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HELMINTHOLOGY - SHORT COMMUNICATION

Identification and molecular characterization of *Echinococcus canadensis* G6/7 in dogs from Corsica, France

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Abstract

Recent surveys at slaughterhouses confirmed the presence of three different species of *Echinococcus granulosus* sensu lato in France: *E. granulosus* sensu stricto, *E. ortleppi*, and *E. canadensis* G6/7. The latter species was only identified on the French Mediterranean island of Corsica, with a high prevalence in pigs and wild boar. In order to investigate the life cycle of *E. canadensis* in this region, dog feces were collected in 31 municipalities, mainly from individual kennels. The analysis of fecal samples from 259 dogs by multiplex real-time PCR shows no infection by *E. granulosus* sensu stricto, but three dogs were infected by *E. canadensis* G6/7. Genetic analyses of mitochondrial genes (*cox1*, *nad1*, *nad3*, *atp6*) revealed in two dogs a haplotype previously identified in pigs. The third dog was infected by a new haplotype differing only from the two others from dogs by two mutations in the *nad3* gene. This latter haplotype is genetically closer to those identified in pigs rather than those from wild boars. Analysis of questionnaires completed by the owners revealed that the sampled dog population was almost exclusively composed of hunting dogs that had been infrequently dewormed. Most of the owners (78%) leave carcasses of hunter-harvested wild boar in close proximity to their dogs. Nevertheless, genetic results seem to indicate that the three dogs were infected due to their consumption of a pig's infected viscera following home slaughtering. This study confirms the role of dogs as definitive hosts of *E. canadensis* G6/7 in Corsica. Further molecular studies, notably in human cases, are needed to assess the zoonotic impact of *E. canadensis* G6/7 in this region.

Keywords *Echinococcus canadensis* G6/7 · Cystic echinococcosis · Dog · Deworming · Corsica

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Introduction

Cystic echinococcosis (CE) is a zoonotic disease found worldwide and transmitted by cestodes of the *Echinococcus granulosus* sensu lato (s.l.) complex. Humans are infected by the larval stage of the parasite, mostly located in a single organ—typically the liver, although the lungs may also be infected (Brunetti et al. 2010). The growth of a hydatid cyst gradually compresses neighboring structures over the course of a long asymptomatic incubation period. The estimated minimum global human burden of CE averages 285,407 disability-adjusted life years (DALYs) or an annual loss of USD 194,000,000 (Budke et al. 2006).

The lifecycle of *E. granulosus* s.l. requires a carnivorous definitive host harboring adult worms in its intestines and an ungulate intermediate host that is infected by the larval stage and consequently forms hydatid cysts in the liver and/or the lungs. Once a definitive host has consumed the infected viscera of an intermediate host, and after a prepatent period of

between 34 and 58 days, intestinal worms result in the parasite's microscopic eggs being excreted into the environment via the feces. Intermediate hosts are subsequently infected through oral ingestion of these eggs. Dogs (*Canis familiaris*) are usually the main definitive host, but other wild carnivorous species can more or less significantly contribute to the parasite's life cycle (Carmena and Cardona 2014). Different species of intermediate hosts are affected depending on the specific parasitic species within the *E. granulosus* s.l. complex: *E. granulosus* sensu stricto (s.s.), *E. equinus*, *E. ortleppi*, *E. canadensis*, or *E. felidis* (McManus 2013; Nakao et al. 2007; Thompson 2008). The taxonomy of *E. canadensis* that regroups the genotypes G6 (previously corresponding to the camel strain), G7 (previously the pig strain), G8 (previously the "American" cervid strain), and G10 (previously the "Fennoscandian" cervid strain) is still controversial (Nakao et al. 2013). Recent phylogenetic studies confirm G6/7 to be a coherent genotypic entity (Addy et al. 2017) and suggest considering G6/7 and G8/10 as two different species (Laurimäe et al. 2018). Nevertheless, this is not in accordance with results obtained by Yanagida et al. (2017) preventing the designation of G6/G7, G8, and G10 as three different species, as had previously been proposed (Lymbery et al. 2015).

