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Original Article

Development of a Biomolecular Assay for Postmortem Diagnosis of *Taenia saginata* Cysticercosis

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Abstract

Bovine cysticercosis is caused by the larval stage of the human tapeworm *Taenia saginata.* According to European data on meat inspection, the prevalence ranges from 0.007% to 6.8%, but the real prevalence is considered to be at least 10 times higher. Laboratory confirmation of the etiological agent is based on gross, stereomicroscopic, and histological examination of submitted specimens. False identifications may occur, possibly because of death and degeneration of cysts, or because taeniid larvae and other tissue parasites, such as *Sarcocystis* spp., may cause similar macroscopic morphological lesions. Therefore, tests that can warrant sure identification of taeniid lesions and calcified cysts in the muscle are needed. The focus of our study was to develop a suitable postmortem test that could be applied on putative lesions by *T. saginata* cysticerci, as ambiguously diagnosed after routine meat inspection. In particular, we proposed a biomolecular assay targeting the mitochondrial cytochrome c oxidase subunit I gene (*COI*). For developing the polymerase chain reaction assay, viable cysts of Cysticercus bovis ($n = 10$) were used as positive reference samples, and those of *Echinococcus granulosus* ($n = 3$), Cysticercus tenuicollis ($n = 3$), and *Sarcocystis spp.* ($n = 4$) as reference negative controls. Further, to evaluate the applicability of the proposed assay, 171 samples of bovine muscular tissue, obtained from local slaughterhouses and containing lesions recognized as *T. saginata* cysticerci by macroscopic examination, were tested. The proposed test confirmed the diagnosis at postmortem inspection in 94.7% (162/171) of samples. In conclusion, the assay developed in this study, amplifying a short fragment from the mitochondrial gene *COI*, showed to be suitable for samples containing both viable and degenerating *T. saginata* cysticerci, yielding an unequivocal diagnosis.

Introduction

Bovine cysticercosis is caused by the larval stage (metacestode) of the human tapeworm *Taenia saginata*, formerly defined as Cysticercus bovis. Intermediate and final hosts are represented by cattle and humans, respectively. Bovine cysticercosis is caused by the infestation and development of cysticerci of *T. saginata* in the muscle tissue of cattle (Pawlowski and Schultz, 1972; Murrell and Dorny, 2005). The eggs reach the animals through contact with contaminated materials or proglottids; after ingestion, the oncospheres can penetrate the intestinal wall and travel via blood to striated muscles (Abuseir *et al*., 2006). Cysts are prevalently found in predilection sites such as the masseter muscles, heart, tongue, and the muscles of the shoulder and diaphragm, although they could also be found in other sites and organs (Minozzo *et al*., 2002; Wilson and Wilson, 2005). The longevity of the cysts ranges from weeks to years (Urquhart *et al*., 1998), and after their death they are usually replaced by a caseous, crumby mass, which may become calcified (McGavin *et al*., 2001). Both living and dead cysts may be found in the same carcass (Gracey *et al*., 1999).

Life cycle is completed with infestation in humans, which may develop taeniosis after consumption of raw or undercooked beef containing viable *T. saginata* metacestodes (Dorny *et al*., 2000). As taeniosis is not a notifiable disease, the incidence is usually estimated from the sale of specific drugs, and prevalence rates ranging from 0.01% to 10% have been reported in Europe (Cabaret *et al*., 2002). Regarding bovine cysticercosis, the prevalence ranges from 0.007% to 6.8% according to European meat inspection data (Pawlowski and Schultz, 1972; Cabaret *et al*., 2002), but the real prevalence is considered to be at least 10 times higher (Onyango-Abuje *et al*., 1996; Dorny *et al*., 2000).

According to the Regulation (EC) No. 854/2004, to determine and, if necessary, assess the level of cysticercosis infestation, all bovines of over 6 weeks of age have to be inspected by incision and visual examination of the masseter muscles

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and heart. In the case of generalized infestation, the carcass and offal are declared unfit for human consumption but, if the infestation is localized, the parts not infected may be declared fit for human consumption after having undergone a cold treatment (Regulation (EC) No. 854/2004).

