

# Multiplex PCR identification of *Taenia* spp. in rodents and carnivores

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**Abstract** The genus *Taenia* includes several species of veterinary and public health importance, but diagnosis of the etiological agent in definitive and intermediate hosts often relies on labor intensive and few specific morphometric criteria, especially in immature worms and underdeveloped metacestodes. In the present study, a multiplex PCR, based on five primers targeting the 18S rDNA and ITS2 sequences, produced a species-specific banding patterns for a range of *Taenia* spp. Species typing by the multiplex PCR was compared to morphological identification and sequencing of *cox1* and/or 12S rDNA genes. As compared to sequencing, the multiplex PCR identified 31 of 32 *Taenia* metacestodes from rodents, whereas only 14 cysts were specifically identified by morphology. Likewise, the multiplex PCR identified 108 of 130 adult worms, while only 57 were identified to species by morphology. The tested multiplex PCR system may potentially be used for studies of *Taenia* spp. transmitted between rodents and carnivores.

## Introduction

Surveying taeniid infections in wildlife definitive hosts is traditionally done by post-mortem examination with morphological identification of intestinal worms (Torgerson and Deplazes 2009), but the morphological criteria used for speciation are complex, labor intensive, and require a

skilled operator which make it less suited for large-scale epidemiological studies. To prevent zoonotic pathogen transmission, fox carcasses are frozen prior to post-mortem examination, which often causes the scolex, the rostellar hooks, and sexually mature proglottids of tapeworms to disintegrate (Borgsteede et al. 2003; Hofer et al. 2000; Wolfe et al. 2001). In rodents, early taeniid larval stages are minute in size and with little or no morphological characteristics, thus often preventing specific diagnosis of liver lesions (Stieger et al. 2002; Reperant et al. 2009).

Several PCR tests/assays are available for differentiating human *Taenia* infections, including a PCR-RFLP system targeting the *cox1* and the first internal transcribed sequence (ITS1) (Bowles and McManus 1994) or the second internal transcribed sequence (ITS2) region (Gasser and Chilton 1995), a PCR based on the sequence of the *cox1* gene (Yamasaki et al. 2002), and a nested PCR system based on the sequence of oncosphere-specific protein (Mayta et al. 2008). For veterinary purposes, a few PCR-based assays are available. The single-strand confirmation polymorphism (SSCP) technique based on the *cox1* gene was used to differentiate between eight *Taenia* species common in canines (Gasser et al. 1999). However, the SSCP technique is complex, and genetic variants could be masked by the presence of less frequent variants; therefore, expensive sequencing is commonly applied with SSCP (Natkaniec et al. 2009). A PCR derived from a non-coding DNA fragment named HDP2 DNA and a PCR-RFLP of the ITS1 and ITS2 regions distinguished between *Taenia* spp. and *Sarcocystis* spp. in pigs and cattle (Gonzalez et al. 2006), while another PCR targeting the 18S ribosomal DNA gene (18S rDNA) discriminated between *Taenia pisiformis* and *Taenia parva* (Foronda et al. 2005). The PCR system of Trachsel et al. (2007) differentiated between zoonotic *Echinococcus* spp. and *Taenia* spp. at the

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genus level, but further speciation relied on sequencing or RFLP. Thus, an unequivocal PCR without a need for confirmatory testing would facilitate large-scale epidemiological studies. The present study aimed to develop a multiplex PCR targeting the 18S rDNA and the ITS2 for species-specific identification of *Taenia* spp. commonly transmitted between carnivores and rodents.

