Towards delimitation of the Echinococcus multilocularis parasite's southernmost range in France

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Short communication

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Abstract:

Alveolar echinococcosis is a severe, potentially fatal, parasitic disease caused by ingestion of microscopic eggs of *Echinococcus multilocularis*. The lifecycle of the parasite is essentially sylvatic, and based on a prey-predator relationship between red foxes and small rodents. A westward expansion from the eastern historical focus has been reported in France, though the parasite has also been detected in the southern Alps. While the focus in the Auvergne region (central France) was described in the 1980s, the southern delimitation of the actual endemic area, especially in the south, was unknown in the absence of dedicated surveys. Red fox samples were collected from 2013 to 2020 in the framework of other transversal epidemiological studies in five sampling areas from southwestern and southeastern France. One hundred and seven intestines were analysed by SSCT, and 221 faecal samples from intestines were analysed by copro-qPCR. None of the 328 foxes exhibited *E. multilocularis* worms or DNA. Although the presence of *E. multilocularis* cannot be totally excluded in the departments from the study areas, the sample size tested argues for an absence of the parasite in these studied areas, which is in accordance with the currently known endemic situation in France. These new data are helpful in determining the southernmost limit of *E. multilocularis* distribution in France. The warm, dry Mediterranean climate in the southeastern areas is less favourable to the transmission of *E. multilocularis* and especially to the survival of eggs in the environment than the climate in the French Alps or Liguria (Italy) climate where the parasite is present. The intermediate area between the southwestern study areas and the historical focus of Auvergne, which is separated by around 150 km, will be investigated in the coming years. Moreover, an ongoing national surveillance programme on *E. multilocularis* in foxes is targeting French departements along the edge of the known endemic area both in the southeast and southwest. The data produced will supplement the results of this study, thus greatly helping to define the current distribution of *E. multilocularis* in France and to target prevention measures to reduce human exposure.

Keywords: *Echinococcus multilocularis*, surveillance, France, red fox

1. Introduction

Alveolar echinococcosis (AE) is a severe parasitic disease fatal to patients if not appropriately diagnosed and treated (Torgerson et al., 2010). The infection is due to oral ingestion of eggs of the tapeworm *Echinococcus multilocularis*, which reaches the liver from the intestines via portal blood flow. The larval stage gradually proliferates and infiltrates the liver parenchyma, thus destroying the surrounding host tissue, and may also affect other organs by infiltration or metastasis (Eckert et al., 2001). After a long asymptomatic period of up to 15 years, human AE may be diagnosed in patients due to a manifestation of symptoms or, increasingly often, coincidentally. The primary lifecycle of the parasite in Europe involves red foxes (*Vulpes vulpes*) and small rodents mainly from the *Arvicolinae* sub-family as respectively definitive and intermediate hosts. Worms in fox intestines release the parasite's eggs into the environment through faeces. Rodents develop alveolar echinococcosis after ingesting the eggs. The life cycle is completed when foxes then predate infected rodents. While the parasite was initially identified in only a few western European countries at the end of the 19th century, its current range now includes 21 European countries (Oksanen et al., 2016) due to expansion through the dispersal movement of foxes from the historical focus (Knapp et al., 2009; Umhang et al., 2021a). In France, the first focus was described at the end of the 19th century in a few departments (French administrative units corresponding to NUTS3 level in European Union NUTS nomenclature) in northeastern France (Vuitton et al., 2015), then a second one was identified more recently in the Auvergne region (Figure 1), central France (Petavy and Deblock, 1980; Petavy and Deblock, 1983). A westward expansion from the historical eastern focus has since been reported (Combes et al., 2012) though assumed to be at least several decades old (Umhang et al., 2014). According to the absence of the parasite in the westernmost department (Morbihan) and detection of infected foxes only in the eastern part of the bordering Brittany (Bretagne administrative region, east of Ille-et-Vilaine department), the northwestern delimitation of the endemic areas to be currently roughly positioned at the east of Brittany (Combes et al., 2013; Combes et al., 2012). More recently, a southward expansion along the Alps was determined by the detection of the parasite's DNA in three fox faecal

