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1 High variability in the number of *E. multilocularis* eggs in cat feces collected in the field

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12

13 Abstract:

14 *Echinococcus multilocularis* is the causative agent of alveolar echinococcosis that is considered as the
15 most severe parasitic disease in Europe. The contribution of cat to environmental contamination by
16 *E. multilocularis* is generally considered as extremely low based on results of experimental infections
17 and worm burden estimations from natural infections. However, the recent collection of numerous
18 cat feces from kitchen gardens in high endemic areas and the detection of *E. multilocularis* DNA in a
19 significant number of these feces raise the question of the risk of human transmission from cats. This
20 study aimed to provide a quantitative estimation of *E. multilocularis* eggs in feces from naturally
21 infected cats. A field sampling conducted in 192 kitchen gardens during a joint study led to the
22 collection and analysis of 597 cat feces, among them 7 (1.2%) yielded positive results for *E.*
23 *multilocularis* real-time PCR. The entire pellets obtained after homogenization, filtration and

24 centrifugation of a 5gr-sample for each of these 7 feces were examined under a stereoscopic
25 microscope. After assessing their number, 20 taeniid eggs were individually isolated and specifically
26 identified by real-time PCR. Morphologically mature *E. multilocularis* eggs were identified in 4
27 samples and the counting of 4 to 43 *E. multilocularis* eggs per gram in these samples, i.e. 62 to 2,331
28 eggs per feces when the total mass of the feces is considered. The number of eggs counted in 2 feces
29 suggests a biotic potential of some naturally infected cats that largely exceed the previous
30 experimental estimations.

31 Keywords: *Echinococcus multilocularis*; domestic cat; biotic potential; eggs; zoonotic risk

32

33 The cestode *Echinococcus multilocularis* is the causative agent of alveolar echinococcosis that is
34 considered as the most severe parasitic disease in Europe [1]. The lifecycle in Europe is essentially
35 sylvatic and based on predation of small rodents by wild canids, mainly red fox (*Vulpes vulpes*). The
36 rodent intermediate hosts are infected after ingestion of microscopic eggs, which are capable of
37 surviving in the environment during months due to their resistant outer envelope [2]. After reaching
38 the liver, the oncospheres will develop into metacestodes leading to production of protoscoleces in
39 around two to four months. The viable protoscoleces are infectious for the definitive host when it
40 ingests the parasitized prey. The intestinal development of protoscoleces into gravid worms with egg
41 production will generally take between 28 to 35 days after infection. The total number of eggs
42 excreted during one infection (i.e. biotic potential) is variable, notably according to host species [3,
43 4]. A comparative study of experimental infection by *E. multilocularis* in definitive hosts described a
44 500-fold higher biotic potential for red foxes, raccoon dogs (*Nyctereutes procyonoides*) and dogs
45 (*Canis familiaris*) than for domestic cats (*Felis catus*) [3]. Very low worm burden (3 to 30-50 worms)
46 were generally found in the intestine of naturally infected cats [5, 6]. A higher worm burden (7,040
47 worms) was described by Umhang et al. [7], but consisted of only immature worms, and only a small
48 proportion of them are assumed to reach the fully mature adults to release eggs. Both because of its
49 low worm burdens and its excretion of a very small number of eggs of unproven infectivity, the cat is
50 generally considered to play an insignificant role in the transmission of *E. multilocularis* [3]. However,
51 the evidence of frequent consumption by cat of *E. multilocularis* intermediate host species [8], the
52 detection of DNA of this parasite in many cat feces [7, 9-13] and the high density of cat feces in
53 kitchen gardens in endemic areas [14] raise concerns about the zoonotic risk associated to cat
54 infections. Information on the biotic potential of naturally infected cats is needed to assess the
55 epidemiological status of this host with respect to *E. multilocularis*.

56 The aim of this study was to estimate the total number of *E. multilocularis* eggs in feces of naturally
57 infected cats from endemic areas. This quantitative estimation was then compared to that obtained
58 from experimental infections to discuss the contribution of the cat to environmental contamination

