Molecular identification of cryptic cysticercosis: *Taenia ovis krabbei* in wild intermediate and domestic definitive hosts

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Short Title: Molecular identification of cryptic cysticercosis in wild and domestic hosts

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Abstract

The complex life cycle of Taeniids represents an ideal model of multi-host system. The complexity of these parasites can therefore cover the epidemiological issues of the interface wild-domestic animals, especially once spatial overlap between wild and domestic definitive and intermediate hosts occurs. Here we use the occurrence of *Taenia ovis krabbei* in two model areas as example of this epidemiological complexity.

In two contiguous areas in Italian Northern Apennines, two hunted roe deer (*Capreolus capreolus*) showed numerous cysticerci in the muscles of their whole body and an adult tapeworm was recorded in a semi-stray dog (*Canis lupus familiaris*). Through molecular typing of the mitochondrial cytochrome c oxidase I (cox1) gene, cysticerci and adult tapeworm of *T. krabbei* were identified. *T. krabbei* cysticercosis was recorded for the first time in Italy. Although the role of dogs in the parasite's life cycle emerges, the overlap between wild and domestic definitive hosts and the increase of wild population densities raise concerns about the temporal (old or new) introduction and the spread of this parasite by one of these canid species (wolf (*Canis lupus*) or dog).

Although *T. krabbei* is not a public health issue, economic concerns for hunters and meat producers related to the cysticerci' damage of carcasses emerged. Therefore, the need is to evaluate the spread of *T. krabbei* in the intermediate and definitive host populations and to ensure a proper sanitary education for hunters in order to avoid practices that could favour the spread and maintenance of its life cycle.

Keywords

Cysticercus; host species; Cervidae; Taeniids; cox1; semi-stray dog

Introduction

In natural conditions, several macro and micro parasites can infect more than a single host species (Morgan *et al*., 2004) creating multi-host systems (Malpica *et al*., 2006; McCormack & Allen, 2007). These complex systems are highly significant for the maintenance of infections since parasites can independently persist in one host population in the absence of the other (Haydon *et al*., 2002; Gortázar *et al*., 2007). However, a relevant distinction should be made between pathogens with a broad spectrum of infection (i.e. Bovine tuberculosis or Brucellosis (Godfroid, 2002; Renwick *et al*., 2007)) and those whose life cycle requires specific different host species (i.e. helminths (Poulin, 2001)). While in the former case the identification of reservoir and dead-end hosts (Haydon *et al*., 2002) among the affected hosts is required, in the latter case the need is to identify species acting as definitive, intermediate and paratenic hosts. In this regard, when there is spatial overlap between more than one definitive or intermediate or paratenic host the epidemiological issue increases, especially in case of pathogens crossing the interface between wild and domestic animals (Miller *et al*., 2013; Guberti *et al*., 2014; Turchetto *et al*., 2014). Moreover, when parasites shared between wildlife and livestock involve highly naturalistic valuable species, such as top predators, conservative issues raise to prevent impacts on endangered species. Taeniids represent an example of these issues.

Taeniids (Cestoda) are a family of tapeworms (Gori *et al*., 2015) with a generalised life cycle where the adult tapeworm infects the small intestines of the definitive host, characteristically carnivores (Willingham *et al*., 1996; Di Cerbo *et al*., 2008; Cantó *et al*., 2011; Haukisalmi *et al*., 2011; Lavikainen *et al*., 2011), while the larval stage (*Cysticercus*) occurs in the musculature (myocardium and skeletal muscle), lung, liver, brain, etc. (extraintestinal sites) of herbivores, that are the intermediate hosts (Goldová *et al*., 2008; Lavikainen *et al*., 2010). Definitive hosts become infected through a predator-prey relationship while the foraging on pasture contaminated with eggs of this parasite, shed through definitive host faeces, is the primary cause of infection in the intermediate hosts. Taxonomic identification of Taeniid species has been traditionally performed by morphological analysis of cysticerci or adult tapeworms. However, in some cases Taeniids are morphologically very similar although genetically distinct from each other (i.e. cryptic species (Perkins, 2000; Chilton *et al*., 2007; Nadler & Pérez-Ponce de León, 2011; Poulin, 2011; Galimberti *et al*., 2012)). Therefore, traditional systematic methods may hardly differentiate these species, while molecular techniques can enable their taxonomic identification (Formenti *et al*., 2016) and phylogenetic analysis can show their genetic diversity/relatedness. Here we use the occasional molecular identification of *Taenia ovis krabbei* (cysticerci and adult tapeworm) in roe deer (*Capreolus capreolus*) and in a semi-stray dog (*Canis lupus familiaris*) in two model areas as example of the epidemiological complexity of monitoring this multi-host infection in areas with spatial overlap between wild and domestic definitive hosts.

Materials and methods

Collection and examination of hosts

Roe deer and dog were from two contiguous areas in Italian Northern Apennines, area 1 (44° 37' N, 9° 20' E) and area 2 (44° 35' N, 10° 44' E) (Fig. 1), which are hunting districts and span 15400 ha and 43573 ha, respectively.

