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SHORT COMMUNICATION

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Local terrestrial snails as natural intermediate hosts of the zoonotic parasite *Angiostrongylus cantonensis* in the new European endemic area of Valencia, Spain

Màrius V. Fuentes[1] | Mercedes Gomez-Samblas[2] | Orly Richter[2,3] | Sandra Sáez-Durán[1] | Rubén Bueno-Marí[1,4] | Antonio Osuna[2] | María Teresa Galán-Puchades[1]

[1]Parasites and Health Research Group, Department of Pharmacy, Pharmaceutical Technology and Parasitology, Faculty of Pharmacy, University of Valencia, Burjassot, Valencia, Spain

[2]Laboratory of Biochemistry and Molecular Parasitology, Institute of Biotechnology, University of Granada, Granada, Spain

[3]Division of Biology and Medicine, Brown University, Providence, Rhode Island, USA

[4]Laboratorios Lokímica, Paterna, Valencia, Spain

Correspondence
Màrius V. Fuentes, Parasites and Health Research Group, Department of Pharmacy, Pharmaceutical Technology and Parasitology, Faculty of Pharmacy, University of Valencia, Burjassot, Valencia, Spain.
Email: mario.v.fuentes@uv.es

Abstract

Aim: The rat lungworm, *Angiostrongylus cantonensis*, has recently been found in the city of Valencia, parasitizing rats, *Rattus norvegicus* and *Rattus rattus*, its natural definitive hosts. This is the first finding of this zoonotic nematode in continental Europe. After informing local and national health authorities, the collection of local terrestrial snails took place with the aim of elucidating their potential role as intermediate hosts of *A. cantonensis*.

Methods and Results: A total of 145 terrestrial snails, belonging to the species *Cernuella virgata*, *Cornu aspersum*, *Eobania vermiculata*, *Otala punctata*, *Pseudotachea splendida*, *Rumina decollata* and *Theba pisana*, were randomly collected between May and December 2022 in public gardens, parks and orchards in six districts of Valencia, in five of which *A. cantonensis* had been reported previously in rats. Once collected and identified, the snails were frozen at −20°C. Subsequently, the DNA was isolated and screened by PCR using specific primers targeting the *A. cantonensis* COI gene. Seven individual snails, belonging to the species *C. virgata*, *C. aspersum* and *T. pisana*, were positive, for an overall prevalence of 4.8%. The PCR product from one of them was sequenced by Sanger sequencing.

Conclusions: The three positive terrestrial snail species are among the edible species that are frequently included in various dishes in Spain. *C. virgata* is reported as a previously unrecorded intermediate host and should be added to the list of more than 200 species of terrestrial snails that have been reported worldwide as intermediate hosts of the rat lungworm. Considering that these terrestrial snails may release infective larvae of *A. cantonensis* on leafy green vegetables on which they feed and during their handling and preparation for consumption, prophylactic measures to prevent human neuroangiostrongyliasis in Valencia and other regions to which this zoonotic parasite may spread are recommended.

Marius V. Fuentes and Mercedes Gomez-Samblas contributed equally to this article.

KEYWORDS
Angiostrongylus cantonensis, intermediate hosts, rat lungworm, terrestrial snails, Spain, Valencia

1 | INTRODUCTION

Angiostrongylus cantonensis, also known as the rat lugworm as *Rattus norvegicus* (Norway rat) and *Rattus rattus* (black rat) are its main definitive hosts, is a zoonotic parasite that can cause neuroangiostrongyliasis in humans (Jarvi & Prociv, 2021). Discovered in rats in the province of Canton, China. in 1933 (Chen, 1935), *A. cantonensis* has spread, over the last century, from Southeast Asia to Australia, various Pacific islands, Central and South America and the USA, as well as the African continent, including Mediterranean countries such as Egypt, the Atlantic Canary Islands and the Balearic island of Mallorca (Da Silva & Mathison, 2018; Delgado-Serra et al., 2022; Foronda et al., 2010; Martin-Carrillo et al., 2023; Paredes-Esquivel et al., 2019; Pozio, 2015; Wang et al., 2008).

