µM or for 1 µ.M. On basis of this information, we choose for the PCR, primers of 0.5 µM besides de other reagents 1.25 U/reaction of Taq polymerase, 4 mM of MgCl2 and 0.2 mM of dNTPs.

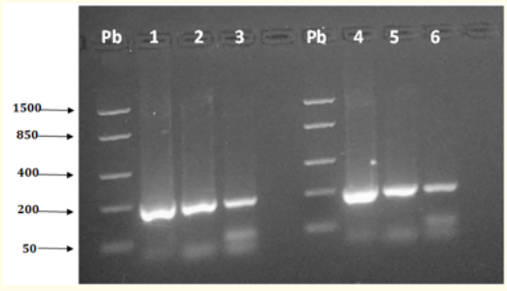

Figure 3: *Standardization of the primer concentrations. Lines 1 and 4: 10 ng/reaction (rx); 2 and 5: 1 ng/rx; 3 and 6: 0.1 ng/rx; Lines 1, 2 and 3: primers at a final concentration of 0.5 µM; Lines 4, 5 and 6: primers at a final concentration of 1 µM.*

Minimal amount of *T. gondii* DNA detected by the nested PCR (sensitivity A)

According to the DNA obtained by the amplification with the protocol IA and IIA (1/10 dilution), we observed an amplified 196 pb at a concentration of 4.3 fg/µL (8.6 fg/reaction) (Figure 4A) and 1 fg/reaction (Figure 4B) respectively.

Minimal amount of *T. gondii* tachyzoites detected by PCR (sensitivity B)

Once that we obtained, by means of several dilutions, at least a tachyzoite/mL, DNA concentration was confirmed. Even when the Fluorometer sensitivity did not detect The DNA in many of the dilutions, (not shown data), given the high sensitivity, the PCR was performed. Under the know conditions it was possible to amplify the DNA from one tachyzoite/mL both for protocol IA as for protocol IIA; in this last protocol, the amplification was higher (Figure 5).

