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SHORT COMMUNICATION

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Crimean–Congo haemorrhagic fever virus in questing non-*Hyalomma* spp. ticks in Northwest Spain, 2021

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Abstract

Crimean–Congo haemorrhagic fever (CCHF) unexpectedly emerged in humans in Northwest Spain in 2021, and two additional cases were reported in the region in 2022. The 2021 case was associated with a tick bite on the outskirts of the city where the patient lived. PCR analysis of 95 questing ticks collected in the outskirts of that city in 2021, none of the genus *Hyalomma*, revealed a prevalence of confirmed CCHF virus (CCHFV) infection of 10.5%. Our results in this emerging scenario suggest the need to consider that CCHFV may be effectively spreading to Northwest Spain and to urgently understand any possible role of non-*Hyalomma* spp. ticks in the eco-epidemiological dynamics of CCHFV.

KEYWORDS
Dermacentor, ecology, *Orthonairovirus*, tick, zoonosis

1 | INTRODUCTION

Crimean–Congo haemorrhagic fever (CCHF) is an emerging disease in Spain. In early June 2021, a new human case of CCHF was confirmed in "El Bierzo" region, in Northwest Spain (42°39′19″ N, 6°36′53″ W) (ECDC, 2023). Unlike the other cases previously reported in the country, this autochthonous case occurred in a region dominated by the influence of the Atlantic climate. On 19 and 22 July 2022, the national health authorities confirmed two more human cases of CCHF with one casualty in "El Bierzo" (ECDC, 2023). These were the last of the 12 clinical cases reported in the country since 2013 (Sánchez-Seco et al., 2022). Previously, in 2017–2018, antibodies to CCHF virus (CCHFV) were detected in a blood donor from a neighbour region, although the authors did not clarify whether the donor had been exposed locally or not (Monsalve Arteaga et al., 2020).

At present, the only proven competent vectors for CCHFV are ticks of the genus *Hyalomma* (Gargili et al., 2017). In the Iberian Peninsula, the two *Hyalomma* species that predominate in terms of abundance and spatial extent are *Hyalomma lusitanicum* and *Hyalomma marginatum* (Valcárcel et al., 2020). The two *Hyalomma* species have been occasionally documented in the northwest of Spain at low frequencies (Mesina & Wint, 2023; Vieira Lista et al., 2022). However, we currently do not know whether they have colonized this region of Spain or whether the reports are the result of occasional introductions by birds or livestock movements from other areas where *H. marginatum* and *H. lusitanicum* are abundant.

Alberto Moraga-Fernández and Alfonso Peralbo-Moreno contributed equally to this article.

"El Bierzo" seems to be an environmentally favourable area for *H. lusitanicum* but not very favourable for *H. marginatum* (Mesina & Wint, 2023). The environmental favourability of the northwest of Spain for *H. marginatum* is low despite global warming (Fernández-Ruiz & Estrada-Peña, 2021). Other tick species are abundant in the region (Ruiz-Fons et al., 2006; Vieira Lista et al., 2022) and could play an unexpected role in CCHFV transmission as previously suggested (Gargili et al., 2017; Lule et al., 2022; Moraga-Fernández et al., 2021).

Recently, animal models on the risk of CCHFV transmission in the Iberian Peninsula have been constructed based on red deer (*Cervus elaphus*; Cuadrado-Matías et al., 2022) and Eurasian wild boar (*Sus scrofa*; Baz-Flores et al., 2024). These studies identified "El Bierzo" as a low-risk region for the transmission of CCHFV. Wild ungulates are important hosts for *H. lusitanicum* in Spain, but they play a minor role as hosts for *H. marginatum* (Peralbo-Moreno et al., 2022; Valcárcel et al., 2020), so these risk models accurately predict the risk of CCHFV transmission by *H. lusitanicum*, but perhaps not by *H. marginatum*. A serological study conducted in 2018 on samples from 326 domestic ungulates and 73 wild ungulates in Northwest Spain estimated a prevalence of antibodies to CCHFV of 7% in domestic ungulates and 3% in wild ungulates (MSBC, 2019). This contrasts markedly with the results obtained in that study for regions in west-central and south-west Spain (16% in domestic and 70% in wild animals) and with the results of a national serological survey in wild boar (Baz-Flores et al., 2024), supporting the estimated low risk of CCHFV transmission in Northwest Spain.

