ORIGINAL ARTICLE

A novel flavivirus strain detected in phlebotomine sandflies in Bosnia and Herzegovina

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ABSTRACT

Aim Phlebotominae sandflies are primary vectors of phleboviruses, causing the sandfly fever disease. The aim of this study was to detect and report the presence of flaviviruses in Phlebotominae sandflies captured in Bosnia and Herzegovina.

Methods After a microscopic and morphometric analysis, the final identification of collected Phlebotomus specimens was confirmed by PCR, using a hemi-nested polymerase chain reaction on extracted and reversely transcribed RNA.

Results We obtained a 155 nt long fragment of the viral non-structural protein 5 (NS5) gene (GenBank accession no. MN090154). The acquired nucleotide sequence, provisionally named as Drežnica, showed a maximum of 70-80% identity in 70-88% (110-137 nucleotides) of the query coverage with several *Anopheles*, *Sabethes*, *Calbertado* and *Culex* flaviviruses. Maximum likelihood phylogenetic analysis showed that the new flavivirus Drežnica clusters together with the flavivirus isolated from *Culiseta annulata* mosquitos.

Conclusion We report the presence of flavivirus in Phlebotominae sandflies, captured in Drežnica, Herzegovina for the first time. The next phase of research will be directed towards virus cultivation, obtaining a longer or complete virus sequence and clarifying the medical and epidemiological importance of the Drežnica virus.

Key words: arboviruses, *Anopheles*, mosquitoes, *Phlebotomus*, sandfly

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INTRODUCTION

Outbreaks of emerging and re-emerging neurotropic viral disease transmitted by arthropods marked the beginning of the 21st century. There are more than 130 arthropod-borne viruses (arboviruses) known to cause human disease, among which the majority belong to RNA virus families: Togaviridae (genus Alphavirus), Flaviviridae (genus Flavivirus), Bunyaviridae (genera Nairovirus, Orthobunyavirus, Phlebovirus, and Tospovirus), Rhabdoviridae (genus Vesiculovirus) and Orthomyxoviridae (genus Thogotovirus) (1-5). Arboviruses are distributed worldwide, causing mild and almost asymptomatic to severe human infections (6-9).

In the last two decades, among all arboviruses, the greatest public attention attracted viruses from genus Flavivirus species Dengue virus (DENV), Yellow fever virus (YFV), Zika virus (ZIKV) and West Nile virus (WNV), transmitted by mosquitoes as well as genus *Phlebovirus* (especially Sandfly fever Naples group and Sandfly fever Sicilian group) transmitted by *Phlebotomus* sandflies.

Sandfly fever, also called "Pappataci fever" or "3-day fever", is characterized with influenza-like symptoms that occur after a 3–6-day incubation period (10). Symptoms include myalgia, physical sensitivity of the eyes to light exposure, headache, abdominal discomfort, fatigue and eating disorder, stiffness of the neck and back, followed with specific blood findings (11,12). Two main serocomplexes associated with sandfly fever are the Naples serocomplex and Sicilian serocomplex, from which Sicilian virus (SFSV), sandfly Cyprus virus (SFCV), sandfly Naples virus (SFNV) and Toscana virus (TOSV) circulate in the Mediterranean Basin (13-16). In endemic areas, infections occur in the summer, with a peak during the month of August, which coincides with the peak activity of sandflies as vectors (17-21). Human infections with the symptoms very similar to sandfly fever can be also caused by many Flaviviruses (e. g. DENV, WNV, YFV, Japanese encephalitis virus or ZIKV). Although the flaviviruses are usually detected in mosquitoes, Moureau et al. for the first time isolated flavivirus RNA from two pools of male *Phlebotomus perniciosus* collected in the territory of Algeria (22). Also, Amaro, Ze-Ze and Alves submitted to the GenBank a flavivirus RNA sequence (HM563684) isolated from Portuguese sandflies (23).

Bosnia and Herzegovina (B&H) is the country with the longest history of publications on sandfly fever virus infections in the Balkans, dating back to the end of the 19th century. In the 1886 year, the Austro-Hungarian military physician Alois Pick described the pathology and treatment of disease that occurred in Herzegovina (24). Only after Pick's discoveries, scientists began to study the disease in more detail, and found that it is prevalent in the regions with a widespread of sandflies, which afterwards led to the renaming of the infection from the "dog disease" to the "sandfly fever". During the second half of the 20th century, the presence of antibodies against SFNV in patients from B&H was reported in two different publications (25,26). In 2009, for the first time in B&H, TOSV infection was confirmed in seven (10.3%) of 68 patients with febrile illness of unknown origin, that were tested for the presence of IgG and IgM antibodies specific for TOSV (19). In 2017 Ayhan et al. reported the presence of the Balkan virus (BALKV) that belongs to the Sandfly fever Naples virus species, in one pool of Phlebotomus neglectus captured in the village of Sovići in Herzegovina (27). The Balkan virus was first detected in Albania, but a former reports confirm the presence of the virus in both B&H and Croatia (27,28). Although BALKV is the first genetically identified phlebovirus in B&H, there is still no evidence that this virus can cause human infections.