## Materials and methods

### Parasite material and DNA extraction

Suspected cysts and/or lesions were collected from visceral organs of naturally infected Danish rodents and insectivores ( $n$ : total=54; *Myodes glareolus*, bank vole,  $n$ =33; *Apodemus flavicollis*, yellow-necked mouse,  $n$ =11; *Microtus agrestis*, field vole,  $n$ =4; *Sorex minutus*, pygmy shrew,  $n$ =4; *Sorex araneus*, common shrew,  $n$ =1, and *Neomys fodiens*, water shrew,  $n$ =1). Fully developed *Taenia* larval stages were morphologically identified to species level based on cyst shape, size, and location in the host as well as the size, number, and shape of rostellar hooks (Loos-Frank 2000). Cyst DNA was extracted by a commercial kit according to the manufacturer's instructions (QIAmp DNA Mini Kit®, Qiagen, Hilden, Germany). For comparison, a previously described multiplex PCR targeting the mitochondrial 12S rDNA was used to amplify the DNA of all cysts to differentiate between *Echinococcus* spp. and *Taenia* spp., and specific diagnosis of *Taenia* was done by the sequencing of a uniform 267-bp band produced for all *Taenia* spp. (Trachsel et al. 2007). Confirmative diagnosis of infections was also done by amplification and partial sequencing of the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) using previously published primers (Bowles et al. 1992). For automated DNA sequencing by a private service company (MWG, Martinsried, Germany), amplicons were purified directly from the PCR product (DNA and Gel Band Purification Kit®, Amersham Biosciences, UK), and sequences were compared to entries in GenBank.

As reference material, *Echinococcus multilocularis* specimens were obtained from experimentally infected gerbils (Zurich, Switzerland), and *Calodium hepaticum* (syn. *Capillaria hepatica*) and *Mesocestoides* spp. from naturally infected Danish rodents.

Adult *Taenia* worms ( $n$ =130) were recovered from 43 infected carnivores collected in Denmark (red foxes,  $n$ =35; feral cats,  $n$ =5; stone marten,  $n$ =2; and pine marten,  $n$ =1). The initial selection of worms was based on the characteristic scolex morphology of *Taenia* spp. Up to ten specimens per host were selected with priority to those with retained rostellar hooks. In either case, all worms were analyzed individually. Morphological diagnosis was done from

shape, size, and number of rostellar hooks (Verster 1969; Loos-Frank 2000). In addition, reference specimens of *Taenia* spp. worms were obtained from collections in Denmark, Switzerland, and Russia: *Taenia taeniaeformis*, *Taenia mustelae*, *Taenia martis*, *Taenia polyacantha*, *Taenia crassiceps*, *Taenia multiceps*, *Taenia pisiformis*, *Taenia hydatigena*, *Taenia ovis*, and *Taenia twitchelli*. DNA of adult worms was extracted by a commercial kit according to the manufacturer's instructions (QIAmp DNA Mini Kit®, Qiagen, Hilden, Germany), with a final elution volume of 300  $\mu$ l. DNA concentrations were measured using a NanoDrop® ND-1000 Spectrophotometer (Saveen Werner AB, Malmö, Sweden). From each of the 43 carnivores, a well-preserved worm specimen was selected for confirmatory sequencing.

### Selection of primers

Putative target sequences of the mitochondrial 16S rDNA, 12S rDNA, *nad1*, *nad4*, and *cox1* genes ( $n$ =35); the nuclear 28S rDNA and 18S rDNA genes ( $n$ =12); and the ITS2 and ITS1 regions ( $n$ =5) of *Taenia* spp. commonly found in carnivores (Loos-Frank 2000) were selected based on sequence availability in GenBank and the level of inter- and intra-specific sequence variations. Selected sequences were then used for designing primers by submitting them to the internet-based free software Multi-Objective PCR Design (muPlex®) (Rachlin et al. 2005). Primers were designed based on selecting the following parameters: they should (1) exhibit at least 2-bp differences from other species, preferably in the last five bases at the third end of the target region, (2) have a predicted melting temperature ranging from 50–63°C, (3) amplify products of sizes ranging from 80–800 bp, and the rest of the parameters were left as default.

### Primer testing and validation

From more than 100 primers designed by *in silico* analysis, one primer pair was manually selected for each of seven *Taenia* species (*T. parva*, *T. multiceps*, *T. crassiceps*, *T. serialis*, *T. pisiformis*, *T. mustelae*, and *T. taeniaeformis*) with respect to differences in PCR band size. The specificity of the selected primers was further assessed *in silico* by blasting all primer sequences against sequences stored in the GenBank database (Basic Local Alignment Search Tool; <http://www.ncbi.nlm.nih.gov:80/BLAST>, access date, 1 November 2009). Additional analysis was done to test absence of hairpins, self-dimers, or heterodimers among the selected primers by the Oligonucleotide Properties Calculator (Oligo Calc. (<http://www.basic.northwestern.edu/biotools/oligocalc.html>)) (Kibbe 2007). Unfortunately, *in vitro* analysis of selected primers showed

that none of the selected primer pairs were species-specific (results not shown). Therefore, primer pairs were selected after the distinctness of the resulting banding pattern for the seven species. This strategy was implemented by removing one primer or primer pair from the above-selected seven primer pairs from the master mix after each multiplex PCR reaction that included the *Taenia* spp. of interest until the desired species-specific banding pattern was produced. Five primers were finally selected (Table 1).