The low apparent abundance of *H. lusitanicum*, the low environmental favourability for *H. marginatum* and the low risk of CCHFV transmission identified in "El Bierzo" did not portend the emergence of local cases. The notification of three CCHF cases suggests that CCHFV might be circulating in the region. Their occurrence demonstrates how little we know about the ecology of CCHFV in western Europe and how this interferes with preventing cases in unexpected locations. Therefore, our aim was to identify whether CCHFV was circulating in questing ticks in the outskirts of the city of Ponferrada that the 2021 patient reported visiting for a walk before the onset of CCHF symptoms.

2 | MATERIALS AND METHODS

2.1 | Tick survey

To estimate the tick species involved in local transmission of CCHFV and to assess the risk they might pose in Northwest Spain, we collected questing ticks in the outskirts of the city of Ponferrada (42°32′47″ N, 6°35′27″ W; Figure 1) on 23–24 June and 29–30 September 2021, after the first case of CCHF in this region. The patient suffering from CCHF reported walking in the countryside outside the city of Ponferrada before the onset of symptoms. We selected an environmentally favourable peri-urban area for wild ungulates, given their relevance as tick hosts (Ruiz-Fons et al., 2006), and which was connected to the city by rural paths that citizens frequently use for sport. We dragged 30 km of vegetation with 1 × 1 m white cotton cloths to collect ticks and estimate tick questing density. Along tick drags, we recorded observations of animal traces: dung, soil overturns, bedding and direct observations. We estimated cattle farm and head density in "El Bierzo" (INE, 2022) as indicators of the potential presence of *H. marginatum* (Figure 1) because cattle are one of its preferred hosts (Valcárcel et al., 2020). Ticks were transported in a controlled environment to our laboratories, where they were morphologically identified (Estrada-Peña et al., 2017) and processed to analyse the presence of CCHFV RNA.

Impacts

- Crimean–Congo haemorrhagic fever (CCHF) is a severe human haemorrhagic disease that is emerging in Northwest Spain, where three clinical cases with a death were reported recently.
- We found that 10% of non-*Hyalomma* questing ticks were infected with CCHF virus (CCHFV) in an area where a tick bite resulted in the first CCHF human case in Northwest Spain.
- The high prevalence of CCHFV found in questing ticks suggests that the virus could be spreading to Northwest Spain and that preventive measures need to be taken in this part of the country.

2.2 | Molecular diagnosis of CCHFV infection in ticks

Each tick was washed in absolute ethanol with vortexing for 1 min before being dissected into two halves in a biosafety level 3 cabinet. One half was preserved at −80°C. The content of the other half was used for nucleic acid purification with Tri Reagent (Sigma-Aldrich, Burlington, MA, USA) in a safety level 3 cabinet (Moraga-Fernández et al., 2021). Purified RNA samples were stored at −80°C until being analysed by conventional nested reverse transcription PCR (nRT-PCR) for the detection of CCHFV RNA in ticks according to the protocol described by Midilli et al. (2009). This nRT-PCR was designed as a dual PCR for the amplification of fragments of the S-segment of CCHFV and Aigai virus, which includes all AP-92-like isolates formerly classified as CCHFV genotype VI (Papa et al., 2022). For our analysis, we only selected the primer pair (Eecf) capable of amplifying a CCHFV-specific S-segment fragment from the nRT-PCR designed by Midilli et al. (2009). The use of this nRT-PCR primer pair allows the amplification of S-segment fragments of CCHFV genotypes III, IV and V (S. Baz-Flores, G. Herrero, R. Cuadrado-Matías, A. Moraga-Fernández, A. Peralbo-Moreno, I. G. Fernández de Mera, M. Frías, & F. Ruiz-Fons, unpublished data; Moraga-Fernández et al., 2021). The nRT-PCR was performed using the Access RT-PCR System (Promega Corporation, Madison, WI, USA) and

