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Potential intermediate hosts of *Angiostrongylus cantonensis* in the European Mediterranean region (Mallorca, Spain)

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ABSTRACT

Angiostrongylus cantonensis is the main etiological agent of eosinophilic meningitis, a disease that often leads to severe neurological manifestations in mammals and birds. In recent years, the prevalence of this zoonotic nematode has dramatically increased as it expands into new territories beyond its native range in Southeast Asia and the Pacific Basin.Its arrival to Europe, the last continent to be invaded, has raised many questions concerning the parasite's life cycle, particularly in the Mediterranean region, where it is now endemic. This study aims to collect evidence about intermediate hosts (snail and slug species) involved in the transmission of the *A. cantonensis* in Mallorca. We have conducted a systematic surveillance of gastropods within 2 km radius areas, surrounding the specific locations where infected hedgehogs were found. We employed a sequence-based detection approach that included a species-specific PCR amplification followed by DNA sequencing of the internal transcribed spacer I (ITS-1). This conservative approach was essential to avoid cross reactions with the numerous metastrongylid species known to be circulating in Mallorca. Overall, we collected and identified 398 gastropods belonging to 17 species, of which 11% tested positive for *A. cantonensis.* These gastropods were collected from touristic settlements to agricultural lands. Five gastropod species: *Theba pisana, Cornu aspersum, Deroceras reticulatum, Milax gagates and Otala lactea,* had been previously incriminated as *A. cantonensis* intermediate hosts, while 6 species: *Papillifera papillaris, Cochlicella acuta, Eobania vermiculata, Ganula lanuginosa, Milax nigricans* and *Rumina decollata,* are newly recorded hosts. The findings of this study have important epidemiological implications, and further measures are discussed to prevent neuroangiostrongylosis cases.

1. Introduction

Neuroangiostrongylosis, also known as rat lungworm disease, is an emerging zoonosis caused by the nematode *Angiostrongylus cantonensis.* Although the parasite's native range is Southeast Asia and the Pacific Basin [1], it can now be found in all continents except Antarctica [2]. The disease is characterized by a wide variety of clinical manifestations, ranging from mild (and possibly asymptomatic) infections that may spontaneously resolve [3] to eosinophilic meningitis and other severe debilitating clinical manifestations of the central nervous system. These appear to result from an inflammatory reaction due to the peculiar brain tropism of the larvae and subsequent death [4]. Extreme pain, vision impairment, paraesthesia, bowel disfunction, encephalitis followed by comma and death have been reported [2,5,6]. In recent years, the prevalence of human neuroangiostrongylosis has increased at an alarming rate [7].

As with many parasitic diseases, understanding the life cycle of *A. cantonensis* is essential to determine the likelihood of human infection [8]. The intermediate hosts of this nematode are gastropods, while rats serve as definitive hosts [9]. However, when it comes to its paratenic hosts, this species has an outstanding capacity to cross phylum barriers, having been reported in planarians, centipedes, crustaceans, fish,

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amphibians and reptiles [8]. Accidental hosts include mammals and birds [10]. In endemic regions, a large number of terrestrial and freshwater gastropod species have been incriminated as possible intermediate hosts, highlighting the low specificity of *A. cantonensis* to these invertebrates [11]. Gastropods primarily become infected through the ingestion of rat faeces containing the first stage larvae (L1), and secondarily through penetration of the gastropod's integument by L1 [12]. Identifying which intermediate host species are associated with the transmission of neuroangiostrongylosis is a critical step prior to designing more focussed detection, monitoring and control strategies [11].

In Europe, the rat lungworm has only been reported in Spain. It was first detected in Tenerife [13], an overseas subtropical Spanish territory, off the coast of Africa. Eight years later, it was found in Mallorca, located in the Mediterranean Basin, approximately 170 km off the coast of Spain [14], supporting the possibility of its introduction into continental Europe [15]. Confirming these suspicions, it was detected in Valencia, mainland Spain in 2022 [16]. Given that Europe has been the last invaded region [17], many aspects of the biology of this parasite are still unknown, especially those regarding the intermediate hosts involved in the transmission. Therefore, the main objective of this study is to collect evidence of which gastropod species may act as intermediate hosts of *A. cantonensis* in the Mediterranean Basin of Europe, since previous studies in the region have focussed only on definitive and accidental hosts [16,18]. We present a strategy consisting of a systematic molecular surveillance of *A. cantonensis* in gastropods collected from regions where *A. cantonensis* positive hedgehogs have been found. Our results will provide the basis for further risk assessment on the rat lungworm in other endemic and potentially endemic regions.

