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1     **Assessment of the exposure to *Echinococcus multilocularis* associated with carnivore**  
2             **faeces using real-time quantitative PCR and flotation technique assays**

3             Abdou Malik Da Silva<sup>a</sup>, Sandra Courquet<sup>a,b</sup>, Francis Raoul<sup>a</sup>, Dominique Rieffel<sup>a</sup>, Patrick  
4                             Giraudoux<sup>a</sup>, Laurence Millon<sup>a,b</sup>, Jenny Knapp<sup>a,b,\*</sup>

5     <sup>a</sup> *Chrono-Environnement, UMR UBFC/CNRS 6249, University of Bourgogne Franche-Comté,*  
6     *25030 Besançon, France*

7     <sup>b</sup> *Department of Parasitology-Mycology, University Hospital of Besançon, 25030 Besançon, France*

8

9     \*Corresponding author. Tel.: +33 363082274. *E-mail address:* [jknapp@univ-fcomte.fr](mailto:jknapp@univ-fcomte.fr)

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17 **Abstract**

18 The eggs of *Echinococcus multilocularis*, the infectious stage, are spread into the environment  
19 through wild and domestic carnivore faeces. The spatial location of the faeces containing infective  
20 *E. multilocularis* eggs is a key parameter for studying areas of exposure and understanding the  
21 transmission processes to the intermediate hosts and humans. *Echinococcus multilocularis* faecal  
22 prevalence is often assessed by detecting *E. multilocularis* DNA, not necessarily eggs. This work  
23 aimed to determine the percentage of faeces containing *E. multilocularis* eggs in a rural town and its  
24 surroundings and whether this level of precision is relevant in assessing exposure to *E.*  
25 *multilocularis*. For this purpose, we developed a combined molecular and microscopic approach to  
26 investigate the *E. multilocularis* exposure of potential hosts in the environment from field-collected  
27 carnivore faeces. Carnivore defecation patterns were then spatialized to study the spatial  
28 distribution of *E. multilocularis*. Faeces were screened for *E. multilocularis* DNA using a specific  
29 real-time quantitative PCR (qPCR). *Echinococcus multilocularis* eggs were morphologically  
30 identified from *E. multilocularis*-specific qPCR-positive faeces after sucrose flotation and  
31 individually confirmed through specific PCR and sequencing. The spatial distribution of *E.*  
32 *multilocularis* was studied using Kulldorff statistics. *Echinococcus multilocularis* eggs were  
33 identified mostly in fox faeces positive for *E. multilocularis* DNA by qPCR ( $n=27/70$ ) and only  
34 from 1 of 15 copro-samples from dogs and 1 of 5 from cats. The faecal prevalence of *E.*  
35 *multilocularis* DNA and eggs was overdispersed, with the same geographical patterns. These data  
36 suggest that *E. multilocularis* DNA and/or egg detection in carnivore faeces, mainly that of foxes, is  
37 appropriate in ecological studies of *E. multilocularis* transmission.

38

39 *Keywords:* *Echinococcus multilocularis*, Egg, Definitive host, Copro-qPCR diagnostic, Flotation,  
40 Spatial distribution

41

## 42 **1. Introduction**

43           The larval stage of the cestode *Echinococcus multilocularis* is responsible for alveolar  
44 echinococcosis (AE), an expanding, potentially lethal, zoonosis that occurs only in the northern  
45 hemisphere, including large areas of Asia, Europe, and North America (Deplazes et al., 2017). The  
46 lifecycle is mainly sylvatic, based on predator-prey interactions between wild and domestic  
47 carnivores acting as definitive hosts, and small rodents as intermediate hosts. The adult worms live  
48 in the small intestine of carnivores and produce the infectious form at sexual maturity, the egg,  
49 eliminated into the environment with faeces during the patent period. Development of the larval  
50 stage of the parasite following the ingestion of eggs causes AE in humans. In Europe, *E.*  
51 *multilocularis* eggs have only rarely been identified in dog and cat faeces (Dyachenko et al., 2008;  
52 Deplazes et al., 2011; Umhang et al., 2012, 2014, 2015; Knapp et al., 2016a). The red fox (*Vulpes*  
53 *vulpes*) has been identified as the main definitive host of *E. multilocularis*, in which the parasite has  
54 experimentally shown a high reproduction rate and high biotic potential, whereas domestic  
55 carnivores, notably the cat, likely excrete few or no eggs (Kapel et al., 2006; Hegglin and Deplazes,  
56 2013; Umhang et al., 2015; Knapp et al., 2018).

57           The use of carnivore stools in ecological investigations on *E. multilocularis* transmission  
58 seems appropriate, as stool contamination is representative of environmental contamination by the  
59 infectious form of the parasite and it is the source of human exposure (Conraths and Deplazes,  
60 2015; Knapp et al., 2018). In addition, the spatial location of the faeces containing infective *E.*  
61 *multilocularis* eggs is a key parameter for studying areas of risk and understanding the transmission  
62 processes to the intermediate hosts and humans, to propose appropriate prevention or control  
63 measures (Knapp et al., 2018). Moreover, the analysis of faecal samples is a non-invasive approach,  
64 allowing the preservation of animal populations, essential when studying pets and ethically more  
65 acceptable for wild animals.

66           The spatial distribution of carnivore faeces and *E. multilocularis* prevalence appear  
67 heterogeneous and aggregate in micro-foci over large areas including historically and newly  
68 endemic rural and urban sites. The reasons are in part linked to the defecation behaviour of the  
69 definitive hosts. For instance, micro-local hotspots of high *E. multilocularis* prevalence in fox  
70 faeces were detected in different rural situations in a study carried out in an eastern region of France  
71 (Knapp et al., 2018). Moreover, at a local level, *E. multilocularis*-positive faeces were found in  
72 19/94 sampled kitchen gardens studied (Pouille et al., 2017). In a North American urban setting, *E.*  
73 *multilocularis* prevalence in the faeces of coyotes varied significantly across the study area (Liccioli  
74 et al., 2014). In a rural context in China, *E. multilocularis* environmental contamination via dog  
75 faeces was focused where dogs defecated inside villages (Vaniscotte et al., 2011). Given the close  
76 proximity of humans and the main definitive host of *E. multilocularis* in rural settings, this  
77 environmental overdispersal can potentially be of major public health importance. However, *E.*  
78 *multilocularis* prevalence is often assessed by detecting *E. multilocularis* DNA, not necessarily  
79 eggs. Furthermore, the sporadic copro-sample collection may lead to failure to detect the parasite  
80 and a misestimation of environmental contamination by *E. multilocularis* via carnivore faeces.

81           The development and optimization of multiplex real-time PCR techniques, based on the  
82 amplification of specific mitochondrial DNA targets, has resulted in the very sensitive and rapid  
83 detection of host species and zoonotic parasites such as *E. multilocularis* in faeces collected from  
84 the environment (Farrell et al., 2000; Nonaka et al., 2009; Knapp et al., 2014, 2016b). Molecular  
85 tools (PCR or real-time quantitative PCR (qPCR)) detect the genetic material in faeces not only  
86 from eggs but also from cells or tissue fragments shed by the worm present in the digestive tract of  
87 the infected animal (Al-Sabi' et al., 2007) and other traces of the parasite in transit. The feeding  
88 behaviour and coprophagia common in certain carnivore species such as dogs may explain the  
89 innocuous passage of parasitic elements, and thus the detection of parasitic DNA, in the digestive  
90 system of these animals (Frenkel and Parker, 1996; Fahrion et al., 2011; Nijssen et al., 2015).  
91 *Echinococcus multilocularis* DNA may also originate directly from the digested metacestode due to

92 predation of infected rodents (Umhang et al., 2015). These events can all potentially lead to  
93 overestimation, to an unknown extent, of the parasitic prevalence in faeces by molecular detection  
94 techniques. Thus, the detection of eggs in stool material is still a key step in the assessment of the  
95 environmental exposure of intermediate hosts or humans to *E. multilocularis*.

