ORIGINAL ARTICLE

MOLECULAR IDENTIFICATION OF *Cysticercus bovis* AT DIFFERENT STAGES OF THE HOST-PARASITE INTERACTION PROCESS

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ABSTRACT

Bovine cysticercosis is considered the main epidemic link of human taeniasis caused by *Taenia saginata* and responsible for great damage in the whole productive chain of meat. The aim of this study was to verify the species-specific molecular identity of bovine cysticerci classified into four stages of the process of host-parasite interaction. The cysticerci were collected during the bovine's post mortem examination by the service of federal inspection in a slaughterhouse in the State of Goiás. Initially, the cysticerci were classified according to anatomopathological criteria. Subsequently, the PCR technique was applied using specific primers for the amplification of fragments of the *Cysticercus bovis* gene LSU RNAr. The results showed that PCR may be applied in the species-specific identification of bovine cysticerci. The assertiveness in its results was larger in DNA samples from cysticerci classified in VS (Vesicular Stage) and CVS (Colloidal Vesicular Stage) in relation to the assertiveness in the results of PCR from DNA samples from cysticerci classified in GNS (Granular Nodular Stage) and CNS (Calcified Nodular Stage). The negativity in the PCR results was larger in DNA samples from cysticerci located in the heart and classified in GNS than the negativity from samples of the same cysticerci stage located in the mastication muscles.

KEY WORDS: Cysticercosis. *Cysticercus bovis. Taenia saginata.* Taeniasis. PCR.

RESUMO

Identificação molecular de Cysticercus bovis em diferentes estágios do processo de interação parasito-hospedeiro

Introdução: A cisticercose bovina é considerada o principal elo epidemiológico da teníase humana provocada por *Taenia saginata* e ocasiona grande prejuízo em toda a cadeia produtiva da carne bovina. Objetivos: verificação espécie-especifica da identidade molecular de cisticercos bovinos classificados em quatro estágios do processo de interação parasito-hospedeiro. Métodos: os

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cisticercos foram coletados durante a inspeção post-mortem de animais sacrificados sob inspeção do serviço de inspeção federal num abatedouro do estado de Goiás. Inicialmente os cisticercos foram classificados de acordo com suas características anatomo-patológicas. Em seguida, a técnica de PCR foi aplicada utilizando-se primers específicos para amplificação do gene LSU RNAr de *Cysticercus bovis*. Resultados e conclusão: o PCR pode ser aplicado para a identificação espécie-especifica de cisticercos bovinos com assertividade maior ($p<0,05$) em amostras de cisticercos classificados no EV (estágio vesicular) e ECV (estágio coloidal vesicular) em relação à assertividade dos resultados de PCR de amostras de DNA de cisticercos nos EGN (estágio granular nodular) e ENC (estágio nodular calcificado). A negatividade dos resultados de PCR foi maior ($p<0,05$) nas amostras de DNA de cisticercos localizados no coração e classificados como EGN em relação à negatividade dos resultados de PCR de amostras de cisticercos do mesmo estágio e localizados em músculos masticatórios.

DESCRITORES: Cisticercose. *Cysticercus bovis. Taenia saginata.* Taeniasis. PCR.

INTRODUCTION

In Brazil, the anatomopathological diagnosis of bovine cysticercosis is performed by veterinarians working as sanitary inspectors on the Federal Inspection Service (SIF) during the *post-mortem* examination. They only evaluate the macroscopic characteristics and classify the cysticerci as "alive" (viable) or "calcified" (dead or nonviable) (Souza et al., 2007). However, through a thorough macroscopic and/or microscopic analysis the cysticerci may be classified into four different stages of the host-parasite interaction (Lino Júnior et al., 2002; Londoño et al., 2002). The optic microscopy still remains as the elective method for parasite's identification because it is based upon a simple and quick technique. However, several restrictions are linked to the use of optic microscopy to perform the parasitological diagnosis such as altered parasite morphology due to the host-parasite interaction or because the cysticercus may present a similar morphology to other species due to problems during the histological fixation of the parasite (Álvarez et al, 2002; Londoño et al., 2002).