2. Material and methods

2.1. Ethics statement

This study did not involve neither sampling protected species nor sampling in protected areas. Sampling of snails was performed under the permission of the Species Protection Service of the Government of the Balearic Islands (CAP 21/2020, CAP 01/2021, CAP 03/2022 and CAP 12/2022).

2.2. Study sites and sampling approach

Mallorca is the largest of the four islands that form the Balearic archipelago, which is situated at the western Mediterranean Sea. Mallorca has a surface of 3635.70 km^2 and a coastline length of 771.83 km. It has a temperate climate and a mean annual rainfall of 450 L/m^2. The Balearic Islands have a tourism-based economy, followed by agriculture, farming and textile and food industries.

Sampling was conducted in six localities where infected North African hedgehogs were previously detected [18]: *Camp de Mar*, a small touristic town located in the south-west of the island; *Son Castelló*, an important industrial park in the capital city, Palma; *Santa Maria del Camí*, a traditional Mallorcan interior town located with a long history of wine production; *Inca*, the third largest city, also located nearby the center of the island; *Sa Pobla*, a small town with large areas dedicated to agriculture; and *Alcúdia*, a family-friendly touristic area located in the north of the island and one of the main ports of the island (Fig. 1).

Gastropod sampling was conducted in a 2 km-radius areas around each site where infected hedgehogs were previously found (Fig. 1) to increase chances of finding positive gastropod hosts. The measurements of these areas were defined according to the approximate *Atelerix algirus* hedgehogs home range [19]. The center of each sampling area was established as the location where infected hedgehogs were collected [18], based on records from the Consortium for the recovery of wildlife of the Balearic Islands (herein COFIB wildlife hospital).

Between 10 and 15 sampling points within these 2 km-radius areas were randomly and opportunistically selected. Each sampling points was visited once and preferably during the rainy days in order to increase the detection of gastropods. Whenever possible sampling took place immediately after positive hedgehogs were found.

Our baseline observations showed that in some sampling areas, no more than a few dozen gastropods could be found (unpublished). Therefore, we set a limit of 10 specimens per species to avoid unnecessary pressure on the gastropod populations, especially when there was no previous knowledge of the gastropod abundance.

