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Metagenomic survey reveals Volzhskoe tick virus in *Hyalomma* ticks for the first time in western Europe, North-Eastern Spain

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ABSTRACT

Ticks are reservoirs and vectors of several emerging arboviruses, yet their associated virome remains poorly characterized. Using meta-transcriptomic sequencing, we surveyed *Hyalomma marginatum* and *Rhipicephalus bursa* ticks collected from areas with contrasting Crimean-Congo haemorrhagic fever virus (CCHFV) seroprevalence in wild ungulates in north-eastern Spain, a region with no reported CCHF cases in humans. While CCHFV RNA was not detected, we identified Volzhskoe tick virus (VTV), a recently described member of the class *Bunyavericetes*, first identified in Russia, in *H. marginatum* from the Ports de Tortosa i Beseit Natural Park—making its first report of VTV in Western Europe. These findings suggest a broader distribution of VTV and raise important questions about its potential interactions with CCHFV, pathogenicity and host range. Moreover, our approach underscores the value of metagenomic surveillance for improving our understanding of tick-borne virus ecology.

1. Introduction

Even though tick-borne pathogens (TBPs) are of public health concern, their ecology is not yet fully understood, primarily due to the complexity of tick ecology and tick-host-pathogen interactions [1]. In the last decades, climatic, environmental and socioeconomic changes have enabled ticks and their associated pathogens to spread into new geographic areas, causing a rise in the number of diagnosed tick-borne diseases, and also leading to the discovery of novel TBPs [2].

In the last decade, metagenomic analysis has become a powerful method for detecting, discovering, characterizing, and classifying TBPs. Metagenomic approaches are highly valuable from a public health perspective, as they can provide an efficient and comprehensive analysis of TBPs, complementary to epidemiological surveillance [3]. Particularly, total transcriptome sequencing, or "meta-transcriptomics", has led to the discovery of an enormous diversity of RNA viruses carried by ticks, even with new viral taxa [4]. Therefore, studying the tick virome is essential for uncovering the full diversity of viruses associated with ticks, including known, novel, and emerging pathogens.

Exploring the virome of *Hyalomma marginatum* ticks is of special interest since this species is the main vector of Crimean-Congo haemorrhagic fever virus (CCHFV) [5]. Further, recent studies have raised concerns about the role of *H. marginatum* in the transmission of other viruses within the class *Bunyavericetes* [6]. In that sense, viromic analyses of *H. marginatum* ticks collected in the Danube Delta (Romania) and in western Hungary resulted in the detection of Volzhskoe tick virus (VTV)

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[7,8], a *Bunyaviricetes* first described in 2020 in a *H. marginatum* tick from the Volga Delta, Russia. Serological surveys revealed the presence of CCHFV antibodies in ruminants from these regions, although CCHFV has never been molecularly detected in local ticks [9,10].

In north-eastern Spain, in close vicinity to the Ebro Delta wetland, a hotspot of high CCHFV seropositivity has been detected in wild boar (*Sus scrofa*) and Iberian ibex (*Capra pyrenaica*) from Ports de Tortosa i Beseit Natural Park (PTiBNP) [11]. Nevertheless, no human cases of CCHF have been reported in the region to date. The present report describes the viruses within the class *Bunyaviricetes* present in *H. marginatum* and *Rhipicephalus bursa* ticks from this area.

2. Methods

Ticks were collected from the vegetation and from wild boars hunted in PTiBNP (40°48′28″N, 0°19′7″E; Catalonia, NE-Spain) as a part of a tick survey carried out in 2023, as described elsewhere and authorized by the Catalan Government [12]. Ticks were morphologically identified using standard taxonomic keys [13]. *Hyalomma marginatum* individuals were further confirmed by molecular identification of mitochondrial markers (12S rRNA and Cytochrome Oxidase 1) [14]. Ticks were divided into 3 pools: the first consisted of three *H. marginatum* feeding from a female wild boar (Sample Name: E24011, SRA: SRS24143219); the second included five *H. marginatum* from another female wild boar (Sample Name: E24002–03, SRA: SRS24143216); and the third with 19 questing (non-fed) *Rhipicephalus bursa* (Sample Name: NF5-E24013–15-17, SRA: SRS24143217). A fourth pool, consisting of two *H. marginatum* feeding on a wild boar from northern Catalonia - over 300 km from PTiBNP - was also included (Sample Name: E24001, SRA: SRS24143220) [11].

Prior to RNA extraction, tick pools were washed three times with 70 % ethanol and once with sterile phosphate-buffered saline (PBS). For RNA extraction, 400 μL of AVL buffer (Qiagen, Hilden, Germany) were added to each tick pool, and the ticks were mechanically disrupted using plastic tissue homogenisers. RNA was extracted from 140 μL of the tick-AVL supernatant using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The extracted RNA was eluted in 50 μL of RNAse-free water and stored at −80 °C prior to molecular analysis.

