Validation of Meat Inspection Results for *Taenia saginata* Cysticercosis by PCR–Restriction Fragment Length Polymorphism

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ABSTRACT

Bovine cysticercosis is a zoonotic disease caused by the larval stage (cysticercus) of the human tapeworm *Taenia saginata*. Infected cattle is an important food safety issue besides an economic concern. Humans get infected by eating raw or undercooked meat containing viable cysticerci. Visual meat inspection of bovines is the only public health measure implemented to control transmission to humans, but it lacks sensitivity and objectivity. It may underestimate the prevalence of the disease by a factor 3 to 10. Furthermore, the success of the method depends on the expertise of the meat inspector as well as on the stage of development of the cysticerci. The focus of this study was to develop and explore the usefulness of a PCR assay as an objective alternative to evaluate the meat inspector’s visual inspection results. Hereto, a PCR was developed for the detection of *T. saginata* DNA in muscle lesions. Based on the laboratory classification of lesions, almost 97% of viable cysts were confirmed by PCR, while for dead cysts, the percentage was approximately 73%. Taken together, these data demonstrate the difficulties of visual meat inspection and their objective interpretation, emphasizing the need to improve current assays to strengthen the control of bovine cysticercosis.

Bovine cysticercosis is a zoonotic disease caused by the larval stage of the human tapeworm *Taenia saginata*. It is a disease notifiable to the World Organization for Animal Health (OIE; former B-list). Infection of cattle with the larval stage is an important food safety issue and of economic concern in different areas of the world. Humans get infected by eating raw or undercooked meat containing viable cysticerci, and transmission to animals occurs upon contamination of food or water by feces of infected humans.

Meat inspection of bovines is the only public health measure implemented to prevent *T. saginata* transmission to people. Based on abattoir surveys, the prevalence of cysticercosis varies in the European Union between 0.01 and 6.8% (2, 7). However, routinely used inspection methods are assumed to detect only relatively heavily infected animals and underestimate the prevalence by a factor 3 to 10 (7). Moreover, in recent years, the prevalence of bovine cysticercosis seems to increase in the European Union, including Belgium, where an increase from 0.27% in 1997 to 0.68% in 2003 has been reported by the Federal Agency for the Safety of the Food Chain (FASFC). These observations, together with the recent extension of the European Community to include countries with a higher prevalence of bovine cysticercosis (7, 11) and the observation that in some abattoirs no cysticercosis at all is detected, has driven national and European authorities to investigate possible measures to strengthen the control of bovine cysticercosis.

The main drawback of routine meat inspection is its lack of sensitivity and objectivity. The procedure is restricted to inspection of predilection sites (heart, masseter muscles, diaphragm, tongue, and oesophagus) in all bovines older than 6 months (6). The success of this method is highly dependent on the expertise of the inspector as well as on the stage of development of the cysts.

A generalized infection of the carcass is declared unfit for human consumption; however, lightly infected carcasses are not condemned but provided long-term storage at low temperatures (–10°C for 10 days) (18). This procedure may greatly affect the value of the meat and hence the profit of the owner (3).

These economical losses, together with the assumption that a diagnosis is not entirely objective, often questions the decision of the meat inspector. Therefore, an objective test to underpin the observations of the meat inspectors is needed. Different immunodiagnostic tests, such as the anti-mortem detection of circulating antibodies or antigens are reported to be 3 to 10 times more sensitive than the visual inspection (8, 9, 14, 15). However, these assays are only suitable for the detection of viable cysticerci (4), whereas carcasses infected with degenerating or calcified cysts should also be condemned.
Consequently, the focus of our study was on an appropriate postmortem test that could be applied on meat samples. A PCR–restriction fragment length polymorphism (RFLP), previously developed to differentiate between *T. solium* and *T. saginata* (16), was modified to detect *T. saginata* DNA in muscle lesions caused by the parasite.