Sampling of gastropods was conducted manually using forceps and

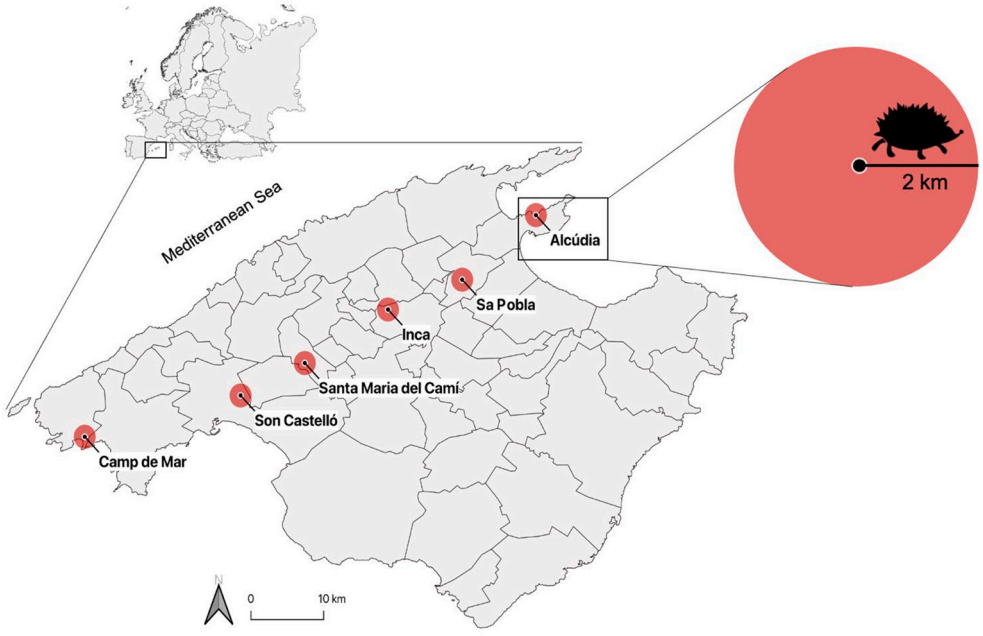

Fig. 1. *Angiostrongylus cantonensis* positive zones of Mallorca (Balearic Islands, Spain) where sampling of gastropods was carried out. Red areas are the 2 km radius sampling areas established according to hedgehog home range. Sampling was performed randomly in different points of these areas. Map created with QGIS 3.16.4-Hannover. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

specimens were placed in individual Ziploc bags to avoid cross-contamination. Both snails and slugs were collected from walls, plants or under rocks (Fig. 2). Gastropods were also collected from areas with trash and litter accumulation. After collection, samples were transported to the Parasitology laboratory at the University of the Balearic Islands for morphological identifications. Protected areas were not included as sampling points. All the collected specimens were stored at −20 °C for further molecular analyses.

2.3. Morphological identification of gastropods

Snails and slugs were identified according to morphological characters following general guides and species descriptions [20–28]. The main morphological character used for snail identification was the morphology of the shell, while some slugs were dissected to examine the internal reproductive organs for the correct identification of cryptic species. All biological material was kept at the laboratory of Parasitology of the University of the Balearic Islands. Valid scientific names were assigned according to [29,30].

2.4. Molecular detection of A. cantonensis in gastropod hosts

We cut a small piece (between 5 and 8 mm) from the posterior end of the gastropod foot and rinsed it in PBS buffer to eliminate the excess of slime and soil. Particular care was taken to avoid cross contamination during the manipulation of the samples in the laboratory, by cleaning and sterilizing tweezers and scissors with 96% alcohol and a Bunsen burner flame. Total DNA was extracted of each sample using the NZY Tissue gDNA Isolation Kit (Nzytech, Portugal). We followed the manufacturer's specifications, except for the pre-lysis step, where samples were incubated 24 h at 56 °C instead of 1–3 h. Additionally, in the final elution step, we used 80 μL elution buffer instead of the 100 μL specified. The DNA obtained was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Saveen Werner ApS, Denmark).

We used a differential PCR amplification method. This consisted of using specific primers targeting the ITS-1 region: ITS1_Canto_F3: AACAACTAGCATCATCTACGTC and ITS1_Canto_R1: CATCCTGTGTATCTCGTTCC. These primers specifically aligned with *A. cantonensis/A. mackerrasae* species, but not with the metastrongylid nematodes: *Angiostrongylus vasorum, Aelurostrongylus abstrusus, Crenosoma striatum* and *Crenosoma vulpis*; theoretically resulting in a 624 bp amplicon [31]. The reaction was carried out in a final volume of 25 μL. The PCR mix contained 1 μL of each primer 10 μM, 1 μL of sample's DNA, 12.5 μL of the Supreme NZYtaq II 2× Green Master Mix (Nzytech, Portugal) and 9.5 μL of MiliQ water. Conditions for the PCR were set as follows: initial denaturalization step at 95 °C for 15 min followed by 35 cycles at 94 °C for 30 s, 57 °C for 90 s, 72 °C for 90 s, and a final extension step at 72 °C for 10 min, according to Izquierdo-Rodríguez et al. [31]. Amplicons were visualized on a 2% agarose gel stained with Pronasafe nucleid acid solution (Conda Laboratories, Spain). Considering that *A. mackerrasae* has never been reported outside Australia, and the adult worms currently circulating in Mallorca have been molecularly and anatomically identified as *A. cantonensis* [14,18], positive samples were identified as *A. cantonensis*.

To confirm the success of the PCR amplification, PCR products were sequenced. First, PCR-positive samples were purified using the NZY Gelpure kit (Nzytech, Portugal) following the manufacturer's instructions. Purified samples were stored in 30 μL of MilliQ water instead of 50 μL elution buffer and sent to Macrogen (Spain) for one-direction Sanger sequencing. All sequences obtained were manually analyzed with CodonCode Aligner version 9.0.1 (https://www.codon-code.com) and MEGA X version 10.2.6 (https://www.megasoftware.net). A BLASTn analysis (https://blast.ncbi.nlm.nih.gov) was performed to confirm the similarity of the resulting sequences with those reported in previous studies. *A. cantonensis* identification was confirmed when the nucleotide identity with GenBank sequences was >99%.