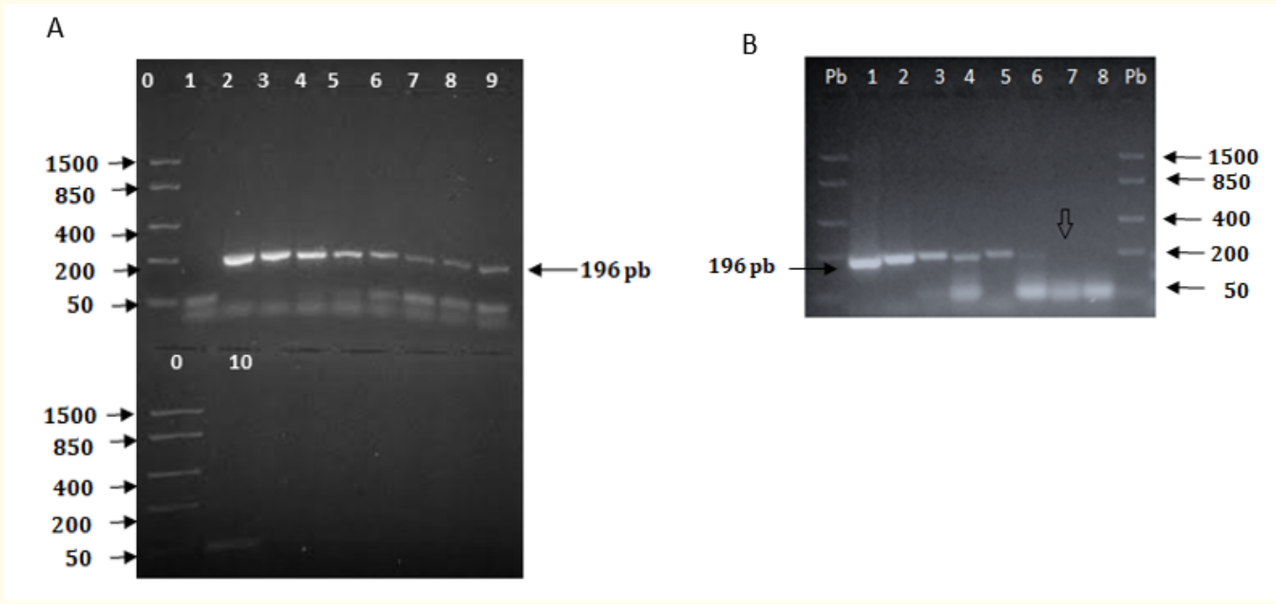

Figure 4: *Sensitivity A of PCR. A. PCR performed with DNA extracted with protocol IA. A. Lines: 0: 50 pb marker; 1: control mix; 2: 43.1 ng/µL; 3: 4.3 ng/µL; 4: 0.43 ng/µL; 5: 43.1 pg/µL; 6: 4.3 pg/µL; 7: 0.43 pg/µL; 8: 43.1 fg/µL (86,2 ng/Rx); 9: 4.3 fg/µL; 10: 0.43 fg/µL. B. Performed with DNA extracted with protocol IIA. Lines: 1: 1 ng/reaction (rx); 2: 0.1 ng/rx; 3: 0.01 ng/rx; 4: 1 pg/rx; 5: 0.1 pg/rx; 6: 0.01 pg/rx; 7: 1 fg/rx; 8: control mix. Arrow in line 7 show the presence of one weak amplifier in one fg/rx.*

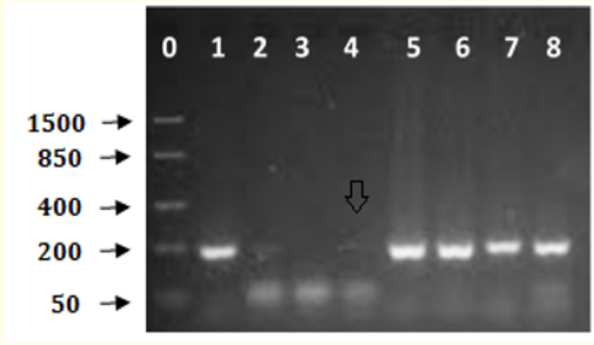

Figure 5: *Electrophoresis of PCR products to evaluate the B sensitivity. Lines 1 - 4. Protocol IA; lines 5 - 8: Protocol IIA. Line 0: 50 pb marker; lines 1 and 5; 1 x 106 tachyzoites (tach.)/mL; lines 2 and 6: 1 x 104 tach./mL; lines 3 and 7 : 1 x 102 tach. /mL; lines 4 and 8: 1 x 100 tach./mL.*

Specificity

After amplification of DNAs from some bacteria and other parasites was found only for *T. gondii* an amplification at 198 pb (Figure 6). The assay with kidney tissue of mice infected with several parasites showed that only the tissues containing *T. gondii* presented an amplified of 196 pb. By the way, DNA obtained from the tissue of mice inoculated with *T. gondii* presented similar concentration independent of the protocol of amplification (Table 1) but the degradation was higher for the protocol IB as compared the IIB (Figure 7). Measure of DNA by the spectrophotometer confirmed that the extracted with the protocol IIB was of better quality, since the absorbance relation, 260/280, was near to 2 (Table 1).

T. gondii detection in meat experimentally infected

The *T. gondii* DNA detection in this material was performed in 203.33 ± 7.76 mg of ground beef with or without the parasite, using protocol IIB. An average of 0.743 ± 0.667 ng of DNA was extracted (Table 2) and the material was almost completely digested (Figure 8A). PCR amplified the DNA in one samples of uninfected beef and in samples inoculated with 10^6 tachyzoites/mL (Figure 8B).

