A highly endemic area of *Echinococcus multilocularis* identified through a comparative re-assessment of prevalence in red fox (*Vulpes vulpes*), Alto Adige (Italy: 2019-2020)

Federica Obber ^{1¶*}, Roberto Celva ^{1¶}, Graziana Da Rold ¹, Karin Trevisiol ¹, Silvia Ravagnan ¹, Patrizia Danesi ¹, Lucia Cenni ²⁻³, Chiara Rossi ², Paola Bonato ¹, Katia Capello ¹, Heidi C. Hauffe ², Alessandro Massolo ³, Rudi Cassini ⁴, Valentina Benvenuti ⁴, Andreas Agreiter ⁵, Davide Righetti ⁵, Marco Ianniello ⁶, Debora Dellamaria ¹, Gioia Capelli ¹, Carlo V. Citterio ¹

Affiliations

1. Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro (PD), Italy

2. Conservation Genetics Research Unit, Centre for Research and Innovation, Fondazione Edmund Mach, San Michele All'Adige (TN), Italy

3. Ethology Unit, Department of Biology, University of Pisa, Pisa, Italy

4. Department of Animal Medicine, Production and Health (MAPS) - University of Padua, Legnaro (PD), Italy

5. Wildlife Management Office, Province of Bolzano, Bolzano (BZ), Italy

6. Ministry of Health, Directorate General for Animal Health and Veterinary Drugs, Rome, Italy

Equal contributions:

[¶]These authors have contributed equally to this work

*Corresponding author: Federica Obber

E-mail: fobber@izsvenezie.it

24 ABSTRACT

25 **Background:** Surveillance of *E. multilocularis* at the edge of its range is hindered by fragmented
26 distributional patterns and low prevalence and burden in definitive hosts. Thus, tests with adequate
27 levels of sensitivity are especially important for discriminating between infected and non-infected
28 areas.

29 **Aim:** We reassessed the prevalence of *E. multilocularis* at the southern border of its distribution in
30 Alto Adige (Italy), to improve surveillance in wildlife and provide more accurate estimates of exposure
31 risk.

32 **Methods:** We compared results from the diagnostic test currently implemented for surveillance
33 (based on Coproscopy+Multiplex PCR - CMPCR), against a real-time quantitative PCR (qPCR) for 235
34 fox faeces collected in 2019-2020. The performances of the two tests were estimated using a scraping
35 technique (SFCT) as the gold standard applied to the small intestines of a subsample (n=123) of the
36 same hosts. True prevalence was calculated and sample size required by each faecal test for the
37 detection of the parasite was then estimated.

38 **Results:** True prevalence of *E. multilocularis* in foxes (14.3%) was definitely higher than reported in
39 the last decade (never >5% from 2012 to 2018). The qPCR also had a higher sensitivity (83%)
40 compared to CMPCR (21%). Agreement with the gold standard was far higher for qPCR (0.816) than
41 CMPCR (0.298) as well, determining a smaller sample size required to detect the disease.

42 **Conclusions:** Alto Adige should be considered a highly endemic area. Surveillance at the edges of *E.*
43 *multilocularis* distribution should adopt qPCR diagnostics on definitive hosts on a small geographic
44 scale.

45

46

47 **AUTHOR SUMMARY**

48 *Echinococcus multilocularis* is an intestinal flatworm, whose adult stage in Europe is harboured mainly
 49 by the red fox (*Vulpes vulpes*), which spreads parasite's eggs by faeces. This parasite is the agent of a
 50 severe and potentially lethal zoonosis, the alveolar echinococcosis, affecting humans after accidental
 51 ingestion of parasite's eggs. In the Italian Alpine area, which represents the southernmost border of
 52 *E. multilocularis* European range, surveillance is hindered by a fragmented distributional pattern,
 53 where presence in foxes has been consistently reported only in few isolated foci in Alto Adige
 54 (Bolzano province – Italy) of low prevalence. In order to improve the efficiency of monitoring efforts,
 55 we tested the performances of two diagnostic protocols on fox faeces (sedimentation, filtration,
 56 counting technique followed by standard PCR, and whole stool real-time PCR) against a benchmark
 57 technique on fox intestines (scraping, filtration, counting technique - considered as the gold
 58 standard). This allowed not only to determine qPCR as a far more sensitive and sample-efficient
 59 diagnostic tool for *E. multilocularis* detection in marginally affected areas, but also to re-assess its
 60 prevalence in Alto Adige, which should be considered a highly endemic area. Consequent actions in
 61 the field and modifications in the surveillance strategy should be therefore considered.