96 Eggs may be concentrated by flotation techniques and identified at the family level  
97 (Taeniidae) based on their morphological characteristics (Mathis et al., 1996; Dryden et al., 2005)  
98 ,and PCR on isolated egg DNA remains the method of choice for the species-level diagnosis  
99 (Gasser et al., 1993).

100 This work aimed to determine the percentage of faeces containing *E. multilocularis* eggs in a  
101 rural town and its surroundings and whether this level of precision is relevant in assessing exposure  
102 of the intermediate hosts to *E. multilocularis*. For this purpose, we periodically collected carnivore  
103 faeces in order to obtain suitable samples for parasite detection. We developed a combined  
104 molecular and microscopic approach to investigate exposure of potential hosts to *E. multilocularis*  
105 in the environment from field-collected carnivore faeces. This strategy is first based on the  
106 screening of *E. multilocularis* DNA in faeces, using qPCR and then the identification of *E.*  
107 *multilocularis* eggs in positive qPCR samples by a flotation technique, confirmed by specific PCR.  
108 Then, carnivore defecation patterns were spatialized to compare the local distribution of *E.*  
109 *multilocularis* DNA and eggs in contaminated faeces.

110

## 111 **2. Materials and methods**

### 112 *2.1. Copro-sampling*

113 Faeces were collected every 2 months between March 2017 and November 2018 in a village  
114 and its surroundings in the eastern part of France (006°26'46"E, 46°56'53"N), in order to regularly  
115 obtain fresh material. This site was located in an historic endemic area for *E. multilocularis*

116 (Combes et al., 2012; Piarroux et al., 2013, 2015). The studied area has a surface of 5.28 km<sup>2</sup>, with  
117 a density of 25 inhabitants per km<sup>2</sup>, and is located at an average altitude of 971 m above sea level in  
118 a predominantly agricultural landscape in which the proportion of permanent grassland to total land  
119 is 52% (see the 2010 General Agriculture Census (RGA) from the Ministry of Agriculture, France,  
120 <http://recensement-agricole.agriculture.gouv.fr/>). It has a continental climate, with long, cold,  
121 snowy winters. Average temperatures range from -2.2 to 15.1°C with average rainfall of 1,317  
122 mm/year ([www.meteofrance.com](http://www.meteofrance.com)). All sampled carnivore faeces were collected from the study area  
123 access roads within the administrative limits (along a predetermined circuit of 25 km, Fig. 1). Fox,  
124 dog, cat, mustelid or undetermined faeces were initially identified in the field by observing their  
125 physical characteristics (shape, size, composition). All sampled faeces were geolocated with a  
126 Global Positioning System (GPS) tracking device, with a precision of ± 10 m. Those were collected  
127 in an individual plastic bags and were stored for 7 days at – 80°C for decontamination to prevent  
128 human infection, and then at – 20°C before laboratory analyses.

129

## 130 2.2. Faecal DNA extraction and qPCR protocols for carnivore host faecal test and *E. multilocularis* 131 diagnostic tests

132 Faecal DNA was extracted from a 0.5 g sample (Knapp et al., 2014) and purified using the  
133 QIAmp Fast DNA Stool Kit (Qiagen, Hilden, Germany) following the manufacturer's  
134 recommendations, performed on a QIAcube automaton (Qiagen, Hilden, Germany), a robotic  
135 workstation for automated purification of DNA.

136 A host faecal qPCR test was performed on the copro-DNA to identify the carnivore host.  
137 The test was carried out as described above and was based on amplification of a fragment of the  
138 *cytB* gene for *Vulpes vulpes* (fox), *Canis familiaris* (dog), and *Felis catus* (cat) (Knapp et al.,  
139 2016b). This molecular identification of host species used as reference was compared with the

140 identification from morphological criteria (described above) in order to verify the accuracy of faecal  
141 identification in the field.

142 DNA extracts were tested in an *E. multilocularis*-specific qPCR for the presence of *E.*  
143 *multilocularis* DNA, in duplicate, based on the amplification of a portion of the mitochondrial *rrnL*  
144 gene (Knapp et al., 2014, 2016b) (*rrn-E. multilocularis* forward primer 5'-  
145 CTGTGATCTTGGTGTAGTAGTTGAGATTT-3', *rrn-E. multilocularis* reverse primer 5'-  
146 GGGGTCAATCACAACAACCC-3', *rrn-E. multilocularis* probe 5'- FAM-  
147 TGGTCTGTTCGACCTTTTTAGCCTCCAT-TAMRA -3') for PCR products of 202 bp.

148 The presence of inhibitory factors was tested in the two previous qPCRs using the Alea  
149 qPCR control tool (Alea forward primer 5'-CCTAAAATGTCTATGATTGGTCCACTA-3', Alea  
150 reverse primer 5'-GGGAGTACCTTGCCATACAAAATT-3', Alea probe 5'-VIC-  
151 TTAAATCAACTCCTAAATCCGCGCGATAGG-TAMRA-3') at a concentration of  $6 \cdot 10^{-6}$  ng/ $\mu$ l,  
152 corresponding to 1,000 plasmid copies/ $\mu$ l of the internal control (Knapp et al., 2016b).

153 DNA-free extraction controls (extraction from only kit reagents), PCR-positive (DNA  
154 extracts from *E. multilocularis* worms) and -negative (ultra-pure water instead of DNA extract)  
155 controls were used to ensure there was no contamination during the DNA extraction and the qPCR  
156 steps.

157 The qPCRs were performed for 45 cycles and reactions with quantitative cycle (Cq)  
158 numbers < 45 cycles were considered to be positive for *E. multilocularis* by qPCR and Cq < 30 for  
159 the species detected among the three targeted carnivore species in the faecal qPCR. The presence of  
160 PCR inhibitors was noted on the basis of the duplex *E. multilocularis* /Alea qPCR results,  
161 considering a Cq > 37 as the threshold beyond which the PCR inhibitors interfered with the PCR  
162 process (Knapp et al., 2016b). All samples were tested in duplicate by qPCR.

163

164 *2.3. Isolation of parasite eggs from faeces*



165 All positive faeces from the *E. multilocularis*-specific qPCR screening and a portion of  
166 randomly selected negative faeces, as controls, ( $n = 25$ ) were subjected to a sucrose flotation  
167 technique as described in Matsuo and Kamiya (2005) and Escotte-Binet et al. (2019) to concentrate  
168 and isolate the parasite eggs, based on differential specific gravity (SG) between the eggs and  
169 particles. Briefly, the protocol consists of dispersing 5 g of faeces in 20 ml of 0.1% Tween 80 by  
170 vortexing to separate the eggs from the organic particles. After a centrifugation step (2,500 g, 10  
171 min), the pellet is resuspended in distilled water and deposited on a sucrose solution (SG = 1.27) to  
172 float the eggs. Then the supernatant is concentrated in a volume of < 1 ml after centrifugation.