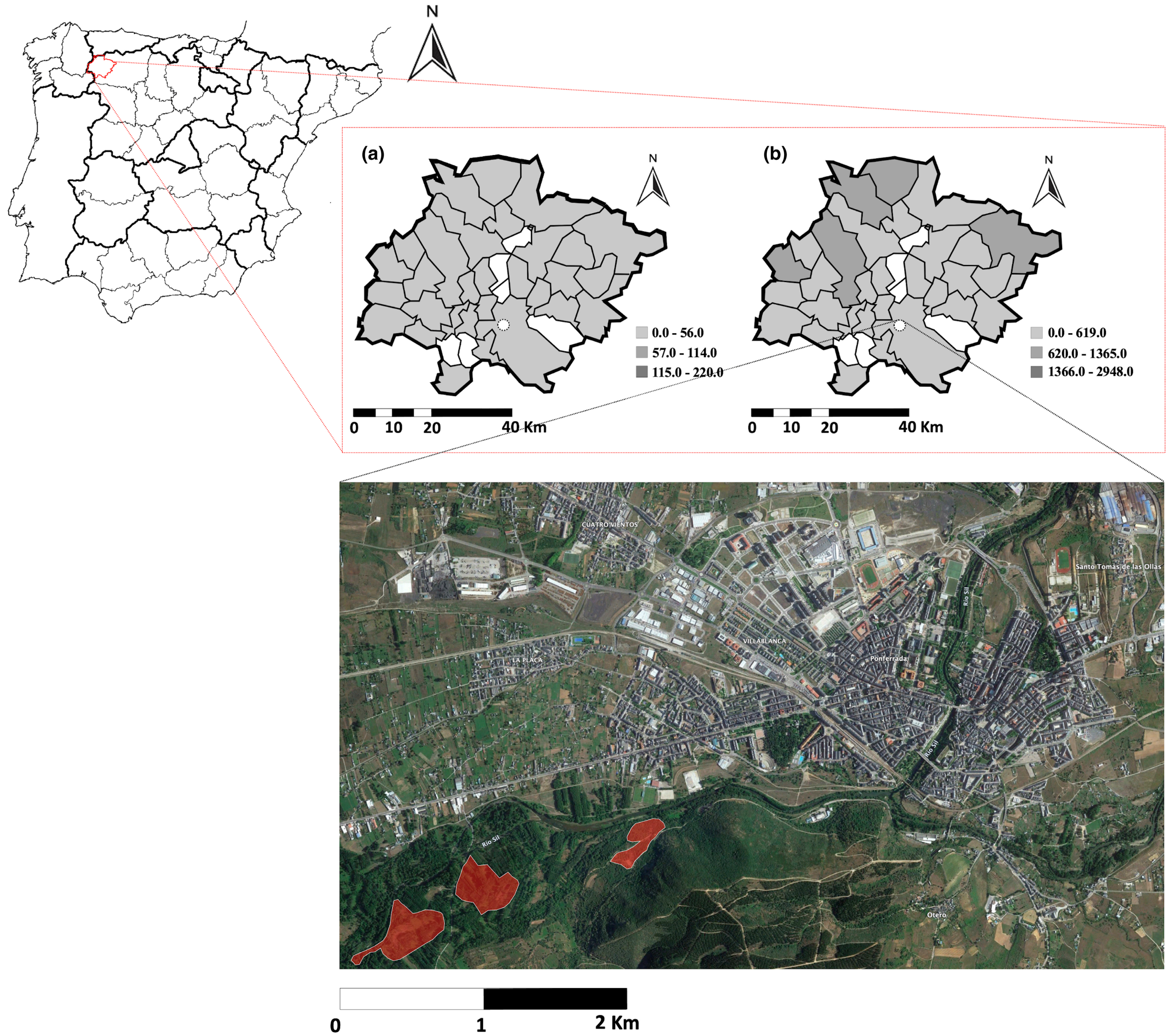

FIGURE 1 Cattle farm (a) and head (b) density (units per Km^2) maps in “El Bierzo” region at the municipality level, based on the 2020 Spanish livestock census (INE, 2022), and questing tick survey areas (red shaded) in the outskirts of Ponferrada city (42°32′47″ N, 6°35′27″ W), Northwest Spain, 2021. The location of Ponferrada city is represented with a white circle in “El Bierzo” region maps.

primers Eecf-F1: TTGTGTTCCAGATGGCCAGC (49–68)/Eecf-R1: CTTAAGGCTGCCGTGTTTGC (356–337) for the first round (307 bp). We employed the Master Mix kit (Promega Corporation, Madison, WI, USA) and primers Eecf-F2: GAAGCAACCAARTTCTGTGC (115–134)/Eecf-R2: AAACCTATGTCCTTCCTCC (326–308) for the second round (212 bp). Amplicons were visualized on 1.5x agarose gels stained with GelRed (Biotium Inc., CA, USA) under UV transillumination. A confirmed CCHFV genotype III positive tick sample (Ramírez de Arellano et al., 2017) was used as the positive control. Positive amplicons were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced (Eurofins Genomics, Ebersberg, Germany). Phylogenetic analyses were carried out on ClustalW to align the obtained CCHFV S segment sequences (212 bp) and reference sequences. The best-fit model of sequence evolution was selected based on corrected Akaike information criterion (cAIC) and Bayesian information criterion (BIC) implemented in Molecular Evolutionary Genetics Analysis 10 (MEGA; http://www.megasoftware.net). Kimura-2 parameter distances were chosen to build the phylogenetic tree. The maximum likelihood method was used to obtain the best tree topologies. Bootstrap confidence limits were calculated based on 1000 replicates.