62

63 **INTRODUCTION**

64 *Echinococcus* spp. (Cestoda, Cyclophyllidea, Taeniidae) are small intestinal tapeworms causing
 65 zoonoses of public health importance worldwide. In the European Union (EU), it is mandatory to
 66 report the detection of these pathogens to national authorities, and their surveillance, prevention
 67 and control are closely regulated for pets, livestock and wildlife.
 68 Collection of relevant metadata is also highly recommended by both the Directive EU 2003/99/EC
 69 (monitoring of zoonoses and zoonotic agents) and the Regulation EU 2016/429 ('Animal Health Law').

70 Among these, *Echinococcus multilocularis* has a complex life-history. The sylvatic cycle depends on a
71 predator-prey system, where the adult (strobilar) stage of the parasite is carried by wild and domestic
72 canids (definitive hosts: DHs), which in turn become infected by ingesting small rodents (intermediate
73 hosts: IHs) carrying the larval stage (metacestode) of the pathogen [1]. *E. multilocularis* is the agent
74 of a severe zoonosis, alveolar echinococcosis [2], which affects more than 18,000 new patients/year
75 worldwide [3] and more than 150–200/year in the endemic area of central-eastern Europe.

76 In 2019, among the 751 human echinococcosis cases reported in the EU, 147 (26.5%) were
77 attributable to *E. multilocularis* [4], with an incidence which has been increasing notably in recent
78 decades [5]. In humans, who act as dead-end IHs, transmission is predominantly food-borne, and
79 infection occurs when eggs (oncospheres) are ingested with water, wild berries and mushrooms or
80 garden vegetables contaminated with DH faeces [6]. Oncospheres hatch in the gut, penetrate the
81 intestinal wall and by the lympho-hematogenous route reach the liver, where they develop into
82 metacestodes. These multiply asexually, infiltrate the liver and can spread to other organs through a
83 metastasis-like process [7]. Alveolar echinococcosis develops very slowly in humans, taking from a
84 few months [5] to 15 years (<https://www.who.int/news-room/fact-sheets/detail/echinococcosis>) to
85 become clinically evident, although immune-suppressed patients show faster proliferation with
86 earlier detection [8]. If untreated, prognosis is poor, and even the treatment itself is burdensome, as
87 it includes a combination of surgery and long-term anti-parasitic therapy [9].

88 In the EU, the DH is predominantly represented by the red fox (*Vulpes vulpes*). From the historically
89 endemic areas of Switzerland, southeast France and southern Germany, *E. multilocularis* has been
90 expanding north, to northern France and Scandinavia, as well as east to the Balkans [4; 10–12].
91 Moreover, in the last two decades this cestode has been found sporadically in southern France [4;
92 13], as well as northern Italy in both the eastern [14] and western Alps [15], which represents at the
93 moment the southernmost border of the parasite's distribution in the EU. Although high spatial

heterogeneity has been noted, in the endemic areas of central and northern Europe the host-parasite-environment pattern seems well established and is expected to be more predictable, whereas the same is not typical of the edges of the distribution [16]. In these areas, long-term surveillance is crucial to assess possible trends in prevalence and spread, as well as the exposure risk for humans. To this aim, sensitivity of the available diagnostic tests is of paramount importance, since worm burden levels are low and the presence of *E. multilocularis* infections in both DHs and IHs may be very hard to detect.