173

#### 174 2.4. Microscopic observation and sampling of individual *E. multilocularis* eggs

175 All flotation supernatants were examined using a Leica DMI3000-B micromanipulator (Leica  
176 Microsystems, Wetzlar, Germany) equipped with an inverted microscope to identify taeniid eggs at  
177 a 400X magnification. The observed taeniid eggs were picked individually with micropipettes  
178 integrated into the micromanipulator and put in approximately 100  $\mu$ l of distilled water in  
179 individual microtubes. For each positive flotation sample, DNA from up to five individual taeniid  
180 eggs was extracted using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) in an elution  
181 volume of 50  $\mu$ l following the manufacturer's instructions. However, the lysis step was extended  
182 from 1-3 h to overnight. The resulting DNA was used to confirm the *E. multilocularis* egg  
183 identification by two PCRs: the *E. multilocularis*-specific qPCR described above and the *E.*  
184 *multilocularis*-specific PCR EMH15/H17, confirmed by electrophoresis. The *E. multilocularis*-  
185 specific PCR EMH15/H17 is based on the amplification of a portion of the mitochondrial 12S  
186 rRNA gene (Dinkel et al., 1998; Stieger et al., 2002; Georges et al., 2004) (EM-H15, forward  
187 primer: 5'-CCATATTACAACAATATTCCTATC-3', EM-H17, reverse primer: 5'-  
188 GTGAGTGATTCTTGTTAGGGGAAG-3') for an expected PCR product size of 200 bp. Then,  
189 several PCR products ( $n = 6$ ) were randomly selected and sequenced by the Sanger method to

190 confirm the *E. multilocularis* diagnosis (Sanger et al., 1992). The nucleotide sequences obtained  
191 were characterized by comparison with an *E. multilocularis* nucleotide sequence reference in  
192 GenBank using the online genetic databases and the Basic Local Alignment Search Tool (BLAST)  
193 available on the NCBI website.

194 In addition, for each negative flotation sample, the supernatants were extracted and  
195 subjected to *E. multilocularis*-specific qPCR to confirm the microscopic observations (see Fig. 2 for  
196 a synopsis of the complete protocol).

197

#### 198 2.5. Determination of flotation technique sensitivity

199 The effectiveness of the sucrose flotation technique followed by microscopic observation  
200 and detection with *E. multilocularis*-specific qPCR (described above) was evaluated by testing it in  
201 duplicate series of faecal-free *E. multilocularis* DNA samples (5 g) spiked with 10 *E. multilocularis*  
202 eggs ( $n=3$ ), five *E. multilocularis* eggs ( $n=3$ ), three *E. multilocularis* eggs ( $n=3$ ) and one *E.*  
203 *multilocularis* egg ( $n=3$ ). These spiked samples were frozen at  $-80^{\circ}\text{C}$  for 7 days before testing. The  
204 faecal samples were obtained from the colon of captive foxes exhibiting no worms of the *Taenia*  
205 genus nor *E. multilocularis* at the experimental station of the ANSES laboratory, France.  
206 *Echinococcus multilocularis* eggs were obtained from a faecal sample from the colon of a naturally  
207 infected fox and confirmed with *E. multilocularis*-specific qPCR.

208

#### 209 2.6. Statistical and spatial data analysis

210 *Echinococcus multilocularis* faecal prevalence between the various carnivore species was  
211 compared using the Fisher's exact test. The non-parametric Mann-Whitney U Test was used to  
212 compare the Cq values between *E. multilocularis* qPCR-positive faeces, with or without *E.*  
213 *multilocularis* eggs detected. A Receiver Operating Characteristic (ROC) curve analysis was used

214 to estimate the ability of the *E. multilocularis*-specific qPCR (*E. multilocularis* DNA screening in  
215 faeces) to differentiate faeces containing *E. multilocularis* eggs or not. An optimal threshold value  
216 was determined by maximizing the Youden index. Faeces with a Cq higher than the optimal  
217 threshold value were classified as *E. multilocularis* egg-negative, whereas samples with a Cq lower  
218 than the optimal threshold value were classified as *E. multilocularis* egg-positive (Fluss et al.,  
219 2005). ROC, area under ROC curve (AUC) and the optimal threshold value were calculated using  
220 the *pROC* R package (Robin et al., 2011).

221 For statistical tests, differences were considered significant for  $P \leq 0.05$ .

222 The spatial distributions of carnivore faeces and *E. multilocularis* faecal occurrence were  
223 mapped on orthophotos (BD Ortho version 2 of the 'Institut Géographique National', France, with a  
224 50 cm resolution as background).

225 The randomness of the distribution of the *E. multilocularis* faecal prevalence was tested  
226 using a spatial scan statistic developed by Kulldorff and implemented in SatScan v9.6 software  
227 (Kulldorff and Nagarwalla, 1995). We tested the null hypothesis that the number of faeces  
228 containing *E. multilocularis* DNA or eggs follows a binomial distribution along the predetermined  
229 circuit. In SatScan, we used the Bernoulli Model, with the cases defined as faeces which were found  
230 to contain *E. multilocularis* DNA or eggs and the controls as negative faeces. Kulldorff's method  
231 places a circular window on the map and moves the circle centre over each point location so that the  
232 window includes different sets of neighbouring points at different positions. At each point location,  
233 the radius of the circle increases continuously from zero to a maximum radius, defined by the user  
234 and defaulted in this study to a radius containing at most 50% of the total number of points.  
235 Potential clusters were detected by calculating a likelihood ratio for each circle, comparing an  
236 expected number of cases (under the null hypothesis) with the observed number of cases in and  
237 outside the window. The most likely cluster is the circle with the maximum likelihood ratio among  
238 all radius circles at all possible locations.

239 Computations were performed and graphical displays were generated using R 3.5.1 (R Core  
240 Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical  
241 Computing, Vienna, Austria, <https://www.R-project.org>) with the packages foreign 0.8-70 (R Core  
242 Team (2017). Foreign: Read Data Stored by 'Minitab', 'S', 'SAS', 'SPSS', 'Stata', 'Systat', 'Weka',  
243 'dBase', ..., <https://CRAN.R-project.org/package=foreign>), rgdal 1.3-9 (Bivand, R., Keitt, T.,  
244 Rowlingson, B., Pebesma, E., Sumner, M., Hijmans, R., Rouault, E., Warmerdam, F., Ooms, J.,  
245 Rundel, C., 2019. rgdal: Bindings for the "Geospatial" data abstraction library) and the  
246 geographical information system Quantum GIS 3.2.3-Bonn (QGIS Development Team, 2018.  
247 QGIS Geographic Information System, <http://www.qgis.com>). Kulldorff's statistics were computed  
248 using SatScan v9.6 March 2018 (<https://www.satscan.org>).

249

## 250 *2.7. Data accessibility*

251 Raw data are available in Mendeley Data, DOI: <https://dx.doi.org/10.17632/bhzccxfy9c.3>.

252

## 253 **3. Results**

### 254 *3.1. Host species identity of faeces collected in the field*

255 We collected 814 faecal samples from March 2017 to November 2018. In total, 763 faecal  
256 samples (93.7% of the sampling) were identified by the host faecal qPCR test, including 278 fox  
257 faeces (34.15%), 431 dog faeces (52.95%), and 54 cat faeces (6.63%).

