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THE VIROME OF *RHIPICEPHALUS*, *DERMACENTOR* AND *HAEMAPHYSALIS* TICKS FROM EASTERN ROMANIA INCLUDES NOVEL VIRUSES WITH POTENTIAL RELEVANCE FOR PUBLIC HEALTH

RUNNING TITLE: NOVEL VIRUSES IN TICKS IN ROMANIA

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Abstract

Ticks are involved in the transmission of various pathogens and some tick-borne diseases cause significant problems for the health of humans and livestock. Despite their obvious importance, the composition of viral communities in ticks, and their interactions with pathogens, is poorly understood, particularly in Eastern Europe that constitutes (via bird

migrations for example) a major hub for animal-arthropod vectors exchanges. The aim of this study was first to describe the virome of *Dermacentor sp., Rhipicephalus sp.* and *Haemaphysalis sp*. ticks collected from poorly investigated regions of Romania (Iasi and Tulcea counties) located at the intersection of various biotopes, countries and routes of migrations. We then focused the study on viruses that could have potential relevance for human and animal health. More than 500 ticks were collected in 2019 from the environment and from small ruminants and analyzed by high-throughput transcriptome sequencing. Among the viral communities infecting Romanian ticks, viruses belonging to the *Flaviviridae, Phenuiviridae* and *Nairoviridae* families were identified and full genomes were derivedPhylogenetic analyses placed them in clades where mammalian isolates are found, suggesting that these viruses could constitute novel arboviruses. We also assessed the bacterial microbiome of the collected ticks. The characterization of these microbial communities increases the knowledge of the diversity of viruses in Eastern Europe and provide a basis for further studies on the relationship between ticks and tick-borne viruses.

Keywords: Eastern Europe, next-generation sequencing, ticks, viruses.

Introduction

Ticks are the second arthropod vectors, after mosquitoes, responsible for the spread of viruses and bacteria from wildlife to domestic animals and humans (Labuda & Nuttall, 2004). The ability of ticks to transmit a wide range of microbial pathogens, combined with their promiscuous feeding and geographical range expansion, makes them a substantial threat to animal and human health (Jongejan & Uilenberg, 2004). Among ixodid ticks, viral disease-causing vectors are found mostly in the following tick families: *Ixodes, Haemaphysalis, Hyalomma, Amblyomma, Dermacentor, Rhipicephalus*, and *Boophilus* (Labuda & Nuttall, 2004). The tick microbiome consists of communities of viruses, bacteria and eukaryotes (Havlikova, Lickova, & Klempa, 2013), among which several pathogens coexist within the

commensal flora. Such pathogens of medical importance include Crimean-Congo hemorrhagic fever virus (CCHFV) (Bente et al., 2013), Kyasanur Forest disease virus (KFDV) (Holbrook, 2012), tick-borne encephalitis virus (TBEV) (Yu et al., 2011), severe fever with thrombocytopenia syndrome virus (SFTSV) (Yu et al., 2011), Alkhurma virus (ALKV) (Labuda & Nuttall, 2004; Yu et al., 2011), and Heartland virus (HRTV) (McMullan et al., 2012); and species of bacteria within the genera *Anaplasma*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Francisella*, *Rickettsia* and *Theileria*. Similarly, several tick-borne viruses also threaten the health of livestock: these include Africa swine fever virus (ASFV), Nairobi sheep disease virus (NSDV), and Louping ill virus (LIV), among others (Dantas-Torres, Chomel, & Otranto, 2012).

The microbiome of most tick species remains unexplored despite the growing number of studies (Brinkmann et al., 2018; Greay et al., 2018a; Maruyama et al., 2014; Meng et al., 2019; Qin et al., 2014; Shi, Lin, Vasilakis, et al., 2016; Temmam, Chretien, et al., 2019). Nevertheless, taking advantage of the rapid development of next generation sequencing (NGS) methods in recent years, many novels viral sequences have been identified in ticks of different species distributed in different regions of the world. For example, Jingmenviruses (JMTV) are a recently reported group of positive sense ssRNA viruses with a genome composed of four segments, two segments coding for nonstructural (NS) proteins and presenting homologies with flavivirus non-structural proteins 3 (NS3) and 5 (NS5) while structural proteins have no known homologous (Shi, Lin, Vasilakis, et al., 2016). The geographical distribution of JMTV-like viruses has rapidly expanded with the identification of closely related viruses in *Rhipicephalus microplus* ticks from Brazil (Souza et al., 2018), China (Xia et al., 2015), Trinidad and Tobago (Sameroff et al., 2019) and the French Antilles (Temmam, Bigot, et al., 2019). Such viruses have been found in China in *Haemaphysalis* sp., *Ixodes* sp., *Dermacentor nuttalli*, and *Amblyomma javanense* ticks (Jia et al., 2019; Qin et al.,

2014) and in *Ixodes ricinus* ticks originating from Finland (Kuivanen et al., 2019). JMTV has been identified in cattle, rodents and primates (Emmerich et al., 2018; Jia et al., 2019; Ladner et al., 2016; Souza et al., 2018), in the urine of bats from Cambodia (Temmam, Bigot, et al., 2019) and more recently in humans suffering from febrile illness with unknown etiology (Wang et al., 2019), revealing how little is known regarding the potential of discovery of new tick-borne arboviruses and suggesting a possible role in public health.