Tick pools were subjected to next-generation sequencing library preparation using the Stranded Total RNA-Seq with Ribo-zero Plus Microbiome kit (Illumina, San Diego, CA, USA). Libraries were sequenced on an Illumina NovaSeq 6000 system in a 2 × 150 base pair format, with a depth of 120 M reads per sample.

Raw sequencing reads were processed for quality control using **fastp,** filtering low-quality reads and removing adapter sequences [15]. Decontamination of non-viral sequences was performed in two steps. First, taxonomic assignment was performed using **Kraken2** with the **Plus PF DB**, which includes sequences from RefSeq of archaea, bacteria, viruses, plasmids, human, protozoa, and fungi. Viral reads were separated for further processing using **KrakenTools** [16]. Second, unassigned reads ("unassigned" and "other" categories) were mapped against host and related reference genomes (*H. marginatum*: GCA_013339685.2; *R. bursa*: GCF_013339695.2 and *Sus scrofa*: GCF_000003025.6) to remove host-derived sequences. Reads that did not align with any reference genome were considered potential viral sequences.

The decontaminated reads were merged with viral reads and assembled into contigs using **SPAdes** in *–rna viral* mode [17]. Relative abundance and sequencing depth for each contig were calculated using custom scripts. Contig depth was measured as the number of mapped reads per contig length, while scaled relative abundance was normalized to the total count of viral and clean reads using the formula: (contig depth/total depths sum) (number of viral reads/total clean reads).

Only contigs ≥500 base pairs in length were retained to ensure assembly quality. Taxonomic assignment of contigs was conducted using **BLASTn** against three databases: (1) the NCBI nt_viruses database containing complete and incomplete viral genomes, (2) the **IMG/VR v4** database, high-confidence only viral genomes [18], and (3) the **Pathoplexus Crimean Congo Hemorrhagic Fever Virus database**, a curated database of Crimean-Congo hemorrhagic fever virus genomes [19]. BLAST results excluded assignments to bacterial or phage viruses (Supplementary Table 1).

We analysed the phylogenetic relationships of VTV by constructing separate phylogenies for segments M and L using viral sequences from sample datasets E24011 and E24002–03. Only contig above 5500 bp for segment L and 2900 bp for segment M were included in the phylogenetic analysis (Supplementary Table 2). All complete reference sequences from segments L (n = 18) and M (n = 15) were retrieved from NCBI (Fig. 1). Datasets were compiled and alignments of each segment were generated using DECIPHER *v.3.0.0* [20]. Final alignment lengths were 5570 bp for segment L and 2933 bp for segment M. Maximum likelihood phylogenetic trees were constructed with model selection via Bayesian Information Criterion (BIC) and bootstrap analysis (1000 replicates), using phangorn *v.2.12.1* [21] and ape *v.5.8* [22] R packages. Phylogenies were visualized in iTOL *v.6* [23].

3. Results

No CCHFV or *Nairoviridae* were identified in any sample (Supplementary Table 1). Only two samples (E24011 and E24002–03), both belonging to pools of *H. marginatum* from PTiBNP, contained contigs assigned to VTV (*Bunyaviricetes*), corresponding to viral segments L and M (Supplementary Table 1). Five contigs across both samples were used for phylogenetic analysis (Supplementary Table 2): In sample E24011, one contig (5570 bp) was identified for segment L and another (2933 bp) for segment M, with scaled relative abundances of 1.21E-07 and 1.80E-07, respectively. These contigs exhibited high sequence identity (93.39 % and 94.15 %) and query coverage (99 % and 98 %) in BLAST analysis, with 422 and 332 mapped reads. In sample E24002–03, three contigs were assigned to *Volzhskoe tick virus*: two for segment L (6,936 bp and 10,418 bp) and one for segment M (4,532 bp). These contigs had higher scaled relative abundances (0.000940911 to 0.000997172) and extensive read support, with over 1.1 million mapped reads for the smaller segment L contig and more than 1.8 million for the larger one. The segment M contig in this sample had 338,189 mapped reads, with 94.07 % identity and 91 % coverage.

VTV was found absent in both remaining samples, one corresponding to the same high seropositivity area but from a pool of unfed *R. bursa* (NF5-E24013–15-17) and the other from a pool of two *H. marginatum* feeding on wild boar outside the CCHFV seropositivity area (E24001) (Supplementary Table 1).