258 The morphological assessment made on fox, dog and cat faeces was confirmed by species  
259 qPCRs for 68%, 86% and 52% of the copro-samples, respectively (Table 1). For mustelid faeces  
260 identified on morphological criteria, 56% and 28% of samples were reassigned to fox and dog

261 stools, respectively, by molecular analysis. Most of the undetermined faeces in the field were  
262 identified by molecular assessment (87.5%).

263 The host faecal test was unsuccessful for 51 faecal samples for which identification was  
264 made based on morphological criteria. This affected 31 fox, 14 dog, one cat, four mustelid, and one  
265 undetermined stools (Table 1).

266 Only four samples (0.5% of all samples analysed) contained PCR inhibitors based on duplex  
267 *E. multilocularis*/Alea qPCR results.

268

### 269 3.2. *Echinococcus multilocularis* in carnivore hosts

#### 270 3.2.1. Validation of the sucrose flotation protocol for frozen *E. multilocularis* eggs

271 When testing naive frozen copro-samples of 5 g spiked with 10 *E. multilocularis* eggs, a  
272 microscopic observation and a positive *E. multilocularis* qPCR signal were obtained for all samples  
273 after the sucrose flotation technique, corresponding to 100% sensitivity (Table 2). Average Cq  
274 values ranged from  $30.73 \pm 0.08$  for 10 eggs to  $37.72 \pm 0.16$  for one egg. When using five eggs, then  
275 one egg, sensitivity decreased from 66.7% to 33.3% for the *E. multilocularis* qPCR.

276

#### 277 3.2.2. *Echinococcus multilocularis* DNA and egg prevalence in carnivore faeces

278 We detected *E. multilocularis* DNA in 96 faeces samples (11.8% of the collection, 95%  
279 Confidence interval (CI): 9.7-14.3%). *Echinococcus multilocularis* DNA was detected significantly  
280 more frequently in fox faeces (25.2%, 95% CI: 20.3-30.8%) than cat faeces (9.3%, 95% CI: 3.5-  
281 21.1%) or dog faeces (3.5%, 95% CI: 2.0-5.8%) (Table 3, Fisher's exact test,  $P < 0.0001$ ).

282 Among the 96 carnivore faecal samples containing *E. multilocularis* DNA, 32 (33.3%) were  
283 found to contain *E. multilocularis* eggs, based on the sucrose flotation technique. The identification

284 of *E. multilocularis* was confirmed by *E. multilocularis* specific qPCR performed on DNA extracts  
285 from five individual taeniid eggs isolated from each flotation-positive faecal sample, for which  
286 between one and five were positive, with a mean Cq of  $34.2 \pm 1.46$  ( $n = 52$ ). All selected PCR  
287 EMH15/H17 products ( $n = 6$ , PCR EMH15/H17 positive from *E. multilocularis* eggs isolated from  
288 one dog, one cat, one undetermined host, and three fox stools) were confirmed to be *E.*  
289 *multilocularis*, with 100% identity to the *E. multilocularis* reference sequence (GenBank accession  
290 number **MK116545.1**).

291 Eggs were observed mostly in fox faeces ( $n = 27/70$ ), one of five cat faeces, one of 15 dog  
292 faeces, and three of six qPCR-undetermined host faeces (Table 3, Fisher's exact test,  $P < 0.0001$ ).  
293 Those three faecal samples were identified morphologically as two fox and one dog stool.

294 All selected *E. multilocularis* qPCR-negative faeces ( $n = 25$ ) did not contain *E.*  
295 *multilocularis* eggs, as their flotation supernatants were also negative by *E. multilocularis* qPCR,  
296 except for one sample that had an average Cq of 37.34.

297

### 298 3.3. Relationship between the *E. multilocularis*-specific qPCRs for *E. multilocularis* egg-positive 299 and egg-negative faeces

300 Comparison of the Cq values obtained from *E. multilocularis*-specific qPCR performed on  
301 carnivore faeces showed that, among positive faeces, the median Cq of the faeces not containing *E.*  
302 *multilocularis* eggs was higher than that of the faeces containing *E. multilocularis* eggs: 38.92  
303 versus 36.9. The distributions of Cq values between *E. multilocularis* qPCR-positive faeces with or  
304 without *E. multilocularis* eggs differed significantly (Fig. 3A, non-parametric Mann-Whitney test,  
305  $W = 1511$ ,  $P < 0.001$ ). All but two faecal samples with a Cq less than 33.62 which had taeniid eggs  
306 observed under a microscope, confirmed *E. multilocularis* by *E. multilocularis*-specific qPCR and  
307 PCR (Fig. 3A). However, the ROC curve showed that the Cq values had only a weak ability to  
308 differentiate samples containing *E. multilocularis* eggs and those without (AUC = 0.73, 95% CI:

309 0.614-0.847). Beyond the optimal threshold C<sub>q</sub> of 38.36, corresponding to a sensitivity of 81%  
310 (95% CI: 66-94%) and a specificity of 69% (95% CI: 58-80%), most of the samples were negative  
311 by flotation (88%) (Fig. 3B).

312

### 313 3.4. Spatial distribution of *E. multilocularis* DNA and eggs via carnivore faeces

314 Domestic carnivore faeces (in particular, dog) were more prominent downtown than outside  
315 the village (Fig. 4C). Fox faeces were mostly found outside the village but some were also found  
316 inside (Fig. 4B).

317 Kulldorff's statistics showed two significant spatial clusters for *E. multilocularis* DNA-  
318 positive faeces and *E. multilocularis* egg-positive faeces for all carnivores pooled, located in the  
319 same areas in both cases. The *E. multilocularis* DNA faecal prevalence was clearly overdispersed,  
320 with a high-rate cluster ( $P = 1.4 \times 10^{-6}$ ) located in the western part of the site but near the centre and a  
321 low-rate cluster ( $P = 0.02$ ) in the eastern part (Fig. 4Aa). The faecal prevalence of *E. multilocularis*  
322 eggs showed significant clusters (high rate:  $P = 10^{-9}$ , low rate:  $P = 4.75 \times 10^{-3}$ ) within the same  
323 geographical locations as those for *E. multilocularis* DNA faecal prevalence (Fig. 4Ab and 4Aa).  
324 For fox, *E. multilocularis* DNA-positive faeces (high rate:  $P = 0.01$ , low rate:  $P = 0.02$ ) and *E.*  
325 *multilocularis* egg-positive faeces (high rate:  $P = 0.002$ , low rate:  $P = 0.01$ ) were also aggregated  
326 (Fig. 4Ba and 4Bb). *Echinococcus multilocularis* contamination in domestic carnivores was not  
327 found to be clustered, likely due to the low number of positive samples (Fig. 4C). Contamination  
328 clusters were due to the high rate of contamination in fox faeces.