Recent studies have indeed reported the identification of novel tick-borne viruses for which the potential zoonotic risk for humans or domestic animals remains unknown. For example, among members of the *Nairoviridae* family, which are primarily transmitted by *Ixodidae* and *Argasidae* ticks for which the natural hosts are birds, bats, rodents, lagomorphs or ungulates (Ladner et al., 2016), a novel virus named Nayun tick nairovirus (NTNV) that clusters within the *Orthonairovirus* genus and that is phylogenetically linked to Crimean-Congo hemorrhagic fever virus was reported in dog-infesting *Rhipicephalus sanguineus* Chinese ticks (Xia et al., 2015). Similarly, among the *Phenuiviridae,* a novel phlebovirus named Tacheng tick virus 2 (TaTV2) was identified in *Dermacentor marginatus* ticks from China and was also detected in one patient's blood (without current information on TaTV2 pathogenicity for humans), suggesting a possible vectorial transmission of this virus (Brinkmann et al., 2018; Dong et al., 2021) and revealing the necessity to monitor the emergence of TaTV2 in humans in contact with ticks. *Flaviviridae*-related tick-borne viruses (e.g., Bole tick virus 4, BTV4) were primarily associated with *Hyalomma asiaticum* and *Rhipicephalus sanguineus* ticks (Shi, Lin, Vasilakis, et al., 2016) and seems to be restricted to ticks, despite a growing number of tick species being susceptible to BTV4 infection. In complement to these viral families known to contain tick-borne viruses, novel families were recently identified. It is the case of the *Chuviridae* family, composed by ssRNA negative-

strand viruses (e.g., *Changping tick virus 2*) for which the ability to infect vertebrates is currently unknown.

Despite the importance of tick-borne diseases in animal and human health, data regarding the virome diversity present in ticks in Eastern Europe is lacking. In this study, we aimed to characterize the virome diversity of *Riphicephalus*, *Dermacentor* and *Haemaphysalis* sp. ticks collected from poorly investigated areas of Romania to increase the knowledge of the diversity of viruses in Eastern Europe, including novel ones that could have potential relevance for human and animal health.

MATERIALS AND METHODS

More than 500 adult ticks belonging to the genera *Rhipicephalus*, *Dermacentor* and *Haemaphysalis* collected in Eastern Romania in the environment or engorged on small ruminants within the Danube delta (Tulcea county) and at the frontier with Moldavia (Iasi county) were analyzed by high-throughput metatranscriptomic analysis. The details of sampling, sequencing and bioinformatics analyses are presented in supplementary materials.

RESULTS

Overview of Romanian ticks microbiome

The taxonomic assignation of sequences revealed that most sequences were assigned to Eucaryota (ranging from 7% to 61%, according to the library sample), viruses (0.4-84%) and bacteria (9-68%), depending on the tick species considered (Table 1). Except for engorged *Rhipicephalus bursa* ticks collected from Tulcea, in which most sequences were derived from JMTV, eucaryote- and bacteria-related sequences were the most abundant, while viral sequences represented less than 2% of total sequences. Results regarding the bacteriome of *D. reticulatus, R. sanguineus, R. bursa* and *H. punctata* are detailed in supplementary data.

Characterization of viral communities

More than 96% of viral sequences were assigned to RNA viruses while few other related viral sequences were identified (1.32% DNA viruses and 2.14% unknown viruses). The detection of DNA viruses in the RNA fraction reflects the transcription activity of these later. Among the RNA viruses, most sequences were assigned to ssRNA+ viruses (61.03%), followed by unclassified RNA (17.24%), unclassified ssRNA+ (11.80%), ssRNA- (9.39%), dsRNA (0.49%) and unclassified RNA- (0.05%) viruses, depending on the tick species considered.

The host spectrum of RNA viruses revealed some differences between *D. reticulatus*, *R. sanguineus*, *H. punctata* and *R. bursa* ticks (Figure 1A). In questing *D. reticulatus, R. sanguineus*, and *H. punctata* ticks, respectively 47%, 67% and 93% of viral reads were assigned to viruses with unknown host while most viral reads (97%) identified in engorged *R. bursa* represented arboviruses (with JMTV being predominant) able to dually infect invertebrate and vertebrate hosts, suggesting that arboviruses observed in engorged ticks may partly reflect the blood virome of sheep and goats on which ticks fed. Similarly, the pattern of hosts for DNA viruses was comparable for questing *D. reticulatus, R. sanguineus*, and *H. punctata* ticks, with a majority of DNA viruses infecting bacteria (57%, 46% and 53%, respectively) followed by vertebrate-infecting viruses, but differed from the host spectrum of DNA viruses of engorged *R. bursa* ticks that mainly infect invertebrates (Figure 1B). These observations suggest a certain degree of specificity of the viromes associated with a given tick species.

The composition of RNA and DNA viral communities infesting *D. reticulatus*, *R. sanguineus*, *H. punctata* and *R. bursa* ticks differed according to tick species (Figure 2) and confirmed the differences observed in host spectrum between the different tick species. The RNA virome of Romanian ticks was composed of 29 families, with some families restricted

to a given tick species (eg., unclassified *Mononegavirales*) while others were shared by all (unclassified *Riboviria*, unclassified *Picornavirales*, unclassified ssRNA+ and *Flaviviridae*) or specific to questing ticks (e.g., *Luteoviridae, Tombusviridae, Marnaviridae)*. The *Flaviviridae* family was the most abundant in engorged *R. bursa* due to the presence of numerous Jingmen tick virus-related reads. The *Phenuiviridae* family was found in *H. punctata*, *R. sanguineus* and *R. bursa* ticks, while *Nairoviridae* family was present in *D. reticulatus* and *R. sanguineus* (Figure 2A).

DNA viruses were classified into 12 viral families. *Circoviridae and Microviridae* represented the majority of DNA sequences, while *Phycodnaviridae, Podoviridae, Siphoviridae,* and *Myoviridae,* were only found in questing ticks (*H. punctata, R. sanguineus, D. reticulatus*) with similar profiles in terms of abundance of each taxon (Figure 2B). Regarding *R. bursa,* the unique engorged tick species, DNA viruses were only represented by *Parvoviridae* and *Genomoviridae* families (Figure 2B).