In spite of the fact that it has been more than 130 years since the sandfly fever was first described in B&H, the disease causing phlebovirus has not yet been isolated from *Phlebotomus* spp. or infected humans, nor genetically analysed in this region. The increase in the number of reported patients with the unclear febrile condition, serous meningitis or encephalitis without identified aetiology, occurring in summer months in B&H, was noticed (19).

The aim of this study was to investigate the presence of phleboviruses (Sandfly virus) in samples of *Phlebotomus* sandflies collected in B&H and the possible presence of flavivirus strains in trapped sandflies, considering the report of flavivirus presence in *Phlebotomus perniciosus* pools trapped in Algeria and also in Portuguese sandflies.

MATERIALS AND METHODS

Materials and study design

In September 2018 *Phlebotomus* sandflies were collected from 44 micro localities in Herzegovina (southern and non-administrative region of B&H) (Stolac, Drežnica, Ljubuški, Mostar and

the village of Blatnica near Čitluk) and 12 micro localities in northeast Bosnia (Živinice and Lukavac). Sandflies were trapped in custom-made traps, set by twilight, and collected in the morning. They were grouped according to the location, into pre-labelled tubes and immediately stored on dry ice. Until sorting and final treatment, all samples were kept at -80 °C. The identification of all collected *Phlebotomus* specimens, based on the microscopic and morphometric analysis, was conducted at the Institute for Biomedical Diagnostics and Research "Nalaz"; Sarajevo, Bosnia and Herzegovina. For molecular analysis the collected sandflies were further divided into 30 pools according to the location with up to 46 phlebotomus per pool.

The Ethics Committee of the Department of Medical Sciences, Academy of Sciences and Arts of Bosnia and Herzegovina, approved the study protocol.

Methods

Individual pools were added into the pre-prepared tubes with approximately 0·5 mL of 1 mm borosilicate beads (LabTIE B.V., Rosmalen, Netherlands) and 700 µL of 20% fetal bovine serum (FBS) in phosphate-buffered saline (PBS) buffer, and homogenized using bead-based homogenization (MagNA Lyser, Roche, Mannheim, Germany) for two rounds at 6500 rpm for 30 seconds with a brief intermitted incubation on ice.

The isolation of RNA was done from 200 µL supernatant using Tri Pure Isolation Reagent (Roche, Mannheim, Germany) according to the manufacturer's protocol.

Reverse transcription (RT) was performed using random hexamers and 200 U/µl M-MLV reverse transcriptase (Promega, Madison, USA) in a two-step protocol. In the first step, 2 µg of isolated RNA (or up to 16 µL) and 1 µL of 500 µg/mL random hexamers in the total volume of 17 µL in ultrapure DEPC-pretreated water, were incubated for 5 minutes at 70 °C, and immediately cooled to 4 °C. In the second step, 8 µl of RT mix was prepared using 5 µL of 5X reaction buffer, 1 µL of 10 mM dNTPs, 1 µL of RNase inhibitor (40 U/mL) and 1 µL of M-MLV reverse transcriptase (200 U/mL) and incubated for 1 minute at 37 °C. RT mix was then added to the annealed hexamers/template, and incubated for 60 min at 37 °C. Enzyme inactivation was performed at 95 °C for 5 min, and then at 4 °C for 5 min, after which the cDNA was stored at -20 °C.

For the virus detection in sandflies, "nested" PCR approaches were applied to detect all Phlebovirus genomes or specifically TOSV genomes. Flavivirus specific genomes were detected utilizing a hemi-nested PCR approach (29,30) (Table 1).

PCR was performed in the volume of 50 µl, using 0·3 µL of Hot Start Taq polymerase (Jena Bioscience, Jena, Germany), 5 µL of 10x PCR buffer, 1 µL of 10 mM dNTPs, 0·4 µL of 50 µM primers (Metabion international, Planegg, Germany) and ultrapure DEPC-pretreated water. As template 5 µL cDNA were used in each case for the first PCR,

Table 1. Primers and assays used for the detection of viral sequences in *Phlebotomus* spp.