All the *E. granulosus* s.l. species have been described in Europe except *E. felidis* which has been reported only in Africa (Hüttner et al. 2008). A combination of slaughterhouse surveys and molecular analysis revealed the presence of three different species in France. *E. granulosus* s.s. was described with very low prevalence levels in sheep and cattle in the south of France (Umhang et al. 2013). The presence of *E. ortleppi* in cattle was identified in two foci grouping seven cases in central and southwestern France, though two human cases from other areas were also diagnosed (Grenouillet et al. 2014). Finally, *E. canadensis* G6/7 was described in pigs and wild boars exclusively in Corsica (Umhang et al. 2014), which is the fourth largest Mediterranean island. It is also the most mountainous and most forested of these islands. While tourism is its leading economic activity, livestock farming (sheep, goats, pigs, and cattle) is also important. Hunting is very popular, especially wild boar hunting which is traditionally carried out using several dogs from different owners. The island is today considered the most endemic area for CE in France. A similar prevalence of *E. canadensis* G6/7 is observed in pigs (5.9%) and in wild boar (4.0%) living on the island (Umhang et al. 2014). In the neighboring island of Sardinia, CE is one of the most widespread parasitic diseases with a very high prevalence of *E. granulosus* s.s. in sheep (75.3%) and cattle (41.5%) compared to *E. canadensis* G6/7 in pigs (0.7%) (Varcasia et al. 2006). The higher prevalence observed in Corsica compared to the south of France was thought to be due to local practices (free-range pig breeding, home slaughtering, and disposal of hunter-harvested wild boar offal) (Umhang et al. 2014). The endemic nature of CE in this area creates economic concerns not only for the production of traditional pork liver sausage (*figatelli*) due to the impact of having infected liver seized, but also for public health, as Corsica is the region with the highest number of human CE incidences in France (1.76 cases/100,000) (Van Cauteren et al. 2016).

While dogs are thought to be the main and almost exclusive definitive host of *E. granulosus* s.l. in France, no recent data about *E. granulosus* s.l. infection in Corsica were available. This study was therefore designed to investigate the infection of dogs by *E. granulosus* s.l. in Corsica, at the same time questioning owners on their deworming routines and the dogs' feeding habits to gain a better understanding of how the CE life cycle is sustained on the island.

Material and methods

Fecal samples from dogs bred in Corsica were collected from September 2013 to March 2014 in the island's two *départements* (a French administrative unit) (Fig. 1). The feces were mainly collected not only in the individual kennels of hunting dogs but also in two veterinary clinics: one in the Haute-Corse *département* and one in Corse-du-Sud.

A questionnaire was given to the owner of each dog with questions on its age and sex, role (sheepdog, hunting dog, pet), the deworming routines to which it is subject (twice a year or more, once a year, less than once a year), its food (leftovers, industrial pet food, carcass remains), contact with other dogs and with humans, and management of offal after home slaughtering of pigs or sheep and wild boar hunting. Owners received the results of their dog's *Echinococcus* copro-diagnosis by mail.

After collection, the feces were stored at − 20 °C and then kept for 1 week at − 80 °C to inactivate *Echinococcus* sp. eggs. DNA was extracted from 500 mg of each fecal sample using the QIAamp DNA Stool Mini Kit (Qiagen). A multiplex Taqman qPCR system was used to detect *E. granulosus* s.s. and *E. canadensis* G6/7. Mitochondrial genes *nad1* and *nad5* were targeted for *E. granulosus* s.s. and *E. canadensis* G6/7, respectively (Table 1). An internal amplification control was included to test the absence of inhibitors. This control involved adding 100 copies of plasmid DNA obtained through artificial construction (Auvray et al. 2009), which was amplified with the same primers as *E. granulosus s.s.* but detected with a different probe to specifically identify each source of DNA. The reaction was performed in duplicate with Maxima Probe qPCR Master Mix (ThermoScientific) in a final volume of 25 μl, including 2 μl of DNA from the fecal sample, and run on a Rotor-Gene thermocycler (Qiagen). The final concentrations of 0.3 μM and 0.2 μM were used for the two primer pairs and the three probes, respectively. The multiplex qPCR program used was 10 min at 95 °C, and then 45 cycles of 15 s at 95 °C and 60 s at 60 °C.