As the polymerase chain reaction (PCR) technique may be performed independently from the integrity of the parasite's morphological structures, it has been frequently used in the identification of the species involved in the taeniases-cysticercosis complex and also in the exclusion of other etiological agents (van der Logt and Gottstein, 2000; González et al., 2004; Nunes et al., 2005; Abuseir et al., 2006; González et al., 2006, Jardim et al., 2006; Flütsch et al., 2008). The World Organization for Animal Health (OIE) recommends the use of PCR for *Taenia* species differentiation and suggests that this technique should be applied to the unmistaken identification of the metacestodes larvae (OIE, 2008).

This study aimed to verify the species-specific molecular identification through PCR of cysticerci isolated from bovines and to correlate this identification with the morphological characteristics of the cysticercotic lesions into the different stages of the host-parasite interaction.

MATERIAL AND METHODS

The collection of the cysticerci was performed in conformity to the routine of the Veterinarian Inspectors from the Federal Inspection Service (Serviço de Inspeção Federal -SIF) from May of 2005 to September of 2006. Therefore, 172 cysticerci were collected during the *post-mortem* examination of muscles and organs from 172 adult bovines from 55 cities of Goias State and slaughtered under the SIF inspection.

Based on the pre-established anatomopathological criteria (Lino Júnior et al., 2002; Londoño et al., 2002) to classify the cysticerci found both in humans and swine, the parasites collected in this study were characterized into four stages related to the bovine host-parasite interaction process: vesicular stage (VS) – transparent vesicular membrane containing translucent liquid with an apparent intact metacestode, colloidal-vesicular stage (CVS) – thicker vesicular membrane containing opaque liquid and an apparent compromised metacestode, granular nodular stage (GNS) – caseous aspect of the vesicular membrane and the metacestode is no longer visualized, and calcified nodular stage (CNS) – the whole cysticerci is a compact nodule with mineralized content. Each of the collected cysticerci was divided into two pieces, one was fixed in 10% formaldehyde solution for paraffin inclusion and staining. The second half had the content of the cysticercotic lesion removed and frozen at -70ºC. The segments that suffered the paraffin inclusion were sliced at 5mm width and stained by the Hematoxilin-Eosin (HE) technique to confirm the classification of the cysticerci into one of the four stages of the host-parasite interaction process.

For the DNA extraction of the cysticerci samples, a phenyl-chloroform protocol was used as described by Van Soolingen et al. (1991). The eluate was submitted to 1% agarose gel and the concentration and integrity of the extracted DNA was visually determined. The molecular mass marker used was λ DNA-*Hind* III (Amershan Biosciences®). Subsequently, DNA amplification PCR was performed using the species-specific oligonucleotides for *Cysticercus bovis* detection of LSU RNAr as described by Jardim et al. (2006).

To determine the specificity of the PCR assays the DNAt from *Homo sapiens, Bos taurus, Sus scrofa, Taenia hydatigena, Taenia taeniaeformis, Hymenolepis diminuta, Anoplocephala magna, Paranoplocephala mamillana* and *Moniezia expansa* were used. As positive control we used the DNAt extracted from proglottids from *T. saginata* and *T. solium*. As negative control we used ultrapure DNA free water. Aiming the molecular identification of the PCR products (amplicons) we proceeded to their sequencing (Megabace 1000 – Amersham Biotech). The obtained sequences were compared to the ones registered in GenBank (access number AB020396 for *T. saginata* and number AB020398 for *T. solium)*."

The concentration of DNAt from the *T. saginata* and *T. solium* positive controls was determined by spectrophotometry (GeneQuant pro RNA/DNA calculator, Amersham Pharmacia Biotech) and by a visual estimative of the 1% agarose gel using as a reference the molecular mass marker λ DNA-Hind III (Amershan Biosciences®). We determined a concentration of 20ng/µl for *T. saginata* DNA and 100ng/µl for *T. solium* DNA. From these samples we performed a serial dilution in a 1:10 scale to determine the PCR sensitivity threshold.