Viruses belonging to the Flaviviridae family

Approximately 97% of reads belonging to the *Flaviviridae* family were assigned to Jingmen tick virus (JMTV) in *R. bursa* engorged ticks collected on sheep and goats from Tulcea area (Table 2). The complete genome of JMTV, obtained directly from the sequencing reads and contigs, presented amino-acid identities ranging from 98.03% to 99.26% depending on the segment considered, with its closest tick-borne Turkish isolate relative (Figure 3A).

Phylogenetic analyses performed on the complete segment 1, encoding the RNA-dependent RNA polymerase, placed Romanian JMTV isolate in a clade encompassing tick-borne and human isolates originating from Eastern Europe, respectively from Turkey and Kosovo, suggesting a possible ability of Romanian JMTV to infect mammals (Figure 3B). However, this later does not belong to the clade of Alongshan virus, which is to date the unique member

of Jingmenviruses that has been proved to be responsible of human pathologies (Wang et al., 2019). Similar topologies were achieved with phylogenetic reconstructions targeting segments 2, 3 and 4 (supplementary data, Figure S5, Figure S6, Figure S7).

Another virus linked to the *Flaviviridae* family (but not yet recognized as so by the International Committee for Taxonomy of Viruses, ICTV) was Bole tick virus 4 (BTV4) (Table 2). Romanian BTV4 was identified in questing ticks originating from Iasi county (*D. reticulatus*) and in ticks from Tulcea county (*R. sanguineus* and *H. punctata*), suggesting that BTV4 is able to infect multiple tick species from various environments. Viruses presented an amino-acid identity ranging from 78.16% to 85.28% with BTV4 previously identified in *H. asiaticum* ticks from China (NC_028371), and a genome coverage comprised between 6.4% (Tulcea isolates) and 99.1% (Iasi isolates), depending on the tick considered (Figure 4), highlighting a difference in abundance of BTV4-related reads between non-engorged (the most abundant) and engorged ticks. Phylogenetic analyses placed Romanian BTV4 in a clade that includes tick-borne isolates from Thailand and Caribbean areas, belonging to *R. sanguineus* and *H. asiaticum* from China (Figure 5). The percentage of nucleotide identities observed between the most covered (Iasi) viruses, ranging from 97.27% to 99.83% along the whole genome, and with closely related BTV4 isolates, is concordant with the phylogeny, showing a high degree of conservation of BTV4 isolates among various tick species (data not shown).

Viruses belonging to the Phenuiviridae family

Three distinct phleboviruses were identified, depending on the tick species and the area considered. Sequences assigned to the *Phenuiviridae* family were represented by Tacheng tick virus 2 (TaTV2), Brown dog tick phlebovirus 2 (BDTPV2) and Changping tick virus 1 (CPTV1) (Table 2), all negative sense bi-segmented ssRNA viruses that missed the M segment coding for the viral glycoprotein.

Romanian BDTPV2 was only detected in questing *H. punctata* and engorged *R. sanguineus* ticks from Tulcea county and presented a horizontal genome coverage ranging from 86.6% to 100% depending on the segment (Figure 6A). Amino-acid identities ranged from 62.3% to 88.4% depending on the segment considered, with its closest tick-borne Turkish isolate relative (MN025508-09). The nucleotide identity observed between the two BDTPV2 Romanian strains ranged from 70.11% (segment S) to 78.87.% (segment L), highlighting differences between BDTPV2 strains carried by two different tick species from the same location, and suggesting a possible host specificity of Brown dog tick phlebovirus 2.

Romanian TaTV2 was only identified in *D. reticulatus* ticks from Iasi county. It presented a horizontal genome coverage comprised between 0% to 100% (Figure 6B), and a nucleotide identity between the different strains ranging from 68.61% to 97.37% for the L segment, and from 61.05% to 74.47% for the S segment; TaTV2/Romania/Iasi22 being the most distant Tacheng tick virus 2 strain among the five Romanian isolates.

As for TaTV2, Romanian CPTV1 was only identified in *D. reticulatus* ticks from Iasi county. It presented lower horizontal genome coverage, between 7.2% to 58% for the L segment and between 0% to 66.2% for the S segment (Figure 6C). Conversely to TaTV2, the nucleotide identity observed between the different strains of CPTV1 was higher and ranged from 90.57% to 98.99% for the L segment, and from 74.44% to 91.82% for the S segment, suggesting a higher degree of conservation between the different CPTV1 strains compared to Romanian TaTV2 isolates.

Phylogenetic analyses performed on the protein sequence of the RNA-dependent RNA polymerase placed Romanian phleboviruses among the *Uukuvirus* genus within the *Phenuiviridae*. Romanian TaTV2 clustered in a clade encompassing tick-borne isolates originating from Eastern Europe, respectively from Anatolia/Turkey (*R. sanguineus*) and isolates originating from ticks (*D. marginatus*) and humans from China, suggesting a

zoonotic potential of Romanian TaTV2 (Figure 7). Romanian CPTV1 placed in the same clade than Romanian TaTV2. One should note that Romanian TaTV2 (except TaTV2/Iasi22) and CPTV1 isolates clustered in a highly supported distinct clade (posterior probability of 1), different from other isolates originating from China, suggesting a possible geographical specificity of these viruses (Figure 7). Conversely, Romanian BDTPV2 clustered in a distinct clade from TaTV2 and CPTV1. It placed within a clade apparently restricted to tick phleboviruses that were primarily identified in *Rhipicephalus* (*bursa* or *sanguineus*) or *H. marginatum* ticks from Turkey or Trinidad and Tobago. In concordance with the level of conservation observed between the two BDTPV2 Romanian strains, BDTPV2/Romania/Tulcea1 and BDTPV2/Romania/Tulcea47 clustered in different subclades, Tulcea47 strain being the most distant and placing at the root of this subclade (Figure 7).