During the routine meat inspection, the diagnosis of *T. saginata* cysts is based on the morphological appearance. Any alteration with identifiable cyst and fluid filled, cheesy, or calcified content found in heart or masseter muscles of slaughtered cattle is assumed to be *T. saginata* (Murrell and Dorny, 2005). Laboratory confirmation of the etiological agent is based on gross, stereomicroscopic, and histological examination of submitted specimens (Ogunremi *et al*., 2004). False identifications may occur, possibly because of death and degeneration of cysts, or because taeniid larvae and other tissue parasites, such as *Sarcocystis* spp., may cause similar macroscopic morphological lesions. Therefore, tests that can warrant sure identification of taeniid lesions and calcified cysts in the muscle are needed (Harrison *et al*., 2005).

Different improved postmortem diagnostic methods have been proposed, such as ELISA methods, detecting antigens in meat juice (Abuseir *et al*., 2007), immunohistochemical techniques, using the avidin–biotin complex immunohistochemical method (Ogunremi *et al*., 2004), and biomolecular assays (Gottstein *et al*., 1991; Abuseir *et al*., 2006; Geysen *et al*., 2007).

DNA-sequence-based primers for the diagnosis of *T. saginata* have been designed mainly to distinguish between taeniosis caused by *T. saginata* or by *Taenia solium*, also considering the severity of *T. solium* infections in humans. The use of polymerase chain reaction (PCR) to confirm or reject the morphological diagnosis of *T. saginata* metacestodes has been described, but an adequate evaluation of the reliability of these used methods is still lacking (SCVPH, 2000; van der Logt and Gottstein, 2000; Geysen *et al*., 2007).

The focus of our study was to develop an appropriate postmortem test that could be applied on lesions suspected to be *T. saginata* cysticerci, ambiguously diagnosed after routine meat inspection. In particular, a biomolecular assay targeting the mitochondrial cytochrome c oxidase subunit I gene (*COI*) was developed.

Materials and Methods

Samples

For the development of the PCR assay, 10 viable cysts of *T. saginata* metacestodes were used as positive reference samples. Reference negative controls were represented by three samples of *Echinococcus granulosus*, three of Cysticercus tenuicollis, and four of *Sarcocystis* spp. To evaluate the applicability of the proposed assay, 171 samples of bovine muscular tissue, containing *T. saginata* cysticerci diagnosed by macroscopic examination, were tested. In particular, these samples, consisting of portions of miocardic and masseter muscles, were collected from local slaughterhouses during routine meat inspection procedures from March 2008 to July 2009. As indicated by Abuseir *et al.* (2006), cysts were examinated macroscopically and classified as (1) viable when they contained protoscolex, (2) calcified with solid, crumby content, cheesy with soft content, and (3) dull when they appeared neither viable nor degenerating and no obvious cyst structure (wall and subsequent layers) was macroscopically detectable.

DNA extraction

For each sample, up to 25 (±5) mg of material was collected from a single excised cyst; effort was made to collect only the content of the cyst, avoiding the cyst wall to reduce the amount of nontarget bovine DNA. When viable cysts were analyzed, only protoscolex was taken.

DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) was used for DNA extraction from all samples. Some minor modification in the protocols consisted of an overnight incubation with Proteinase K and a decrease in the volume of the final elution step (100 μL).

Extracted DNA was quantified with a NanoDrop 2000 spectrophotometer (ThermoScientific, Wilmington, NC).

Primers

For the specific amplification of *T. saginata*, the forward primer (Tsag) designed by Yamasaki *et al.* (2004) was used in conjunction with a newly designed reverse primer.

For this purpose, the *COI* gene sequences of several *T. saginata*, available in the GenBank (AB066495, AB107237–AB107247, AB271695, AB275143, AB465231–AB465248, and AY195858), were aligned using ClustalV software (Higgins *et al*., 1992). Moreover, the sequences of other Taeniidae species (*T. solium*, *Taenia hydatigena*, *Taenia crassiceps*, and *E. granulosus*) were included in the alignment to evaluate possible cross reactions.

Reverse *COI* primer was designed as follows: 5′-ACGT AAATAAATAAGCCCACAATATT-3′, matching positions 574–549 of *T. saginata* sequence (GenBank accession no. AB465248).

Finally, based on the previously produced alignment, a set of internal primers were designed to develop a nested PCR approach. Nested *COI* primers were designed as follows: 5′-GGGTGCTGGTATAGGGTGGACT-3′ (forward) and 5′-ATTAATAGAACTAAAAATTCTAGACG-3′ (reverse), corresponding to positions 363–384 and 492–467 of *T. saginata* (GenBank accession no. AB465248), respectively (Fig. 1).