The chi-square (Ξ^2) test was used to evaluate the correlation and distribution of the PCR results (positivity and negativity) in the molecular identification of the cysticerci that were classified in different stages of the host-parasite interaction process as well as to their anatomical location in the carcasses (Sampaio, 1998).

RESULTS

It was possible to collect 172 cysticerci of which 48.3% (n=83) were located in the masticatory muscles, 44.8% (n=77) in the cardiac muscles, 2.9% (n=5) in the liver, 1.7% (n=3) in the esophagus, 1.2% (n=2) in the diaphragm pillar, and 1.2% (n=2) in the tongue.

In the cysticerci classified in the VS, the cystic membrane and the vesicular fluid were transparent and the metacestode presented a white coloration (Table 1, Figure 1A, 3A). In the cysticerci classified in the CVS, the opaque vesicular fluid and the yellowish metacestode inside the vesicle were observed (Figure 1B, 3B). In the cysticerci classified in the GNS, the cysticercotic lesion was surrounded by a white-yellowish tissue and the internal area of the vesicle was filled with caseous material (Figure 1C, 3C). Finally, in the cysticerci classified in the CNS the lesion was surrounded by a whitish tissue which reminded of collagen and the internal area of the vesicle was filled with compact structures similar to calcified granules (Figure 1D, 3D).

Table 1. Relative frequency of the general pathological processes found in cysticerci classified into the four stages of the host-parasite interaction process.

	Pathological alterations				
Stages of the host-parasite interaction process	Chronic inflammation	Eosinophils	Necrosis	Fibrosis	Calcification
VS	100%	26,27%	0%	100%	0%
CVS	100%	17,24%	6,90%	100%	0%
GNS	100%	11,79%	35,29%	100%	26,47%
CNS	100%	69,23%	61,54%	100%	100%

Legend: VS – vesicular stage; CVS – colloidal-vesicular stage; GNS – granular nodular stage; CNS – calcified nodular stage

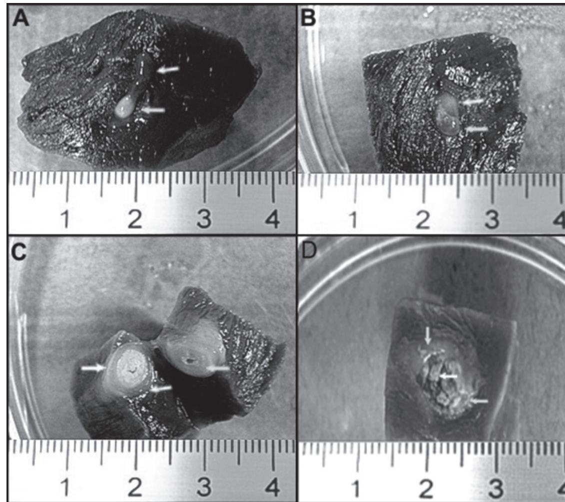

Figure 1. Macroscopic aspects of bovine cysticerci classified into four stages of the host-parasite interaction process. A. Vesicular stage with transparent cystic membrane and vesicular fluid (arrow) containing a whitish metacestode within the lower region of the vesicle (arrow). B. Colloidal-vesicular stage with an opaque cystic fluid (arrow) and yellowish metacestode within the lower region of the vesicle (arrow). C. Nodular granular stage with the cysticercotic lesion surrounded by a whitish tissue (arrows) almost totally filled with an amorphous yellowish substance. D. Calcified nodular stage with the cysticercotic lesion surrounded by a whithish tissue (arrows) and completely filled with compact and amorphous structures (arrow).