329

## 330 4. Discussion

331 This study focused on collecting evidence of *E. multilocularis* eggs in carnivore faeces in  
332 which *E. multilocularis* DNA was first detected by *E. multilocularis*-specific qPCR. The *E.*

333 *multilocularis* DNA and egg detection methods are notably effective in the patent phase of infection  
334 (Al-Sabi' et al., 2007). DNA detection is possible but remains uncertain during the pre-patent and  
335 post-patent periods due to irregular excretion of tissue or eggs (Deplazes et al., 2003). Here, the *E.*  
336 *multilocularis* qPCR Cq values were significantly lower for samples in which eggs were present  
337 than for flotation-negative samples, implying a larger amount of *E. multilocularis* DNA in *E.*  
338 *multilocularis* qPCR-positive faeces. Our results suggest that in our protocol (DNA extraction and  
339 qPCR protocol), faeces for which the *E. multilocularis* qPCR Cq values were less than 33.62 were  
340 likely to contain *E. multilocularis* eggs. The effectiveness of the key step of DNA extraction from  
341 taeniid or *E. multilocularis* eggs using a kit with only chemical lysis was previously demonstrated  
342 (Štefanić et al., 2004; Davidson et al., 2009; Øines et al., 2014; Umhang et al., 2017). This Cq value  
343 is very close to the mean *E. multilocularis* qPCR Cq of a DNA extract from one *E. multilocularis*  
344 egg ( $34.2 \pm 1.46$ ). In a previous study, the detection limit of PCR targeting mitochondrial DNA (*E.*  
345 *multilocularis*, *Echinococcus granulosus*, and that from single eggs of other tainiids), was estimated  
346 to be 33 copies (Trachsel et al., 2007). Conversely, no conclusion can be drawn concerning the  
347 presence/absence of *E. multilocularis* eggs in faeces with *E. multilocularis* qPCR Cq values ranging  
348 from 33.62 to 38.36, the maximum beyond which most of the samples were negative. This  
349 maximum corresponds to a mean *E. multilocularis* qPCR Cq of a DNA extract of faeces (500 mg)  
350 experimentally spiked with one *E. multilocularis* egg ( $37.6 \pm 1.34$ ) (Knapp et al., 2014). The  
351 absence of eggs in this interval could be justified by a high concentration of *E. multilocularis*-free  
352 DNA. This is probably related to random sampling bias. Indeed, the *E. multilocularis* qPCR was  
353 performed from only 0.5 g of faeces and the flotation technique from 5 g. In cases of low-grade  
354 infection, this small subsample size would be more likely to contain no eggs. This could also  
355 explain the weak ability of the *E. multilocularis* qPCR to differentiate samples containing *E.*  
356 *multilocularis* eggs and those without. The *E. multilocularis* qPCR performed directly on carnivore  
357 faeces probably detects more *E. multilocularis*-free DNA than *E. multilocularis* eggs. It appears that  
358 flotation is necessary to assess exposure to *E. multilocularis* associated with carnivore faeces in the



359 environment. The sucrose flotation method combined with microscopical observation and qPCR  
360 detection used in this study proved to have sensitivity of 100% for the detection of 10 *E.*  
361 *multilocularis* eggs in 5 g of frozen copro-samples. Additionally, the sucrose flotation method  
362 enabled the potential recovery of only one egg in 5 g of faeces. Moreover, a flotation sample with  
363 no eggs gave a mean Cq of 37.34, demonstrating the presence of trace amounts of *E. multilocularis*  
364 DNA in the supernatant. The choice of the sucrose solution was justified by its safety and neutrality  
365 on parasitic elements, which can facilitate the recognition of eggs under the microscope after  
366 flotation. For instance, zinc chloride (SG=1.42), although more effective in floating eggs, could  
367 potentially alter the structural integrity of oocysts (Escotte-Binet et al., 2019) and frozen taeniid  
368 eggs (our personal observations). It is also very hazardous to human and environmental health  
369 (National Center for Biotechnology Information. PubChem Database., 2005. Zinc chloride,  
370 CID=5727. [https://pubchem.ncbi.nlm.nih.gov/compound/Zinc-chloride#section=Safety-and-](https://pubchem.ncbi.nlm.nih.gov/compound/Zinc-chloride#section=Safety-and-Hazards)  
371 Hazards). On the other hand, some *E. multilocularis* qPCR results on isolated taeniid eggs were  
372 negative, likely related to the fact that other taeniid species can be found in carnivore intestines  
373 (Umhang et al., 2012; Li et al., 2013; Umhang et al., 2014, 2015; Karamon et al., 2019; Tylkowska  
374 et al., 2019). The individual isolation of eggs offers the possibility to non-invasively study the  
375 genetic diversity of the parasite in the same carnivore host faeces using the tandemly repeated  
376 microsatellite marker EmsB (Bart et al., 2006; Knapp et al., 2007, 2009; Umhang et al., 2014; Valot  
377 et al., 2015) and avoid complex analysis of egg or DNA pools (Carlsgart et al., 2008).

378 The use of species-specific qPCRs made it possible to identify the carnivore species for 763  
379 of the 814 faeces samples collected, for a success rate of 93.7%. According to this molecular  
380 method, dog and fox faeces were better identified in the field than cat and mustelid faeces. Our  
381 results indicate that morphological identification of copro-material remains uncertain. Confirmation  
382 by a molecular reference technique is advisable to understand the relative contribution of each  
383 definitive species to environmental contamination (Pouille et al., 2017; Knapp et al., 2018). Only  
384 0.5% of all analysed samples contained PCR inhibitor agents. Knapp et al. (2018) reported a similar

385 success rate (94.3%) but a higher rate of faeces containing inhibitors (9.7%). In our study, the  
386 frequent sampling of faeces probably allowed us to obtain fresher samples, less subject to  
387 environmental hazards and DNA degradation than copro-samples obtained by sporadic collection  
388 (Kovach et al., 2003; Nonaka et al., 2009). Improved sampling conditions and the use of specific  
389 kits for DNA extraction reduce the presence of PCR inhibitors and thus increase the sensitivity and  
390 specificity of qPCR methods for species identification from stools (Al-Sabi' et al., 2007; Knapp et  
391 al., 2014).

392 More than half of the faeces collected among domestic carnivores were from dogs versus  
393 only 7% for cats. These faeces were found mostly in the centre of the village near houses. Fox  
394 faeces accounted for 1/3 of all samples and were found in the centre and surrounding area. The  
395 presence of fox faeces in proximity to humans is not surprising, as several studies have reported that  
396 foxes approach human dwellings at night in rural areas to feed (Janko et al., 2012; Krauze-Gryz et  
397 al., 2012; Scott et al., 2014), which was especially noticed in the study site (Knapp et al., 2018).  
398 However, *E. multilocularis* DNA was detected in 25% of fox faeces (70/278) versus only 4%  
399 (15/431) of dog faeces and 9.3% (5/54) of cat faeces. This relatively high prevalence of *E.*  
400 *multilocularis* DNA in cat faeces was observed (4/43; 9.3%) in an endemic area in the north-eastern  
401 part of France (Knapp et al., 2016a). This observation could be due to the small number of samples  
402 collected or a possible multiple copro-sampling of the same or a few infected cats. In foxes and  
403 dogs, similar findings were reported for the same study site in 2012 and 2013 (Knapp et al., 2018).  
404 Indeed, *E. multilocularis* DNA was detected in 25.85% (61/236) of fox faeces compared with  
405 1.46% (3/205) of dog faeces. Fox faeces collected from 94 vegetable gardens located in the  
406 northeast of France were also reported to be the most contaminated, with a rate of 35% (24/69)  
407 versus 11% (2/18) for dog faeces and 7% (6/86) for cat faeces (Pouille et al., 2017). In light of the  
408 reported prevalence of *E. multilocularis* DNA, these studies, as well as ours, confirm the high risk  
409 of transmission of *E. multilocularis*, associated mainly with foxes, the role of cats and dogs being  
410 considered marginal (Hegglin and Deplazes, 2013). We confirmed these hypotheses by the

411 detection of *E. multilocularis* eggs after flotation, mainly in fox faeces in this study (27/32) and  
412 only in 1/15 copro-samples from dogs and 1/5 from cats. Despite a low prevalence of *E.*  
413 *multilocularis* DNA and eggs in dog faeces, the contribution of dogs in the transmission of *E.*  
414 *multilocularis* to humans may be sizable due to their proximity with them and the presence of a  
415 large amount of dog faeces downtown in the village studied (Liccioli et al., 2015a; Knapp et al.,  
416 2018). Thus, the exposure risk related to domestic carnivores has to be more carefully considered.  
417 We are planning further studies based on in vivo experiments in mice to assess the infectivity of *E.*  
418 *multilocularis* eggs originating from cat and dog faeces from our study site.