Fig. 2. Examples of different areas where snails and slugs were collected. A, urban area (U); B, rural area (R); C, suburban area (S); D, agricultural area (A).

3. Results

Based on the present survey, seventeen gastropod species (14 snail species and 3 slug species), belonging to 9 families were found and identified in the areas previously reported as positive. All snails and the slug species *Deroceras reticulatum* could be identified to the species level using external morphological characters. Slug species *Milax gagates* and *Milax nigricans* could only be differentiated by the morphology of the internal reproductive organs (the atrial gland and the horn-shape stimulator), observed via necropsy. Sampling goal ($\geq$ 10 individuals/ species/ area) could only be achieved for the most abundant snail species: *Cornu aspersum, Eobania vermiculata* and *Theba pisana* while <10 specimens were found for the rest of the species (Table 1). In total, we examined 398 gastropod specimens. The species *C. aspersum*, *E. vermiculata* and *T. pisana*, were the most widely distributed, being present in all sampling sites. These same species, in addition to *Cochlicella acuta*, *M. gagates* and *Rumina decollata*, were present in all habitat types: Agricultural area (A), Urban area (U), Suburban area (S) and Rural area (R) (Table 1, Fig. 2).

We obtained band sizes compatible to *A. cantonensis*, as described by Izquierdo-Rodríguez et al. [31] in 60 of the 398 gastropod specimens examined. However, successful sequences (>99% similarity to *A. cantonensis/A. mackerrasae* from GenBank) were only possible in 43 specimens. The alignment of the sequences obtained, representing more than one specimen per species of mollusk, did not contain any variable position according to MEGA X. We only observed scattered microsatellites with minor differences in the number of repetitions. We have identified three *A. cantonensis* haplotypes present in specimens from Mallorca (GenBank Ac. N° OR119900 - OR119902), which have been previously reported in other parts of the world.

Based on our sequence-based diagnosis, the overall prevalence of *A. cantonensis* in gastropods examined in this study was 10.8% (43/398). These results allow us to confirm the molecular detection of *A. cantonensis* in 11 of the 17 species examined in Mallorca (Table 1 and Fig. 3). The negative species coincidently were the least abundant ones, with all but one (*Ferussacia folliculum*), represented by <10 specimens. In snails, the prevalence ranged from 2.6% in the snail *Theba pisana* to 22.6% in *R. decollata*, while the prevalence in slugs ranged from 8.3% in *M. nigricans* to 21.4% in *D. reticulatum.* Positive PCR amplifications were obtained in gastropods from 5 of the 6 previously identified endemic foci in Mallorca, but successful sequencing could only be obtained in gastropods from 4 endemic foci. The prevalence rates per localities were as follow: 21.42% (12/56) in *Santa Maria del Camí*, 15.15% (10/66) in *Sa Pobla*, 16.09% (14/87) in *Camp de Mar*, 8.33% (7/84) in *Inca*, 0% (0/58) in *Son Castelló* (Palma) and 0% (0/47) in *Alcúdia*.

4. Discussion

Since the first detection of *A. cantonensis* in Mallorca [14] and its subsequent detection in the east of mainland Spain [16], no information has been available concerning the possible intermediate hosts of this parasite in the Mediterranean region. This study reports that at least eleven gastropod species could act as intermediate hosts of *A. cantonensis* in the Mediterranean island of Mallorca, representing 65% of the seventeen gastropod species found in the six endemic localities studied. We have previously used hedgehogs to trace the path of the disease in Mallorca [18], but given this species' 2 km home range, it was not clear if these animals could be used to determine the specific spots on the island where the parasite was circulating. PCR-positive gastropod species have been found in all but one locality, demonstrating that our approach, which consisted in sampling gastropods within a 2 km radius from the point where positive hedgehogs were found, is effective in elucidating the possible intermediate hosts of the rat lungworm. We confirm that in Mallorca, the rat lungworm circulates in gastropods from previously identified endemic foci on the island, ranging from coastal touristic localities, to agricultural settings.

