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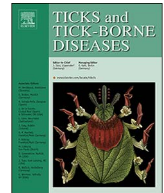

Short communication

Molecular identification of novel phlebovirus sequences in European ticks

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ABSTRACT

In recent years the number of newly described tick-borne phleboviruses has been steadily growing. Some of these novel viruses are highly pathogenic in humans, e.g. the Heartland and severe fever with thrombocytopenia syndrome virus. We aimed to analyse ticks sampled across Europe to investigate the diversity of phleboviruses using a comprehensive PCR-based screening approach. A total of 4387 ticks were collected from the vegetation in regions of France, Belgium, Germany, Sweden, and Estonia, respectively. Ticks were pooled and 22/979 pools tested positive using a PCR targeting the large (L) segment of phleboviruses. Phylogenetic analysis of a 500-bp fragment of the L segment showed a distinct novel clade provisionally named Glabbeek/Osterholz group (Belgium and Germany). In addition, sequences from ticks sampled in France clustered together with the recently described Antigone virus from Greece and AnLuc from Portugal. Our results extend the current diversity of phleboviruses in Europe. Future research should address the ecological processes driving the occurrence of phleboviruses and the impact of these novel phleboviruses for public health.

1. Introduction

The phlebovirus genus comprises arthropod-borne viruses and belongs to the family Bunyaviridae. Traditionally and depending on their vectors they can be grouped into the sandfly fever virus group (sandflies and mosquitoes) and the Uukuniemi-like virus group (ticks) (Elliott and Brennan, 2014). Of note, this classification is challenged by the steadily growing diversity of phleboviruses. Phleboviruses are distributed worldwide and capable of infecting humans and livestock. Notably, recent technological advancements, e.g. next-generation sequencing, led to the description of a number of novel phleboviruses worldwide. Among these, two novel phleboviruses emerged which can cause severe disease in humans: severe fever with thrombocytopenia syndrome virus (SFTSV) identified in China and Heartland virus (HRTV) found in the United States (McMullan et al., 2012; Yu et al., 2011). However, for most of the newly described phleboviruses a clear association with human disease has not been established yet. Of note, previous studies on the detection of tick-borne phleboviruses largely depended on well-defined geographic regions. In Europe, novel phleboviruses have been described in Greece and Portugal, respectively (Papa et al., 2016; Pereira et al., 2017). Here, we aimed to analyse ticks sampled across a wide geographic gradient of Europe using a consistent methodology to test for known and potentially novel phleboviruses.

2. Material and methods

Questing ticks were collected in the framework of the smallFOREST-project with sampling sites in eight regions in rural landscapes of Europe (southern and northern France, Belgium, western and eastern Germany, southern and central Sweden, and Estonia) in 2013 (Valdés et al., 2015). Ticks were collected by flagging with a 1 × 1 m cloth over different vegetation and placed in 80% ethanol. Identification of ticks was done morphologically using standard taxonomic keys (Babos, 1964). For further analysis, ticks were pooled according to stage and sex (Supplementary Table S1). Larvae were excluded and each pool comprised up to 5 adult ticks (separated into males and females) and up to 10 nymphs, respectively.

Prior to the nucleic acid extraction ticks were air-dried to evaporate remaining ethanol. A total of 0.4 ml MEM medium (ThermoFisher, Darmstadt, Germany) with 0.5% bovine serum albumin (Biochrom AG) and 1% Penicillin- Streptomycin (ThermoFisher) was added to each pool. Mechanical homogenization was done using lysing matrix H in a Fastprep-24 instrument (MP Biomedicals, Eschwege, Germany). Homogenates were centrifuged for 5 min at 3000*g* and 200 µl of supernatant was subjected to total nucleic acid extraction using the Total NA kit on a MagnaPure instrument according to the manufacturer's instructions (Roche, Mannheim, Germany). The nucleic acids were

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eluted in a volume of 100 µl and stored at −20 °C until PCR analysis. A one-step RT-PCR was done using primers HRT-GL2759F (CAGCATGGIGGIYTIAGRGAAATYTATGT) and HRT-GL3276R (GAWGTRWARTGCAGGATICCYTGCATCAT) targeting the large (L) segment of phleboviruses (Matsuno et al., 2015). Amplicons of the expected length (approximately 500 bp) were purified and directly sequenced. In addition, a RT-PCR targeting the small (S) and medium (M) segment was done on all L segment-positive samples as described (Lambert and Lanciotti, 2009; Liu et al., 2003). The nucleotide sequences were aligned using the aligner MAFFT (Multiple Alignment using Fast Fourier Transform)(Katoh and Standley, 2013). Phylogenetic trees were constructed using the maximum likelihood method PhyML 3.1 under the GTR substitution model with 1.000 bootstrap replicates (Guindon et al., 2005). The GTR (+G + I) substitution model was selected using the automated "Smart Model Selection" tool (available at http://www.atgc-montpellier.fr/phyml-sms/).

3. Results

A total of 4387 ticks of *Ixodes ricinus* and 37 *Dermacentor* ticks were collected. *Ixodes* was analysed in 958 pools and *Dermacentor* in 21 pools, respectively (Supplementary Table S1). Overall, a positive result using the L segment RT-PCR was obtained in 22/979 (2.2%) pools, with 18 pools containing *Ixodes* (Supplementary Table S1). Of the RT-PCR positive pools, 7/22 (32%) were successfully sequenced. Three of the sequenced pools contained *Dermacentor* and four pools contained *Ixodes ricinus*. The seven pools stemmed from ticks collected in southern France, Belgium, and northern Germany from April to June 2013 (Table 1). In a subsequent analysis of the seven L segment RT-PCR positive pools, four pools tested positive using the S segment RT-PCR and were successfully sequenced. None yielded an amplification product using the M segment RT-PCR despite several attempts.