HERE Fig. 1

Several wild ungulates (wild boar (*Sus scrofa*), roe deer, fallow deer (*Dama dama*), red deer (*Cervus elaphus*), small game (pheasants (*Phasianus colchicus*), partridges (*Perdix perdix*) (only in area 2), red partridges (*Alectoris rufa*), hares (*Lepus europaeus*)) and foxes (*Vulpes vulpes*) are present. Roe deer is the most abundant among wild ruminants with densities reaching 18-20 subjects/100 ha in area 1 and 2, respectively. A recently, re-established population of wolves (*Canis lupus*) is present in both areas.

In both areas, the hunting of ungulates and small game is a traditional and regulated activity. Indeed, hunters and their own dogs (pointing and searching dogs, bloodhounds) regularly attend the area. Moreover, in accordance with the Italian Law (157 of 11/02/1992), hunters have to carry culled game to the control centres where, for each subject, age, sex, shooting site, morphobiometric measures are registered.

While in both areas the presence of hikers and walkers with their own domestic dogs cannot be excluded *a priori* considering the proximity with built-up areas, in area 1 semi-stray and shepherd dogs have a stable presence associated with small farms and zoo-agricultural activities.

Following the field detection of parasites, adult tapeworm and cysts (from both thigh and back muscles) were collected and stored at 4°C for successive parasite examination. In laboratory, both adult and larval stages were analysed by visual examination under both optical and dissecting microscope (40x magnification).

Molecular analysis

The DNA of both adult tapeworm and cysts was extracted using a commercial kit according to the manufacturer's instructions (QIAmp DNA mini kit®, Qiagen, Hilden, Germany). A fragment of 450 bp of the mitochondrial cytochrome c oxidase I (cox1) gene was amplified with specific primers JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') and JB4.5 (5'-TAAAGAAAGAACATAATGAAAATG-3') (Bowles *et al*., 1992) and the PCR protocol was performed according to Gasser *et al*. (1999) and Galimberti *et al*. (2012). Amplicons size was assessed by electrophoresis in 1.5% agarose gels stained with ethidium bromide, and PCR products were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA) and directly sequenced. Sequencing was performed in an ABI Prism 3130 genetic analyser (Applied Biosystems). Sequences were deposited in GeneBank under accession numbers KY498632-4, and analysed by ClustalW and MegAlign software (DNAStar Inc.,

Madison, WI, USA). Sequences (335 nt) were compared with other cox1 sequences of *Taenia* spp. present in Genbank to evaluate sequence similarity, expressed as percentage of nucleotide identity. In particular the following sequences were analysed: *T. krabbei* (JX560319, EU544573, JF261321, JF261327, JF261322), *T. multiceps* (GQ228818), *T. regis* (AM503330), *T. solium* (AB086256), *T. ovis* (JX134122), *T. arctos* (GU252130), *T. saginata* (AB645845), *T. serialis* (AM503322), *T. hydatigena* (JN831308, EU544551), *T. taeniaeformis* (AB221484). *Echinococcus oligarthrus* (AB208545) and *T. mustelae* (EU544571) were used as outgroups (Lavikainen *et al*., 2011). Phylogenetic analysis was performed by using the neighbor-joining method p-distance model and bootstrap test of 1,000 replicates in MEGA 5.

Results

Two adult female roe deer, hunted in March 2015 and March 2016, respectively, in apparently good body condition showed numerous oval, white cysts of approximately two to four mm in diameter (Fig. 2) in the muscles of their whole body. In July 2015 an adult tapeworm was observed in the faeces of a semi-stray dog.

HERE Fig. 2

Adult tapeworm was damaged and thus morphologically indistinguishable; macroscopic analysis of roe deer thigh and back muscle samples showed cysticerci with a bladder membrane and watery, transparent fluid.

Taenia spp. DNA was isolated and the partial sequence of cox1 gene was obtained. BLAST analysis showed *T. krabbei* for both cysticerci and adult tapeworm. Phylogenetic analysis (Fig. 3) showed that the Italian isolates grouped together with other isolates of *T. krabbei* from both intermediate (roe deer, Denmark) and definitive (wolves, Finland and Sweden, and arctic fox, Norway) European hosts. This group was genetically similar to *T. multiceps* (95.2-95.8% of nucleotide identity), *T. saginata* (94.0-94.6%) and *T. serialis* (92.8-93.1%) while it was genetically distant from the morphologically similar *T. ovis* (87.2-87.5%) and *T. arctos* (88.7-89.3%). Sequence distances among Italian isolates of *T. krabbei* and the other Taenia species are reported in the Supplementary Table S1 (available on the Journal's website (http://journals.cambridge.org/jhl)).