Galán-Puchades et al. (2022) reported finding adult *A. cantonensis* parasitizing *R. norvegicus* and *R. rattus* in the city of Valencia (Spain) in 2021, the first report of the rat lungworm in continental Europe.

The first stage larvae of *A. cantonensis* shed in rat faeces are eaten by snails and slugs, its intermediate hosts, in which they mature to the infective third larval stage (L_3). When rats ingest L_3 in infected snails/slugs, they in turn become infected. Paratenic hosts may also participate in the life cycle of *A. cantonensis*, including freshwater shrimp, crabs and frogs (Turck et al., 2022), as well as endemic lizards in the Canary Islands (Anettová et al., 2022), by ingesting infected intermediate hosts. L_3 larvae may also be released by infected snail/slugs in their slime and could therefore contaminate vegetables (Rollins et al., 2023); they may also be released into water (Rivory et al., 2023).

Several studies suggest that the rapid spread of the rat lungworm has coincided with globalization, that is through the unintended transport of infected rats and, perhaps to a lesser extent, snails or slugs associated with commerce, and because of global warming and changes in eating habits (Eamsobhana, 2014; Kliks & Palumbo, 1992). Once *A. cantonensis* has reached a new area, it establishes its life cycle. As Wang et al. (2008) proposed, when the rat lungworm has been detected in its definitive natural hosts in a specific area, the area should be considered endemic for *A. cantonensis*, as is the case of Valencia and its metropolitan area.

The most common way of *A. cantonensis* transmission to humans is food-borne through eating raw or undercooked infected snails or perhaps vegetables contaminated with L_3 released in the intermediate hosts' slime (Cowie, 2013). Therefore, it is of public health importance to know the identity of these intermediate hosts in the endemic areas (Wang et al., 2008). In this context, and as part of a multidisciplinary project on *A. cantonensis* in the city of Valencia, the aim of this study was to analyse the potential role of several snail species as intermediate hosts of the rat lungworm.

Impacts

- Three species of terrestrial snails, the vineyard snail, *Cernuella virgata*, the garden snail, *Cornu aspersum*, and the white garden or Mediterranean coastal snail, *Theba pisana*, are reported as intermediate hosts of *Angiostrongylus cantonensis* for the first time in continental Europe; among them, *C. virgata* is reported as a new intermediate host.
- The three snail species are eaten as part of typical Spanish dishes, although they are not usually consumed raw.
- Handlers of these terrestrial snail species should take special preventive measures to avoid accidental human neuroangiostrongyliasis.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Collection of terrestrial snails from public gardens, parks and orchards in the city of Valencia did not involve protected species. Collecting was conducted by Laboratorios Lokimica, the company in charge of pest control in the city, with the permission of the Pest Control Section of the Health Service of Valencia City Council.

2.2 | Collection of snails

A total of 145 terrestrial snails were randomly collected in public gardens and parks, as well as orchards, located in six of the 19 districts (Figure 1) of Valencia, between May and December 2022.

In five of the six districts where snails were collected, *A. cantonensis* had previously been reported in rats, namely districts 4-Campanar, 11-Poblats Marítims, 16-Benicalap, 18-Pobles de l'Oest and 19-Pobles del Sud; in the sixth district, district 14-Benimaclet, it had not been found in rats (Figure 1).

Snails were collected using tweezers to avoid contact with them and were then placed in individual bags with the place and date of collection and preserved at 4°C at the Laboratory of Parasitology at the University of Valencia until their identification. Identification was based mainly on shell morphology following various general guides (Altaba, 1991; Martínez-Ortí, 1999; Robles-Cuenca, 1989; Ruiz-Ruiz et al., 2006). The classification of snails was made according to the MolluscaBase (www.molluscabase.org).