Viruses belonging to the Nairoviridae family

Nairoviridae-related sequences belonging to the *Orthonairovirus* genus were distantly related to Nayun tick nairovirus (NTNV), a nairovirus-related that was primarily identified in *Rhipicephalus* sp. ticks from China (KP141755). Romanian NTNV differed from its Chinese counterpart by presenting 73% of amino-acid identity in the S segment. Unfortunately, the sequence of the L segment of Chinese NTNV was not available in public databases (Xia et al., 2015), so we were not able to identify sequences related to this segment in the dataset, despite comprehensive analyses with the use of HMM profiles (data not shown). Surprisingly, Sanger sequencing performed to fill the gaps between NGS reads revealed the presence of an internal stop in one of the two Sanger sequences compared to the reference Chinese sequence (Figure 8A). This observation suggests the presence of two populations of viral RNAs in the tick pool: one coding for the full length of NTNV nucleoprotein, and the other presenting an internal TGA stop codon that could result in the possible presence of an

endogenous viral element (less subject to selection pressure than an exogenous virus) in the genome of Romanian *R. sanguineus* ticks. As for *Argasidae*-related tick-borne nairoviruses, Romanian and Chinese NTNV placed at the root of arbo-nairoviruses infecting mammals (for example CCHFV or NSDV, Figure 8B), either suggesting that NTNV could represent a novel virus species within the *Orthonairovirus* genus or, as it was proposed for phleboviruses (Matsuno et al., 2018) it could constitute an ancestral sequence reflecting the origin of the *Orthonairovirus* genus.

Discussion

Although global warming is often cited as the underlying mechanism favoring the spread of tick-borne diseases, climate is just one of many factors among others (for example their population density, the likelihood that they will be infected with pathogens for humans and the frequency of tick-human contacts) that determine which tick species are found in a given geographic region (Estrada-Pena & de la Fuente, 2014). The distribution of ticks and their vectored pathogens is also affected by a plethora of biological and environmental determinants, including deforestation and urbanization, which may together favor the spread and establishment of selected vectors into previously tick-free areas (Colwell, Dantas-Torres, & Otranto, 2011; Estrada-Pena, Ayllon, & de la Fuente, 2012; Ostfeld & Brunner, 2015). However, until a decade ago, only a few tick-borne viruses were known, among which flaviviruses were the most characterized (Gould & Solomon, 2008; Hermance & Thangamani, 2017; Yoshii, 2018). Interestingly, recent studies have made significant inroads characterizing the microbiome of ticks, which is a rich mixture of viruses, eukaryotes and bacteria (Bonnet, Binetruy, Hernandez-Jarguin, & Duron, 2017; Greay et al., 2018b; Harvey et al., 2019; C. X. Li et al., 2015; Moutailler, Popovici, Devillers, Vayssier-Taussat, & Eloit, 2016; Narasimhan & Fikrig, 2015; Narasimhan et al., 2014; Pettersson et al., 2017; Sakamoto et al., 2016; Shi, Lin, Tian, et al., 2016; Tokarz et al., 2014; Vanmechelen, Laenen, Vergote,

& Maes, 2017) and revealed the tick-born origin of unknown human febrile illnesses (Wang et al., 2019) highlighting the importance of virus discovery in ticks.

Ecology of sampling sites and tick microbiomes

The aim of our study was to describe the virome of *Dermacentor sp., Rhipicephalus sp*., and *Haempahysalis sp*. ticks collected from Eastern Romania, as the first key step preceding the identification of arboviruses of medical and veterinary significance. We focused our attention on two areas due to their potential importance in the spread of pathogens. The first area studied, the Danube Delta Biosphere Reserve (DDBR) is the second largest wetland in Europe, predominantly located in Eastern Romania, with some parts also located in Ukraine. The DDBR shows a high degree of biodiversity and functions. It serves for example as breeding sites for sheep and goats, and also constitutes a major hub for bird migration (Hanganu et al., 2002) from Africa and Asia, leading to high risk of introduction of animal pathogens, including zoonotic and vectored agents (Tomazatos et al., 2019; Tomazatos et al., 2021). The biological richness of this area is represented by 30 types of ecosystems comprising 2391 species of flora and 6197 species of fauna (including fish, amphibians and reptiles) but also mammalian terrestrial wildlife and domestic animals (such as rodents, insectivores, carnivores, bats, artiodactyles and lagomorphs) (Authority, 2007). In such environment, ticks may constitute the bridge that could lead to interspecies transfer of pathogens, including to humans. The second area studied is represented by suburban sites intended for recreational activities in Iasi county, a different region in terms of biodiversity because deciduous trees and meadows are the predominant vegetation at this area (Pavel et al., 2014) situated at the border with the Republic of Moldova. The fauna on this site is mainly represented by domestic animals (pets but also small and large ruminants).

Ticks are obligate blood-feeding ectoparasites that belong to the class *Arachnida*, that is further divided into three families, *Ixodidae* (hard ticks), *Argasidae* (soft ticks) and *Nuttalliellidae* (Anderson & Magnarelli, 2008). *Ixodidae* is the largest family and includes four genera and over 700 species of ticks distributed around the globe (Brites-Neto, Duarte, & Martins, 2015). *Dermacentor reticulatus* is the second most widespread hard tick species of Europe, after *Ixodes ricinus* (Paulauskas et al., 2018). The distribution range of *D. reticulatus* has been reported to recently expand to higher latitudes and altitudes throughout Central Europe (Germany, Poland, Hungary, Slovakia, and Romania) (Mihalca et al., 2012; Siroky et al., 2011). The most common vertebrate host reported are domestic animals as sheep, goats, cattle, horses, cats, dogs, and pigs (Foldvari, Siroky, Szekeres, Majoros, & Sprong, 2016). It is considered the main vector of *Babesia canis, Rickettsia slovaca, R. raoultii* (Rudolf et al., 2016) and is a carrier of other pathogens such as *Anaplasma spp., Bartonella spp., Coxiella burnetti, Francisella spp.* (Foldvari et al., 2016), and tick-borne encephalitis and Omsk haemorrhagic fever viruses (Ruzek, Yakimenko, Karan, & Tkachev, 2010). In our study, *D. reticulatus* ticks collected from Iasi county were mainly infected by two members of the *Phenuiviridae* viral family: Changping tick virus 1 (CPTV1) and Tacheng tick virus 2 (TaTV2); by a *Flaviviridae*-related virus (Bole tick virus 4, BTV4), and by several bacteria as *Francisella persica, Candidatus Coxiella mudrowiae* and *R. raoultii*.