**MATERIALS AND METHODS**

**Sample collection.** From March to December 2003, a study was conducted in collaboration with three large Belgian slaughterhouses, located in Zottegem (SL1, East Flanders), Eeklo (SL2, East Flanders), and Mouscron (SL3, Hainaut), respectively. Meat inspectors operating in these abattoirs were requested to dissect lesions, shortly upon detection, which to their judgment were considered viable or dead was also included. Viability was based on the softness to touch and the presence of the fluid content with or without a viable protoscolex. Dead cysticerci showed a hard feeling when touched and a creamy, greenish or yellowish discoloration (13). Upon arrival, the samples were thawed and photographed, and a second visual inspection was performed blind by the same person for all samples, sometimes with the aid of a dissection microscope. Lesions were classified as i) viable (referring to their stage before being frozen), ii) degenerating, iii) calcified cysts, or iv) nonspecific lesions. Thereafter, the lesions were dissected, put in 250 μl of lysis buffer 2 × (60 mM Tris-HCl, pH 7.4, 60 mM EDTA, 10% Tween 20, 5 mM MgCl₂, 1% Triton X-100, and 1.6 M guanidine-HCl) and stored at −20°C until further processing.

**DNA extraction.** The DNA was extracted using a modified Boom method (1). In short, this method is based on the lysis and nuclelease-inactivating properties of guanidine thiocyanate combined with the DNA-binding properties of diatomaceous earth. The final pellet was resuspended in Tris-EDTA (10 mM Tris, 1 mM EDTA) and stored at −20°C until use for PCR amplification.

**Choice of primers.** The primers, used previously for the 12S rDNA-PCR (16) and designed based on the 12S rDNA gene of *Taenia solium* (GenBank AB 086256) (12), were not optimal for the amplification of *T. saginata* DNA. Therefore the PCR protocol as described by Rodriguez-Hidalgo et al. (16) was slightly adapted. Based on conserved regions between a partial *T. saginata* sequence for large subunit ribosomal RNA (LSU rDNA) adjacent to a partial sequence for the *T. saginata* 12S ribosomal RNA (12S rDNA) (GenBank AB020396 and AB031355, respectively) and the *T. solium* 12S rDNA sequence (12), more stringent annealing primers were designed with the aid of the programs Amplify (5) and Right Primer, version 1.25 (10). The specificity of the primers was checked by doing a BLAST search against all DNA sequences present in GenBank. This approach resulted in one reverse primer (ITM TnR) and two forward primers (TnF and Tn2). The combination of ITM TnR and Tn2 gave less satisfactory result on amplification of *T. saginata* DNA and therefore only the primer set ITM TnR-TaenF was used.

DNA isolated from the *T. saginata* sample E2 (16) was cloned and the obtained sequence was used to design new primers. Based on the conserved regions between the obtained *T. saginata* sequence and that of the original *T. solium*, a second forward primer (nTAE) was designed to develop a seminested PCR approach (17).

**DNA amplification and RFLP.** A total volume of 25 μl, containing 5 μl of *Taenia* spp. DNA was used for amplification in a seminested PCR. Amplifications were carried out using a PTC-100 thermal cycler (M. J. Research, Inc., Waltham, Mass.). In the first round, primers ITM TnR (5′-CTCAATAATAATCGAGGTTGACGG-3′) and TaenF (5′-GTGGTCCCA-CCTCGATGTGACT) were used to amplify 846 nucleotides. A second round was performed using ITM TnR and nTAE (5′-CCTGAGCCAGGTCGTTCTTAT-3′) to amplify a sequence of 766 nucleotides.

The amplicons were analyzed by electrophoresis on 2% (wt/vol) agarose gels, followed by ethidium bromide staining and photography under UV illumination.

The amplification products (6 μl) were digested with restriction endonuclease *Ddel* (10 U/μg of DNA) (5,000 U/ml; New England Biolabs, Inc., Ipswich, Mass.) in a final volume of 15 μl for 4 h at 37°C following the manufacturer’s recommendations. The digested product was then analyzed by electrophoresis on a 10% polyacrylamide gel. A DNA size marker of 100 bp was included for size identification of the bands. The gel was silver stained and then preserved at ambient temperature. Cyst lesions described as viable samples were retested to confirm the PCR result, when tested negative the first time.

**RESULTS**

**Sample characteristics.** A total of 399 suspected *C. bovis* lesions from 335 carcasses were sent by the abattoirs and dissected in the laboratory. When multiple samples from one animal were analyzed, only one result (positive if one of the samples tested positive, otherwise negative) was considered for further analysis. Lesions were taken from different predilection sites (masseter [n = 206], heart [n = 23], and diaphragm [n = 6]). The highest number of lesions originated from abattoir SL1 (n = 296). From abattoirs SL2 and SL3, where the sampling only started in the second half of the study period, 21 and 18 samples were sent, respectively. From the 335 samples, 78 lesions were classified by the meat inspectors as viable cysts and 239 lesions as dead cysts; the samples from SL3 (n = 11) were sent without any classification.