For all the samples that showed negative result with the PCR assays described above, further tests were carried out to verify (1) positivity for *Sarcocystis* spp. with genus-specific primers (Vangeel *et al*., 2007); (2) misdiagnosis with the larval stage of cestodes other than *T. saginata*, using specific primer sets for Taeniidae (von Nickisch-Rosenegk *et al*., 1999); and (3) the presence of detectable DNA and the absence of PCR inhibitors, by means of bovine-specific primers (Bottero *et al*., 2003).

The primers were synthesized by Sigma Aldrich (St. Louis, MO).

PCR procedure

Both primers for the *COI*-PCR and the nested PCR were carried out in a final volume of 25 μL containing 20 mM Tris–HCl (pH 8.4); 50 mM KCl; 0.5 U of recombinant Taq DNA polymerase (Invitrogen, Paisley, United Kingdom); 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Invitrogen); 2 mM di $MgCl_2$; and 12.5 pmol of primers. DNA was added with a concentration of 100 ng/μL for the *COI*-PCR, and 2.5 μL of amplification product for the nested PCR.

COI-PCR amplification was performed with an initial step of 94°C for 5 min, followed by 20 cycles of 94°C for 30 sec, with annealing temperatures starting at 65°C for 1.30 min

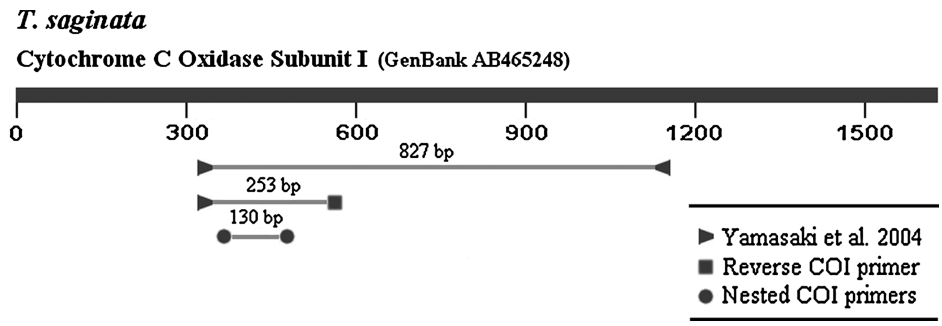

FIG. 1. Polymerase chain reaction strategy outline.

(decreasing 0.5°C/cycle) and 72°C for 30 sec for extension. This step was followed by 20 cycles of 94°C for 30 sec, 55°C for 1.30 min, 72°C for 30 sec, and finally 72°C for 5 min.

Nested PCR conditions consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycle of 94°C for 30 sec, 1.30 min annealing at 54°C, and 30 sec extension at 72°C. The final extension was carried out at 72°C for 5 min.

Bovine, *Sarcocystis* spp., and taeniid species-specific PCR assays were performed following the author instructions (von Nickisch-Rosenegk *et al*., 1999; Bottero *et al*., 2003; Vangeel *et al*., 2007).

Amplimers were resolved by electrophoresis on a 2% agarose gel (Invitrogen), run in Tris–acetate–ethylenediaminetetraacetic acid buffer for 70 min at 110 V, and stained with ethidium bromide (0.4 ng/mL) for 20 min.

Confirmatory sequencing of the *COI* and nested-PCR-amplified fragments was carried out for all samples. Amplified products were purified with Exo-Sap treatment according to the manufacturer's recommendations (USB Europe, Staufen, Germany). Forward and reverse sequencing reactions were performed using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1 (Applied Biosystems, Foster City, CA). Sequenced fragments were purified by DyeEX (Qiagen) and resolved by capillary electrophoresis using an ABI 310 Genetic Analyser (Applied Biosystems). The nucleotide sequences were analyzed using the BLASTN sequence similarity search at the NCBI database (Altschul *et al*., 1990).