Rhipicephalus bursa is a hard tick distributed in Europe around the Mediterranean basin while it seems restricted in Romania to the Southern lowland region, despite sporadic reports from Central and Northwestern parts of Romania, suggesting a possible colonization mediated by the transport of livestock (Mihalca et al., 2012). *R. bursa* usually feed on ruminants or other domestic animals, wildlife or human hosts. It is recognized as the vector of many important pathogens of livestock, including *Babesia ovis* (Erster et al., 2016), *Coxiella burnetti* (Raele, Galante, Pugliese, De Simone, & Cafiero, 2015), *Theileria spp.*

(Ferrolho et al., 2016) *Anaplasma marginale* (Ferrolho et al., 2016) and *Anaplasma ovis* (Renneker et al., 2013), *Ehrlichia canis* (Aktas, 2014; Masala et al., 2012), and of viruses such as CCHFV nairovirus (Mehravaran et al., 2013). In the present work, *R. bursa* was the only engorged tick species studied, collected on sheep and goats, and was mainly infected by Jingmen tick virus (JMTV), a recently reported *Flaviviridae*-related segmented virus.

Rhipicephalus sanguineus, the brown dog tick, primarily feeds on dogs and can occasionally infest a wide range of domestic and wild hosts, including cats, rodents, birds, and humans (Dantas-Torres et al., 2010; Dantas-Torres, Figueredo, & Brandao-Filho, 2006; Estrada-Pena & Jongejan, 1999; Iori, Lanfranchi, & Manilla, 1996; Saxena & Maheshwari, 1985). *R. sanguineus* is implicated in the transmission of various pathogens to dogs (Dantas-Torres, 2008; Ewing, Mathew, & Panciera, 2002; Otranto, Dantas-Torres, & Breitschwerdt, 2009) and recent studies have shown that *R. sanguineus* ticks exposed to high temperatures are more prone to bite humans (Parola et al., 2008).

Haemaphysalis punctata is generally assumed to be a species with a Mediterranean distribution (Estrada-Pena et al., 2013) whose main hosts are wild and domestic ruminants, carnivores, hedgehogs, rodents and birds (Nosek, 1971). *H. punctata* is the most widespread species of the genus in Romania (Mihalca et al., 2012) and constitutes a vector for major zoonotic viruses such as CCHFV or TBEV (Estrada-Pena & Jongejan, 1999).

Relevance of Romanian tick-borne viruses for human and animal health

We performed a viral meta-transcriptomic analysis of *D. reticulatus*, *R. bursa*, *R. sanguineus* and *H. punctata* that identified many viral families, including viruses infecting plants, invertebrates and bacteria, and especially viruses belonging to three viral families known to comprise arboviruses (*Flaviviridae, Phenuiviridae* and *Nairoviridae*). These results show that these ticks harbor a high richness of viruses, including viruses that could be relevant for

human and animal health. Members of these families were previously reported in other tick species and genera elsewhere, suggesting different degrees of host permissiveness of these viruses that could reflect their ability to ensure interspecies transmission (invertebrate and even vertebrate).

It is the case of Jingmen tick virus, a recently identified segmented ssRNA+ virus first detected in *R. microplus* ticks in the Jingmen region of Hubei province in China in 2010. This virus has recently raised animal and public health concern because of its ability to infect a wide variety of tick vectors (e.g., *Haemaphysalis sp., Ixodes sp.,* or *Dermacentor sp.)* and mammals (bats, rodents, cattle, primates) and its ability to cause disease in humans (Wang et al., 2019). We detected JMTV in *R. bursa* ticks, engorged in sheep and goats from Tulcea county, suggesting that ruminants may play a role in the life cycle of Jingmenviruses in Romania, as previously suggested in China, Brazil and in Trinidad and Tobago (Maruyama et al., 2014; Qin et al., 2014; Sameroff et al., 2019). In addition, the phylogenetic positioning of Romanian JMTV, close to human Kosovar isolates with a history of tick bite (Emmerich et al., 2018) questioned on the ability of Romanian JMTV, vectored by *R. bursa* ticks, to infect humans. These observations pave the way for additional studies aiming at deciphering the epidemiology and possible clinical impact of JMTV for ruminants and humans in the area, and evaluating its risk of spreading to different locations, via migratory birds for example.

Similarly but to a lesser extent, the identification of Tacheng tick virus 2 in *D. reticulatus* ticks that is phylogenetically close to a human clinical isolate with a history of tick bite in northwestern China (Dong et al., 2021) raised concerns about the risk of Romanian ticks to harbor novel *Phenuiviridae* arboviruses. Indeed, *Phenuiviridae* members include arboviruses pathogen for humans and animals, transmitted by phlebotomine sandflies, mosquitoes and ticks (Matsuno et al., 2015; Palacios et al., 2013). Tick-borne phleboviruses are mainly vectored by *Ixodidae* ticks, and birds and small mammals were proposed to act as reservoirs

of these viruses (Z. Li et al., 2016) while humans and domestic animals are considered as accidental hosts (Hubalek, Rudolf, & Nowotny, 2014). They are currently classified into three main serogroups: SFTS and Bhanja viruses infecting humans and Uukuniemi virus (including TaTV2), in the *Uukuvirus* genus, infect ruminants (Palacios et al., 2013). In our study, TaTV2 placed in a clade encompassing tick-borne isolates originating from Eastern Europe, respectively from Anatolia/Turkey, and isolates originating from ticks and humans from China, suggesting that the virus could be able to infect animals and cause disease in humans (Dong et al., 2021), especially in areas where vectors (ticks), reservoirs (birds and smalls mammals) and domestic animals are concomitantly present. The establishment of a One Health surveillance network of such viruses at risk of emergence would be key to monitor the diffusion of TaTV2 in the area and the adaptation of TaTV2 to mammals, as it was suggested that TaTV2 is currently not well adapted to humans (Dong et al., 2021).