#### Polymerase chain reaction conditions

The applied multiplex PCR reaction mixture comprised: 3  $\mu$ l (DNA concentrations >20 ng/ $\mu$ l) template DNA in a final volume of 25  $\mu$ l. The multiplex PCR reactions contained 1.5 mM magnesium chloride (MgCl<sub>2</sub>), 40 mM of each deoxynucleotide triphosphate, 50 pmol of each primer, and 1 U Phusion© DNA Polymerase together with its specific 5 $\times$  Phusion HF buffer© (Finnzymes, Espoo, Finland). Bovine serum albumin (1  $\mu$ g/ $\mu$ l) and dimethyl sulfoxide (2%) were supplemented to the multiplex PCR mixture to enhance the reaction. Negative control reactions without template DNA were included in each reaction. The cycling conditions were: denaturation at 94°C for 1 min, 40 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 120 s, and a final extension at 72°C for 7 min. The reactions were done using a thermal cycle (GeneAmp PCR system, 9600, version 2.01, PerkinElmer). Amplicons were detected on 1.5% agarose gels stained with Envision DNA Dye© (N313-1ml, Amersco, Solon, USA).

To test if intermediate host DNA would react to the designed primers, DNA from kidneys of two rodent species (*A. flavicollis* and *M. glareolus*) and one insectivore species (*Talpa europaeae*) were isolated as described above. Also, to test the possible negative effect on multiplex PCR products by the presence of DNA of intermediate hosts, increasing concentrations of *A. flavicollis* DNA (up to 55 ng/ $\mu$ l) were mixed with DNA of four *Taenia*

spp. commonly found in rodents (*T. taeniaeformis*, *T. mustelae*, *T. polyacantha*, and *T. crassiceps*).

#### Statistics

Confidence intervals of 95% were calculated on the basis of binomial distribution.

## Results

#### The multiplex PCR

The designed primers targeting the nuclear 18S rDNA gene and the ITS2 region (Table 1.) reacted to DNA of *Taenia* spp. and produced a distinct species-specific banding pattern (Fig. 1). Combining DNA of different *Taenia* species in the same reaction produced an unclear banding pattern (not shown). DNA of *E. multilocularis* and *C. hepaticum* produced a distinct band at 300 bp by PCR (Fig. 1), but the two species can be easily differentiated by another produced band of 700 bp for *C. hepaticum* and two bands of around 500 and 550 bp for *E. multilocularis*.

DNA from *M. glareolus* kidney was not amplified by the designed primers, but the DNA of the common mole and *A. flavicollis* produced a single band of approx. 300 bp (not shown). Mixing intermediate host DNA in increasing concentrations of several *Taenia* spp. did not affect the distinct diagnostic bands of four *Taenia* spp. commonly found in rodents (Fig. 2).

Many adult worms came from road-killed and hunted foxes that were left out in the environment for days and could have suffered from partial autolysis. Therefore, samples of different DNA concentrations were examined, and the minimum DNA concentration of a sample needed to obtain amplified products by multiplex PCR was determined as 20 ng/ $\mu$ l.

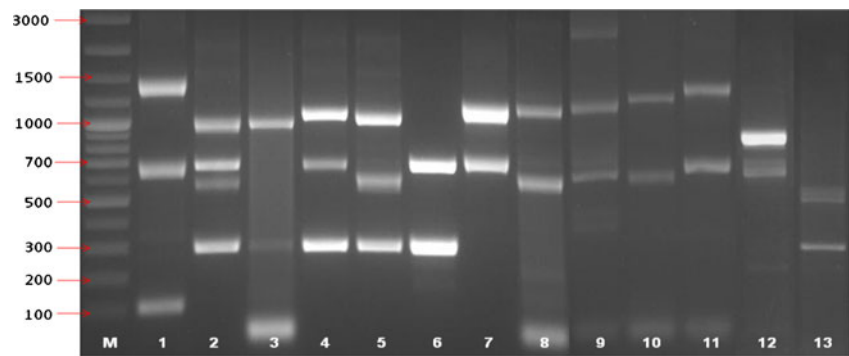
#### Multiplex PCR on *Taenia* larval stages or lesions from rodents