We report an overall *A. cantonensis* prevalence of 11% in gastropod intermediate hosts at the Mallorcan endemic foci. However, the prevalence is likely to be higher, considering that our approach relied on successful amplification and subsequent sequencing, which cannot always be achieved. In fact, we obtained characteristic PCR positive bands (624 bp) for 60 gastropod specimens, increasing prevalence to 15%, but

Table 1
Prevalence of gastropods infected with *Angiostrongylus cantonensis* sampled from the six areas: INC (Inca), SAP (Sa Pobla), CAM (Camp de Mar), SAN (Santa Maria del Camí), SON (Son Castelló), ALC (Alcúdia). Habitat type: Agricultural area (A), Urban (U), Suburban (S), Rural area (R). Diet information obtained from [32].

Family	Species	Diet	Positives/n (%)	Zones where sampled	Habitat type
Slugs			10/63 (15.87%)		
Agriolimacidae	*Deroceras reticulatum* (O.F. Müller, 1774)	Phytophagous and saprophytic	6/28 (21.42%)	ALC, CAM, INC*, SAN*, SAP*	A, S*, R
Milacidae	*Milax gagates* (Gray, 1855)	Phytophagous and saprophytic	2/11 (18.18%)	CAM, SAP*, ALC, INC, SON	A*, U, S*, R
	Milax nigricans (Philippi, 1836)	Phytophagous and saprophytic	2/24 (8.33%)	CAM, SAP, ALC, INC*, SON	A, U, S*, R
Snails			33/335 (9.85%)		
Clausiliidae	*Papillifera papillaris* (O.F. Müller, 1774)	Phytophagous and saprophytic	1/10 (10%)	ALC, CAM, INC*	A*, S, R
Ferussaciidae	*Ferussacia folliculum* (Schröter, 1784)	Phytophagous and saprophytic	0/14 (0%)	CAM, SON	U, S
Helicidae	*Cornu aspersum* (O.F. Müller, 1774)	Phytophagous and saprophytic	5/53 (9.43%)	ALC, CAM*, INC, SAN*, SAP*, SON	A*, U, S*, R
	Eobania vermiculata (O.F. Müller, 1774)	Phytophagous and saprophytic	12/66 (18.18%)	ALC, CAM*, INC, SAN*, SAP*, SON,	A*, U*, S*, R*
	Otala lactea (O.F. Müller, 1774)	Phytophagous and saprophytic	4/27 (14.81%)	INC, SAN*, SON	A*, S, R*
	Otala punctata (O.F. Müller, 1774)	Phytophagous and saprophytic	0/3 (0%)	ALC	S
	Theba pisana (O.F. Müller, 1774)	Phytophagous and saprophytic	2/78 (2.56%)	ALC, CAM*, INC, SAN*, SAP, SON	A*, U*, S, R
	Cochlicella acuta (O.F. Müller, 1774)	Phytophagous and saprophytic	1/20 (5%)	CAM*, INC, SON	A, U*, S, R
	Cochlicella barbara (Linnaeus, 1758)	Phytophagous and saprophytic	0/9 (0%)	INC, SAP	A, U, S
Geomitridae	*Trochoidea elegans* (Gmelin, 1791)	Phytophagous and saprophytic	0/5 (0%)	ALC, INC	S, R
	Xerotricha conspurcata (Draparnaud, 1801)	Phytophagous and saprophytic	0/9 (0%)	SON	U
Hygromiidae	*Ganula lanuginosa* (Boissy, 1835)	Phytophagous and saprophytic	1/9 (11.11%)	CAM, INC, SAP*	U, S*, R
Achatinidae	*Rumina decollata* (Linnaeus, 1758)	Phytophagous, saprophytic and carnivorous	7/31 (22.58%)	ALC, CAM*, INC, SAN*, SAP*	A*, U*, S*, R*
Trissexodontidae	*Caracollina lenticula* (Michaud, 1831)	Phytophagous and saprophytic	0/1 (0%)	CAM	S
	Total		**43/398 (10.80%)**		

* Localities where positive gastropods were found.