419         Despite the low number of faeces containing *E. multilocularis* eggs, their spatial distribution  
420 was also overdispersed in the study area, with the same hotspot as faeces detected with *E.*  
421 *multilocularis* DNA. Such *E. multilocularis* environmental contamination is likely to persist for  
422 several months. Indeed, *E. multilocularis* eggs can survive for more than 1 year in cold conditions  
423 (Veit et al., 1995). Our study confirms that contamination via the definitive host faeces on a local  
424 scale is generally overdispersed (Giraudoux et al., 2002; Vaniscotte et al., 2011; Liccioli et al.,  
425 2014; Knapp et al., 2018). The activity patterns and defecation behaviour of the definitive hosts  
426 have been suggested as possible causes of such micro-local hotspots of *E. multilocularis*  
427 transmission to intermediate hosts and humans (Robardet et al., 2011; Giraudoux et al., 2013; Raoul  
428 et al., 2015; Knapp et al., 2018). It cannot be excluded that, in the present study, only a small  
429 number of individuals deposited multiple faeces collected in the field. It is therefore possible that  
430 such spatial heterogeneity of environmental contamination by *E. multilocularis* was the  
431 consequence of a very few individuals in the definitive host population (notably foxes) harbouring  
432 almost the entire parasitic biomass (Raoul et al., 2001; Fischer et al., 2005). Individual  
433 identification of collected faeces could thus allow us to know the true *E. multilocularis* prevalence  
434 in a definitive host population in a non-invasive manner and to identify the contribution of each  
435 individual to the environmental contamination (Liccioli et al., 2015b).

436 Our data suggest that *E. multilocularis* DNA and/or egg detection in carnivore faeces  
437 (mainly that of foxes) is appropriate in ecological studies of *E. multilocularis* transmission, as their  
438 spatial distribution presented the same characteristics. According to our results, *E. multilocularis*  
439 egg detection from *E. multilocularis*-positive qPCR samples is especially useful in evaluating  
440 exposure due to infected domestic animals which have close contact with humans.

441

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449

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654 **Figure legends**

655

656 **Fig. 1.** The predetermined circuit (red dash) of carnivore faeces collection on the study site (a rural  
657 town in the eastern part of France (006°26'46"E, 46°56'53"N)).

658

659 **Fig. 2.** Workflow of *Echinococcus multilocularis* DNA and egg detection in carnivore faeces.

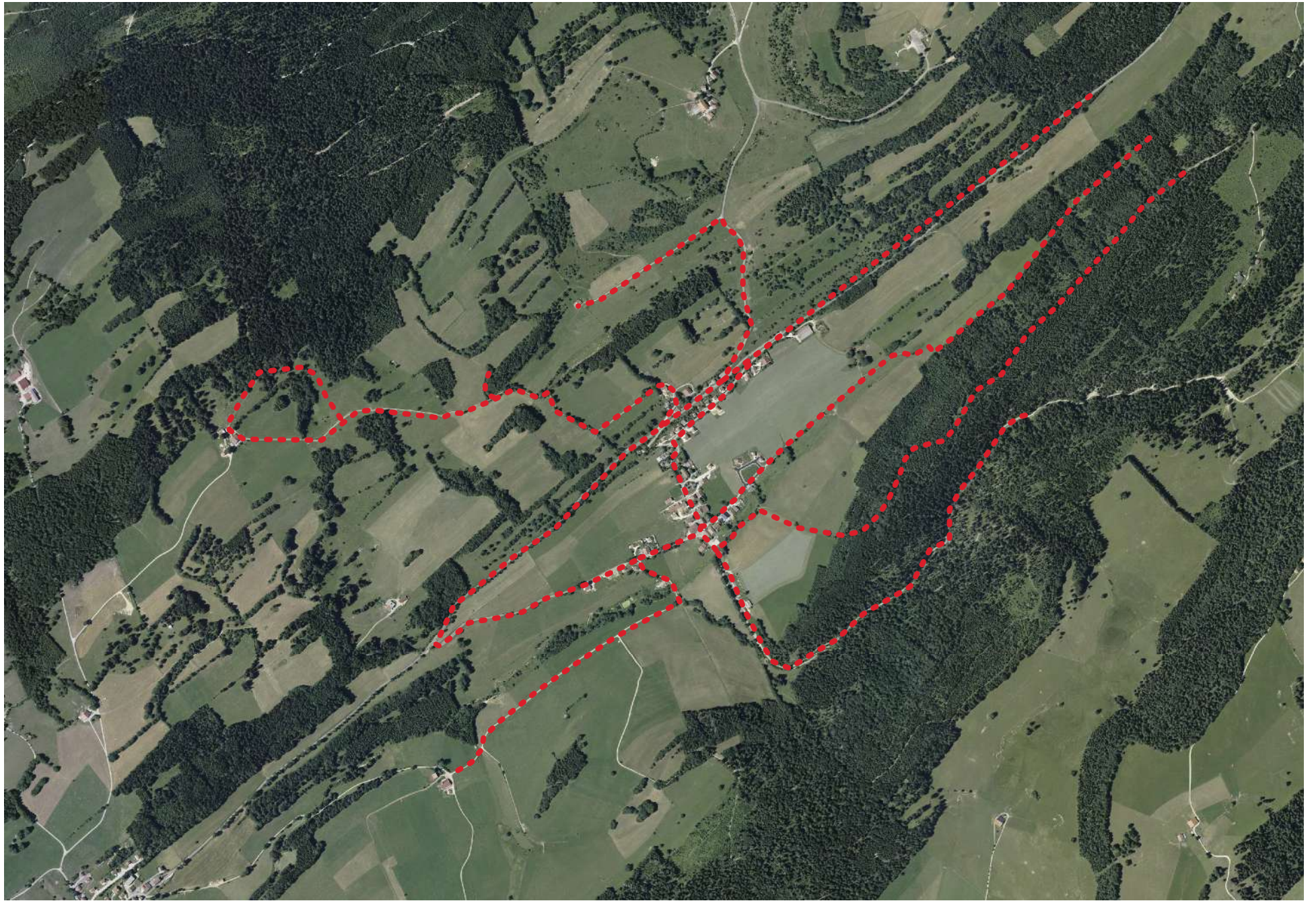
660 Screening and flotation were performed using 0.5 and 5 g of faeces, respectively. qPCR, real-time  
661 quantitative PCR.