In the same *Phenuiviridae* family, we identified Changping tick virus 1 and Brown dog tick phlebovirus 2 that also clustered in the *Uukuvirus* genus. For now, these viruses were only identified in ticks (C. X. Li et al., 2015; Sameroff et al., 2019), so their potential ability to infect multiple hosts is questionable. Romanian CPTV1 was only identified in *D. reticulatus* ticks from Iasi county while two variants of BDTPV2, that clustered in two different subclades, were respectively identified in *R. sanguineus* and *H. punctata* ticks from Tulcea county, suggesting a possible specialization of these viruses to their tick host. In addition, the fact that CPTV1 isolates cluster in a specific Romanian clade, distinct from other isolates originating from China, suggests a possible geographical specificity of these viruses and reinforce the hypothesis that CPTV1 and BDTPV2 may belong to the commensal flora of Romanian ticks.

In the same way, among the *Flaviviridae* family, a virus not yet recognized by the International Committee for Taxonomy of Viruses was identified. Different Romanian BTV4

strains presenting with a high degree of genome conservation were detected in four questing *D. reticulatus* pools from Iasi county but also in *R. sanguineus* and *H. punctata* ticks from Tulcea county. This suggests that very closely related BTV4 strains infect multiple tick species from many geographical areas, as previously reported for *H. asiaticum* ticks from China (Shi, Lin, Vasilakis, et al., 2016) and in *R. sanguineus* from Thailand (Temmam, Chretien, et al., 2019) or Trinidad and Tobago (Sameroff et al., 2019). This high degree of genome conservation observed between different tick species from various environments and geographical origins is puzzling and questions on the origin of BTV4. Further studies are needed to determine the origin of BTV4 and its ability to infect vertebrate hosts in the addition of numerous tick species.

The detection of Nayun tick nairovirus-related S sequences in the virome of *R. sanguineus* ticks from Tulcea county is also of concern. Belonging to the *Nairoviridae* family, most *Orthonairovirus* members with known vectors are transmitted by ticks to mammalian hosts such as bats, rodents and ungulates, and infection of their mammalian hosts are generally asymptomatic (Garrison et al., 2020). In the present work, we were able to detect only sequences related to the S segment of Nayun tick nairovirus, which questions on the presence of an exogenous virus in the tick pool. The search for endogenous viral elements (EVE) presenting homologies with NTNV was negative, suggesting that NTNV may not constitute an EVE integrated in the genome of *R. sanguineus*. However, the presence of an internal stop in the RNA populations suggest that defective viruses may be present and complement intact viruses. Romanian NTNV clustered within the *Orthnairovirus* genus, in a sister clade of Chinese NTNV, but was distantly related and placed at the root of the known arbo-nairoviruses infecting mammals. Its ability to infect vertebrate hosts is still unknown and need further characterization to determine if NTNV could be considered as a novel arbovirus, or if it constitutes the tick origin of nairoviruses, as proposed for phleboviruses (Matsuno et

al., 2018). To conclude, our study increases the knowledge of the diversity of viruses in Eastern Europe, including novel ones, which is a prerequisite for monitoring viruses at potential risk for animal and human health.

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Data availability statement

The genome of viruses described in the study were deposited into the GenBank database under the accession numbers MW561132-MW561159 (Table 2).

Conflict of Interest

The authors declare no competing interests.

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Table 1. Number of reads provided by Kraken2 tool and the distribution of these reads according to eucaryota, viruses and bacteria

Library ID	Ticks number	Sampling origin	Localisation	No. of raw reads	No. total of Kraken2 reads	Eucaryota	Viruses	Bacteria
IASI20	7	Environment	IASI	54 500 645	181259	88511 (49%)	1721 (0.9%)	90281 (50%)
IASI21	10	Environment	IASI	45 974 122	129917	62641 (48%)	1144 (0.9%)	65475 (50%)
IASI22	10	Environment	IASI	42 420 208	123191	54817 (44%)	2281 (2%)	65403 (53%)
IASI23	5	Environment	IASI	62 115 189	181832	79240 (44%)	2178 (1%)	99655 (55%)
IASI50	379	Environment	IASI	45 695 030	75464	28644 (38%)	663 (0.9%)	45856 (61%)
TULCEA49	10	Environment	TULCEA	89 613 642	329590	202254 (61%)	2930 (0.9%)	123187 (37%)
TULCEA1	80	Sheep/ goat	TULCEA	49 898 354	1201578	82712 (7%)	1005342 (84%)	111884 (9%)
TULCEA47	5	Environment	TULCEA	87 951 679	611147	187229 (31%)	2194 (0.4%)	417439 (68%)