Data obtained from the FASFC demonstrate that during the period of our survey, not all cysticercosis-positive carcasses were sampled and sent for analysis to our laboratory (296 of 433 for SL1, 21 of 85 for SL2, and 18 of 125 for SL3). The monthly percentage of carcasses condemned because of *T. saginata* infection differed between the three slaughterhouses, with a lower prevalence in SL3 (range, 0.1 to 1.3%) compared to SL1 (range, 0 to 2.4%) and SL2 (range, 0.5 to 2.9%). Also, monthly detection varied less in SL3 as compared to SL1 and SL2. No relationship was observed between the muscle type and the percentage of viable or dead cysts (data not shown).

**PCR-RFLP.** The 12S rDNA-PCR is genus specific and yields a band of 766 bp for all *Taenia* species (Fig. 1). The RFLP with *Ddel* was merely performed to confirm the hypothesis that the observed amplicon by PCR was caused by the presence of *Taenia* DNA. Based on the RFLP profiles, a distinction between *T. solium* and *T. saginata* is possible; however, the profiles observed following restriction of *Taenia crassiceps* and *T. solium* DNA are similar.
FIGURE 1. PCR results of *T. saginata* lesions. Amplification was performed in two sequential rounds. In the first round, primers ITM TnR (5’CTCAATAATCGAGGGTGACGG 3’) and TaenF (5’ GTTGCCACCTCGATGTTGACT) were used to amplify 846 nucleotides. A second round was performed using ITM TnR and nTAE (5’ CGTGAGCCAGGTCGGTTCTTAT 3’) to amplify a sequence of 766 nucleotides. Lanes 4, 6 to 18, and 20 to 26 display a band of approximately 800 bp and are positive PCR results. Lanes 3 and 5 are PCR-negative sample results. Lanes 27 and 29 are negative control samples (Milli Q instead of extracted DNA) and lanes 28 and 30 are positive control samples (*T. crassiceps* DNA). Lanes 2 and 19 are DNA size markers.

(data not shown). We used *T. crassiceps* DNA as positive control in the assay (a successful restriction yields band of 469 and 324 bp). A successful restriction of *T. saginata* DNA with *Dde*I yields three bands: one of 469 bp and two smaller bands of 164 and 158 bp. The latter two bands cannot be differentiated with the resolution of a 10% acrylamide gel and appear as a single band following silver staining (Fig. 2).

**Comparison between PCR and visual inspection.**

Classification of the results was done by considering the fact that the more degenerative the lesion was, the less probable the PCR would be positive due to the disappearance of parasitic DNA from the lesion. Overall, 257 (76.7%) of the 335 lesions gave a positive PCR result.

If we consider the results of the viable cysts as defined in the laboratory, 63 (96.9%) of 65 were found positive, whereas a smaller number of 71 (91%) of 78 viable cysts as defined by the meat inspectors were found positive (Table 1). Analysis of the “dead cysts” differed between abattoir and laboratory. In the abattoir, no further differentiation was done, whereas in the laboratory, cysts were further differentiated between degenerating and calcified cysts.

Using the most straightforward classification (viable versus dead cyst), 169 (70.7%) of 239 cysts classified as dead in two slaughterhouses (SL1 and SL2) had a positive PCR result (data not shown). From these 239 dead cysts, 2 were classified as viable in the laboratory and found positive on PCR. However, by considering only degenerating lesions as defined in the laboratory, 137 (79.1%) of 173 were found positive against only 52 (59.7%) of 87 that were found calcified. Ten lesions were classified as nonspecific lesions in the laboratory, although five samples yielded a positive PCR result (Table 1).