3 | RESULTS AND DISCUSSION

In the 14 km of vegetation dragged in June, we captured 52 ticks (37.1 ticks/ha), including 12 *Dermacentor marginatus*, 29 *Ixodes ricinus*, nine *Rhipicephalus sanguineus* sensu lato, one *Dermacentor*

TABLE 1 Detailed molecular results of Crimean–Congo haemorrhagic fever virus RNA detection in adult questing ticks, Northwest Spain, 2021.

Survey dates and surface	Tick species	No. of ticks collected	No. of ticks positive/analysed nRT-PCR[a]	nRT-PCR estimated prevalence (95% confidence interval)
23 and 24 June 2021 (14,000 m^2)	*Ixodes ricinus*	29	4/29	13.8% (3.9–31.7)
	Dermacentor marginatus	12	2/12	16.7% (2.1–48.4)
	Rhipicephalus sanguineus s.l.	9	1/9	11.1% (0.3–48.3)
	Rhipicephalus bursa	1	0/1	0.0% (0.0–97.5)
	Dermacentor reticulatus	1	0/1	0.0% (0.0–97.5)
Subtotal June		52	7/52	13.5 (5.6–25.8)
29 and 30 September 2021 (16,000 m^2)	*D. marginatus*	43	5/42	11.9% (3.4–25.6)
	I. ricinus	1	0/1	0.0% (0.0–97.5)
Subtotal September		44	5/43	11.6% (3.9–25.1)
Total (30,000 m^2)		96	12/95	12.6% (6.7–21.0)

[a]nRT-PCR: nested conventional reverse transcription PCR employed as screening method (Midilli et al., 2009).

