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ORIGINAL PAPER

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Visual diagnosis of *Taenia saginata* cysticercosis during meat inspection: is it unequivocal?

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Abstract A total of 267 cysts were collected from March to December 2004 from two main abattoirs in northern Germany. The cysts were classified by the usual organoleptic methods during meat inspection as *Cysticercus bovis*. The reported prevalence of cysticercosis in the abattoirs was 0.48 and 1.08%, respectively. The cysts were examined macroscopically for description of their morphology and constituents and classified as viable or degenerating (dead). The DNA was extracted from these cysts and subjected to polymerase chain reaction (PCR) for evaluation of the detection methods used and to make certain that the cysts did indeed belong to *C. bovis*, as indicated at the slaughterhouses. Two sets of primers were used with different sensitivity levels. The first, HDP1, was able to detect 200 fg of *Taenia saginata* DNA and 100 pg of *C. bovis* DNA. The other primer set, HDP2, was able to detect 1 pg of *T. saginata* DNA and 1 ng of *C. bovis* DNA. No more than 52.4% of the samples tested positive for *C. bovis* in the PCR using both primers, while 20% of the viable cysts and 49.2% of the degenerating cysts tested negative with both primers.

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Introduction

Cysticercus bovis is the larval stage of *Taenia saginata*, the bovine tapeworm. Humans are the final host and bovines the intermediate host to this infection. Human taeniosis, or infection with the adult *T. saginata*, is characterized by the presence of up to 30-meter-long worm in the small intestine of the infected person (Gracey et al. 1999), who may pass millions of eggs daily(Urquhart et al. 1988). These eggs can survive up to 7 months in the appropriate environment (Rommel et al. 2000) and can be transmitted to the intermediate host. Estimates based on computations from incidence indicate that 2% of the human population in Europe is infested with *T. saginata* (SCVPH 2003).

In the meat industry, economic losses are closely associated with the status of infe ction. If a heavy infestation or generalized cysticercosis is found in a carcass, it must be totally condemned. Light infection or localized cysticercosis leads to condemnation of the infected parts; furthermore, the carcass must be kept in cold storage at a temperature not exceeding −7°C for up to 3 weeks to inactivate the parasites(Gracey et al. 1999). In England alone, the costs of refrigeration, handling, and transport are estimated at £100 per carcass, or £4.0 million annually (Gracey et al. 1999). Africa suffers great losses due to bovine cysticercosis estimated to be $1.8 billion annually (Harrison 1996). According to meat inspection data, the prevalence of bovine cysticercosis varies between 0.01 and 6.8% in various European countries (SCVPH 2000), but the actual prevalence is expected to be between three and ten times higher (Geerts et al. 1981; van Knapen and Buys 1985; Onyango-Abuge et al. 1996; Dorny et al. 2000).

Protection of human health against zoonoses is one of the key objectives of consumer-relevant legislation in the European Community. In its opinion on zoonoses adopted on 12 April 2000, the Scientific Committee on Veterinary Measures Relating to Public Health considered that the measures then in place for the control of food-borne zoonotic infections were insufficient. It further considered that the epidemiological data collected by Member States

were incomplete and not fully comparable. As a consequence, the Committee recommended improved monitoring arrangements and identified risk management options.

To improve this situation, the European Parliament and Council announced Directive 2003/99EC on the monitoring of zoonoses and zoonotic agents. The purpose of this Directive is to ensure that zoonoses and zoonotic agents are properly monitored, and that food-borne outbreaks are subjected to proper epidemiological investigation. *C. bovis* was identified as one of a number of relevant pathogens which should be included in monitoring systems.

Objectives

The routine inspection procedure for bovine cysticercosis consists of visual inspection of the slaughtered animal, in particular, the inspection of the cut muscles of the split carcass and of several specific locations (predilection sites: external and internal masseter muscles, tongue, heart, and diaphragm) after incision. This post-mortem examination procedure has been shown to detect only 20% of all macroscopic lesions (Berends et al. 1993; Harbers 1991). It has been shown that the currently established inspection methods are not capable of sensitively detecting low levels of *T. saginata* metacestodes, which, in many cases, are found in tissues other than the predilection sites (Minozzo et al. 2002; SCVPH 2000; SCVPH 2003; Wanzala et al. 2002). In addition to the probable underestimation of the real prevalence in bovine populations, the precision of the visual identification is questionable, as the cysticerci can be confused with lesions caused by infections with *Sarcocystis* and *Actinobacillus* or with other local alterations (Ogunremi et al. 2004; Gracey et al. 1999).