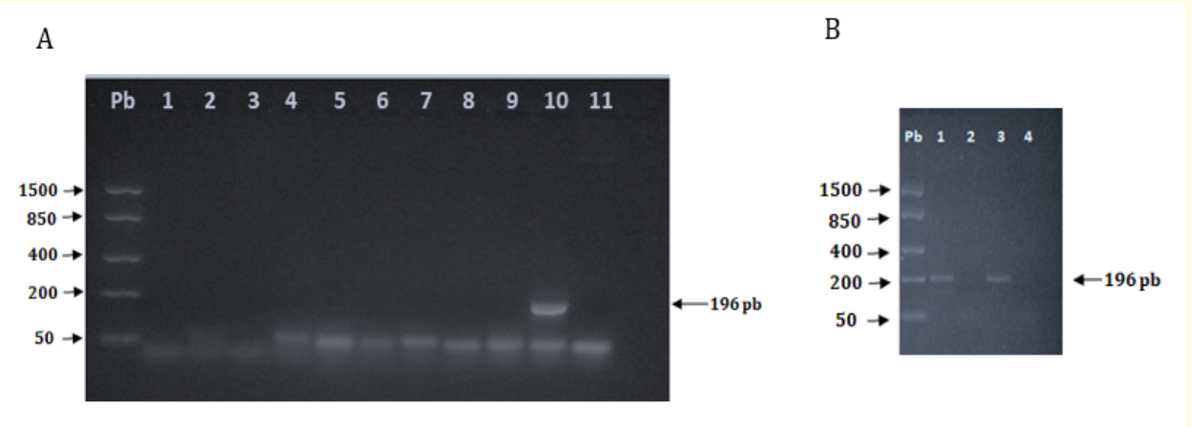

Figure 6: *PCR Specificity for T. gondii detection. A. Specificity against the DNA of bacteria or parasites of the culture in vitro. Lines: 1. Control mix; 2. Escherichia coli; 3. Proteus vulgaris; 4. Salmonella sp.; 5. Staphylococcus aureus; 6. Listeria sp;.7. Plasmodium berghei; 8. Trypanosoma cruzi; 9. Leishmania mexicana; 10. Toxoplasma gondii; 11. Pseudomonas aeruginosa; B. Specificity against DNA extracted from the kidneys of mice infected with other parasites. Lines: 1. T. duttoni plus 105 tachyzoites de T. gondii Cepa RH; 2. T. duttoni; 3. P. berghei plus 105 tachyzoites de T. gondii. 4. P. berghei. Pb: marker of 50 pb.*

Figure 7: *Detection by PCR of T. gondii in mouse muscle. A. Muscle DNA electrophoresis. Lines: 1 and 4: muscle of the hind leg; 3 and 4: diaphragm muscle; 6 and 7: Pectoral muscle: I. IB protocol; II: Protocol IIB.*

			Fluorometer	Spectrophotometer UV-visible			
Tissue	**Quantity (mg)**	**Protocol**	**DNA (ng/µL)**	**260 nm**	**280 nm**	**260/280**	**DNA (µg/mL)**
Hind leg	70	I	15.0	0.168	0.118	1.4	16.8
	70	II	9.7	0.097	0.046	2.1	9.7
Diaphragm	70	I	28.8				
	80	II	22.2				
Pectoral Muscle	40	I	11.4				
	40	II	12.0				