HERE Fig. 3

Discussion

In the present study we identified cysticercosis of *Taenia ovis krabbe*i (Moniez, 1879) for the first time in Italy, to the best of our knowledge. This Taenia species is morphologically similar to *T. ovis*, although they are biologically distinct (Priemer *et al*., 2002). Indeed, wild cervidae have been considered the principal intermediate hosts for *T. krabbei* (Al-

Sabi *et al*., 2013), while cattle, goats, sheep and pigs have been shown to be refractory to the parasite (Lavikainen *et al*., 2013; Al-Sabi *et al*., 2013). On the contrary, the life cycle of *T. ovis* includes *Bovidae*, preferably sheep but also goats (Flueck & Jones, 2006). This biological distinction is furthermore confirmed by the results of our phylogenetic analyses that showed the genetic distance between *T. krabbei* and *T. ovis*, as previously highlighted (Al-Sabi *et al*., 2013). On the other hand, the clustering of European *T. krabbei* isolates (Italy – present study, Denmark, Norway, Sweden, and Finland) supports their common origin although more loci and isolates of several geographical origins should be analysed to define the population genetics of this species (Lavikainen *et al*., 2011).

The recorded *T. krabbei* cysticercosis supports the role of roe deer as intermediate host of this parasite in Italy. In this regard, the occurrence of the adult parasite in dog pointed out the epidemiological involvement of this species in *T. krabbei* life cycle, as previously highlighted by other authors (Sawyer *et al*., 1976; Letková *et al*., 2008; Lavikainen *et al*., 2013; Al-Sabi *et al*., 2013). Indeed, although wolf (*Canis lupus*) has been reported as original definitive host of *T. krabbei* (Priemer *et al*., 2002; Bagrade *et al*., 2009; Lavikainen *et al*., 2013), domestic populations 'at risk' (i.e. hunting dogs, shepherd dogs) can be infected and contribute to spreading the infection (Otranto *et al*., 2015). However, the coexistence of wild and domestic definitive hosts within the study areas does not allow to determine which species has first introduced the parasite. On one hand, the recent occurrence of the parasite's eggs in wolves from a contiguous area (Gori *et al*., 2015) supports their role. On the other hand, the increasing movements of dogs, particularly due to hunting travels to Eastern Europe, where the infection has been recorded for a long time (Goldová *et al*., 2008; Letková *et al*., 2008; Al-Sabi *et al*., 2013), may suggest a role of this species. Moreover, as this is the first report of *T. krabbei* in the study areas, to the best of our knowledge, alternative hypotheses about an old, undetected occurrence or a new introduction of *T. krabbei* in the study areas can be proposed. In particular, this parasite might have been already present but, until now, never detected. Minor infection can indeed often go unnoticed (Laaksonen & Paulsen, 2015). On the other hand, as morphological inspection of Taeniid species can lead to misidentification or to not conclusive results (Loos-Frank, 2000; Priemer *et al*., 2002; Flueck & Jones, 2006; Goldová *et al*., 2008; Lavikainen *et al*., 2010; Lavikainen *et al*., 2013) with both dog and roe deer serving as hosts for multiple Taenia species (Murai & Sugár, 1979; Letková *et al*., 2008), a misidentification with other taeniid species may have occurred. A previous study based on morphological analyses highlighted, indeed, *T. ovis* in wolves of the area (Guberti *et al*., 1993). In this regard, molecular techniques are useful tools to distinguish cysticerci of different species, but it is only in recent years that these methods have been routinely performed. Alternatively, a recent introduction of the parasite in the study areas cannot be ruled out. In particular, an infected definitive host may have spread *T. krabbei* eggs and the parasite's life cycle may have been favoured by the abundant hosts' species community present in the study areas ascribed to a recent increase of population of both intermediate (cervids) and definitive hosts (wolves, foxes). While our data do not allow us to

determine which mechanism is really occurred in our population, these occasional first findings of cysticerci highlight how passive surveillance is more likely than active surveillance to detect a “new” wildlife disease (Guberti *et al*., 2014). Although *T. krabbei* is not a public health issue (Hoberg, 2002), severe cysticercosis causes the rejection of the meat as foodstuff for aesthetic reasons (Laaksonen & Paulsen, 2015). Considering the economic concerns for hunters and meat producers related to the cysticerci’ damage of carcasses, our results highlight the need to define the spread of this parasite and identify definitive and other intermediate host species involved in the local life cycle of *T. krabbei*. Moreover, the need is to plan and ensure a proper sanitary education for hunters in order to avoid the spread and maintenance of the parasite. Indeed, in addition to the feeding of dogs with raw viscera/meat, the risk of maintaining its life cycle is related to the discard in the ground of viscera of hunted animals.

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Statement of interest

None.

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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Figure captions

Fig. 1 Map of Northern Italy to show sampling area 1 (horizontal lines) and area 2 (vertical lines) and locations of roe deer with cysticercosis (insert a black triangle) and the dog with the adult tapeworm (insert a black circle).

Fig. 2 Cysticerci (arrowed) in the thigh muscle of roe deer**.**

Fig. 3 Phylogenetic relationship based on the cox-1 sequences of *T. krabbei* recorded in our study (bold type) and selected reference sequences of *T. krabbei* and other related Taeniids. Phylogenetic tree was constructed by using the neighbor-joining method in MEGA 5, and boostrap values > 70% (1,000 replicates) are indicated. Reference sequences are identified by scientific name, host, country of origin and GenBank accession number. The scale bar indicates nucleotide substitutions per site.