In the phylogenetic reconstructions of both M and L segments, the same two clades of VTV with high bootstrap support emerged: in one clade, we found all sequences belonging to China and in the other, all sequences from Spain, Hungary and Russia (Fig. 1). The VTV identified in this study felt closer to Hungary, except for one sequence of L segment from sample E24002–03, which appears more closely related to a strain from Russia (Fig. 1, A).

4. Discussion

The detection of VTV in southern Catalonia represents the first report of this virus in Western Europe. This finding suggests that VTV might be more widely distributed than previously documented and highlights the importance of metagenomic-based surveillance for further characterizing the remarkable diversity of tick viruses. The origin of VTV is still unknown, however, its detection near wetlands and migratory bird stopovers reinforces the previous hypothesis that birds may play a role in its spread [7].

There is no information regarding the role of tick hosts in the ecology of VTV, but current evidence suggests that VTV is mostly *Hyalomma*-specific [24]. Indeed, we did not find VTV sequences in the pool of *R. bursa* ticks collected in the same area. As VTV has been detected so far

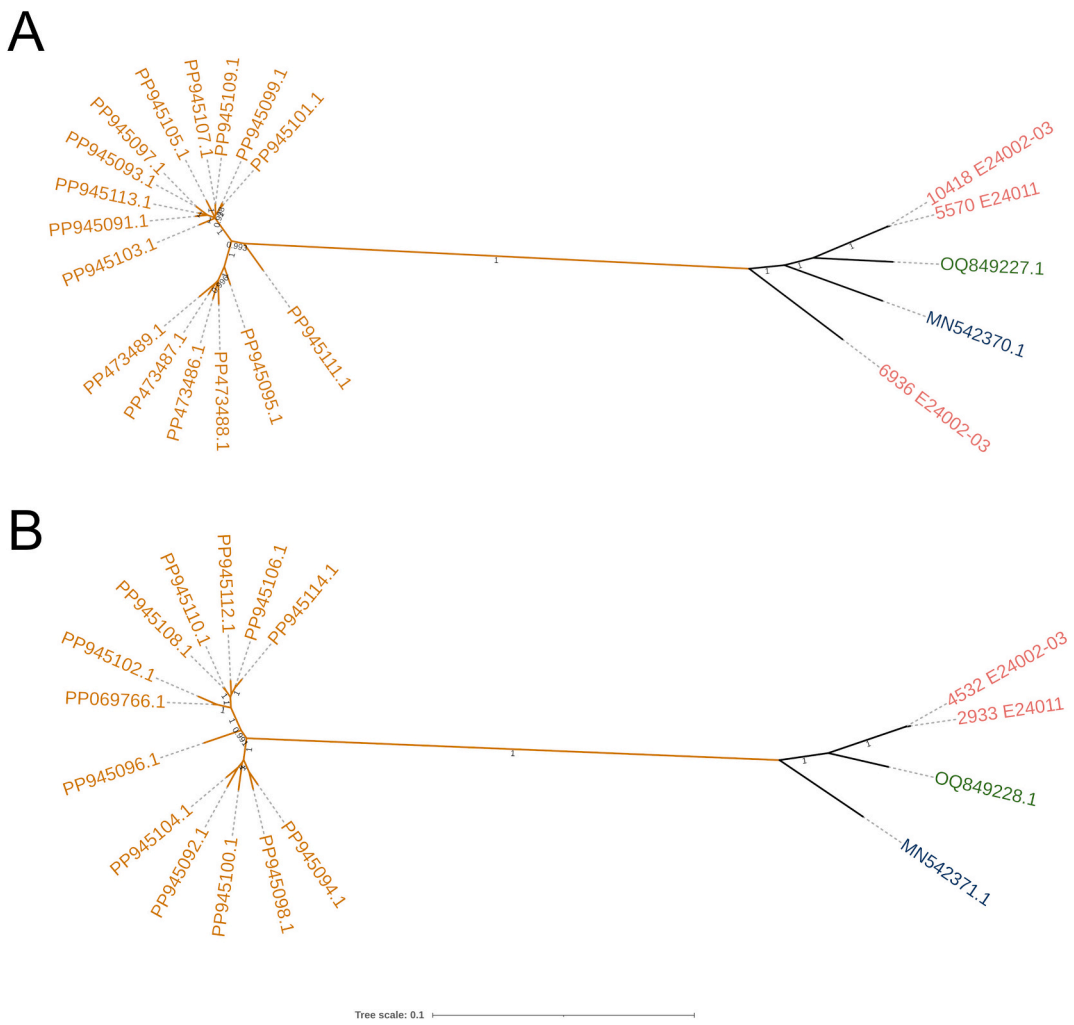

Fig. 1. Phylogenetic reconstruction of *Vozhskoe tick virus* with 1000 bootstraps were performed and bootstrap >0.90 are shown for both A. Segment L and B. Segment M. Sequences from China (yellow), Russia (blue) and Hungary (green) are named after their NCBI accession numbers. And samples from Spain (red), from this study, belong to final contigs from samples E24011 and E24002–03. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

exclusively in ticks, the capacity of VTV to infect vertebrate hosts, including humans, remains unconfirmed. Moreover, the pathogenic potential of VTV and its impact on hosts is unknown, as well as whether animals such as wild boars could act as reservoirs or amplifying hosts of VTV.