In the Province of Bolzano (Alto Adige, northeastern Alps, Italy), *E. multilocularis* in red foxes was first detected in early 2000 at a prevalence of about 13%, estimated using a nested PCR on DNA extracted from fox faeces [14]. In the following years, in order to increase the surveillance area and sample, another test (Coproscopy+Multiplex PCR [CMPCR] on parasite's eggs) was used to analyse 2872 faecal samples across northeastern Italy. While optimizing the cost/benefit ratio [17], this test was known to have a good specificity (93.4%) but a low sensitivity (54.8%), relying on a preliminary screening by flotation of cestodes' eggs, so that their burden could drop under the detection threshold [18]. CMPCR was used to confirm the persistence of the Alto Adige focus from 2012 to 2018, with an increasing trend in prevalence in later years, although never higher than 5% annually [17]. Considering the persistence and severity of this zoonosis, the current work aimed to enhance the sensitivity of *E. multilocularis* surveillance in red foxes, re-assess its prevalence in Alto Adige, and provide guidelines for a more effective surveillance strategy at the southern edge of its distribution.

113

114 Methods

In 2019 and 2020, 235 red foxes that were legally culled or found as carcasses were collected across Alto Adige by Provincial wildlife technicians and transported to the Bolzano Laboratory of Istituto Zooprofilattico Sperimentale delle Venezie (IZSVE). To achieve an even sampling regime across the

territory, one to four animals were collected from each hunting area (Fig 1). At necropsy, a faecal sample was taken from the rectum by a sterile glove. Moreover, when it was intact and not damaged (e.g. by the gun shot), the small intestine was tied off at both ends, removed and stored. Both faecal samples and small intestines were frozen at -80°C for at least 72h to inactivate *Echinococcus* eggs. Each faecal sample was then divided into two equal parts and tested for the presence of *E. multilocularis* by two methods:

1. CMPCR, following Citterio et al. 2021 [17] (at the Parasitology laboratory of IZSVE): briefly, 2 g of faecal matter was examined for *Taeniidae* eggs by flotation, filtration and sieving. Multiplex PCR amplification and Sanger sequencing were then performed on harvested eggs.
2. qPCR following Knapp et al. 2016 [19], with minor modifications (at the Animal Genetics laboratories and Sequencing Platform of the Fondazione E. Mach): in brief, whole DNA was extracted from 50 mg of faecal sample as described in Santa et al. 2019 [20], followed by qPCR amplification (Viia 7 Real-time PCR System, ThermoFisher Scientific, Waltham, MA) targeting the mitochondrial DNA marker *rrnL*, using 10 pmol of species-specific primers for *E. multilocularis* (202 bp) and 0.2 pmol of hydrolysis probe. The qPCR-positive samples were amplified with the same primer pair and sequenced with the dideoxy chain-termination protocol on an ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems) using the BigDye Terminator cycle sequencing kit (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA). Sequences were aligned with an *E. multilocularis* reference fragment (GenBank acc. n. AB018440) using BioEdit 7.0.9 [21].

In order to evaluate the performances of the two diagnostic tests, a subsample of fox intestines, was examined for adult worms by the Scraping, Filtration and Counting Technique (SFCT), considered as the gold standard for *E. multilocularis* detection, following Santa et al. 2018 [22]. We performed SFCT on the available small intestines of the foxes that had tested positive for one or both diagnostic tests