samples in the Hautes-Alpes department (Umhang et al., 2016) and later of infected *Arvicola terrestris* (Umhang et al., 2021b). Furthermore, the parasite's DNA has been identified even further south, near the French-Italian border, in both wolf and dog faeces (Massolo et al., 2018). No surveys have yet investigated southwestern France, and the parasite has not been reported in Spain or Portugal (Oksanen et al., 2016). However, the parasite's current geographical distribution in southern France is thought to lie further south than the range currently indicated by reported cases. The aim of this study was to use convenience sampling of red fox samples in order to explore the parasite's potential presence far beyond the known endemic areas in southeastern and southwestern France.

2. Materials and Methods

Samples were collected from 2013 to 2020 in the framework of other transversal epidemiological studies in five sampling areas (Figure 1). Two areas, A and B, are military camps located in the Var and Bouches-du-Rhône departments (southeastern France, 350 km² and 15 km² respectively) where foxes (n=77) were sampled for investigations from 2013 to 2020 on vector-borne pathogens (Medkour et al., 2020). The three other areas are located in Charente (C: 539 km²), Dordogne (D: 525 km²) and Landes (E: 504 km²) departments (southwestern France) where foxes were sampled for research on *Mycobacterium bovis* from 2017 to 2020 (n=30, n=147 and n=74 respectively) (Richomme et al., 2020). Foxes were trapped throughout the years for areas A and B and between October and March for areas C, D and E. All fox carcass used in the present study were obtained in agreement with national regulations. As the study did not involve invasive procedures on live animals, no ethical approval was necessary. The fox intestines from areas A, B and C were collected during the necropsy and frozen at -20°C. For foxes from areas D and E, faecal samples were taken from the rectum and frozen at -20°C. All the intestinal and faecal samples were frozen at -80°C for a week prior to laboratory analyses in order to prevent any risk of zoonotic infection.

97 The intestines from areas A, B and C were analysed according to the SSCT (Segmental Sedimentation
98 and Counting Technique) method (Umhang et al., 2011). Briefly, intestine of red foxes were divided in
99 five equal parts and the first (from anterior to posterior parts) and fourth were scrapped to remove
100 the intestinal content which is diluted with water to look for the presence of *E. multilocularis* worms
101 with a binocular. This method is very sensitive (98.4%) regarding the intestinal reference method SCT
102 (Sedimentation and Counting Technique) which is considered the gold standard. For foxes from area
103 D, DNA was extracted from 500 mg of faeces with a KingFisher Flex automated extraction system
104 (Thermo Scientific) according to the manufacturer's instructions after supplemental mechanical lysis
105 using an LSI MagVet Universal Isolation Kit (Life Technologies). Five samples of fox faecal samples
106 previously identified as naturally infected by *E. multilocularis* were used as a positive control for this
107 automated DNA extraction step. For foxes from area E, the QIAamp Fast DNA stool mini kit (Qiagen)
108 was used, again according to the manufacturer's recommendations. All the copro-DNA samples were
109 submitted for *E. multilocularis* detection by a real-time PCR based on the amplification of a part
110 of the mitochondrial rrnL gene including an internal control (Knapp et al., 2016). A final qPCR volume
111 of 20µL was used, containing 5µL of DNA, 10µL of Master Mix Maxima Probe, 50 copies of the internal
112 control plasmid, 1.5µM of primers for *E. multilocularis* and internal control, 0.2µM and 0.1µM of *E.*
113 *multilocularis* and internal control probe, respectively. The qPCRs were performed in duplicate and run
114 on a Mx3005P thermocycler (Agilent) was used with a program that consists of 10 min at 95°C and 45
115 cycles of 15 s at 95°C and 60 s at 60°C. In the event of inhibition, the DNA samples were diluted to
116 1/10th before being re-tested.
117
118 3. Results and discussion
119 Previous knowledge on the distribution of *E. multilocularis* in France was mainly based on the parasite's
120 detection in foxes with a sampling effort ranging from about 1 to 2 foxes per 100 km² in each of the
121 departments investigated (Combes et al. 2012, Combes et al. 2013, Umhang et al. 2016) (Figure 1). In