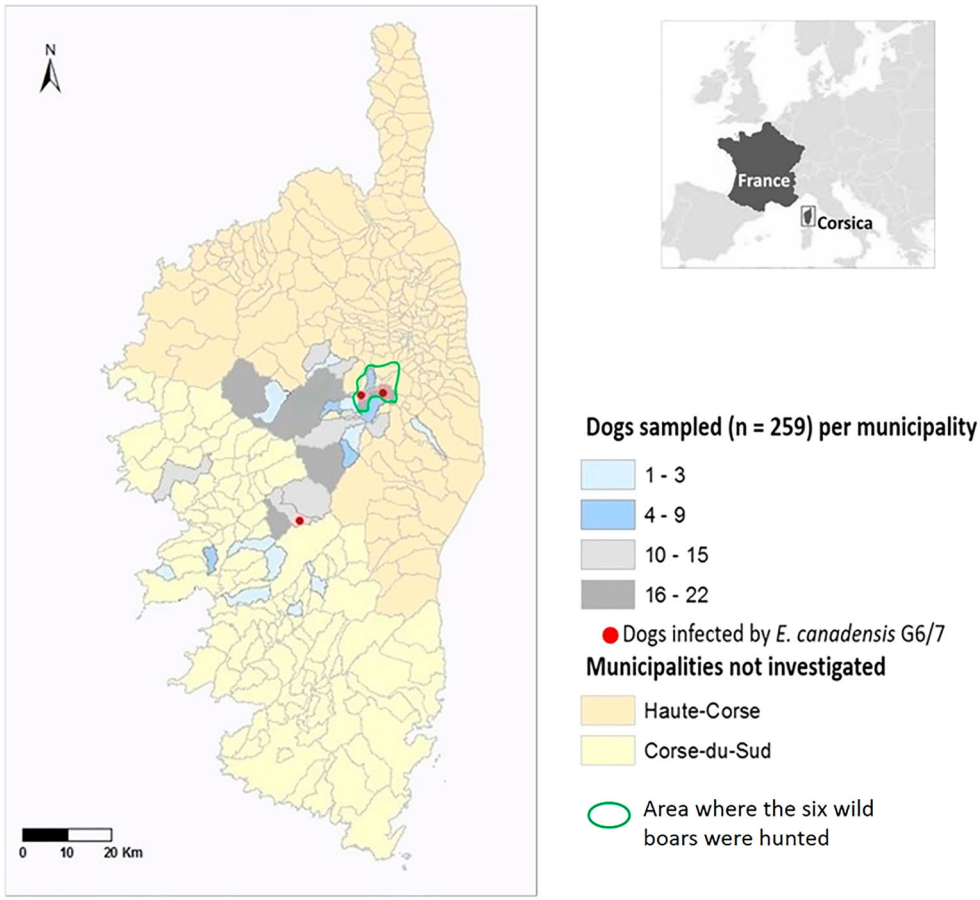

Fig. 1 Dogs sampled per municipality and location of dogs infected by *E. canadensis* G6/7

In order to undertake phylogenetic analyses enabling comparisons with previous identifications of *E. canadensis* G6/7 in Corsica, four mitochondrial genes (*cox1*, *nad1*, *nad3*, *atp6*) were fully or partially sequenced as previously described (Umhang et al. 2014). Additionally, the presence of *Taenia* species was detected using PCR primers Cest4–Cest5 targeting the *12S* mitochondrial gene as described by Trachsel et al. (2007).

A private company (Beckmann Coulter Genomics, UK) sequenced the amplicons obtained through conventional PCR, while the Vector NTI software program (Invitrogen, France) was used to align nucleotide sequences. A haplotype network was constructed for *E. canadensis* G6/7 samples using TCS 1.2 software (Clement et al. 2000) and modified using tcsBU software (Múrias dos Santos et al. 2016). The network inferred from the concatenated sequences of the four mitochondrial genes was analyzed. The nucleotide sequences of *E. canadensis* G6/7 from 19 pigs and two wild boars sampled in Corsica in a previous study (Umhang et al. 2014) were also incorporated in the haplotype network alongside those of

Table 1 Description of the different primers and probes used in multiplex qPCR to detect *E. granulosus* s.s. and *E. canadensis* G6/7 using an internal control

qPCR assay	Gene targeted	Position	Oligonucleotide sequences (5′-3′)	Labelling dye	
				5′	3′
E. granulosus sensu stricto	*nad1*	303–318	AGGCCTCTCCGTGTTG	–	–
		326–344	TGGCTGCCGCCAGAACATC	FAM	BHQ1
		371–348	CAACCAGTACACAACAAAGAATAC	–	–
Internal control	Plasmid DNA	–	CAAGGCGACAAGGTGCTGATGCCG	Cy5	BHQ3
E. canadensis G6/7	*nad5*	1239–1261	TCTTTCTGATAGACGAGGTTAGG	–	–
		1294–1269	CACCAAACTCACACTACAAACCACCG	HEX	BHQ1
		1319–1298	TCCATAAAGCCAAAAATTGTAC	–	–

the dogs. Additionally, the nucleotide sequences from four other infected wild boars found in the same area as those from the above mentioned study, and sampled during the period when the dog feces were being collected, were also added to the network.