2.3 | Sample preparation and DNA isolation

Once identified, the 145 terrestrial snails were frozen for subsequent use. Each snail was thawed and longitudinally cut to ensure a thorough dissection of the snail's body, increasing the probability of detecting *A. cantonensis* L_3 larvae. One part was stored at −20°C, while the other part was homogenized in tubes containing beads (MN Bead Tubes Type D, Macherey-Nagel, catalogue number 740814.5) and 600 μL of PBS in a Precellys instrument at 6500 rpm twice for 30 s. The homogenate was then frozen at −20°C. The samples were handled under hygienic and sterile conditions. Tweezers and scissors were flamed and cooled between each snail.

Four hundred microlitre of the previous homogenate was used to isolate total DNA using the NucleoSpin Tissue Kit (Macherey-Nagel, catalogue number 740952.250) following the provided protocol with modifications. To each sample, 200 μL of T1 buffer and 25 μL of proteinase K were added, and the mixture was incubated overnight at 56°C. The following day, 200 μL of T3 buffer was added and the mixture was incubated for 10 min at 70°C. DNA precipitation and washing steps were performed according to the kit protocol, and the elution was carried out using 70 μL of milliQ water. The quality and concentration of DNA was measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

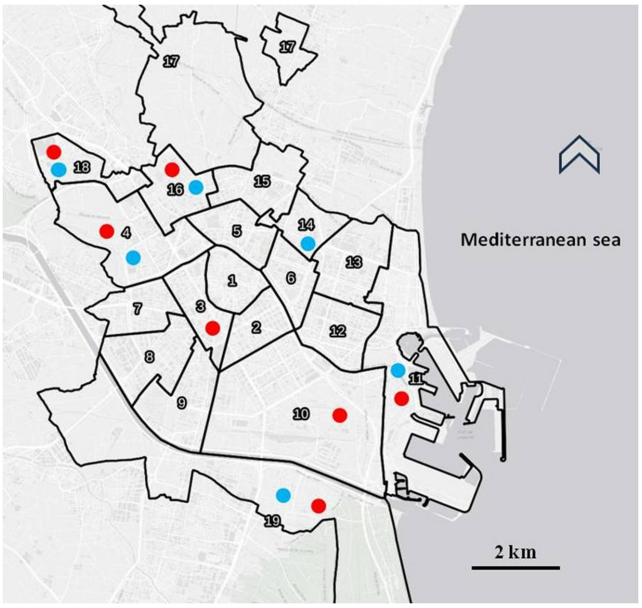

FIGURE 1 Map of the districts of Valencia; blue dots show the six districts in which terrestrial snails were collected. Red dots show those districts in which *Angiostrongylus cantonensis* had been found previously in rats.

2.4 | Screening snails for *A. cantonensis*

Snails were subjected to PCR and sequencing of the cytochrome c oxidase subunit 1 (COI) *A. cantonensis* partial gene (Galán-Puchades et al., 2023; Rodpai et al., 2016) using the primers AngiCOIF 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′ and AngiCOIR: 5′-CGAGGATAACCATGTAAACCAGC-3′. PCRs were prepared in a total volume of 50 μL, including 1× buffer (containing 1.75 mM $MgCl_2$), 200 μM dNTP mix (Canvax, N0030), 0.2 μM of each primer, 1× Platinum SuperFi II DNA Polymerase (Invitrogen, 12361050) and 1 μL of 1/10 DNA dilution. PCR conditions were as follows: 1 min at 98°C, followed by 40 cycles of denaturation at 98°C for 20 s, annealing at 60°C for 20 s and extension at 72°C for 30 s, with a final extension step at 72°C for 5 min. PCR products were visualized in 1% agarose gel. Subsequently, the PCR product was extracted following the NucleoSpin Gel and PCR Clean-up kit protocol (Macherey-Nagel, catalogue number 740609.250) and subjected to Sanger sequencing at the Granada Institute of Parasitology and Biomedicine 'López-Neyra' (IPBLN) genomic facilities. The sequences were quality-trimmed, aligned and analysed using Geneious software (Kearse et al., 2012).