From the 172 samples submitted to PCR, 145 were positive (84.3%) and 27 were negative (15.7%). To verify if the histopathological lesions due to the host-parasite interaction process influenced or not the PCR results it was necessary to analyze the results obtained from the classification of the cysticerci into one of the four different stages. From this analysis we observed that 43 cysticerci were at the VS and from these 42 (97.67%) were PCR positive and 1 (2.3%) was negative. Another 43 cysticerci were classified as CVS from which 39 (90.7%) were PCR positive and 4 (9.3%) were negative, 43 cysticerci were classified as GNS and from these 32 (74.4%) were PCR positive and 11 (25.6%) were negative while from the 43 cysticerci classified as CNS, 32 (74.4%) were positive and 11 (25.6%) were negative.

When analyzing the macroscopic characteristics of the cysticerci and the PCR results, it was observed that the positivity of the PCR was significantly higher in the VS and CVS cysticerci (94.2%) ($p<0.05$) and significantly lower in the GNS and CNS cysticerci (74.4%) ($p< 0.05$).

The microscopic analysis and classification of the cysticerci was performed according to the histopathological quality of the samples, 86 slides were analyzed. Of these, 12 were classified as VS and had 100% PCR positivity, 21 were classified as CVS and had 95.2% (n=20) PCR positivity, 29 were classified as GNS and had 75.9% (n=22) PCR positivity, and 24 were classified as CNS and had 79.2% (n=19) PCR positivity. The analysis of the microscopic classification and the PCR results, showed that the positivity of the PCR results was significantly higher in the VS and CVS cysticerci (97%) ($p<0.05$) and significantly lower in the GNS and CNS cysticerci (79.2%) ($p< 0.05$) (Figure 2).

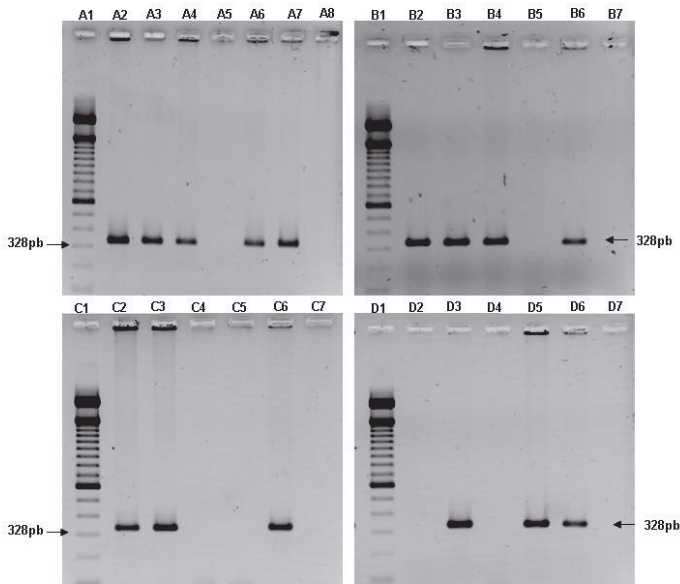

Figure 2. Specific PCR amplification of genic fragments of 328pb of LSU RNAr gene of *Cysticercus bovis* from cysticerci from the following stages of the host-parasite interaction: Vesicular stage: A1. molecular marker of 100bp, A2, A3, A4 and A6 were PCR positives, A5 was negative, A7. positive control, A8. negative control; Colloidal vesicular stage: B1. molecular marker of 100bp, B2, B3 and B4 were PCR positive, B5. PCR negative, B6. positive control and B7. negative control. Granular nodular stage: C1. molecular marker of 100bp, C2 and C3 were PCR positive, C4 and C5 were PCR negative, C6. positive control, C7. negative control. Calcified nodular stage: D1. molecular marker of 100bp, D3 and D5 were PCR positive, D2 and D4 were negative, D6. positive control, D7. negative control.