Results and discussion

Feces were sampled from a total of 259 dogs from 59 owners in 31 municipalities (the smallest administrative unit in France) (Fig. 1). Each owner had 4.3 dogs on average. Twenty-four (7.3%) fecal samples were obtained from veterinary clinics. Among these dogs, 86% were adults (more than 1 year old), 60% were male, and 77% ($n = 200$) were kept purely for hunting. Almost 19% ($n = 49$) were hunting dogs that lived on a pig farm and were also used as sheepdogs for pig breeding, whereas six were exclusively sheepdogs and four were pets living in rural areas.

Of all the dogs sampled, 41% were dewormed twice a year or more, 45% once a year, and 14% less than once a year or never. Due to a certain proximity between kennels and hunts, each dog was in contact with 10.3 other dogs on average. Two to three people (average 2.6) were in regular contact with each dog. Almost half of the dogs sampled (49%) were fed with a mix of leftovers and/or industrial pet food, and 18% were only fed with industrial pet food. One third (33%) of the dogs were given pig carcass remains (including viscera) after home slaughtering and/or the carcass remains of hunter-harvested wild boar.

Of the hunting dogs included in this study ($n = 249$), 78% were owned by hunters who reported having left wild boar carcasses in the countryside, the others depositing them in public trash bins or communal graves.

Molecular diagnostics revealed the presence of *E. canadensis* G6/7 DNA in three fecal samples (TK7806, TK7823, and TK7855), but *E. granulosus* s.s. was not detected. The prevalence of *E. canadensis* G6–7 was thus estimated at 1.2% ($CI_{95\%}$ 0.2–3.3%). The three infected dogs were adults from three different municipalities; two in Haute-Corse and one in Corse-du-Sud (Fig. 1). All three were hunting dogs and one of them lived on a pig farm. Two were dewormed twice a year and the other once a year. With respect to *Taenia* sp., two infections by *Taenia ovis* and one by *Taenia hydatigena* were detected in three other dogs belonging to three different owners.

The sequencing of the three *E. canadensis* G6/7 fecal samples for the four mitochondrial targets revealed that samples TK7806 and TK7855 indicated the same concatenated haplotype, G6/7 C, previously described in pigs (Table 2 and Fig. 2). From fecal sample TK7823, nucleotide sequences identical to haplotypes G6/7 C were obtained for *cox1*, *nad1*, and *atp6*, but another haplotype for the *nad3* locus was identified with two mutations (116C/T, 337T/G) compared to haplotype G6/7 C. Taking into account the four loci investigated, fecal sample TK7823 corresponds to a newly identified haplotype in Corsica named G6/7 E. The six wild boars appeared to be represented by a further three different concatenated haplotypes named G6/7 F (sample TK3916), G6/7 G (sample TK3918), and G6/7 H (samples TK8564, TK8566, TK8573, TK8574), even if wild boars mostly share similar haplotypes to pigs in terms of individual genes. The nucleotide sequences corresponding to haplotypes of the four targeted genes were registered in GenBank with accession numbers MG808393 to MG808404 for the three dogs and MH823705 to MH823728 for the six wild boars.

The parsimony-based haplotype network indicates that the infected fecal samples from dogs TK7806 and TK7855 correspond to the most commonly represented haplotype in pigs, whereas the other one (TK7823) differed by two to four mutations from other haplotypes found in pigs. For the samples collected, the three haplotypes identified in wild boars differ slightly from those identified in pigs and dogs.

This study is the first to provide molecular data about the infection of dogs by *E. granulosus* s.l. in Corsica, and more widely in France. Only a few studies on canine infection by *E. granulosus* s.l., and particularly by *E. canadensis* G6/7, are available elsewhere. The presence in Europe of this latter species in dogs has only been described in eastern European countries, with a single report of canine infection in Lithuania (Bruzinskaite et al. 2009). The prevalence of *E. canadensis* G6/7 in dogs observed here (1.2%; $CI_{95\%}$ 0.2–3.3%) is slightly below that observed in Lithuania (3.8%; $CI_{95\%}$ 1.7–7.0%), which is coherent with the fact that the prevalence in pigs (5.9%; $CI_{95\%}$ 5.0–6.8%) in Corsica (Umhang et al. 2014) is also below that described in Lithuania (13.2%; $CI_{95\%}$ 10.7–16.2%).