on faeces (n= 23), plus on a manageable number of fecal-negative samples (n=100), for a total of 123. Briefly, the pylorus to caecum section of the small intestine was cut into 30 cm lengths, each of which was then opened longitudinally and rinsed with tap water to remove loose faecal content. The intestine was then scraped, the resulting wash water was poured through a series of sieves (1000 µm, 212 µm and 75 µm), and the respective filtrates were collected separately into three distinct beakers. The filtrates were analysed by stereomicroscope (10X-63X) in 5 cm gridded petri dishes, to count the adult helminths. Using the SFCT results, sensitivity (Se) and specificity (Sp) with 95% exact binomial confidence intervals were calculated for each test, and their agreement (Cohen's Kappa) to the standard was provided. True prevalence (TP) was then estimated, using the adjustment to the Rogan-Gladen formula as proposed by Lang and Reiczigel for confidence intervals [23]. Finally, the sample size needed for the detection of *E. multilocularis* by each of the two tests was assessed at the estimated TP and according to varying expected prevalence using the modified binomial approximation analysis method implemented in EpiTools (<https://epitools.ausvet.com.au/freecalctwo>). We set a target population size of 300 individuals on a local scale (hypothetical epidemiological unit), based on the results of unpublished data from wildlife management offices in different areas of the northeastern Italian Alps, providing estimated densities of 2.1, 3.4 and 7.5 foxes/km².

159

160 Results

161 The distribution of the 235 fox faecal samples and of the 123 fox small intestines is shown in in Fig 1.
162 Of the 235 fox faecal samples, seven (2.9%) were CMPCR-positive and 34 (14.4%) were qPCR-positive.

163

164 Fig 1 Distribution of individual fox faecal samples from Alto Adige in 2019-2020 (n=235) and subset
165 examined for adult worms by SFCT (n=123)

166 Adult *E. multilocularis* were found by SFCT in 24/123 (19.5%) fox small intestines (Fig 2) while, for the
167 same animals, five (4.1%) and 23 (18.7%) faecal samples resulted as CMPCR and qPCR positive,
168 respectively. Positive outcomes for the three diagnostics are summarized in Table 1.

169

170 Fig 2 Results of the fox fecal samples collected in 2019-2020 (n=123) tested by Coproscopy+Multiplex
171 PCR (CMPCR), qPCR and Scraping, Filtration and Counting Technique (SFCT- gold standard) for *E.*
172 *multilocularis* in Alto Adige, Italy

173

174 **Table 1 Matrix of the results of the two faecal tests (CMPCR and qPCR) and the gold standard**
175 **(SFCT) for the detection of *E. multilocularis* in 123 foxes**

Test	Coproscopy + Multiplex PCR (CMPCR)	qPCR	Scraping, filtration counting technique (SFCT)
CMPCR	5 (4.1%)		
qPCR	5	23(18.7%)	
SFCT	5	20	24 (19.5%)

176

177 Compared to SFCT, CMPCR detected 'true' infections in 5/24 (4.1%) cases, resulting in very poor
178 sensitivity (Se) (0.21; Table 2), and far lower than previously reported (about 0.55 [18]). Conversely,
179 qPCR confirmed the SFCT results in 20/24 (86.9%) of the cases, translating into a much higher Se
180 (0.83; Table 2). The qPCR test also identified three positive samples that were negative for SFCT, and
181 thus considered as false positives.

182

183 Table 2 Sensitivity, specificity and agreement (K) for two diagnostic methods (Coproscopy+Multiplex
184 PCR [CMPCR] and qPCR) for detecting *E. multilocularis* in fox fecal samples compared to the Scraping,
185 Filtration and Counting Technique (SFCT - gold standard)

Index	CMPCR versus SFCT (95% CI)	qPCR versus SFCT (95% CI)
Sensitivity (Se)	0.21 (0.07 - 0.42)	0.83 (0.63- 0.95)
Specificity (Sp)	1 (0.96 - 1)	0.97 (0.91 - 0.99)
Agreement (K)	0.30 (0.09 - 0.50)	0.82 (0.68 - 0.95)

186

187 Based on the Se and Sp calculated from these results (Table 2), the TP estimated for the whole sample
188 set (n=235) was 14.30 % (CI95%: 0-29.14) for CMPCR and 14.24% (CI95%: 7.04 -22.64) for qPCR. The
189 sample size required for the detection of *E. multilocularis* in a fox population of 300 individuals with
190 various expected prevalences is shown in Fig 3. For example, if the expected prevalence is 15%, about
191 90 individuals (about one third of the population) should be tested when using CMPCR, whereas
192 about 60 individuals (20%) would be enough to detect parasite presence by qPCR.