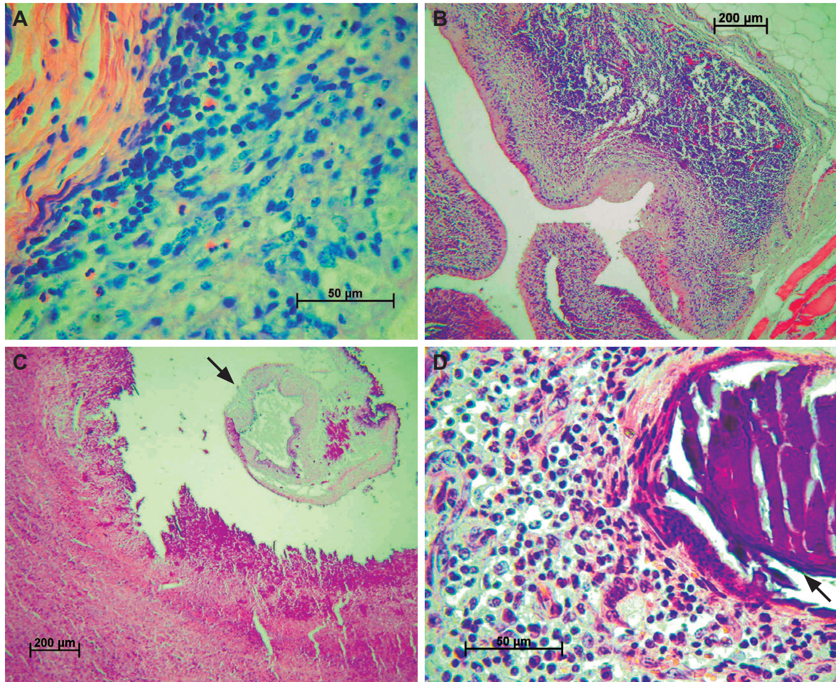

Figure 3. (A) Photomicrograph of a VS cysticercus, surrounding the parasite it is possible to observe fibrosis and an intense inflammatory infiltration composed by macrophages, lymphocytes and eosinophils (Giemsa, scale 50µm). (B) Photomicrograph of a CVS cysticercus, it is possible to observe in the host tissue an intense inflammatory infiltration and fibrosis surrounding the parasite (HE, scale 200µm). (C) Photomicrograph of a GNS cysticercus, it is possible to observe morphological alterations of the metacestode (arrow) and host tissue with inflammation and fibrosis (HE, scale 200µm). (D) Photomicrograph of a CNS cysticercus, it is possible to observe calcification through out the parasite (arrow) and an intense inflammatory reaction in the surrounding tissue (HE, scale 50µm).

In relation to the anatomical location of the cysticerci in the carcasses, from the 86 that were microscopically analyzed, 37 (44.6%) were located on the masticatory muscles and 46 (55.4%) were located on the cardiac muscles. Thirty-four (92%) of the cysticerci from the masticatory muscles were PCR positive and 3 (8.1%) were negative, in the cardiac muscles 36 (78.3%) were positive and 10 (21.7%) were negative. It was observed that the PCR negativity was significantly higher ($p<0.05$) in the cysticerci located on the cardiac muscles than in the masticatory ones. In addition, from the 34 PCR positive cysticerci located on the masticatory muscles,

5 (14.7%) were classified as VS, 8 (23.5%) as CVS, 14 (41.2%) as GNS, and 7 (20.6%) as CNS. From the 3 PCR negative cysticerci from the same location, none was at the VS, 1 (33.3%) was at the CVS, 1 (33.3%) at the GNS and 1 (33.3%) at the CNS. Furthermore, 7 (19.4%) cysticerci from the cardiac muscles location and PCR positive were classified as VS, 10 (27.8%) as CVS, 7 (19.4%) as GNS, and 12 (33.3%) as CNS. From the 10 PCR negative cysticerci from the same location, none was classified as VS nor as CVS, 6 (60%) were at the GNS, and 4 (40%) at the CNS.