The genetic analyses we carried out using mitochondrial genes revealed the same haplotypes in pigs as in two of the three infected dogs in Corsica. A low genetic diversity of *E. canadensis* was previously observed in 19 pigs when only four concatenated haplotypes (1748 bp) were described using the four mitochondrial genes (Umhang et al. 2014). Sequencing the full *cox1* and *nad1* genes has also exposed very low polymorphism levels in the Corsican pig subpopulation compared to others in Europe, sub-Saharan Africa, and the Middle East (Addy et al. 2017). This can be explained by the geographic segregation of the island's parasite population in addition to a low level of polymorphism in the founder population. Interestingly, sequencing complete mitochondrial genomes from worldwide samples of *E. canadensis* G6/7 has resulted in the identification of two sub-clusters for genotype G7 of *E. canadensis*. One sub-cluster is composed of samples obtained around the world, including from Corsican pigs, while the other is exclusively composed of samples from Corsica and the neighboring island of Sardinia (Laurimäe

Table 2 Description of the different haplotypes obtained for each locus of the mitochondrial genes sequenced in the three dogs infected by *E. canadensis* G6/7

	Host species	*nad1*	*cox1*	*nad3*	*atp6*	Final haplotype
TK7806	Dog	A/B/C/D	C/D	C	C	C
TK7823	Dog	A/B/C/D	C/D	E	C	E
TK7855	Dog	A/B/C/D	C/D	C	C	C
TK3916	Wild boar	A/B/C/D	A/B	A/B	B	F
TK3918	Wild boar	A/B/C/D	C/D	D	C	G
TK8564, TK8566, TK8573, TK8574	Wild boar	A/B/C/D	C/D	D	H	H

Haplotypes A to D refer to those previously described in pigs in Corsica by concatenated sequences of the four genes (Umhang et al. 2014). This study is the first to describe concatenated haplotypes E to G from wild boars. The same nucleotide sequences were obtained when more than one haplotype is mentioned. A haplotype other than A to D was mentioned only when a specific sequence was identified in a new haplotype

et al. 2018). A shared evolution involving exchanges between the parasite populations of these two islands, 11 km apart, may be suspected and needs to be further investigated.

With regard to the production of traditional pork sausage "charcuterie," the importation of live pigs is very limited and they are not usually mixed with local populations. Furthermore, the importation of pig carcasses is not thought to contribute to the parasite's life cycle. Even so, one of the infected dogs harbored a genetic variant due to mutations in the *nad3* gene which had not previously been detected. It should be noted here that only the following swine were tested for the additional genes *nad3* and *atp6*: 19 pigs of the 180 found to be infected in a previous study (Umhang et al. 2014) and six wild boars in the present study. We can imagine that the new haplotype described here in a dog was also present in pigs and/or wild boar.

The detection of the same haplotype among the four mitochondrial targets in both pigs and dogs indicates the presence of a complete *E. canadensis* G6/7 life cycle in Corsica. In light of the samples obtained during our research, the three dogs appear to have been infected following their consumption of infected viscera from a pig rather than from a wild boar, even though all three dogs were considered hunting dogs and two of the dogs were sampled in the area where wild boar were hunted.

The answers to the dog owner questionnaire clearly highlight risky practices that play a role in sustaining the life cycle of *E. canadensis* G6/7. The common hunting practice in which wild boar carcasses are left in the countryside, as well as the home slaughtering of pigs that is still practiced on the island, provides opportunities for dogs to become infected. Furthermore, the dogs we sampled were dewormed twice a year at most, which is too infrequent to prevent infection and the subsequent excretion of infectious eggs. As the infection of pigs by *E. canadensis* G6/7 in Corsica is relatively homogeneous throughout the island (Umhang et al. 2014), we can hypothesize that the canine prevalence observed in the present study would be similar in other pig-breeding areas in Corsica where practices of pig or wild boar slaughter waste deposits are similar. In addition to the present study, a previous survey on a small sample ($n = 38$) of foxes (*Vulpes vulpes*) collected in various areas of Corsica did not indicate the presence of *E. granulosus* sp. in this other carnivorous species (Richomme 2009). Even when found to be infected, foxes are considered to play a minimal role in contaminating the environment with *E. granulosus* s.l. eggs (Mackenstedt et al. 2015). As previously hypothesized, dogs thus appear to be the main and maybe the sole definitive host of *E. canadensis* G6/7 in Corsica (Umhang et al. 2014). In order to reduce the spread of this parasite on the island and its