The main objective of this study was to examine cysts classified at the slaughterhouse as *C. bovis* and to determine the accuracy of this identification made by the classical methods of meat inspection. The suspected cysticerci were submitted to PCR for confirmation as a step toward developing a more reliable method for the identification of *C. bovis* in bovine tissues.

Materials and methods

The cysts were collected between March and December 2004 from 250 animals slaughtered at two abattoirs in northern Germany. The average age of the affected animals was 58 months (between 13 and 192 months). The cysts were collected during routine meat inspection procedures. A special documentary form was completed for each sample with animal-related information including its age and sex and the sites and number of cysts. Each sample was kept in a special container and stored at −18°C until transfer to the laboratories of the Centre for Food Sciences in Hannover for further examination.

Morphological classification and identification:

The cysts were examined macroscopically and classified accordingly as viable or degenerating (Minozzo et al. 2002). Fluid-filled, viable cysts were considered mature when they contained a protoscolex. Those without a distinct protoscolex were considered immature. Degenerating cysts were classified as calcified when their contents were solid, as cheesy when smooth, or dull when they contained no contents and were apparently neither viable nor degenerating.

Extraction of DNA

The samples were cut into small pieces, then ground with liquid nitrogen. Proteinase-K (Macherey-Nagel, Dueren, Germany) was added to each sample, which was then incubated at 37°C for about 3 h. A NucleoSpin Tissue Kit (Macherey-Nagel, Dueren, Germany) was used according to the manufacturer's recommendations to extract genomic DNA from four field-derived positive *C. bovis* cysts, from *T. saginata* that was collected from naturally infected person at northern Germany in 2003. These were sent to our laboratories, conserved in 70% ethanol through Professor Gürtler/University of Greifswald. Samples were also taken from bovine meat from known non-infected calves.

PCR test

PCR was carried out using two sets of primers as described by Gonzales et al. (2000, 2002a,b). Both primers were synthesized by Invitrogen Life Technologies (Karlsruhe, Germany). *T. saginata* DNA and the field-derived cysts were used as a positive control and to determine the sensitivity of each primer set so that at least 8 pg of *T. saginata* genomic DNA was detected (data not shown), which is the amount contained in a single cestode egg. Negative bovine meat DNA was examined with both primers and was used as negative control. *T. saginata* DNA and DNA from the positive cysts were tested with bovine meat DNA using both primers to guarantee the absence of inhibitors.

HDP1 primer set

HDPI primer set is based on the published HDP1 *T. saginata* DNA fragment (Harrison et al. 1990). From this sequence, two oligonucleotides that were selected PTs4F1 (5′-CAG TGT GCT GAA GAT GAA TA-3′) and PTs4R1 (5′-GAA TTT GGC TCT CAC TGA ATG-3′) specifically amplified genomic DNA from *T. saginata* (Gonzales et al. 2000, 2002a,b). PCR was carried out in a total volume of 25 µl containing PCR buffer (10X, containing 15 mM $MgCl_2$, Qiagen, Hilden, Germany), each deoxynucleoside triphosphate (between 10 and 11 mM; ROTI-MIX, Carl

Table 1 Location, size, and morphological classification of the examined cysts (n=267)

Site of cyst[a]	M. masseter (%)	M. pterygoides (%)	Heart (%)	Diaphragm (%)	
Number per site(%)	230 (86.1%)	5 (1.9%)	32 (12%)	–	
Size	> 5×5 mm	2×2–5×5	< 2×2 mm		
Number (%)	25 (9.4%)	167 (62.5%)	75 (28.1%)		
Contents	Viable cysts		Degenerative cysts		Dull[d] (%)
	Mature[b](%)	Immature[c] (%)	Calcified (%)	Cheesy (%)	30 (11.2%)
	17 (6.4%)	8 (3%)	128 (47.9%)	84 (31.5%)	

[a] Other sites like anterior or posterior muscles are not examined at the slaughterhouse
[b] Fluid-filled cysts with protoscolex
[c] Fluid-filled cysts without a clear protoscolex
[d] With neither fluid nor pus

Roth & Co. KG, Karlsruhe, Germany), 0.25 µM primer PTs4F1, and 2.5 U of *Taq* polymerase (HotStarTaq DNA Polymerase, Qiagen, Hilden, Germany). Sample DNA was added at a concentration of between 60 and 100 ng, based on the amount of extractable DNA from the samples. PCR conditions were 94°C for the initial denaturation followed by 35 cycles at 94°C for 60 s, 60°C for 30 s, 72°C for 30 s, and at 72°C for 10 min for final extension. PTs4R1 was added at a concentration of 0.5 µM to the reaction mixture at the beginning of the 25th cycle. The PCR was repeated with the same conditions using 1 µl from the first PCR for amplification of more DNA.