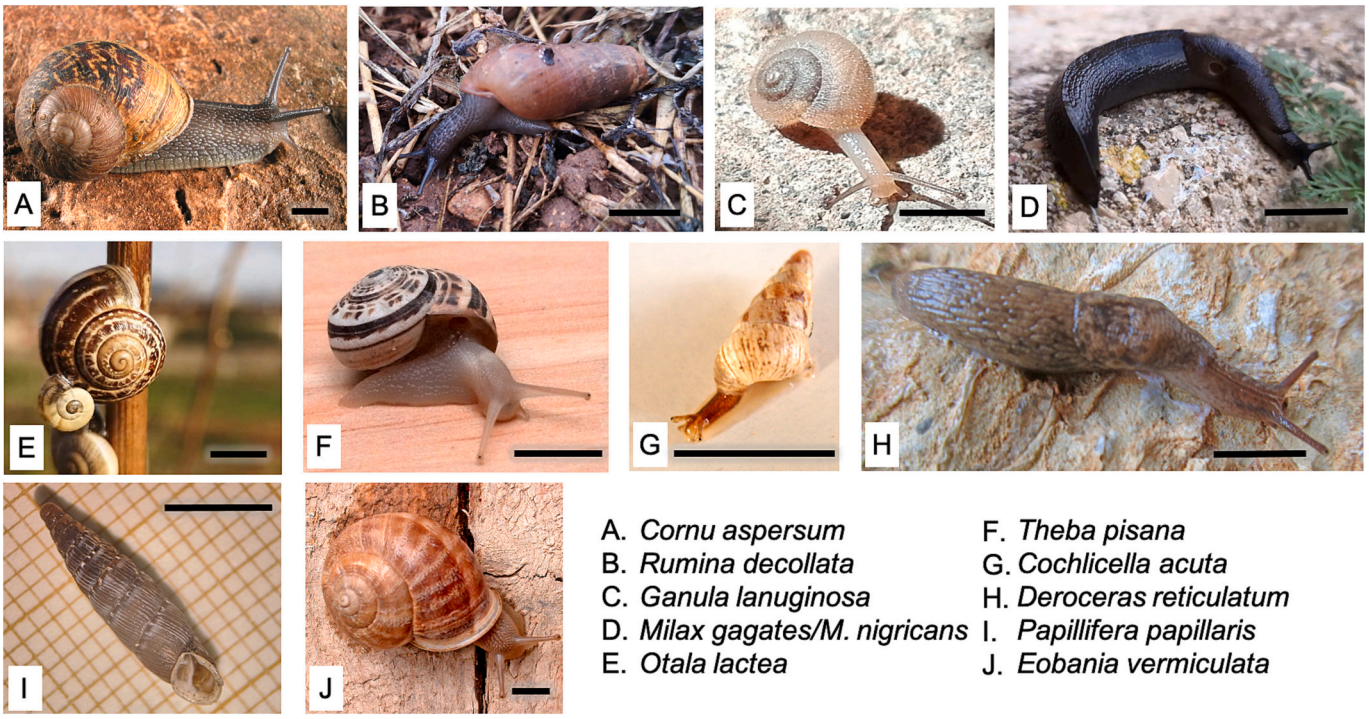

Fig. 3. Snail and slug species found infected with *Angiostrongylus cantonensis* in Mallorca. Scale bars = 1 cm.

not all sequences were of sufficient quality to confirm them all as *A. cantonensis*. We should take into consideration that the sensitivity of the sequence-based identification of *A. cantonensis* is limited when it comes to mild infections [33]. Furthermore, recent studies suggest that the parasite may be at higher densities in the anterior part of the gastropod [34]. However, these data should be taken cautiously as explained by Cowie et al. [35]. Despite its limitations, the sequence-based identification approach that we have followed is a reliable method to identify the potential intermediate hosts of the rat lungworm in the Mediterranean region. Other diagnostic techniques, such as multiplex PCR and real-time PCR offer more sensitive detections; however, they have been optimized for other regions of the world [33,36]. Given the long list of metastrongylid nematodes known circulating in gastropods from the Balearic Islands [37,38], it is still necessary to optimize these techniques to rule out cross reactions. We have found three *A. cantonensis* haplotypes in the Mallorcan gastropods, however, these may not represent different populations, since intragenomic ITS-1 divergence commonly occurs in many taxa [39].