193

194 Fig 3 Sample size required to detect *E. multilocularis* at various expected prevalences (with 95% CI)
195 in a population of 300 foxes, based on Sensitivity and Specificity of CMPCR and qPCR estimated here.
196 Regression was obtained by interpolating 13 prevalence values.

197

198 Discussion

199 Our results lead to the conclusion that the true prevalence of *E. multilocularis* in Alto Adige has been
200 higher than previously reported in the last decade (<5%). According to the standards proposed by
201 Casulli et al. (2015), this region should now be considered as highly endemic (prevalence >10%) [24],
202 suggesting that surveillance should be intensified both locally and in the bordering territories.

203 The methods most widely used in the EU for detecting *E. multilocularis* in animal carcasses are
 204 modifications of techniques targeting adult parasites, such as the sedimentation and counting (SCT)
 205 and intestinal scraping (IST) [24]; the copro-antigen ELISA test [25]; or PCR [26] for species
 206 determination. However, methods aimed at detecting adult helminths have practical limitations,
 207 since they require the collection and necropsy of a consistent number of DHs throughout a study
 208 area, storage capacity of large volumes of organs temporarily at -80°C (for ensuring positive samples
 209 are not infective) and long-term at -20°C (for storage), as well as being time-consuming (at least 3
 210 hours for each sample). Therefore, methods on faeces remain the best option for surveillance, given
 211 also that faecal samples can be collected in the field using a standardized protocol, and many samples
 212 can be screened simultaneously and rapidly. Surveillance coupling monitoring faeces of both fox
 213 carcasses and scats would better encompass the life cycle of both the parasite and its main definitive
 214 host throughout the year, and faecal prevalence could be used as a proxy for human risk of exposure.
 215 In addition, non-invasive collection and genotyping of scats would allow repeated sampling on the
 216 same fox social groups, tracking the seasonal pattern of infective eggs shedding at an individual level.
 217 Antigenic and molecular tests are rapid and useful in regions characterized by a high parasitic
 218 presence [27], but copro-antigen ELISA has the disadvantage of a low sensitivity, especially in the
 219 case of low parasitic burden [24]: molecular methods remain therefore the best option. Based on our
 220 results, tests directly applied on faeces, as qPCR, are recommended: in fact, besides reducing the
 221 number of samples needed to detect the infection (Fig 3) and consequently, the sampling effort and
 222 the cost of analyses, qPCR would also be the best option for detecting parasite DNA from scats
 223 collected in the field, since these generally result in poor quality DNA compared to fresh fecal samples
 224 taken during necropsies [28]. The higher sensitivity of qPCR is offset by an apparent decrease in
 225 specificity, suggested by the three positive samples not identified by SFCT (see also [29]). However,
 226 since SFCT is designed to detect adult worms, it may be that the extra positive samples detected by

227 qPCR were indications of late infections, when traces of parasite DNA may still be present in the
228 intestine but adult helminths are no longer macroscopically detectable or, on the contrary, of early
229 infections, when immature parasites (scolices) are present but are very difficult to identify
230 microscopically [14].

231 At the edges of *E. multilocularis* distribution, an efficient strategy for surveillance would be to
232 progressively include areas bordering the foci, in which the parasite could have gone undetected, or
233 in which *E. multilocularis* had been found occasionally, but no further confirmed, using qPCR as the
234 main diagnostic tool. In such a framework, we suggest that surveillance in foxes should be performed
235 on a small scale (e.g. considering an area similar to an Alpine valley as the basic epidemiological unit)
236 rather than a larger one (e.g. using the NUTS at level 1 or 2, as recommended for *E. granulosus* by
237 Tamarozzi et al. 2020 [30]). This would optimize efforts by increasing the chances of detecting *E.*
238 *multilocularis* in areas previously considered free from the parasite. Moreover, such a detailed
239 surveillance would be more informative of the actual risk of exposure for humans.