the present study, the sampling areas were smaller due to the use of convenient sampling, but the sampling effort was much higher, ranging from 5 to 400 foxes per 100 km² in the five sampling areas (Table 1). None of the 328 foxes analysed had either *E. multilocularis* worms or their DNA. Considering the density of collected foxes, this result argues in favour of an absence of the parasite in the studied areas, even if the presence of *E. multilocularis* cannot be totally excluded from a statistical point of view (upper limit of the confidence interval at 95% of the prevalence: 2.5% in area D in the southwestern, and 4.7% in the southeastern areas). These new data, which show that no parasites were detected, are in accordance with previous knowledge on the endemic situation in France and are helpful in delimiting the southernmost range of the geographical distribution of *E. multilocularis* in France.

In 2016, *E. multilocularis* DNA was detected from fox faecal samples in the Hautes-Alpes (Umhang et al., 2016) in southeastern France, approximately 100 kilometres north of area A (Var). According to EmsB microsatellite analyses obtained from infected *Arvicola terrestris* specimens in the same Alpine area (Umhang et al., 2021b), the presence of the parasite there is thought to be the result of an expansion from the northeastern historical focus. Furthermore, the parasite's DNA was also detected in wolf and dog faeces in Italy (Massolo et al., 2018), suggesting the cestode's presence in the French-Italian border region (Alpes-Maritimes department). The southernmost positive wolf faecal sample from Italy were from the Province of Imperia (western part of the Liguria region), only 110 km northeast of Area A, found negative for the parasite in the current study. This could be explained by the warm, dry Mediterranean climate of area A, which is less favourable to the transmission of *E. multilocularis* and especially to the survival of eggs in the environment than the climate of the French Alps or Liguria.

The most southwestern *E. multilocularis* reports concern the historical focus of the Auvergne region (central France) (Figure 1). The first reports were made in the 1980s (Petavy and Deblock, 1980; Petavy and Deblock, 1983). By the early 2000s, the prevalence in foxes was estimated to stand at 9% (95% CI:

5%-17%) in the Cantal department (southern Auvergne) (Combes et al., 2012). An ongoing survey in this region aims to assess the presence of the parasite among foxes and its origin using the EmsB microsatellite approach. The areas of southwestern France investigated in the present study (areas C, D and E) are 150 km west of the westernmost cases reported in Auvergne. This intermediate area will be investigated in the coming years as part of the above-mentioned survey in Auvergne. Moreover, a national surveillance programme on *E. multilocularis* in foxes is under way and will target departments along the edge of the known endemic area, both in southeastern and western France. The data thus produced will supplement our own study results, thereby advancing efforts to define the actual distribution of *E. multilocularis* in France today, and to target preventive measures to reduce human exposure.

Declaration of Competing Interests

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

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Table 1: Prevalence and confidence interval of *E. multilocularis* in the five study areas in southern France according to sample type and analysis method.

Areas	Departments	Nb of samples	Type of samples	Diagnostic method	Prevalence [95% confidence interval in %]	Density of fox sampled/100 km²
A	Var	17	intestines	SSCT	0 [0-19.5]	5
B	Bouches-du-Rhône	60	intestines	SSCT	0 [0-6.0]	400
C	Charente	30	intestines	SSCT	0 [0-11.6]	6
D	Dordogne	147	faeces	copro-qPCR	0 [0-2.5]	28
E	Landes	74	faeces	copro-qPCR	0 [0-4.9)	15

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Table 2: Helminths found at a macroscopic level during the SSCT analyses (which focus on the first and fourth segments of intestine when divided in five equal parts) and rate of infection in foxes in three areas of the study where this research was implemented. The specific determination of nematodes has not been performed.