59 by *E. multilocularis*. A field sampling was conducted from January 2014 to December 2015 by Bastien
60 et al. [15] in 192 kitchen gardens located in high endemic regions for *E. multilocularis* in northeastern
61 France. It resulted to the collect of 1,016 carnivore feces. Real-time coproPCR assays identified 597 of
62 these feces as being from cats and detected *E. multilocularis* DNA in seven of them [9]. All the seven
63 feces originated from different kitchen gardens. These feces were stored frozen since collection
64 including one week at -80°C for decontamination. Five grams were homogenized in 50ml of distilled
65 water and then filtered through a 120µm nylon mesh. After centrifugation, the supernatant was
66 discarded and the pellets completed again with 50ml of distilled water before to be homogenized.
67 These steps were repeated at least two times to clear the pellets. The entire pellets were then
68 examined under a stereoscopic microscope (x200) and all the taeniid eggs observed during this
69 examination were counted. When present, 20 taeniid eggs were individually isolated for each fecal
70 sample in order to be submitted to individual DNA extraction (Nucleospin Tissue, Macherey-Nagel). A
71 real-time PCR assay was performed to detect DNA of *E. multilocularis* with sequencing confirmation
72 [16]. Five microliters of DNA were used in a final volume of 20µl using Maxima Probe master mix and
73 an internal control. All samples were tested in duplicate. The real-time PCR assays were realized
74 using a Mx3005P thermocycler (Agilent) with an initial step of 95°C for 10min and 45 cycles of 95°C
75 for 15s and 60°C for 60s. When a Ct value was obtained for at least one duplicate, sequencing was
76 realized from the PCR products to confirm identification of *E. multilocularis*. If no DNA of *E.*
77 *multilocularis* was detected, the DNA samples were submitted to an endpoint PCR using primer
78 Cest4-Cest5 in order to identify the Taenidae species involved after sequencing [17]. In absence of
79 detection for the two PCR assays (*E. multilocularis* or Taenidae species), the eggs were excluded for
80 the calculation of the number of eggs per gram (epg). We used the ratio of *E. multilocularis* eggs
81 versus other Taenidae species obtained from the successful amplification among the 20 eggs isolated
82 to estimate the number of *E. multilocularis* eggs per gram and calculate the total number of these
83 eggs in the cat's feces.

84 No taeniid eggs were observed after filtration for 2/7 fecal samples from which *E. multilocularis* DNA
85 was detected. Nevertheless, *Toxocora* sp. eggs were present for these two fecal samples confirming a
86 correct flotation. Identification of *E. multilocularis* eggs was obtained for 4/5 samples with taeniid
87 eggs, including one with coinfection by *Hydatigera taeniaeformis*. In the only fecal sample free of *E.*
88 *multilocularis* eggs among these five samples, taeniid eggs were all corresponding to the cat
89 tapeworm *Hydatigera taeniaeformis* (Table 1). The lowest average Cq values from fecal samples
90 were obtained from the four samples with *E. multilocularis* eggs (30.6 to 35.8) compared to the three
91 without eggs (36.9 to 39.2). The microscopic observation revealed morphologically mature *E.*
92 *multilocularis* eggs with the presence of embryophores surrounding the oncosphere in the four
93 cases. When present, the number of *E. multilocularis* eggs per gram (epg) was ranging from 4 to 43.
94 Considering the total mass of each fecal sample, 62 to 2,331 *E. multilocularis* eggs per fecal sample
95 were estimated.

96 Our finding of a slightly lower amount of DNA in feces without eggs than in feces with eggs confirms
97 that a substantial quantity of the copro-DNA from the parasite may not originated from eggs but
98 rather from worm tissue cells. In addition, parasitic DNA detected in definitive host (i.e. cat) feces can
99 also come from ingestion of infected prey if the feces were produced within a few days of that
100 ingestion as it has been demonstrated for *Toxoplasma gondii*, [18]. Worm tissues or parasitic DNA
101 from infected prey can explain alone the detection of *E. multilocularis* in the three feces where no
102 eggs were observed. Regarding feces with *E. multilocularis* eggs, the DNA from these eggs comes in
103 addition to the worm tissue source facilitating the molecular detection. A significant difference
104 between distribution of Cq values from fox feces with or without eggs was also previously reported
105 by Da Silva et al. [19].

106 Based on an experimental infection with 20,000 protoscoleces, the mean biotic potential of cats was
107 estimated of 573 eggs excreted during an entire patent period estimated at 13 days in cats [3].
108 However, a highest daily eggs excretion of 856 eggs at 28 dpi was described for one cat in the same
109 study. By comparison, the estimation of the number of eggs excreted in two fecal samples from our