Results and Discussion

Several different diagnostic tests have been described to support visual diagnosis of *T. saginata* cysticercosis. Serological methods, such as detecting circulating antigens or antibodies, both in live and slaughtered animals (Dorny *et al*., 2000; Abuseir *et al*., 2007) have been studied. However, this technique failed to detect many light infections (Van Kerckhoven *et al*., 1998), and the possibility of cross reaction with other parasites indicates the need for further investigation (Brandt *et al*., 1992). Also, an immunohistochemical method has been proposed as a promising alternative by Ogunremi *et al.* (2004), but results show that cross reaction with other taeniid species may occur.

Therefore, histological evaluation of *T. saginata* cysticerci-suspect lesions is still considered the only laboratory diagnostic tool suitable for routine confirmation of undiagnosed lesions during inspection procedures at slaughterhouse. The morphology and cellularity of these putative lesions can be used to distinguish them from other commonly encountered and somewhat similar lesions. Demonstration of a cysticercus-specific host structure is therefore necessary for a definitive diagnosis (Silverman and Hulland, 1961), but an accurate identification cannot always be made, as such features are often not obvious in degenerated specimens and especially if the sample contains calcified tissues (Schandevyl and Vercruysse, 1982).

Molecular diagnosis has therefore been considered, considering the high sensitivity, objectivity, and rapidity of most molecular tools. Protocols using multiplex PCR for the molecular differentiation of adult *T. saginata*, *T. saginata asiatica*, and *T. solium* have been developed and successfully applied (Gonzalez *et al*., 2000; González *et al*., 2004). However, these same primers yielded unreliable results on both viable and degenerated bovine cysticerci when tested on naturally infected cattle (Abuseir *et al*., 2006).

Geysen *et al.* (2007) designed a PCR assay, targeting mitochondrial DNA, that was able to amplify the expected fragment (846 bp) in 97% of viable cysts, but only in 60% of calcified specimens. These results have been partially explained by authors with the lacking of amplifiable DNA in positive but degenerated specimens.

The onset of degeneration in mature cysts depends on the inflammatory response of the host but generally consists of the breakdown of the cyst wall and cuticle, followed by the disappearance of following layers and eventually in the dissolution of parasite remnants (Silverman and Hulland, 1961). Detectable DNA is therefore likely to be present in a degraded form and in low amounts. Several studies suggested that degraded DNA can still be amplified by PCR if the primers are designed so to recognize and amplify a shorter target sequence, between 100 and 300 bp, preferably of mitochondrial genes (Dalmasso *et al*., 2004; Hui, 2006). In fact, the circular structure of mtDNA has been shown to have a superior preservation to degradation, and because of its presence in multicopy, detection of target DNA is possible even from a small amount of starting material (Fondevila *et al*., 2008).

On the basis of these considerations, a primer set targeting the mitochondrial *COI* gene was chosen, based on the literature (Yamasaki *et al*., 2004). The *COI* gene has been particularly targeted for phylogenetic studies, and thus several sequences are available in nucleotide databases. In particular, in this study a new reverse primer was designed to amplify a shorter fragment.

Specificity tests were conducted and all positive reference samples yielded amplicons of the expected size (253 bp). In addition, no cross reaction was observed with samples of phylogenetically correlated species that could affect cattle,

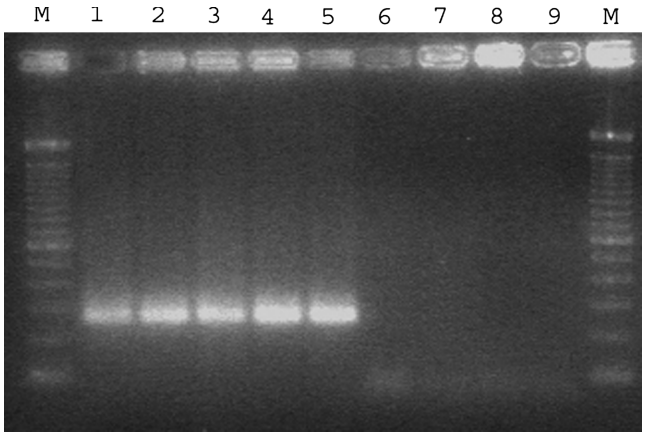

FIG. 2. Specificity of polymerase chain reaction amplification of the *COI* gene of *Taenia saginata* cysticerci in beef. Lanes 1–5, DNA extracted from viable *T. saginata* cysticerci; lane 6, *Echinococcus granulosus*; lane 7, *Taenia hydatigena*; lane 8, *Sarcocystis* spp.; lane 9, reagents control; M, 100-bp ladder.

such as *E. granulosus* and *T. hydatigena* (Euzéby, 1998), and with samples of a coccidian protozoa, such as *Sarcocystis* spp., that can cause muscular lesions easily misidentified as *T. saginata* cysticerci (González *et al*., 2006) (Fig. 2).