Tick species	Order	Family	Genus	Best hit NCBI	No. of NGS reads provided by Microseek	AA identity %	Reference accession number	Location	Virus	Genbank accession number
D. RETICULATUS **(IASI20)**	Unclassified	*Flaviviridae*	Unclassified	Bole tick virus 4	201174	85.28%	NC028371	**IASI**	BTV4/Romania/Iasi20	MW561133 (Polyprotein gene)
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	Changping tick virus 1	32169	50-79.12%	KM817665 (L) KM817732 (S)		CPTV1/Romania/ Iasi20_segment_L	MW561141 (L)
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	Tacheng tick virus 2	28411	68.1%	KM817684 (L) KM817744 (S)		CPTV1/Romania/ Iasi20_segment_S	MW561142 (S)
									TaTV2/Romania/ Iasi20_segment_L	MW561154 (L)
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	Pacific coast tick phlebovirus	444	87-100%	KU933937 (S) KU933936 (L)			
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	American dog tick phlebovirus	408	54-80%	KJ746901 (L) KJ746902 (S)			
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	Tick phlebovirus	282	66%	KY979166			
	Mononegavirales	*Rhabdoviridae*	Unclassified	American dog tick rhabdovirus 2	1560	63-69%	MF962661			
	Mononegavirales	*Rhabdoviridae*	Unclassified	Tacheng tick virus 3	147	87%	NC031268			
	Mononegavirales	*Rhabdoviridae*	Unclassified	Taishun tick virus	375	68%	NC031273			
	Mononegavirales	*Rhabdoviridae*	*Vesiculovirus*	Chandipura vesiculovirus	138	50%	NC020805			
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	Tacheng tick virus 2	84052	68.87%	KM817684 (L) KM817744 (S)	**IASI**	TaTV2/Romania/ Iasi21_segment_L TaTV2/Romania/ Iasi21_segment_S	MW561155 (L) MW561156 (S)
	Unclassified	*Flaviviridae*	Unclassified	Bole tick virus 4	24720	79.24%	NC028371			MW561134 (Polyprotein gene)

D. RETICULATUS **(IASI21)**	*Bunyavirales*	*Phenuiviridae*	*Phlebovirus*	Changping tick virus 1	52770	48.91-73.32%	KM817665 (L) KM817732 (S)		BTV4/Romania/Iasi21 CPTV1/Romania/ Iasi21_segment_L CPTV1/Romania/ Iasi21_segment_S	MW561143 (L) MW561144 (S)
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	Pacific coast tick phlebovirus	948	57-85%	KU933937 (S) KU933936 (L)			
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	American dog tick phlebovirus	150	52%	KJ746901 (L) KJ746902 (S)			
	Mononegavirales	*Rhabdoviridae*	Unclassified	American dog tick rhabdovirus 2	1629	48-61%	MF962661			
	Mononegavirales	*Rhabdoviridae*	Unclassified	Tacheng tick virus 3	267	86-92%	NC031268			
	Mononegavirales	*Rhabdoviridae*	Unclassified	Taishun tick virus	93	71%	NC031273			
	Mononegavirales	*Chuviridae*	Unclassified	Changping tick virus 2	1410	58%	NC028260			
D. RETICULATUS **(IASI22)**	*Amarillovirales*	*Flaviviridae*	*Flavivirus*	New Mapoon virus	144	52%	NC032088	**IASI**	TaTV2/Romania/ Iasi22_segment_L TaTV2/Romania/ Iasi22_segment_S CPTV1/Romania/ Iasi22_segment_S	MW561157 (L) MW561158 (S) MW561145 (S)
	Amarillovirales	*Flaviviridae*	*Flavivirus*	Wesselsbron virus	138	82%	NC012735			
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	Tacheng tick virus 2	16935	59-98.96%	KM817684 (L) KM817744 (S)			
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	Changping tick virus 1	3972	51.19-65.61%	KM817665 (L) KM817732 (S)			
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	Pacific coast tick phlebovirus	294	87%	KU933937 (S) KU933936 (L)			
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	American dog tick phlebovirus	273	53%	KJ746901 (L) KJ746902 (S)			
	Mononegavirales	*Rhabdoviridae*	Unclassified	Tacheng tick virus 3	327	100%	NC031268			
	Mononegavirales	*Rhabdoviridae*	Unclassified	American dog tick rhabdovirus 2	234	71%	MF962661			
	Mononegavirales	*Rhabdoviridae*	Sprivivirus	Pike fry sprivivirus	288	54%	NC025356			
	Mononegavirales	*Chuviridae*	Unclassified	Changping tick virus 2	108	47%	NC028260			
D. RETICULATUS (IASI23)	Unclassified	*Flaviviridae*	Unclassified	Bole tick virus 4	318931	84.05%	NC028371		BTV4/Romania/Iasi23 TaTV2/Romania/ Iasi23_segment_L	MW561135 (Polyprotein gene) MW561159 (L)
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	Tacheng tick virus 2	28323	62.04%	KM817684 (L)			