110 field sampling is 2 to 4-fold higher than this estimated biotic potential. The availability of only one
111 fecal sample for each infection prevent us to assess duration of the patent periods in these natural
112 infections, as well as the total number of eggs excreted in the course of the whole patent period.
113 However, one can speculate that in each infection related to these two cases, several others feces
114 from the same cats during the patent period will also containing eggs, potentially even more than
115 described in the analyzed feces. Therefore, our data suggest that the biotic potential of some cats
116 may be largely higher than expected even if the null or low eggs production associated with the three
117 others positive fecal samples are in accordance to the very low biotic potential generally described
118 for the species. The estimation of 440 eggs per gram reported for a cat from Hokkaido Island, Japan
119 [20] which is largely (i.e. 10 fold) exceeding our highest estimations argues to excretion of large
120 quantities of *E. multilocularis* eggs by cats from natural infections not being a very rare event. The
121 high excretion of eggs by some cats may explain the detection of *E. multilocularis* DNA from soil
122 samples of kitchen gardens where no fox feces were observed, including fenced kitchen gardens [9].
123 In a general way, natural infection in cats by *E. multilocularis* is generally restricted to a molecular
124 diagnostic, and the number of eggs is rarely estimated when the presence of *E. multilocularis* eggs is
125 confirmed resulting to scarce data related to the biotic potential. The data obtained from
126 experimental infections are very informative regarding the global epidemiological situations but may
127 underlooked some specific situations. Combination of different cat species, quantities of
128 protoscoleces, *E. multilocularis* strains, ecological contexts and immunological expositions will results
129 to a large variety of possibilities regarding the success of natural infections. The strong predation of
130 rodents by cats can notably increase the chance of repeated infections and raise the need to assess
131 the potential development of an immunity decreasing the worm burden and patent period, as
132 described for foxes and dogs [21, 22]. It also has to be noted that the high number of eggs excreted
133 by infected cats in this study remains very inferior to the biotic potential of canids, as notably red
134 foxes and dogs can excrete 279,910 and 346,473 eggs per individual, respectively [3]. Furthermore,
135 the existence of a zoonotic risk associated to *E. multilocularis* eggs excreted via cat feces is currently

136 not confirmed. Eggs obtained from feces of a cat experimentally infected by a North American strain
137 of *E. multilocularis* has developed to metacestode after ingestion by a brown lemming [23] but those
138 obtained after experimental infection of cats by an European strain has not been successful to infect
139 mice, unlike those obtained from foxes, dogs and raccoon dog feces [3].

140 In conclusion, our data show that some cat feces may contain high numbers of *E. multilocularis* eggs,
141 although the overall contribution of cats to environmental contamination by this parasite is probably
142 extremely low compared to that of foxes. The presence of high numbers of *E. multilocularis* eggs in
143 some cat feces reinforce the need to test the infectivity of these eggs obtained in natural conditions,
144 which has not yet been reported. Specific protocols coupling rapid molecular diagnostic from
145 decontaminated part of the cat fecal sample potentially followed by isolation of taeniid eggs are
146 required. In absence of current in vitro test for evaluation of the infectivity, in vivo assays in rodents
147 remains the only way to confirm the infectivity, and then a zoonotic potential.

148

149 **Declaration of Competing Interests**

150 The authors declare no competing interests in association with this study.

151

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155

156 **References**

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228

229 **Table 1:** Information and coprologic results of the seven cat feces with detection of *E. multilocularis*

230 DNA by copro real-time PCR.

Table 1: Information and coprologic results of the seven cat feces with detection of *Echinococcus multilocularis* DNA by copro real-time PCR.

Sample ID	Village	Kitchen garden ID	Collection date	Total weight of the fecal sample (g)	Average Cq values of <i>E. multilocularis</i> qPCR	Presence of taenid eggs	Proportion of <i>E. multilocularis</i> among taenid eggs	Number of <i>E. multilocularis</i> eggs/g	Total number of <i>E. multilocularis</i> eggs/fecal sample	Proportion of <i>H. taeniaeformis</i> among taenid eggs	Number of <i>H. taeniaeformis</i> eggs/g
10426	Faxe	FAX08	07/11/2014	54.2	30.6	yes	18/20	43	2331	0/20	0
10471	Grandpré	GRA07	23/01/2015	36.3	35.5	yes	6/20	33	1198	2/20	22
10371	Lorry-mardigny	LOR09	18/12/2014	8.4	34.8	yes	15/20	14	118	0/20	0
10459	Boult-aux Bois	BAB13	21/01/2015	15.4	35.8	yes	15/20	4	62	0/20	0
10550	Avricourt	AVR12	10/03/2015	28.6	36.9	yes	0/20	0	0	15/20	10
10390	Avricourt	AVR08	18/12/2014	52.5	37	no	/	/	/	/	/
11885	Faxe	FAX08	16/12/2015	42.4	39,2*	no	/	/	/	/	/

*Cq value obtained for only one duplicate.