Some species are considered particularly important in the transmission of *A. cantonensis.* These include *Parmarion martensii* in Hawaii [11], *Lissachatina fulica* in Asia and Brazil and *Pomacea canaliculata* in China [40,41]. None of these species are present in the Spanish endemic regions. In this sense, we agree with Prociv et al. [42], who maintain that the role of L. *fulica* in the dispersal and colonization of *A. cantonensis* has been overemphasized and it is not critical in the completion of the life cycle of the parasite. The transmission risk of neuroangiostrongylosis is most likely determined by its surrounding environment [43].

In Mallorca, all gastropod species were found in or nearby human settlements. Of the 11 positive species, the snails *T. pisana, Otala lactea* and *C. aspersum* and the slugs *D. reticulatum* and *M. gagates* had been previously incriminated as *A. cantonensis* intermediate hosts [11,33,44–46], while we report here six species as newly recorded hosts: *Papillifera papillaris, Cochlicella acuta, Ma. vermiculata, Ganula lanuginosa, M. nigricans* and *R. decollata*. All these species are widely distributed not only in the Mediterranean Basin of Europe, but also in the Iberian Peninsula [22,26,28,47]. Among these, *C. aspersum, O. lactea T. pisana* and *Ma. vermiculata* are some of the edible species typically used in the Mediterranean cuisine [48]. The higher infection rates found in the achatinid *R. decollata* might merit more focus, as this species can be found in high population densities, in biotopes frequented by both rodents and insectivores [49]. This gastropod is a great colonizer and is known to play a role in the transmission of the terrestrial life cycle of brachylaimid trematodes infecting small mammals in Spain [50].

It is important to emphasize that PCR-based methods cannot differentiate between nematode's larval stages; therefore, we cannot confirm that *A. cantonensis* infective stages (third stage larvae, L3) were present in all the positive species of this study. Nevertheless, L3 have been observed in a large diversity of gastropods [11]. Hence, the gastropods incriminated in our study are likely to allow the full completion of the *A. cantonensis* larvae. However, more studies focused on the detection of the L3 by microscopy or experimental infections are needed to confirm this assumption.

Extrapolating our results to the risk of human neuroangiostrongylosis infection remains controversial given the complexity of the *A. cantonensis* transmission. In Shenzhen, China, where surveillance included healthy human populations, 1.87% of healthy individuals resulted seropositive for *A. cantonensis*. In comparison, prevalence in gastropods was 10.67% and 6.29% in L. *fulica* and *P. canaliculata,* respectively [51], values that are similar than those found here in Mallorca (11%). However, these authors used tissue digestion followed by morphological identification which has proven less sensitive than PCR-based techniques [52]. Furthermore, raw snail consumption in China increases the chances of human infection. An arguably closer situation to the one in Mallorca is observed in Sidney (Australia), where raw snails are not used for human consumption. In this region, a 3% prevalence has been found in gastropods [33], which is lower than the one in Mallorca, and interestingly, several human cases have been reported [53]. A lower prevalence in gastropods was also found in Hawaii (6%) [11], where neuroangiostrongylosis is endemic with several cases reported each year.

In Western Mediterranean regions, temperatures have risen steadily in the last 40 years, and this trend is expected to continue in the coming years [54]. Mathematical models have shown that in the next few years, Europe will become more suitable for the establishment of *A. cantonensis*

[55]. Further studies are needed to determine the overall prevalence of *A. cantonensis* in other Mediterranean regions, as early detection of this zoonotic agent reduces the chances of severe infection and improves prognosis of affected individuals. We believe that prevention strategies, including raising medical awareness and providing health education to the population should be prioritized in Spain and other Mediterranean regions.

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CRediT authorship contribution statement

Sebastià Jaume-Ramis: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Alberto Martínez-Ortí:** Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. **Sofía Delgado-Serra:** Data curation, Methodology, Writing – review & editing. **María Dolores Bargues:** Data curation, Formal analysis, Methodology, Validation, Visualization, Writing – review & editing. **Santiago Mas-Coma:** Validation, Visualization, Writing – review & editing. **Pilar Foronda:** Validation, Visualization, Writing – review & editing. **Claudia Paredes-Esquivel:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Data availability

Data will be made available on request.

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