Notably, in regions such as Hungary and the Danube Delta in Romania, VTV has been detected in tick populations without concurrent findings of CCHFV [7,8]. However, serological surveys indicate the circulation of CCHFV antibodies in ungulates within these regions [9,10], paralleling our observations in southern Catalonia. The reasons behind this intriguing pattern are unclear, and more studies are needed to elucidate the factors influencing the presence or absence of specific viruses in tick populations and their wild hosts. Likewise, even though no CCHFV sequences were identified in this study, additional research focusing on *Bunyaviricetes*, including CCHFV, in ticks from PTiBNP, with a larger sample size, should be performed to assess the ecology of *Bunyaviricetes* in the region.

The discrepancy between high CCHFV seropositivity and the lack of CCHFV detection in ticks, combined with the identification of VTV in ticks in a region, could be due to several reasons. A plausible explanation is antibody cross-reactivity between CCHFV and VTV, leading to false-positive serological results. The fact that no VTV was found in our *H. marginatum* pool from north Catalonia, an area lacking CCHFV seropositivity or CCHFV detection, adds weight to this hypothesis. However, to confirm this hypothesis, it is necessary to enhance metatranscriptomics analyses in ticks and their wild hosts. Further studies, including VTV isolation in tick-derived and mammalian cells lines, virus neutralization tests and, ultimately, experimental infections in several ungulate species are needed to fully elucidate VTV ecology, host range and zoonotic potential. Nevertheless, our results highlight the importance of interpreting serological test results with caution, especially in multi-host and vector-borne epidemiological systems, where the whole viral diversity is unknown, leading to higher chances of cross-reactivity and misinterpretations [25]. Thus, this study underscores the importance of metagenomics for unravelling the “virosphere” in each particular system as a first step to help better interpret diagnostic tests results and epidemiological scenarios.

Commonly, viruses within the class *Bunyaviricetes* have three genome segments - large (L), medium (M), and small (S) segments [26]. Our metagenomic analysis successfully assembled the L and the M genome segments of VTV but it was unable to identify the S segment. This could indicate that the S segment is either highly divergent, making it difficult to classify as a viral segment, or that VTV lacks an S segment entirely. Interestingly, other unclassified tick-specific bunyaviruses, such as *Ixodes ricinus* bunyavirus-like virus 1 and Bronnoya virus, also seem to lack identifiable S segments, suggesting this may be a common feature among certain tick-associated bunyaviruses [27,28]. In fact, some authors propose a new family within the *Bunyaviricetes* class, including

VTV, Bronnoya and other similar tick-specific bunyavirus-like viruses [29].

The epidemiological relationship within *Bunyaviricetes* still remains unknown, and further interdisciplinary studies are needed to understand whether the presence of VTV in an area could interfere with CCHFV surveillance programs and even reduce CCHFV circulation in ticks-host interface by inducing the production of cross-protective antibodies against CCHFV infection in animals. This hypothesis, if confirmed, could have important implications for interpreting serological data and understanding virus–virus interactions in complex multi-host systems.

In conclusion, our study expands the known range of VTV and raises important inquiries about its ecology, origin, and potential interactions with CCHFV. Ongoing multidisciplinary research is needed to answer these questions and understand tick-specific viruses' transmissibility, ecology, seroreactivity and potential pathogenicity. Overall, this study highlights the importance of using metagenomic approaches for viral surveillance, providing essential baseline data for One Health programs by revealing the viral diversity circulating in vectors and informing the interpretation of serological surveys.

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CRediT authorship contribution statement

Laura Carrera-Faja: Writing – original draft, Methodology, Investigation, Conceptualization. **Mariette Viladomat Jasso:** Visualization, Software, Formal analysis, Data curation. **Iris Sarmiento:** Methodology. **Jordi Manuel Cabrera-Gumbau:** Formal analysis. **Johan Espunyes:** Writing – review & editing, Conceptualization. **Jaime Martínez-Urtaza:** Writing – review & editing, Supervision. **Oscar Cabezón:** Writing – review & editing, Conceptualization.

Declaration of competing interest

We declare no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2025.101279.

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