Table 1: *Tissue characteristics, extraction protocol and DNA quantification.*

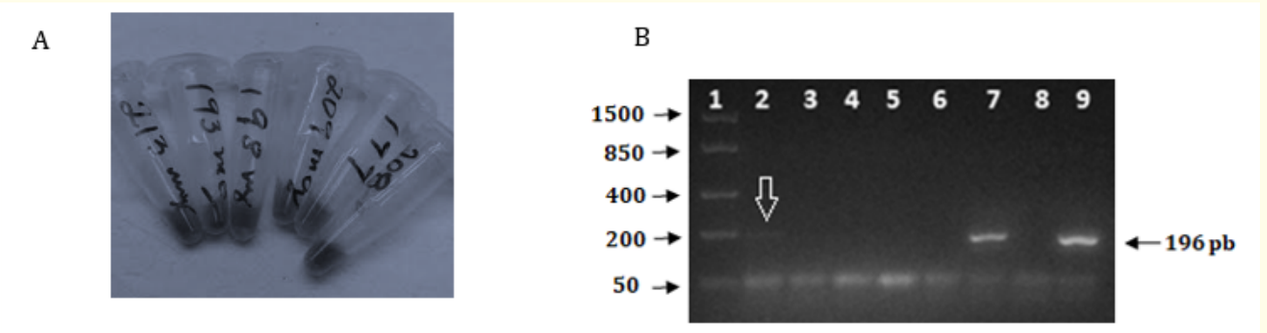

Figure 8: *T. gondii detection in meat experimentally infected. A. Waste from the digestion of meat with the protocol IIB. B. PCR done with inoculated or not inoculated meat. Lines. 1: 50 pb marker; 2 and 3: non-inoculated meat. Lines 4, 5, 6 and 7: meat inoculated with 1 tach./mL, 1 x 10^2 tach./mL, 1 x 10^4 tach./mL and 1 x 10^6 tach./mL, respectively. 8: control mix; 9: positive control (DNA of T. gondii 1 x 10^6 tach./mL). The arrow shows the weak amplification (8B).*

Number	Meat (mg)	Tachyzoites/mL	ADN (ng/mL)
1	213	0	0,248
2	208	9 x 10^6	1,980
3	199	1x 10^6	1,010
4	209	1 x 10^4	0,317
5	198	1 x 10^2	0,115
6	193	1 x 10^0	0,879

Table 2: *Processed meat weight, number of inoculated tachyzoites and DNA quantity.*

T. gondii detection in chicken meat and pork experimentally infected

DNA extraction of infected and not infected meat was performed, under the protocol IIB. DNA amplification occurred only in the infected material (Figure 9A) but a positive amplification was found in the uninfected pork (Figure 9B). In infected chicken meat, the test detected 10^6 tachyzoites/mL while in pork it was possible to detect 10^4 and 10^6 parasites/mL.

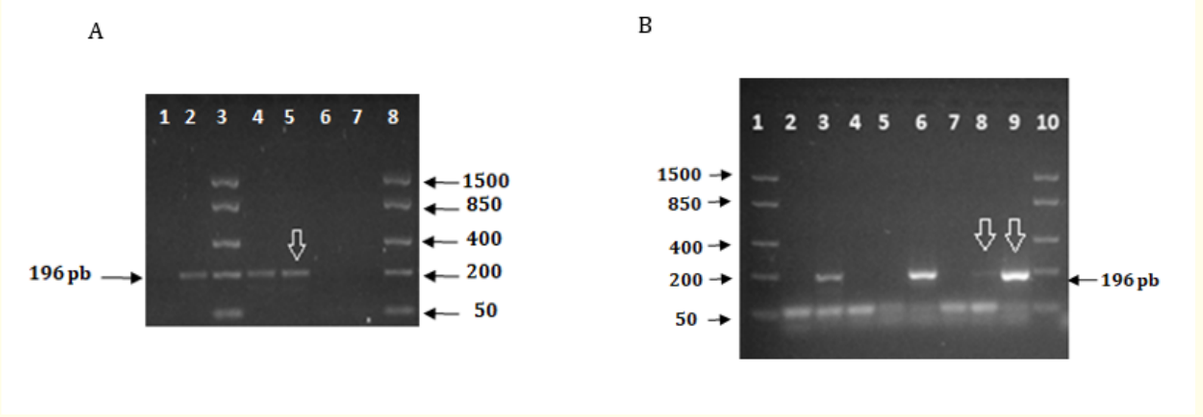

Figure 9: *T. gondii detection in chicken meat and pork experimentally infected. A. Chicken meat infected or not with tachyzoites (tach.). Lines: 1. Control mix; 2. Positive control (ADN from T. gondii tachyzoites); 3. 50 pb marker; 4, 5: chicken meat inoculated with 4,5 x 10^6 tach./mL and 1 x 10^6 tach./mL; 6 y 7: non-infected meat; 50 pb marker. B. Pork. Lines: 1. Pb marker. 2. Control mix; 3, 4, and 5. Pork non-infected meat; 6. positive control; 7, 8 y 9: infected pork with 1 x 10^2 tach./mL, 1 x 10^4 tach./mL and 1 x 10^6 tach./mL, respectively. Arrow shows the presence of an amplification in chicken meat inoculated with 1 x 10^6 tach./mL (9A), while in pork, this amplification occurs when the inoculum was 1 x 10^4 y 1 x 10^6 tach./mL (9B).*