Further, the specificity of the amplified fragments was confirmed when nucleotide sequences of all reference strains were submitted to BLASTN sequence similarity search (Altschul *et al*., 1990).

As regard to assay applicability, the number of samples that yielded a positive result to *COI*-PCR was 154 out of 171 (Table 1). When different stages of degeneration were taken in account, differences were observed as resulted positive 11 out of 12 viable cysts, 42 out of 45 cheesy cysts, 93 out of 101 calcified cysts, and 8 out 13 dull cysts, thus confirming greater difficulties in identification when degraded samples were tested (Abuseir *et al*., 2006; Geysen *et al*., 2007).

To improve the sensitivity of the assay, as suggested by previous studies (Yamasaki *et al*., 2006; Geysen *et al*., 2007), we designed two internal primers on the amplified *COI* and developed a nested approach, which has been used to test negative samples. The nested PCR approach allowed the identification of 8 more samples as positive among the 17 formerly tested as negative. Results have been confirmed by sequencing of specific fragments. For the remaining nine negative samples, seven resulted positives to the specific PCR for the identification of *Sarcocystis* spp., confirming the possibility of misdiagnosis between degenerating *T. saginata* cysticerci and lesions caused by *Sarcocystis* spp. (González *et al*., 2006).

Table 1. Results of Applicability Test

Cyst stage	*No. of samples*	*Positive samples*		
		COI-*PCR*	*Nested PCR*	Sarcocystis *spp.*
Viable	12	11	1	0
Cheesy	45	42	2	1
Calcified	101	93	4	4
Dull	13	8	1	2[a]
Total	171	154	8	7

[a]Two samples negative to all the test performed were positive for the presence of bovine DNA, demonstrating the absence of PCR inhibition.

PCR, polymerase chain reaction.

The two remaining negative samples also gave negative results when further analyzed with a specific PCR for taeniid species, and used to determine if the misdiagnosed lesions could be ascribed to the presence of erratic cyst belonging to species other than *T. saginata* (Euzéby, 1998). These two samples were subsequently tested with a PCR targeting bovine DNA, and gave positive results, demonstrating the presence of detectable DNA and the absence of PCR inhibitors (van der Logt and Gottstein, 2000; Abuseir *et al*., 2006). These findings suggest that nonspecific lesions can be possibly misdiagnosed as parasitic (Pawlowski and Schultz, 1972; Gracey *et al*., 1999; van der Logt and Gottstein, 2000).

Overall, the proposed test was able to confirm the diagnosis made at postmortem inspection in 94.7% (162/171) of samples, and when considering degenerating specimens, in 94.3% (150/159) of samples. These findings underline the robustness of the developed test and showed its suitability for the identification of degraded cysticerci, which proved to be the main issue to overcome (Abuseir *et al*., 2006; Geysen *et al*., 2007).

Meat inspection of bovines is the only public health measure performed to prevent *T. saginata* from entering the food chain (Dorny *et al*., 2000). The main shortcomings of routine meat inspection may be found in its low sensitivity and that its success heavily depends on the expertise of the inspector. Moreover, the stage of development of the cyst has to be taken into careful consideration because it is difficult to distinguish between old lesions caused by cysticerci and other lesions (Geysen *et al*., 2007).

The assay developed in this study, amplifying a short fragment from the mitochondrial *COI* gene, yielded an unequivocal diagnosis, showing to be species specific and suitable both for samples containing viable and degenerating *T. saginata* cysticerci.

Rapidity and objectivity in the interpretation of results prove this molecular assay to be a useful, high-throughput laboratory tool for the confirmation of macroscopic diagnosis of ambiguous lesions.

Further studies, comparing the proposed method with histological and immunohistochemical techniques, would be of great importance to assess the full performance potential of the developed *COI*-PCR for the identification of *T. saginata* cysticerci, assessing the actual sensitivity and specificity of the assay.

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Disclosure Statement

No competing financial interests exist.

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