HDP2 primer set

The primers were derived from another *T. saginata* DNA sequence (Harrison et al. 1990), which specifically amplified *T. saginata* with an amplification product of 600-bp band pattern. From this sequence two oligonucleotides were selected, PTs7S35F1 (5′-CAG TGG CAT AGC AGA GGA GGA A-3′) and PTs7S35R1 (5′-GGA CGA AGA ATG GAG TTG AAG GT-3′). PCR was carried out in a total volume of 25µl containing PCR buffer, each deoxynucleoside triphosphate at a concentration of between 10 and 11 mM, 0.5 µM primer PTs7S35F1, 0.5 µM primer PTs7S35R1, and 2.5 U *Taq* polymerase. Sample DNA was added at a concentration of 60-100 ng, based on the amount of extractable DNA from the samples. PCR conditions were 94°C for the initial denaturation, followed by 35 cycles at 94°C for 60 s, 56.5°C for 30 s, 72°C for 30 s, and at 72°C for 10 min for final extension.

Each sample was tested in duplicate including positive and negative samples. The products of the amplification were fractionated on 2.5% agarose gel and visualized under UV light by ethidium bromide staining. The amplification procedure was carried out in a GenAmp PCR System 9700 (Applied Biosystems, Foster City, USA).

Results

Morphological results

Of the 267 cysts examined, only 25 (9.4%) were viable. Most (86.1%) of the cysts were found in the masseter muscle. In the present study, a single cyst was reported in 94% of the examined cases, two cysts in 5.6%, and three cysts in just 0.4%. The classification of the examined cysts is summarized in Table 1.

PCR results

The average amount of DNA that was extracted from each of the four positive field cysts was 5.83 µg/cyst. The PCR results are summarized in Table 2.

HDP1

Results were ladder pattern of between 10 and 11 bands, approximately 50 bp apart. HDP1 primers detected *T.*

Table 2 Comparison of the PCR results obtained using HDP1 and HDP2 primers for the detection of bovine cysticercosis

Morphological classification	Number	HDP1+ (%)	HDP2+ (%)
Viable cysts:			
Mature[a]	17	15 (88.2%)	7 (41.2%)
Immature[b]	8	5 (62.5%)	3 (37.5%)
Total	25	20 (80%)	10 (40%)
Degenerative cysts:			
Calcified	128	64 (50%)	1 (0.8%)
Cheesy	84	47 (56%)	1 (1.2%)
Dull[c]:	30	9 (30%)	1 (3.3%)
Total	242	120 (49.6%)	3 (1.2%)
Total:	267	140 (52.4%)	13 (4.9%)

[a] Fluid-filled cyst with protoscolex
[b] Fluid-filled cysts without a clear protoscolex
[c] With neither fluid nor pus

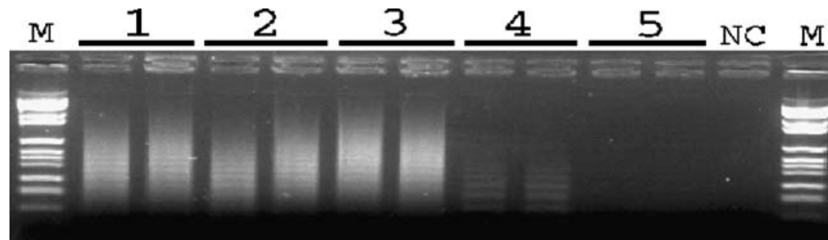

Fig. 1 Sensitivity of HDP1-based primer set. Different DNA concentrations from positive *C. bovis* samples. Each concentration was tested in duplicate. One nanogram (1), 500 pg (2), 250 pg (3), 100 pg (4), 50 pg (5), and from negative control sample (NC). The amplification product was fractionated using 2.5% agarose gel with ethidium bromide. DNA size standard (Cibus)(M)

saginata DNA at a sensitivity of 200 fg and *C. bovis* cysts' DNA at 100 pg (Fig. 1).