Discussion

Studies on relation to *T. gondii* transmission in Costa Rica demonstrate that the ingestion of raw meat as well as some sausage, are important mechanisms in the epidemiology of toxoplasmosis both in Costa Rica [11,12], and in other countries [24]. In Costa Rica, there is veterinary inspection that evaluate some parasitological and bacteriological aspects. However, the diagnosis do not include microscopic parasites as *T. gondii, Sarcocystis* spp., *Neospora* spp. and *Trypanosoma cruzi* that can be transmitted by raw meat ingestion [31]. Since there is not any standard protocol for the detection of *T. gondii* in human consuming meats, we do not know the infection percentages and the potential risk of infection through the ingestion of these foods. Therefore, the molecular studies in this field are necessary in order to clear these aspects. DNA parasite detection by PCR is a relatively simple method that in addition, is sensitive, reproducible and low cost [16] as compared with the biologic model with mice that lasts between 3 to 6 weeks or cellular culture that takes 4 to 10 days to show the results [17]. Sensitivity and sensibility of this method depends on the DNA extraction technique, sample processing, nature of the chosen sequence and the parameters used in amplification reaction [18].

As proved by the analytic study with the two commercial kits, it was demonstrated that a better quality and less degraded DNA was obtained with the protocol II. It probably occurs because this method uses a silica column for DNA purification, while in the protocol I, this procedure is done by proteins precipitation with EDTA, a chelating agent. These results agree with those already reported by Dhaliwal and Carbajal [32], who demonstrated that the purification with silica columns gives a low quality.

In this study, we demonstrated that, independent of the matrix employed, standarized endpoint PCR presented a high sensitivity. In this sense, Burg., *et al.* [19] who designed a PCR for the detection of *T. gondii* gene *B1*, detected 1 parasite in a sample free of any other matrix, or 10 parasites in presence of 105 leukocytes utilizing a nested PCR. After this publication other authors [20], working with this procedure, obtained a sensitivity of 10 fg when *T. gondii* DNA alone was analyzed and of 1pg when 300 µL of blood was added. We were able to detect one fg of DNA, 10 times lower than that described by Ponce and Gomez [20], and similar to that reported by Burg., et al [19]. However, the sensitivity diminished when meat was used as the matrix. Although there are not reports related to this parameter, we consider that the sensitivity in these experiments (between 104 and 106 parasites) would permit to detect a *T. gondii* cyst; these cysts, according to their size can contain a similar number of bradyzoites as reported by Watts., *et al* [33].

The conditions applied in this work, let us to have a PCR more sensitive, similar to the nested PCR, lower spending in reagents and contamination risk in less time. Therefore, this PCR can be used for *T. gondii* DNA in meat and other products using the external primers of the nested PCR previously described [19].

The standardized PCR described herein was 100% specific, with a low probability of false positive results, which agree with studies of Ponce and Gomez [20], who found a high specificity of the nested PCR for Toxoplasma detection as compared with DNA of *Mycobacterium bovis, Taenia solium larvae, Plasmodium falciparum* and *Cryptococcus neoformans*. In the same sense Burg., *et al*. [19], demonstrated the specificity against *Sarcocystis, Neospora, Plasmodium, Aspergillus, Candida, Cryptococcus and Absidia* spp.

Conclusion

In conclusion, the PCR technique for diagnosis of toxoplasmosis means an important progress, specially, in those cases where the clinic and serologic exams are not clear. In addition, the results can be obtained in 24h [34]. On the other hand, molecular detection by means of endpoint PCR of T. gondii in food products open a possibility for molecular epidemiological studies of parasite transmission in Costa Rica. There are similar reports in other countries [16,21,22] for diagnosis of T. gondii in cerebrospinal fluid [20] or in amniotic fluid [35].

Acknowledgement

This work was partially supported by the Universidad de Ciencias Médicas (UCIMED). We thanks to Laura Valerio Campos, Jimmy Ramirez Monge and Jose Bolaños Jimenez for laboratory assistance and animal care.

Competing Interests

We declare that there is no financial interest or conflict of interest.

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