662

663 **Fig. 3.** Relationship between quantitative cycle (Cq) values obtained from *Echinococcus*  
664 *multilocularis*-specific real-time quantitative PCR (*E. multilocularis* qPCR) performed on carnivore  
665 faeces containing *E. multilocularis* eggs (*E. multilocularis* egg (+)) or not (*E. multilocularis* egg (-  
666)). (A) Distribution of faeces containing *E. multilocularis* eggs, or not, according to the Cq values of  
667 *E. multilocularis*- specific qPCRs. N, n: sample size. Boxes represent the inter-quartile distances;  
668 thick lines, the medians; whiskers, the extremes values; crosses, the means; and circles, outliers.  
669 Statistical significance was determined using the non-parametric Mann-Whitney U Test. \*\*\**P* =  
670 0.001. (B) Receiver Operating Characteristic (ROC) curve of the *E. multilocularis*-specific qPCR  
671 Cq values to predict the presence/absence of *E. multilocularis* eggs (area under ROC curve (AUC)  
672 = 0.73). The optimal threshold by the Youden index Cq = 38.365, corresponding to a sensitivity of  
673 81% (95% Confidence interval (CI): 66-94%) and a specificity of 69% (95% CI: 58-80%).

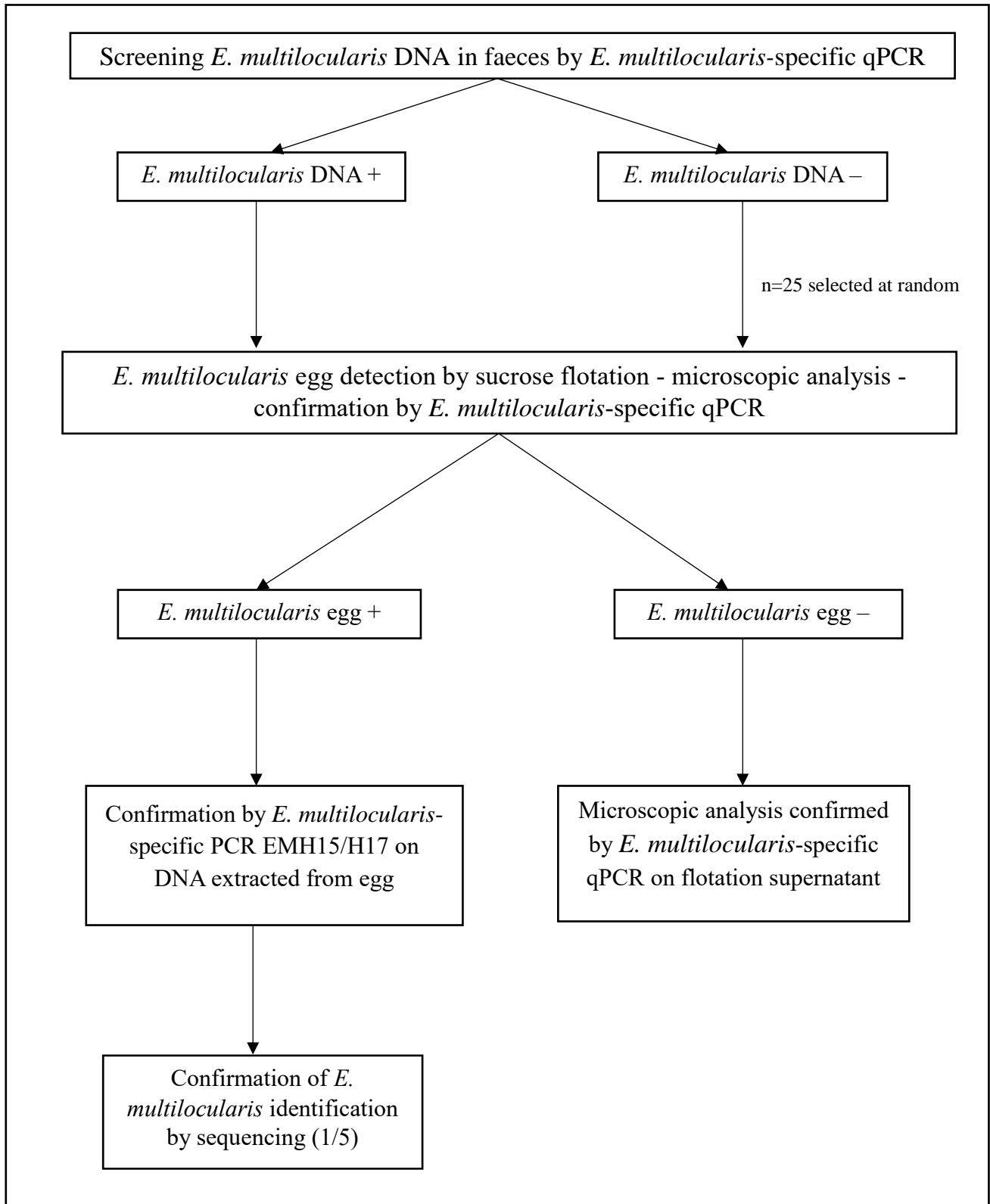
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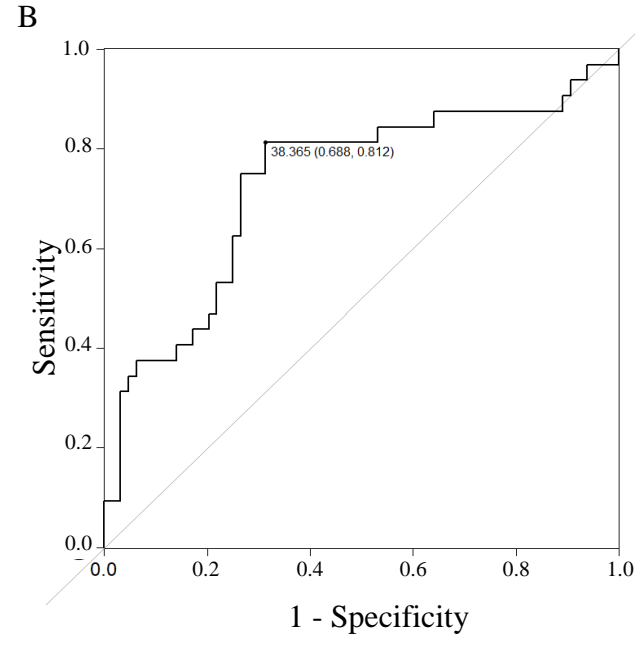
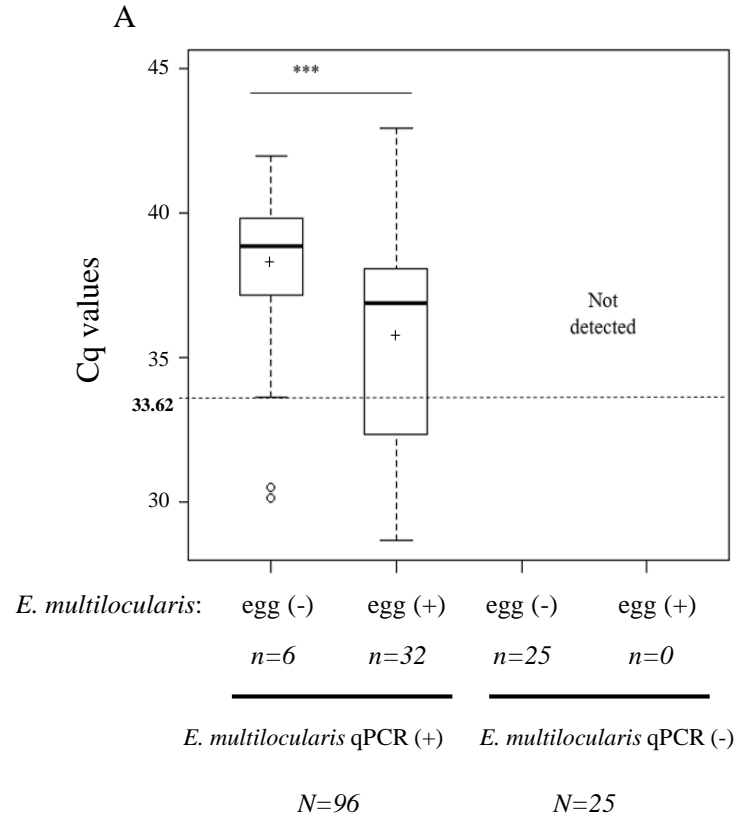
675 **Fig. 4.** Kulldorff statistics on the distribution of carnivore faeces in the studied site in France, from  
676 *Echinococcus multilocularis* DNA-positive faeces (Aa, Ba, Ca) and *E. multilocularis* egg-positive  
677 faeces (Ab, Bb, Cb). (A) All carnivore faeces collected; (B) fox faeces; (C) dog and cat faeces.