							KM817744 (S)	**IASI**		
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	Changping tick virus 1	2883	56.85-84%	KM817665 (L) KM817732 (S)		CPTV1/Romania/ Iasi23_segment_S	MW561146 (S)
	Mononegavirales	*Rhabdoviridae*	Unclassified	Tacheng tick virus 3	1536	79-94%	NC031268			
	Mononegavirales	*Rhabdoviridae*	Unclassified	American dog tick rhabdovirus 2	1188	60-69%	MF962661			
	Mononegavirales	*Rhabdoviridae*	Unclassified	Taishun tick virus	387	58-74%	NC031273			
	Mononegavirales	*Rhabdoviridae*	Unclassified	Parage aegeria rhabdovirus	288	60%	KR822826			
***D. RETICULATUS* (IASI50)**	*Bunyavirales*	*Phenuiviridae*	*Phlebovirus*	Tacheng tick virus 2	42912	49-100%	KM817684 (L) KM817744 (S)	**IASI**	TaTV2/Romania/ Iasi50_segment_L TaTV2/Romania/ Iasi50_segment_S	MW561152 (L) MW561153 (S)
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	Changping tick virus 1	30411	69.4%	KM817665 (L) KM817732 (S)		CPTV1/Romania/ Iasi50_segment_L	MW561140 (L)
	Unclassified	*Flaviviridae*	Unclassified	Bole tick virus 4	45237	76.16-83.28%	NC_028371		BTV4/Romania/Iasi50	MW561132 (Polyprotein gene)
***R. SANGUINEUS* (TULCEA47)**	Unclassified	*Flaviviridae*	Unclassified	Bole tick virus 4	2223	75-92%	NC_028371	**TULCEA**		
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	Brown dog tick phlebovirus 2	28716	62.29-87.35%	MN025508 (L) MN025509 (S)		BDTPV2/Romania/ Tulcea47_segment_L BDTPV2/Romania/ Tulcea47_segment_S	MW561138 (L) MW561139 (S)
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	Tick phlebovirus Anatolia 1	270	91%	MG764521			
	Bunyavirales	*Nairoviridae*	*Orthonairovirus*	Nayun tick nairovirus	2421	73.08%	KP141755		NTNV/Romania/ Tulcea47_segment_S	MW561151 (S)
	Mononegavirales	*Rhabdoviridae*	Unclassified	Taishun tick virus	3225	81-83%	NC031273			
	Mononegavirales	*Rhabdoviridae*	Unclassified	Bole tick virus 2	324	74-85%	NC031079			
	Mononegavirales	*Rhabdoviridae*	Unclassified\	American dog tick rhabdovirus 2	123	63%	MF962661			
	Mononegavirales	*Rhabdoviridae*	Unclassified	Wuhan house fly virus 1	31	71%	NC031282			
	Mononegavirales	*Rhabdoviridae*	*Ledantevirus*	Yongjia tick virus 2	147	63%	NC031305			
***H. PUNCTATA* (TULCEA49)**	Unclassified	*Flaviviridae*	Unclassified	Bole tick virus 4	813	71-90%	NC028371	**TULCEA**		
R. BURSA		*Flaviviridae*		Jingmen tick virus	2521170	96.77% 99.26% 98.03%	MN486263 (S1) MN486270 (S2) MN486266 (S3)		JMTV/Romania/ Tulcea1_segment_1 JMTV/Romania/ Tulcea1_segment_2 JMTV/Romania/ Tulcea1_segment_3	MW561147 (1) MW561148 (2) MW561149 (3)

(TULCEA1)						98.80%	MN486270 (S4		JMTV/Romania/ Tulcea1_segment_4	MW561150 (4)
	Bunyavirales	*Phenuiviridae*	*Phlebovirus*	Brown dog tick phlebovirus 2	33093	89-100%	MN025508 (L) MN025509 (S)	**TULCEA**	BDTPV2/Romania/ Tulcea1_segment_L BDTPV2/Romania/ Tulcea1_segment_S	MW561136 (L) MW561137 (S)
	Bunyavirales	*Phenuiviridae*	*Bandavirus*	Bhanja virus	9099	94-100%	NC027142 (S) NC027141 (M) NC027140 (L)			

Table 2. Viral sequences identified in ticks from South-Eastern Romania by high-throughput sequencing

Figure legends:

Figure 1. Classification of identified viruses by host spectrum. Viruses were classified according to the known host of the family they belong: arboviruses (dual arthropod and vertebrate hosts); vertebrates; plants; fungi; algae; amoeba; protozoans; protists; invertebrates; bacteria or unknown host. A) RNA viruses B) DNA viruses. (*D. reticulatus, R. sanguineus* and *H. punctata*- questing ticks; *R. bursa*- engorged ticks)

Figure 2. Classification by viral family according to tick species identified by NGS in Romanian ticks in A) in 29 RNA families B) in 12 DNA families. (*D. reticulatus, R. sanguineus* and *H. punctata*- questing ticks; *R. bursa*- engorged ticks)

Figure 3. Characterization of Romanian Jingmen tick virus. A) Schematic organization of JMTV/Romania/Tulcea genome identified in *R. bursa* ticks. The open reading frames (ORFs) are indicated with yellow arrows, and genome coverage is indicated in pink. Segmented viruses were presented as concatenated sequences for better clarity (blue arrows represent the different segments). B) Phylogenetic relationship of JTV/Romania/Tulcea segment 1 identified in Romanian *R. bursa* ticks with others tick-borne (black), primate-borne (blue), ruminant-borne (green), rodent-borne (red) and bat-borne (purple) Jingmenviruses

Figure 4. Schematic organization of BTV4 identified in *D. reticulatus, R. sanguineus* and *H. punctata* ticks from Romania. The open reading frames (ORFs) are indicated with yellow arrows, the gaps are indicated in dark green and genome coverage is indicated in pink

Figure 5. Phylogenetic relationship of the RNA-dependent RNA polymerase domain of BTV4 identified in Romanian *D. reticulatus, R. sanguineus* and *H. punctata* ticks with *Flavivirus, Hepacivirus, Pegivirus* and *Pestivirus* genera of *Flaviviridae*

Figure 6. Schematic organization of A) BDTPV2 B) TAaTV2 C) CPTV1 identified in D. *reticulatus, R. bursa and sanguineus* and *H. punctata* ticks from Romania. The open reading

frames (ORFs) are indicated with yellow arrows, the gaps are indicated in dark green and genome coverage is indicated in pink. Segmented viruses were presented as concatenated sequences for better clarity (blue arrows represent the different segments)

Figure 7. Phylogenetic relationship of TaTV2, CPTV1 and BDTPV2 RNA-dependent RNA polymerase identified in Romanian *D. reticulatus, R. bursa* and *sanguineus)* ticks with others viral families (*Nairoviridae, Hantaviridae* and *Peribunyaviridae*) among the *Bunyavirales*

Figure 8. A) Schematic organization of NTNV identified in *R. sanguineus* ticks from Romania. The open reading frames (ORFs) are indicated with yellow arrows, the gaps are indicated in dark green, genome coverage is indicated in pink and the internal stop are indicated with a blue arrow. B) Phylogenetic relationship of NTNV S amino-acid sequence with other orthonairoviruses