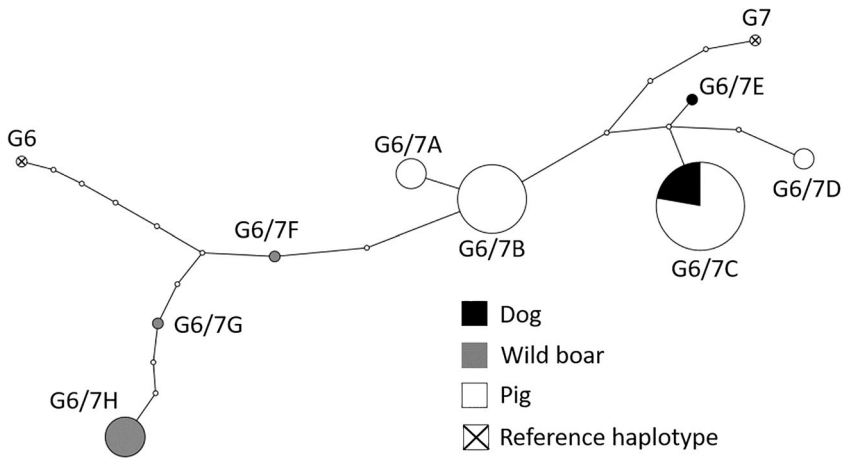

Fig. 2 Parsimony-based haplotype network of mitochondrial DNA (*cox1*, *nad1*, *atp6*, and *nad3* corresponding to 1748 bp) obtained from CE cyst samples from 19 pigs (Umhang et al. 2014), six wild boars and the three fecal samples from dogs in this study. The size of the circles roughly indicates the number of individuals (only one for the two reference sequences of *E. canadensis* G6 and G7), and each mutation event is represented by a white circle on the lines

economic impact on pig breeding, a more appropriate deworming frequency and a strict restriction on dogs' access to pig and wild boar viscera are still necessary.

The absence of infection by *E. granulosus* s.s. observed in the present study in dogs is consistent with results from a previous survey in various slaughterhouses that targeted not only pigs but also sheep, cattle, and goats (Umhang et al. 2014). Nevertheless, most of the sheep and cattle—the main intermediate hosts of *E. granulosus* s.s.—investigated during this earlier survey were young animals. Further investigations focusing on *E. granulosus s.s.* in sheepdogs would be relevant even if it affects a smaller population than hunting dogs. The lack of physical evidence for this *Echinococcus* species in Corsica is intriguing. There is still a low prevalence in the south of France (Umhang et al. 2013). In a previous French national survey of *E. granulosus* at the slaughterhouse, data from Corsica were reported generally as "CE infection in cattle" (Soulé et al. 1989). As no appropriate tools were available in the 1990s, no molecular data are available, but it seems highly probable that these data concerned infection by *E. granulosus* s.s. rather than *E. canadensis* G6/7. An older survey in 1959 described *E. granulosus* prevalence at 22% in cattle, 24% in sheep, and 46% in pigs after morphological inspection at the slaughterhouse (Deschiens 1960), which strengthens the hypothesis of the coexistence at that period of both *E. granulosus* s.s. and *E. canadensis* G6/7 in Corsica. The identification of two dogs infected by *T. ovis* indicates that dogs are also accessing and consuming viscera from sheep, revealing their capacity to sustain another parasite with similar life-cycle traits.

The high human incidence of CE in Corsica compared to mainland French regions (Van Cauteren et al. 2016) raises questions about the zoonotic potential of *E. canadensis* G6/7. This matter cannot yet be investigated because no molecular diagnosis of CE human cases originating from Corsica has so far been possible because human cases are not registered in France. Furthermore, surgery or percutaneous treatment for a CE cyst on patients originating from Corsica are often performed in different hospitals across the continent, which prevents the acquisition of any genotyping data.

Finally, despite the data obtained from definitive and intermediate hosts, the absence of *E. granulosus* s.s. or its presence in livestock, still at a low prevalence in Corsica, will not be conclusively proven without implementing further molecular studies in definitive and intermediate hosts as well as in patients affected by CE.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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