0 500 1000 m

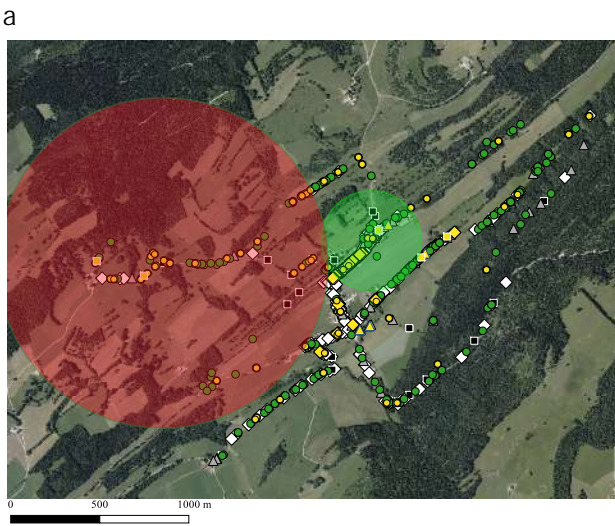






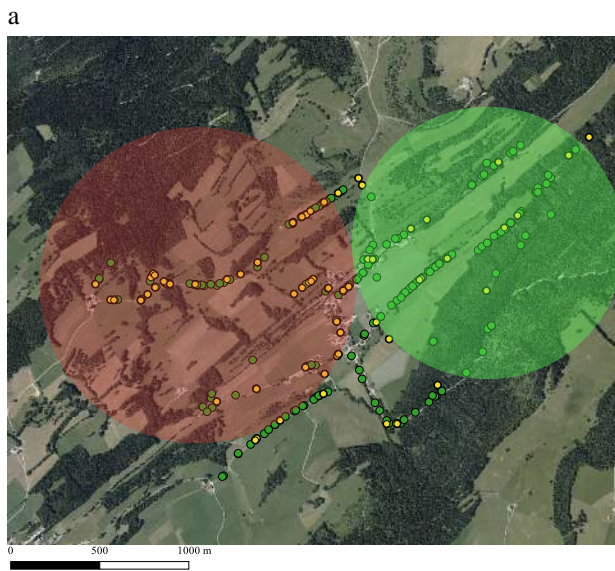


**A**



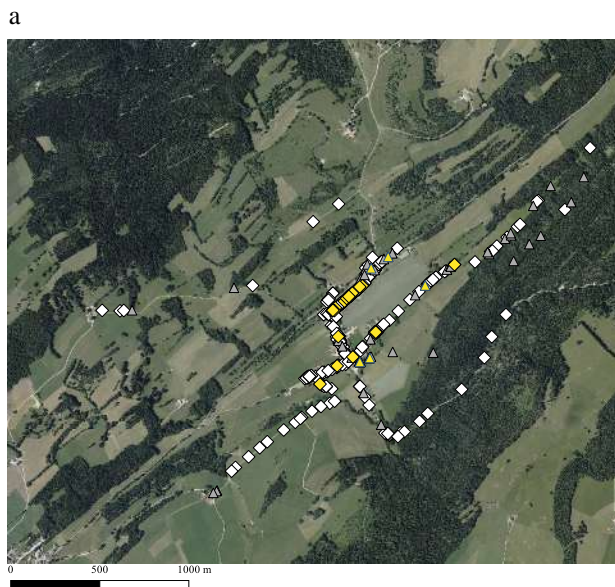
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**B**



b

**C**

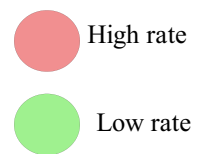


b

**Faeces :**

	Negative	<i>E. multilocularis</i> DNA +	<i>E. multilocularis</i> eggs +
Fox	●	●	●
Dog	◇	◇	◇
Cat	△	△	△
Undetermined	▪	▪	▪

**Clusters :**



**Table 1.** Comparison between faeces identification based on morphological criteria (shape, size, and composition) and faeces identification based on molecular analysis (by species-specific real-time quantitative PCRs)

Morphological identification	Molecular identification				Total
	Fox	Dog	Cat	Undetermined	
Fox	<b>220 (68.3%)</b>	39 (12.1%)	32 (10.0%)	31 (9.6%)	322
Dog	38 (8.7%)	<b>374 (85.8%)</b>	10 (2.3%)	14 (3.2%)	436
Cat	5 (21.7%)	5 (21.7%)	<b>12 (52.2%)</b>	1 (4.4%)	23
Mustelids	14 (56.0%)	7 (28.0%)	0 (0.0%)	4 (16.0%)	25
Undetermined	1 (12.5%)	6 (75.0%)	0 (0.0%)	1 (12.5%)	8
<b>Total</b>	278	431	54	51	814

Bold values indicate occurrence of faeces per species for which morphological identification was confirmed by molecular analysis.

**Table 2.** Method sensitivities (in %) of the sucrose flotation technique combined with microscopic observation and detection by specific real-time quantitative PCR (qPCR) for *Echinococcus multilocularis* in frozen copro-samples (5 g). For each number of *E. multilocularis* eggs, three samples were used.

<b>Number of eggs</b>	<b>Microscopic observation</b>	<b>qPCR <i>E. multilocularis</i></b>
0	-	00.0% (0/3)
1	00.0% (0/3)	33.3% (1/3)
3	00.0% (0/3)	33.3% (1/3)
5	33.3% (1/3)	66.7% (2/3)
10	100.0% (3/3)	100.0% (3/3)

**Table 3.** Detection of *Echinococcus multilocularis* DNA and eggs in carnivore host faecal samples collected between March 2017 and November 2018 in a rural town in the eastern part of France.

<b>Host identification</b>	<b>Fox</b>	<b>Dog</b>	<b>Cat</b>	<b>Undetermined</b>	<b>Total</b>
<b>Total sampled</b>	278	431	54	51	814
<b>qPCR <i>E. multilocularis</i> (number of positive samples)</b>	70	15	5	6	96
Prevalence %	25.2	3.5	9.3	11.7	11.8
95% CI	(20.3-30.8)	(2.0-5.8)	(3.5-21.1)	(4.9-24.6)	(9.7-14.3)
<b><i>E. multilocularis</i> egg-positive samples</b>	27	1	1	3	32

qPCR, real-time quantitative PCR; CI, Confidence Interval.

*Echinococcus multilocularis*  
eggs confirmation by PCRs

Flotation, taeniid egg sampling

Hosts and *Echinococcus multilocularis* real-time PCRs

Copro-sampling, geolocation



*Echinococcus multilocularis*  
spatial distribution