Subsequently, a phylogenetic tree was created using MrBayes version 3.2.6, employing Bayesian inference as described by Tian et al. (2023). The GTR substitution model was used for the most accurate fit. *A. costaricensis* (NC013067) was chosen as outgroup.

3 | RESULTS

3.1 | Snail species

Seven snail species were collected from the six districts of Valencia and identified (Table 1): *Cernuella virgata* – Geomitridae and Helicellinae; *Cornu aspersum*, *Eobania vermiculata*, *Otala punctata*,

TABLE 1 Snail species and number of specimens collected and analysed by district. 4-Campanar, 11-Poblats Marítims, 14-Benimaclet, 16-Benicalap, 18-Pobles de l'Oest and 19-Pobles del Sud.

Snail species/district	4	11	14	16	18	19	TOTAL
Cernuella virgata	–	–	–	3	2	7	12
Cornu aspersum	–	–	–	–	3	–	3
Eobania vermiculata	1	12	6	9	2	3	33
Otala punctata	–	–	–	–	1	1	2
Pseudotachea splendida	15	6	1	7	2	–	31
Rumina decollata	–	–	–	1	2	–	3
Theba pisana	18	2	25	5	1	10	61
TOTAL	34	20	32	25	13	21	145

Pseudotachea splendida and *Theba pisana* – Helicidae and Helicinae; and *Rumina decollata* – Achatinidae and Rumininae.

3.2 | *A. cantonensis*-infected snails

Seven of the 145 snails tested positive for *A. cantonensis* when the DNA was amplified (Figure S1). However, only two of them, the O3 and U5 snails corresponding to *T. pisana* and *C. virgata* collected in Poblats Marítims and Pobles del Sud districts, respectively, were of sufficient quality to be sequenced by Sanger sequencing with GenBank accession numbers OR960500 and PP468354, respectively. The phylogenetic tree (Figure S2) clusters one of the amplified sequences in the snail sample O3 alongside sequences previously published corresponding to *A. cantonensis* individuals isolated from definitive hosts in the city of Valencia (*R. rattus* or *R. norvegicus*) and other isolates from Spanish islands (Majorca and Tenerife). The sequence corresponding to snail U5 diverges from the former group to cluster with *A. cantonensis* isolates from Thailand.

The infected terrestrial snails were as follows: the vineyard snail, *C. virgata*, the garden snail, *C. aspersum*, and the white garden snail or Mediterranean coastal snail, *T. pisana*, collected in three of the six surveyed districts of Valencia (Table 2).

Prevalence of positive snails among the 145 analysed was 4.8%, with the most prevalent species being *C. aspersum* (67.7%), although only three individuals were analysed.

4 | DISCUSSION

Regarding the validity of the positive results obtained, given that the sampled individuals live in contact with soil, to mitigate the risk of potential cross-contamination with free-living nematodes in the molecular analysis, newly designed primer sequences, adapted from Rodpai et al. (2016), were employed. These modifications were implemented to prevent non-specific and cross-amplifications during *A. cantonensis* screening.

We opted for the COI gene over ITS1 due to findings reported by Rodpai et al. (2016), building upon research by Jefferies et al. (2009) and Liu et al. (2011). They highlighted that while ITS2 sequences provide sufficient variability for molecular species distinction in *Angiostrongylus*, the ITS2 region within the same species can exhibit either high variability or lack thereof. Our choice of the COI gene was driven by the aim to not only conduct an epidemiological study on snails but also investigate the potential presence of multiple *A. cantonensis* haplotypes.

According to the Bayesian phylogeny conducted in this study, it is possible that a new haplotype may emerge in Spain, further removed from the previous group corresponding to the sequence of *A. cantonensis* isolated from snail U5 and more closely resembling nematodes isolated from Thailand, Cambodia, Vietnam and Laos.

In the three districts of Valencia in which infected snails were found, *A. cantonensis* had previously also been found parasitizing rats (Galán-Puchades et al., 2022, 2023).