Out of the 54 lesions, 32 were diagnosed as *Taenia* spp. by sequencing. The new multiplex PCR identified 31 of these at the species level, whereas only 14 lesions were identified by morphology (*T. taeniaeformis*,  $n=11$ ; *T. mustelae*,  $n=2$ ; and *T. polyacantha*,  $n=1$ ). One rodent liver had mixed infection of *T. taeniaeformis* and *Mesocostoides* spp. based on multiplex PCR assay (confirmed by sequencing of both the 12S rDNA and *cox1* genes). Twenty-two liver lesions were not identified as *Taenia* infection by any of the applied methods and were classified as other pathologic changes.

**Table 1** Sequence of primers used for developing species-specific banding patterns for *Taenia* spp. by PCR

Target gene	Primer name	Sequence 5' to 3'
18S	TparvF	TCTGCCTACCATGGTGAT
	TparvR	AGGACGGTATCTGATCGT
	TmultF	TCCGATAACGAACGAGAC
	TmultR	TCAGCGCAAGCTTATGAC
ITS2	TcrasR	GCTACTGATTGGTGCGTT GTTGAT

**Fig. 1** PCR banding patterns for differentiating 1 *T. taeniaeformis*, 2 *T. mustelae*, 3 *T. polyacantha*, 4 *T. ovis*, 5 *T. hydatigena*, 6 *C. hepaticum*, 7 *T. pisiformis*, 8 *T. twitchelli*, 9 *T. multiceps*, 10 *T. martis*, 11 *T. crassiceps*, 12 *Mesocestoides* spp., 13 *E. multilocularis*, M 100-bp DNA marker



### PCR on *Taenia* worms from carnivores

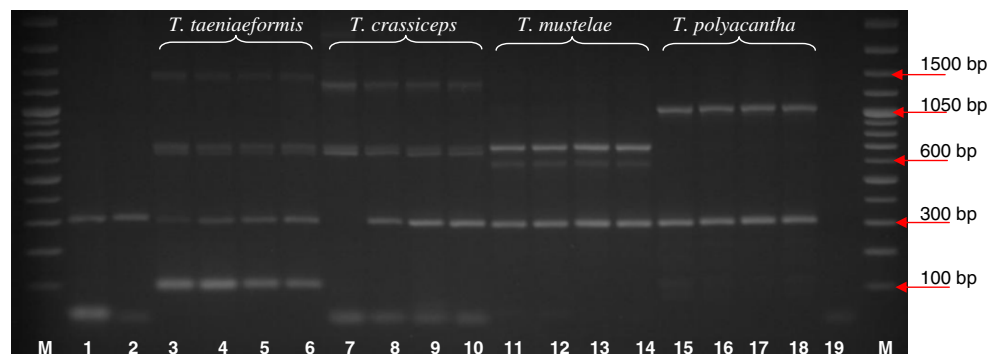
The designed multiplex PCR identified 108 of 130 *Taenia* worms at the species level. The identified *Taenia* spp. were *T. polyacantha* ( $n=91$ ), *T. taeniaeformis* ( $n=16$ ), and *T. mustelae* ( $n=1$ ). A non-specific band of around 300-bp length was amplified for 19 worms, which were identified by sequencing of the *cox1* gene as *T. polyacantha* ( $n=17$ ), *T. crassiceps* ( $n=1$ ), and *T. martis* ( $n=1$ ). From worms where DNA was not amplified ( $n=3$ ), only one could be identified by morphology (*T. polyacantha*). Among the collected worms, only 34 contained mature segments (26.2%); therefore, only hook morphology was used for morphological identification. By morphology of rostellar hooks, 57 worms could be identified at the species level (43.9%), which were *T. polyacantha* ( $n=41$ ) and *T. taeniaeformis* ( $n=16$ ).