PCR confirmed 140 cysts as *C. bovis* (52.4%), of which only 20 (14.3%) were viable. The others were degenerating and of different morphological appearance.

HDP2

Results were a 600-bp band pattern. HDP2 primers detected *T. saginata* DNA with a sensitivity of 1 pg and *C. bovis* cysts' DNA at 1 ng (Fig. 2). PCR determined that 13 (4.9%) cysts were *C. bovis*, ten of which (77%) were viable.

Five (1.9%) viable cysts tested negative with both PCR methods. Two-thirds (67.1%) of the positive cysts measured between 2×2 and 5×5 mm. Smaller cysts were also not unusual. All cysts identified as positive using HDP2 primers were also positive using HDP1 primers.

Discussion

The use of PCR-HDP1 primers is considered to be more sensitive than that of PCR-HDP2 primers, but it is also more time-consuming (because of the necessity to use double-folded PCR and the addition of the second primer at the 25th cycle). Furthermore, the ladder pattern of the amplification results is sometimes difficult to interpret, and the PCR test was repeated for several samples.

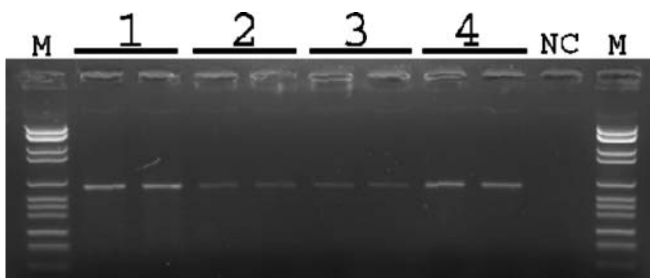

Fig. 2 Sensitivity of HDP2-based primer set. Different DNA concentrations from positive *C. bovis* samples. Each concentration was tested in duplicate. Fifty nanograms (1), 10 ng (2), 1 ng (3). *T. saginata* DNA with a concentration of 50 pg (4), and from negative control sample (NC). The amplification product was fractionated using 2.5% agarose gel with ethidium bromide. DNA size standard (Cibus)(M)

The fact that there were five viable cysts that were negative with both primers can be attributed to the proposal that these can be cysts or lesions of other parasites, which can be found in muscles. The tests of cysts collected from New Zealand and Switzerland between February 18, 1995 and July 1, 1996, with PCR, showed that cysticerci, which appeared to be not caused by *T. saginata*, can be found in cattle, although they resemble *C. bovis* both in gross pathology and histology (van der Logt and Gottstein 2000). This alerted regulatory authorities to the possible existence of another cestode.

Although both primers were tested for specificity and sensitivity (Gonzales et al. 2000, 2002a,b), the appearance of these negative samples raises the possibility of false negative results using both primer sets, presence of inhibitors that interfere with PCR amplification, or it may be attributed to insufficient amplifiable DNA in positive, but degenerate, specimens.

Anterior or posterior muscles are not examined during ordinary meat inspection despite the fact that these muscles can contain high numbers of cysts (Minozzo et al. 2002; Wanzala et al. 2002).

Monitoring the parasitic life cycle of *T. saginata* in the slaughterhouse is limited by the poor efficiency of visual detection after palpation and incision. Despite this underestimation of cysticercosis, which was clear through many serological studies (Hughes et al. 1993; Onyango-Abuge et al. 1996; SCVPH 2000, 2003). On the opposite side, its prevalence may also be overestimated through misdiagnosis of other morphological alterations in affected muscles.

Application of serological methods for the detection of cysticercosis at the abattoir level would greatly improve the efficacy of efforts to eliminate human taeniosis and, thus, bovine cysticercosis. Many serological methods are now available for the differentiation between viable and degenerative cysts (SCVPH 2000, 2003; Wanzala et al. 2002; Onyango-Abuge 1996; Hughes et al. 1993). As the meat safety system based only on conventional post-mortem inspection at slaughterhouse is apparently of relatively low efficacy both for preserving public health and for epidemiological studies, alternative methods of meat inspection should be studied thoroughly so that they can be used to support traditional meat inspection methods.

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