Among the three terrestrial snail species that tested positive for *A. cantonensis* in Valencia, two of them, *T. pisana* and *C. aspersum*, were previously reported as intermediate hosts in the Spanish islands of Tenerife (Canary Islands) (Martin-Alonso et al., 2015) and Mallorca (Balearic Islands) (Jaume-Ramis et al., 2023). However, *C. virgata* (Figure S3) is reported here as a new intermediate host that should be added to the list of more than 200 species of terrestrial snails reported worldwide as intermediate hosts of the rat lungworm (Cowie et al., 2023). Three of the four negative species analysed, *E. vermiculata*, *O. Punctata* and *R. decollata*, had previously been reported as intermediate hosts in Mallorca (Jaume-Ramis et al., 2023), but with the exception of *E. vermiculata*, the low number of individuals analysed in the present study could explain these negative results.

Concerning the distribution, habitat and culinary use of the three terrestrial snail species (Altaba, 1991; Martínez-Ortí, 1999; Robles-Cuenca, 1989; Ruiz-Ruiz et al., 2006) identified as intermediate hosts of *A. cantonensis* in Valencia, it is important to highlight that:

- These snails are present in Mediterranean and Western European countries.
- Their wide distribution in continental Europe may increase the risk of dispersal of *A. cantonensis*, not only through its natural definitive hosts, *Rattus* spp., but also through its intermediate hosts, which can travel associated with soil and vegetables that are exported to other areas, in both the Iberian Peninsula and the rest of Europe.
- They can live in a wide variety of habitats, including vegetable fields, presenting a serious hazard for both local consumers and consumers in other areas to which these products are exported.
- All three species are edible and ingredients of the popular Valencian dish 'caragolà' (snail stew) and are also the most widely consumed snails in various parts of the Iberian Peninsula. Therefore, people who gather the snails in the wild or workers who handle them in snail farms could be at risk of contracting the disease from dirty hands that, at some point, have come into contact with infected snail slime.

Snail species/district	11	18	19	Internal code
Cernuella virgata	–	–	1	U5
Cornu aspersum	–	2	–	W1, W2
Theba pisana	1	–	3	O3, Q6, U4, U6

TABLE 2 Snail species, number of positive specimens and prevalence by districts with a PCR positive result for *Angiostrongylus cantonensis*. 11-Poblats Marítims, 18-Pobles de l'Oest and 19-Pobles del Sud.

Infective larvae of *A. cantonensis* can be released, especially by stressed, dead snails (Cowie et al., 2022; Rollins et al., 2023), onto green leafy vegetables on which they feed or even larvae could be released during their collection or handling and preparation for their consumption. Therefore, considering that the three species of terrestrial snails that were positive are edible, the following preventive measures to avoid human neuroangiostrongyliasis in Valencia, but also in other European regions, are recommended (Fuentes et al., 2023):

- Consuming snails only when well cooked or previously frozen at −15°C for 12–24 h (Pozio, 2015).
- Inspecting and cleaning thoroughly all vegetables meant to be consumed raw or cooked below a temperature of 60°C and washing for several minutes under cold running water with the aim of removing all snails and/or slugs and their body parts or slime, which could contain infective parasite larvae (Da Silva & Mathison, 2018).
- Informing consumers about these preventive measures through educational campaigns on the biology of terrestrial snails, mainly on these species so far recognized as intermediate hosts.

New analyses of more individuals belonging to snail and slug species both already analysed and newly collected in all districts of Valencia and its metropolitan area should be carried out aiming to ascertain which other species are involved as intermediate hosts of the rat lungworm in this new endemic area.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Màrius V. Fuentes https://orcid.org/0000-0002-9508-6390
Mercedes Gomez-Samblas https://orcid.org/0000-0003-4801-528X
Sandra Sáez-Durán https://orcid.org/0000-0002-7537-4176
Rubén Bueno-Marí https://orcid.org/0000-0002-4898-8519
Antonio Osuna https://orcid.org/0000-0002-3818-1471
María Teresa Galán-Puchades https://orcid.org/0000-0002-6712-9461

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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