240 Finally, it is worth noting that a high endemicity of *E. multilocularis*, as that found in Alto Adige, should
241 be a mandate for increased surveillance of possible human cases, as well as infections in domestic
242 dogs. As dog infection could definitely increase the risk to humans, we suggest that this surveillance
243 also use the most sensitive molecular tools available.

244

245 **Conflict of interest:** None declared

246 **Funding statement:** This work received funding from the Italian Ministry of Health (project
247 codes: IZSve RC 03/11; IZSve RC 18/2016; IZSve RC 05/19).

248

249

250 Authors' contributions

Conceptualization	Carlo Vittorio Citterio, Heidi Christine Hauffe, Alessandro Massolo, Federica Obber, Roberto Celva, Lucia Cenni
Data Curation	Federica Obber, Roberto Celva, Lucia Cenni
Formal Analysis	Katia Capello, Roberto Celva, Carlo V. Citterio, Federica Obber, Rudi Cassini, Alessandro Massolo
Funding Acquisition	Carlo V. Citterio
Investigation	Lucia Cenni, Roberto Celva, Graziana Da Rold, Valentina Benvenuti
Methodology	Silvia Ravagnan, Patrizia Danesi, Alessandro Massolo, Chiara Rossi, Rudi Cassini
Project Administration	Carlo V. Citterio
Resources	Karin Trevisiol, Andreas Agreiter, Davide Righetti, Debora Dellamaria
Supervision	Gioia Capelli
Visualization	Federica Obber, Roberto Celva, Paola Bonato
Writing – Original Draft Preparation	Federica Obber, Roberto Celva, Carlo V. Citterio
Writing – Review & Editing	Federica Obber, Roberto Celva, Carlo V. Citterio, Alessandro Massolo, Heidi C. Hauffe, Rudi Cassini, Debora Dellamaria, Marco Ianniello, Gioia Capelli

251

252 References

- 253 1. Romig T, Deplazes P, Jenkins D, Giraudoux P, Massolo A, Craig PS, et al. Ecology and life cycle
254 patterns of *Echinococcus* Species. Adv Parasitol. 2017; 95:213–314.
255 doi:10.1016/bs.apar.2016.11.002.
- 256 2. Bouwknegt M, Devleeschauwer B, Graham H, Robertson LJ, van der Giessen JW. The Euro-Fbp
257 Workshop Participants. Prioritisation of foodborne parasites in Europe, 2016. Euro Surveill. 2018;
258 23(9):17-00161. doi:10.2807/1560-7917.ES.2018.23.9.17-00161.
- 259 3. Torgerson PR, Keller K, Magnotta M, Ragland N. The Global Burden of Alveolar Echinococcosis.
260 PLoS Negl Trop Dis. 2010; 4(6): e722. doi:10.1371/journal.pntd.0000722