The phylogenetic analysis showed that the French sequences (provisionally named Saint Lys 2 and 3) cluster together with those recently described in Greece (Antigone virus) and in Portugal (AnLuc). The nucleotide identity among Antigone virus and Saint Lys 2 and 3 was 80.3% and 80.6%, respectively. The nucleotide identity among AnLuc (GenBank accession number LC146410) and Saint Lys 3 was 92.7%. Another sequence from France (Saint Lys 1) clustered together with the prototype Bhanja virus with an identity of 78.8% at the nucleotide level. All of the French sequences were detected in *Dermacentor* ticks. A total of four sequences could not be assigned to an already existing group and were thus classified as a novel group provisionally named Glabbeek/Osterholz group after the region in which the ticks were sampled (Fig. 1). All these sequences stemmed from *Ixodes ricinus* ticks collected in Belgium (Glabbeek) and two distinct regions (Osterholz and Prignitz) in northern Germany. Amplification and sequencing of partial S segment was successful for all of these sequences. The nucleotide sequence identity of this novel group varies between 94.7%-96.8%. Interestingly, in the phylogentic trees of the L segment and the S segment these sequences were more related to mosquito-borne phleboviruses. All sequences were submitted to GenBank and were assigned the accession numbers KX964666 to KX964676. Virus isolation using Vero E6 cells failed due to the previous ethanol preservation.

4. Discussion

Previous studies on phleboviruses were largely confined to regions with limited geographic extent. In our study, we used a consistent screening approach on samples collected on an extensive transect across Europe. Herewith, we were able to provide molecular evidence of a novel phlebovirus clade and to extend the geographic distribution and phylogenetic relationship of the recently described Antigone virus from Greece and AnLuc from Portugal.

Notably, we detected a presumably novel phlebovirus clade which was present in three distinct geographic regions in Belgium and northern Germany. All sequences of this clade were found in *Ixodes ricinus*. It is well-known that ticks of the genus *Ixodes* are able to harbour a variety of pathogens but data on the prevalence and diversity of tick-associated viruses is rather limited (Diuk-Wasser et al., 2016; Rizzoli et al., 2014). Interestingly, this novel clade clustered between the mosquito-borne and the tick-borne phleboviruses using partial L and S segment sequence information. It should be noted that this classification is historically based and supports the notion that it needs to be reassessed in light of newly described phleboviruses (Tokarz et al., 2014). Full genome sequencing of our samples is envisaged and may shed more light on the phylogenetic relationship of this novel clade. However, phylogenetic analysis based on a 500-bp fragment seems to be robust enough to assign a tentative taxonomic position (Matsuno et al., 2015). Of note, we used a recently described RT-PCR approach using degenerated primers to detect phleboviruses, which was successfully used by different groups and yielded a number of novel phleboviruses to date (Matsuno et al., 2015; Papa et al., 2017; Pereira et al., 2017). This rather easy to perform and cost-effective approach may set the stage to extend our current understanding of tick-associated phleboviruses.

Moreover, we were able to detect phlebovirus sequences in *Dermacentor* ticks from France, which were closely related to sequences of the recently described Antigone virus from Greece and AnLuc virus from Portugal (Papa et al., 2016; Pereira et al., 2017). The detection of genetically related sequences in unconnected and distinct geographic regions has been observed before and is characteristic for all known phlebovirus groups to date (Matsuno et al., 2015). Interestingly, the sequences from Greece and Portugal were detected in ticks of the genus *Rhipicephalus* and were predominantly collected from animals. In contrast, we analysed ixodid ticks from the vegetation only. It should be noted that ecological factors, such as vegetation height and forest habitat properties influence tick abundance as recently demonstrated in a Swedish study (Asghar et al., 2016). In addition, community composition (including host biodiversity) and climate have also an effect as shown for *Ixodes ricinus* (Medlock et al., 2013). Thus, to better appreciate the overall prevalence of tick-associated phleboviruses in

Table 1
Data on phlebovirus-positive pools with sequencing results.

Name	Country	Geographic coordinates	Number of ticks/ pool	Tick species	Stage/sex	Collection date	GenBank acc. no. L segment	GenBank acc. no. S segment
Saint- Lys 1	France	43.52114°N,1.22414°E	2	*Dermacentor* spp.	adult/female	02/05/2013	KX964666	na
Saint- Lys 2	France	43.509991°N,1.240531°E	2	*Dermacentor* spp.	adult/female	01/05/2013	KX964667	na
Saint- Lys 3	France	43.49617°N,1.24321°E	5	*Dermacentor* spp.	adult/female	30/04/2013	KX964668	na
Prignitz	Germany	53.240550°N,12.252130°E	10	*Ixodes ricinus*	nymph/ unknown	31/05/2013	KX964669	KX964673
Osterholz	Germany	53.20478°N,8.67842°E	2	*Ixodes ricinus*	adult/female	03/06/2013	KX964670	KX964674
Glabbeek 1	Belgium	50.87472°N,4.93944°E	10	*Ixodes ricinus*	nymph/ unknown	15/06/2013	KX964671	KX964675
Glabbeek 2	Belgium	50.87583°N,4.93861°E	10	*Ixodes ricinus*	nymph/ unknown	15/06/2013	KX964672	KX964676

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