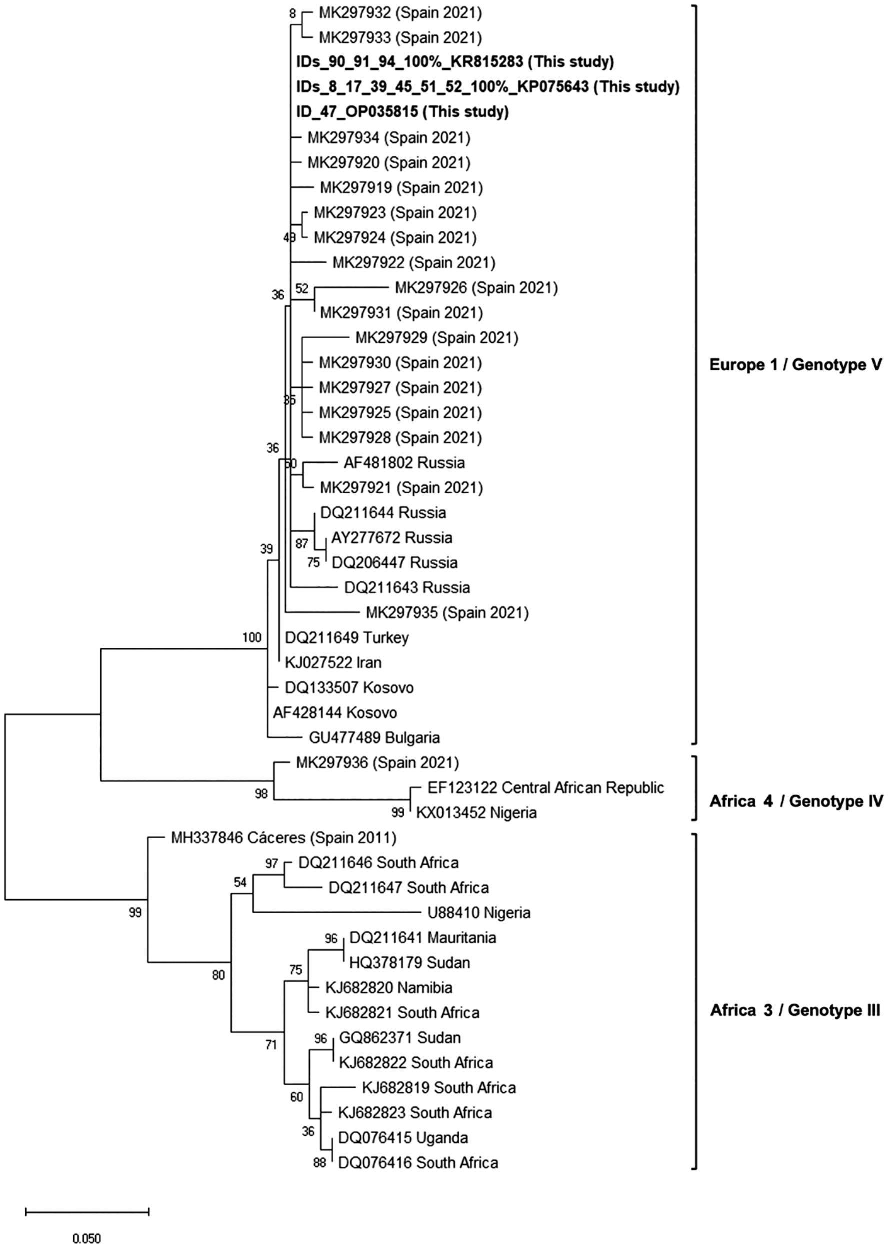

FIGURE 2 Phylogenetic analysis of CCHFV S segment sequences displaying the position of new identified CCHFV sequences. A maximum-likelihood tree was built using the S segment (212-bp partial sequence). Newly identified sequences in this article were named as ID and GenBank accession number and are shown in bold type in the tree.

reticulatus and one *Rhipicephalus bursa*. In September, we collected 44 ticks in 16 km (27.5 ticks/ha), including 43 *D. marginatus* and one *I. ricinus* (Table 1). No *Hyalomma* spp. ticks were collected locally even though the first survey was performed during the activity peak of *Hyalomma* spp. (Valcárcel et al., 2020) and with an appropriate method for capturing questing *Hyalomma* spp. ticks (Cuadrado-Matías et al., 2024). The absence of *Hyalomma* spp. in the area where the 2021 patient presumably could have been bitten by a CCHFV-infected tick suggests that they may not have been involved in the transmission of CCHFV to the patient. However, in May 2023, we were notified of the presence of questing adult *Hyalomma* spp. ticks 11.5 km southeast of our study sites (Coordinates: 42°28′8.48″ N, 6°31′21.24″ O). We confirmed that the ticks were *Hyalomma* spp. on pictures taken after collecting them but did not have access to the ticks ourselves. This finding may suggest that *Hyalomma* spp. are present in highly favourable environmental foci in this region, perhaps at low abundance according to existing evidence.

A positive PCR result was obtained for 12.6% (12/95; 95% confidence interval (CI): 6.7–21.0) of the ticks (seven *D. marginatus*, four *I. ricinus* and one *R. sanguineus* sensu lato) with the PCR used as the screening method (Table 1). Ten of the 12 positive amplicons were sequenced with good quality. Six and three of the sequences showed 100% homology with Russian and Iranian genotype V isolates (Europe 1 clade) with GenBank Accession Numbers KP075643 and KR815283, respectively (Figure 2). Sequence ID_47 displayed 99.5% homology with isolate KR815283, and it was uploaded to GenBank with Accession Number OP035815.

Indirect indices of Eurasian wild boar presence were frequently recorded. We observed scarce traces of roe deer (*Capreolus capreolus*) and no livestock traces along the 30 km, which agrees with the low cattle density in the municipality of Ponferrada as shown in Figure 1. These results indicate that cattle were not involved in the circulation of CCHFV in the study area and that wild animals were probably responsible for the local circulation of the virus. Many people practising sports along paths were observed during the surveys.

It was striking to confirm, by PCR and sequencing, that 10 of the 95 questing non-*Hyalomma* spp. ticks (10.5%; CI: 5.2–18.5) were infected by CCHFV, perhaps one of the highest infection rates ever documented in questing and fed ticks (Orkun et al., 2017; Sánchez-Seco et al., 2022). Another surprising finding was the absence of *Hyalomma* spp. ticks in the study area, despite the high prevalence of CCHFV found in questing ticks of species considered not competent for CCHFV (Gargili et al., 2017). However, the limited extent of our survey area might have overlooked very favourable foci for *Hyalomma* spp. ticks in the region, so we cannot rule out that they might have played a role in the human cases of CCHF reported in "El Bierzo." An interesting finding was the low questing tick density in the study area despite its epidemiological link with the 2021 case of CCHF (ECDC, 2023). Although only the genotype V was identified, the determination of the specific genotype and the possibility of reassortment events will require future efforts to obtain complete sequences of the virus in this region of Spain.

A tick species abundant in the region, *D. marginatus*, has previously been suggested as potentially competent for CCHFV transmission (Orkun et al., 2017), but its precise role has not been elucidated to date. Since our findings were obtained from questing and not from fed ticks, the observed positivity rate indicated that there was a high rate of CCHFV circulation in 2021 in the study area. These results support that it is essential that we estimate the vector capacity for CCHFV of these species to accurately predict the spatiotemporal foci of CCHF emergence.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

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

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