- 261 4. EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and
262 Control), 2021. The European Union One Health 2019 Zoonoses Report. EFSA J. 2021; Feb 27; 19(2):
263 e6406. doi:10.2903/j.efsa.2021.6406.
- 264 5. Vuitton DA, Demonmerot F, Knapp J, Richou C, Grenouillet F, Chauchet A, et al. Clinical
265 epidemiology of human AE in Europe. Vet Parasitol. 2015; Oct 30; 213(3–4): 110-20. doi:
266 10.1016/j.vetpar.2015.07.036
- 267 6. Lass A, Szostakowska B, Myjak P, Korzeniewski K. The first detection of *Echinococcus multilocularis*
268 DNA in environmental fruit, vegetable, and mushroom samples using nested PCR. Parasitol Res. 2015;
269 114(11):4023–9. doi:10.1007/s00436-015-4630-9
- 270 7. Kern P, Menezes da Silva A, Akhan O, Müllhaupt B, Vizcaychipi KA, Budke C, et al. The
271 Echinococcoses: Diagnosis, Clinical Management and Burden of Disease. Adv Parasitol. 2017; 96:259-
272 369. doi:10.1016/bs.apar.2016.09.006
- 273 8. Chauchet A, Grenouillet F, Knapp J, Richou C, Delabrousse E, Dentan C, et al. Increased incidence
274 and characteristics of alveolar echinococcosis in patients with immunosuppression-associated
275 conditions. Clin Infect Dis. 2014; 15; 59(8):1095-104. doi:10.1093/cid/ciu520
- 276 9. Brunetti E, Kern P, Vuitton DA. Writing Panel for the WHO-IWGE. Expert consensus for the diagnosis
277 and treatment of cystic and alveolar echinococcosis in humans. Acta Trop. 2010; Apr; 114(1):1-16.
278 doi:10.1016/j.actatropica.2009.11.001
- 279 10. Combes S, Comte S, Raton V, Raoul F, Boue F, Umhang G, et al. Westward spread of *Echinococcus*
280 *multilocularis* in foxes, France, 2005–2010. Emerg Infect Dis. 2012; Dec 18: 2059-62. doi:
281 10.3201/eid1812.120219
- 282 11. Deplazes P, Rinaldi L, Alvarez Rojas CA, Torgerson PR, Harandi MF, Romig T. Global distribution of
283 alveolar and cystic echinococcosis. R.C.A. Thompson, P.D., A.J. Lymbery (Eds.), Adv. Parasitol. 2017;
284 95: 315-493. doi:10.1016/bs.apar.2016.11.001

- 285 12. EFSA AHAW Panel (EFSA Panel on Animal Health and Welfare), 2015. Scientific opinion on
286 *Echinococcus multilocularis* infection in animals. EFSA J. 2015; 13(12):4373, 129 pp.
287 doi:10.2903/j.efsa.2015.4373
- 288 13. Umhang G, Comte S, Hormaz V, Boucher JM, Raton V, Favier S, et al. Retrospective analyses of
289 fox feces by real-time PCR to identify new endemic areas of *Echinococcus multilocularis* in France.
290 Parasitol Res. 2016; Nov 115 (11): 4437-41. doi:10.1007/s00436-016-5220-1
- 291 14. Casulli A, La Rosa G, Pozio E, Manfredi MT, Di Cerbo AR, Genchi C et al. *Echinococcus multilocularis*
292 in red foxes (*Vulpes vulpes*) of the Italian Alpine region: Is there a focus of autochthonous
293 transmission? Int J Parasitol. 2005; Sep 35 (10):RC 1079–83. doi:10.1016/j.ijpara.2005.04.005
- 294 15. Massolo A, Valli D, Wassermann M, Cavallero S, D’Amelio S, Meriggi A, et al. Unexpected
295 *Echinococcus multilocularis* infections in shepherd dogs and wolves in south-western Italian Alps: a
296 new endemic area? Int J Parasitol Parasites Wildl. 2018; Aug 13; 7 (3): 309–16. doi:
297 10.1016/j.ijppaw.2018.08.001
- 298 16. Santa M, Musiani M, Ruckstuhl K, & Massolo A. A review on invasions by parasites with complex
299 life cycles: The European strain of *Echinococcus multilocularis* in North America as a model.
300 Parasitology. 2021; 1-13. doi: <https://doi.org/10.1017/S0031182021001426>
- 301 17. Citterio CV, Obber F, Trevisiol K, Dellamaria D, Celva R, Bregoli M, et al. *Echinococcus multilocularis*
302 and other cestodes in red foxes (*Vulpes vulpes*) of northeast Italy, 2012-2018. Parasit Vectors. 2021;
303 Jan 7; 14(1):29. doi: 10.1186/s13071-020-04520-5
- 304 18. Otero-Abad B, Armua-Fernandez MT, Deplazes P, Torgerson PR, Hartnack S. Latent class models
305 for *Echinococcus multilocularis* diagnosis in foxes in Switzerland in the absence of a gold standard.
306 Parasit Vectors. 2017; 10(1):612. doi: 10.1186/s13071-017-2562-1
- 307 19. Knapp J, Umhang G, Pouille ML, Millon L. Development of a real-time PCR for a sensitive one-step
308 copro-diagnosis allowing both the identification of carnivore feces and the detection of *Toxocara spp.*