It was observed significant negativity ($p<0.05$) of the PCR for the cysticerci located on the cardiac muscles and classified as GNS (60%) when compared to the negativity from the cysticerci located on the masticatory muscles and classified as the same stage of the bovine host-parasite interaction process (33.3%).

DISCUSSION

This study correlated the molecular identification of *C. bovis* from bovine carcasses with the macroscopic and microscopic classification into four stages of the host-parasite interaction process.

As to the location of the collected cysticerci our results are in accordance to Fernandes and Buzetti (2001), Moreira et al. (2001), Almeida et al. (2002), Moreira et al. (2002) and Souza et al. (2007) which also found more than 90% of the cysticerci located both on the masticatory and cardiac muscles from the inspected bovine carcasses. However the frequency of the location of the cysticerci found in our study is different from the location reported by Minozzo et al., (2002) and Nunes et al., (2005) which submitted bovines to experimental infection of *C. bovis* and after a rigorous inspection of the organs found that other muscle groups that are not routinely inspected were also infected such as skeleton and thoracic and pelvic muscles.

The macroscopic characteristics observed in the bovine cysticerci were similar to the ones described in the human cysticerci by Escobar-Izquierdo (1988), Del Brutto (1999) and Lino Júnior et al. (2002), and in swine cysticerci by Alvarez et al. (2002) and Londoño et al. (2002). However it was not possible to find in the literature this classification being used for bovine cysticerci analysis.

As the histopathological lesions occurred with greater intensity in GNS and CNS classified cysticerci we suppose that this process may have influenced the presence of *C. bovis* DNA in the analyzed cysticerci. However, it is important to highlight the negative PCR results from VS and CVS classified cysticerci because according to the adopted classification criteria the metacestodes were visualized inside those cysticercotic lesions. Abuseir et al., (2006) also found that the host-parasite interaction process may influence the parasite's DNA detection which is in accordance to this study results. González et al., (2006) and Chiesa et al., (2010) reported other parasites that may cause macroscopic lesions that may be confused with *C. bovis*, such as *Taenia hydatigena* and *Sarcocystis* spp. Maybe the negative results found in our study were related to those other parasites.

The macroscopic classification of the cysticerci is not as reliable as the microscopic analysis of the host-parasite interaction process which allows the visualization of the morphologic characteristics of the parasite and the consequent reaction to its presence by the host tissue. However, as it was possible to detect the parasite's DNA from cysticerci classified into all four stages of the host-parasite interaction process, we suppose that other variable may have influenced the detection of the parasite's DNA from the PCR negative samples. The same hypothesis, i.e., the epidemiological possibility of the presence of another etiological agent in those bovine cysticercotic lesions, was proposed by Gibson (1959), Biondi et al. (2000), Gusso et al. (2000), Minozzo et al. (2002) and Abuseir et al. (2006).

As to the greater PCR negativity in the cardiac muscles located cysticerci, similar results were observed by Sterba and Dyková (1978), Sterba et al. (1979), Blazek et al. (1986) and van der Logt and Gottstein (2000). In addition, it is possible that the cardiac tissue may react with a differentiated inflammatory response to the infection due to *C. bovis* which may alter the course of the bovine host-parasite interaction process when facing cysticercotic lesions.

In conclusion, the cysticerci were mainly found at the masticatory and cardiac muscles which indicate the *C. bovis* preference to this location. From the study of the cysticerci in relation to their macroscopic and microscopic characteristics and the *C. bovis* PCR positivity we conclude that this technique may be applied to the molecular identification of the bovine cysticercosis. Besides, this technique may be used in the molecular identification of cysticerci classified into all four stages of the host-parasite interaction process. Also, the PCR positivity was higher in the DNA samples from VS and CVS classified cysticerci and the PCR negativity was higher in the DNA samples from cardiac muscles cysticerci classified as GNS. Therefore, molecular studies correlating macroscopic and microscopic analysis of cysticerci may improve the identification of the bovine cysticercosis and the carcasses inspection process.

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