### Discussion

The presented multiplex PCR produced distinct banding patterns for the tested *Taenia* species (Fig. 1) and may

represent a step in the development of a simple tool for speciation of taeniid worms from carnivores and cysts in viscera of intermediate hosts. Even though the DNA of the intermediate host and some *Taenia* spp. share a 300-bp product, this band appears not to interfere with the diagnostic bands of *Taenia* spp. On the other hand, a *T. pisiformis* sample contaminated by host DNA (two parasite bands + one host band) could be misinterpreted as *T. ovis* (three parasite bands). Similarly, banding patterns of *T. twitchelli*, *T. multiceps*, and *T. martis* may be hard to distinguish if they are not run side by side. However, data on the animal source of parasite material and location in the host can aid in the final diagnosis of the multiplex PCR products. The multiplex PCR may potentially generate distinct bands for other *Taenia* spp., but this remains to be proven.

For previously published taeniid PCR tests, specific diagnoses of carnivore infections were largely based on copro-PCR methods requiring expensive cloning and/or sequencing of the amplified DNA fragment (Trachsel et al. 2007; Dyachenko et al. 2008). The present multiplex PCR produced distinct bands from the majority of both adult worms and larval cysts in comparison to morphological



**Fig. 2** Testing competition between intermediate host DNA and taeniid DNA using the newly designed primers. Lanes 1, 4, 8, 12, and 16 DNA from *A. flavicollis* (Y) (18 ng/ $\mu$ l); lanes 5, 9, 13, and 17 Y (37 ng/ $\mu$ l); lanes 2, 6, 10, 14, and 18 Y (55 ng/ $\mu$ l); lanes 3–6 *T.*

*taeniaeformis*, lanes 7–10 *T. crassiceps*, lanes 11–14 *T. mustelae*, lanes 15–18 *T. polyacantha*, lane 19 negative control (milli-Q  $H_2O$ ), M 100-bp DNA marker. DNA samples of *Taenia* spp. were obtained from worms. The gel is 2% run at 90 V for 3 h

examination, facilitating a higher number of definitive diagnoses and thereby a higher utilization of collected wildlife samples in epidemiological studies.

The smeared banding pattern for a few samples, confirmed as *Taenia* by sequencing, underlines that DNA quantity and quality affect the performance of the PCR; moreover, samples of freshly isolated cysts and worms with more than 20 µg/µl DNA produced distinct banding patterns. Although many of the foxes were partly autolyzed, causing the disintegration of the worms and degradation of DNA (Huttner et al. 2009), the present multiplex PCR amplified DNA from the majority of the recovered worms.

The obstacles experienced in designing species-specific primers for distinguishing *Taenia* spp. were also encountered by Trachsel et al. (2007) who attempted developing species-specific *Taenia* spp. primers targeting the mitochondrial *cox1* gene. Future attempts may explore other target sequences for the primer development.

As the majority of the *Taenia* worms from carnivores in the present study were immature, such infections would be missed by copro-PCR based on fecal eggs (Ziadinov et al. 2008). Hence, PCR on recovered intestinal worms provides a more sensitive prevalence assessment. On the other hand, copro-PCR techniques are non-invasive indicators of patent infections which are valuable for the assessment of environmental egg contamination.

In the rodents, the developed multiplex PCR identified twice the number of *Taenia* lesions as compared to morphological identification. A closely related parasite to *Taenia* spp., *E. multilocularis*, and a distantly related nematode, *C. hepaticum*, were included in the analysis of this study, since both species at early stages of their development produce liver lesions that could hardly be distinguished from those of *Taenia* spp. With the exclusion of the 300-bp band, the 700 bp of *C. hepaticum* is also produced by other *Taenia* spp., while the banding pattern of *E. multilocularis* is clearly distinct from that of other species, two bands positioned at 500 and 550 bp. As PCR has mainly been used to diagnose infections with *Taenia* spp. in animal definitive hosts (Mathis and Deplazes 2006), the present multiplex PCR can be used for specific diagnosis of ambiguous liver lesions in rodents and thereby contribute to our understanding of the transmission dynamics of *Taenia* spp. in communities of intermediate hosts.

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**Conflict of interest** All authors declare no conflict of interest.

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