			Number of fox found infected (rate of infection)			
Areas	Departments	Number of fox analysed	*Mesocestoides sp.*	*Taenia sp.*	*Amoebotaenia sp.*	Nematoda (unregards the species)
A	Var	17	7 (41%)	6 (35%)	0	1 (6%)
B	Bouches-du-Rhône	60	26 (43%)	26 (43%)	1 (2%)	16 (27%)
C	Charente	30	20 (67%)	8 (27%)	2 (7%)	3 (10%)

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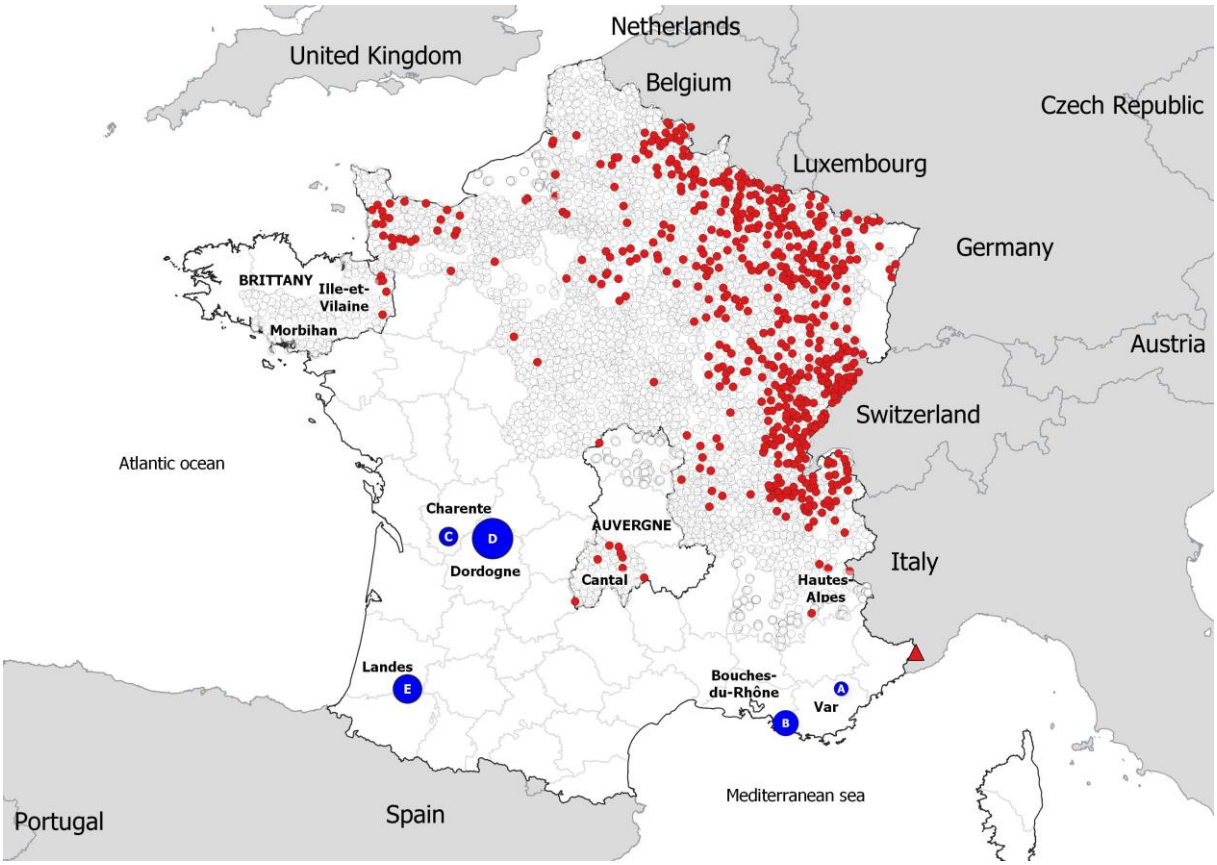

Figure 1: The five study areas are indicated by blue circles; the sample size in each area is proportional to the diameter of the circle and previous data on the distribution of *E. multilocularis* among foxes in France (red circle for individual positive fox, white circle for negative individual fox) and at the Italian border (red triangle for positive individual fox or dog) among dogs and wolves. The names of the French regions (in upper case) and departments (in lower case) mentioned in the article are indicated on the map.

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