A species-specific RFLP to confirm the presence of *T. saginata* DNA. RFLP was performed on a batch of randomly selected PCR-positive samples only (33 of 335). Within this group of 33, DNA from three of the six PCR positives, but “unlikely to be cysticerci,” was also further submitted to a restriction analysis with *Dde*I. Figure 2 shows the pattern of PCR-positive samples following restriction digestion with *Dde*I. Lane 6 shows the digestion profile of the positive control sample (*Taenia crassiceps* DNA) yielding bands of 469 and 324 bp. In lanes 2, 5, and 7, examples are shown of samples that were classified in the laboratory as “unlikely to be cysticerci,” but that yielded positive PCR results. Their restriction profile is identical to that of *T. saginata* DNA, yielding one band of 469 bp and two small bands of 158 and 164 bp (appearing as one band). Of these three samples, the one in lane 7 had been classified as a living cyst in the slaughterhouse, whereas those in lanes 2 and 5 were classified as dead. All other examples shown originate from samples that were not considered to be live cysts, neither by the slaughterhouse nor following inspection in the laboratory. Lanes 1, 4, 8, and 10 had been classified as calcified cysts, whereas lanes 9, 11, 12, 13, and 14 were classified as degenerating cysts.

**DISCUSSION**

Overall, 76.7% of the diagnoses of cysticercosis made by the meat inspectors were confirmed by PCR. But one has to be careful with the interpretation of these results. Viability as defined in the laboratory could in this study be...
considered as indisputable and should yield 100% positive results in any PCR assay. The results on viable cysts in this study showed that 96.9% were positive, meaning that two lesions diagnosed as viable cysts were not cysticerci. Moreover, the visual inspection of these negative samples revealed nothing atypical. If we consider the 78 viable cysts as characterized by the meat inspectors, 71 samples or 91% were yielding positive results. The difference between this observation and the 96.9% positive viable cysts as defined in the laboratory could be explained by the fact that in a small proportion of samples, the viable cyst has been cut open during inspection and the parasite material would not be present for DNA extraction. A less likely explanation could be the event of a change in the characteristics between collection and analysis due to storage.

Another important observation is the fact that the proportion of PCR-confirmed diagnosis of dead cysts (degenerating and calcified cysts) was still substantially high, being 70.7%. This suggests that even upon calcification of cysts, parasite material can be isolated. Admittedly, a macroscopic examination of cysts is not really suitable for an evaluation of the degenerating or calcified status of a cyst. But it can be inferred from the results that all degenerative lesions would still contain sufficient parasite DNA to yield in all cases a positive PCR. This means that approximately 25% of the degenerative lesions are not from cysticerci. Several lesions can be confounded with cysticerci. Of 10 viable or unclassified samples found negative on PCR in this study, 3 were most likely old abscesses, 3 were unstructured pieces of fat, 2 were larger fluctuating structures, 1 resembled a cut through a venule, and 1 a leftover of muscle fascic. This reasoning can also be expanded to the interpretation of the results from calcified cysts. The fact that 60% of calcified cysts still yielded a positive PCR could mean that from the 40% negatives, 25% were not cysticerci, but the remaining 15% were cysticerci in which no DNA could be detected anymore.

We could conclude that from the 335 samples defined
as cysticerci lesions by the meat inspectors, 3% are most likely not from cysticercus lesions but from other lesions. Even 5 (50%) of 10 of the lesions that could not be classified to any group in the laboratory, yielded a positive PCR result, showing the difficulty in differentiating older cysticerci lesions.

In the slaughterhouse where the majority of the samples came from (SL1), almost three times more dead than living cysts were detected, whereas in the other two slaughterhouses, an almost equal proportion of living and dead cysts were detected. Obviously, viable cysts are easier to detect than calcified cysts; consequently, the lower number of dead cysts diagnosed in SL2 and SL3 might result from missed diagnoses.

Although the assay we used has proven to be successful for an objective confirmation of *T. saginata* cysticercosis, we have to stress that this test is not suitable as a rapid on-site diagnostic test. The focus of the present study concentrates on the postmortem diagnosis. The development of an on-site assay, suitable for a postmortem diagnosis of cysticercosis, would provide an instant and “objective” feedback to meat inspectors, but to date, there is no such test available.

In conclusion, the results of this study indicate that *T. saginata* cysticercosis is commonly diagnosed in the Belgian cattle population. From the 335 samples diagnosed as cysticerci by the meat inspectors at three slaughterhouses, only an estimated 3% of samples were wrongly attributed to cysticercus lesions. Further analysis shows that it is very difficult to differentiate between old lesions caused by cysticerci and other lesions. Taken together, these data demonstrate the difficulty of visual inspection as a diagnostic method and emphasize the need for a more objective and sensitive assay. Such an assay would greatly contribute to strengthen the control of *T. saginata* cysticercosis.

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