309 and *Echinococcus multilocularis*. Appl Environ Microbiol. 2016; 82(10): 2950-58. doi:
310 10.1128/AEM.03467-15

311 20. Santa MA, Pastran S, Klein C. et al. Evaluation of an automated magnetic bead-based DNA
312 extraction and real-time PCR in fecal samples as a pre-screening test for detection of *Echinococcus*
313 *multilocularis* and *Echinococcus canadensis* in coyotes. Parasitol Res. 2019; 118. 119-25. doi:
314 10.1007/s00436-018-6125-y

315 21. Hall TA. BioEdit a user-frendly biological sequence alignment editor and analysis program for
316 Windows 95/98/NT. 1999. In (pp. 95-98). Nucleic Acids Symposium Series: Oxford University Press.

317 22. Santa MA, Pastran SA, Klein C, Duignan P, Ruckstuhl K, Romig T, et al. Detecting co-infections of
318 *Echinococcus multilocularis* and *Echinococcus canadensis* in coyotes and red foxes in Alberta, Canada
319 using real-time PCR. Int J Parasitol Parasites Wildl. 2018; Mar 7(2): 111-15. doi:
320 10.1016/j.ijppaw.2018.03.001

321 23. Lang Z, Reiczigel J. Confidence limits for prevalence of disease adjusted for estimated sensitivity
322 and specificity. Prev Vet Med 2014; 113: 13-22. doi: 10.1016/j.prevetmed.2013.09.015

323 24. Casulli A, Possenti A, La Torre G et al. *Echinococcus multilocularis* infection in animals
324 (GP/EFSA/AHAW/2012/01). EFSA supporting publication. 2015; EN-882, 168 pp.

325 25. Deplazes P, Alther P, Tanner I, Thompson RC, Eckert J. *Echinococcus multilocularis* coproantigen
326 detection by enzyme-linked immunosorbent assay in fox, dog, and cat populations. J Parasitol. 1999;
327 Feb; 85(1):115-21.

328 26. Bretagne S, Guillou JP, Morand M, Houin R. Detection of *Echinococcus multilocularis* DNA in fox
329 faeces using DNA amplification. Parasitology. 1993; Feb; 106 (Pt 2):193-9. doi:
330 10.1017/s0031182000074990

- 331 27. Eckert J. Predictive values and quality control of techniques for the diagnosis of *Echinococcus*
332 *multilocularis* in definitive hosts. Acta Trop. 2003; Feb; 85:157–63. doi: 10.1016/s0001-
333 706x(02)00216-4
- 334 28. Taberlet P. and Luikart G. Non-invasive genetic sampling and individual identification. Biol. J. Linn.
335 Soc. 1999; 68: 41-55. <https://doi.org/10.1111/j.1095-8312.1999.tb01157.x>
- 336 29. Da Silva AM, Courquet S, Raoul F, Rieffel D, Giraudoux P, Millon L, et al. Assessment of the
337 exposure to *Echinococcus multilocularis* associated with carnivore faeces using real-time quantitative
338 PCR and flotation technique assays. Int J Parasitol. 2020; Dec; 50(14): 1195-1204. doi:
339 10.1016/j.ijpara.2020.07.008
- 340 30. Tamarozzi F, Legnardi M, Fittipaldo A, Drigo M, Cassini R. Epidemiological distribution of
341 *Echinococcus granulosus s.l.* infection in human and domestic animal hosts in European
342 Mediterranean and Balkan countries: A systematic review. PLOS Negl. Trop. Dis. 2020; 14(8). doi:
343 10.1371/journal.pntd.0008519

Fig1

Fig2

Fig3