Primer name	Sequence (5'- 3')	Function	Band size (bp)	Reference
Pan-Phlebovirus PCR				
NPhlebo1+	ATGGARGGITTTGTIWSICIICC	outer forward	550	(29)
NPhlebo1-	AARTTRCTIGWIGCYTTIARIGTIGC	outer reverse		
NPhlebo2+	WTICCIAAICCIYMSAARATG	inner forward	250	
NPhlebo2-	TCYTCYTTRTTYTTRARRTARCC	inner reverse		
TOSV S segment PCR				
TosN123	GAGTTTGCTTACCAAGGGTTTG	outer forward	660	(29)
TosN829	AATCCTAATTCCCCTAACCCCC	outer reverse		
TosN234	AACCTTGTCAGGGGNAACAAGCC	inner forward	520	
TosN794	GCCAACCTTGGCGCGATACTTC	inner reverse		
TOSV L segment PCR				
NPhlebo1+	ATGGARGGITTTGTIWSICIICC	outer forward	550	(29)
NPhlebo1-	AARTTRCTIGWIGCYTTIARIGTIGC	outer reverse		
NPhlebo2+	WTICCIAAICCIYMSAARATG	inner forward	130	
ATos2-	RTGRAGCTGGAAKGGIGWIG	inner reverse		
Pan-Flavivirus PCR				
MAMD	AACATGATGGGRAARAGRGARAA	outer forward	260	(30)
cFD2	GTGTCCCAGCCGGCGGTGTCATCAGC	outer reverse		
MAMD	AACATGATGGGRAARAGRGARAA	outer forward	210	
FS778	AARGGHAGYMCDGCHATHTGGT	inner reverse		

whereas 1 µL first-round amplicon was added into the nested respectively hemi-nested PCR.

Amplification with NPhlebo1 primers was carried out at 94 °C for 2 min, followed with 40 PCR cycles at 94 °C for 30 secs, 45 °C for 60 secs and 72 °C for 30 secs, extension at 72 °C for 5 min, and held at 4 °C for the infinite time. Nested PCR with NPhlebo2 FW/REV primers or NPhlebo2 and ATos2 primers were carried using 35 cycles and the same cycling conditions as used for NPhlebo1 primers. Amplification with TosN123 and TosN829 outer primers was carried out at 95 °C for 90 secs, followed with 40 PCR cycles at 95 °C for 30 secs, 51 °C for 30 secs and 72 °C for 40 secs, extension at 72 °C for 5 min, and held at 4 °C for the infinite time. Amplification with TosN234 and TosN794 was carried out at 95 °C for 90 secs, followed with 35 cycles at 95 °C for 30 secs, 56 °C for 30 secs and 72 °C for 30 secs, final extension at 72 °C for 5 min and 4 °C for the infinite time.

Amplification of Flavivirus RNA with MAMD and cFD2 primers was carried out at 94 °C for 2 min, followed with 40 PCR cycles at 94 °C for 30 secs, 53 °C for 30 secs and 72 °C for 45 secs, one step of final extension at 72 °C for 5 min, and held at 4 °C for infinite time. Heminested PCR with MAMD and FS 778 primers was carried using 35 cycles and same cycling conditions as used for first PCR.

Of all performed PCRs, 10 µL were combined with 2 µL of 6x Loading dye and analysed using electrophoresis on 1% or 2% agarose gel, depending on amplicon size, prepared in SBA buffer and 0·025% of Roti-GelStain (Carl Roth, Karsruhe, Germany), run alongside 6·5 µL of DNA ladder (GeneRuler DNA Ladder Mix, ThermoFischer Scientific, Waltham, USA). Electrophoresis was conducted at 120 V for 22 min (Cleaver Scientific LTD, Rugby, United Kingdom). Visualization of bands was done under a UV transilluminator (omniDOC Gel Documentation System, Cleaver Scientific LTD, Rugby, United Kingdom).

For sequencing, bands of according size were gel-excised and purified by AccuPrep Gel Purification Kit (Bioneer Corporation, Daejeon, South Korea) and protocol recommended by the manufacturer.

Sequencing was done using Big Dye Terminator v1.1 Cycle Sequencing kit or Big Dye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems). After purification of extensions products by ethanol / EDTA / sodium acetate precipitation according to the manufacturer's recommendation amplicons were analysed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems), using compatible POP-6 Polymer and 50 cm well-to-read capillary of the same manufacturer. Visualization of obtained nucleotide sequence was determined using Sequencing Analysis Software v5.2 (Applied Biosystems), and settlement and comparative sequence analysis using the Bio Edit Sequence v.7.2.5.0 (31).

The obtained nucleotide sequences were compared with sequences stored in the NCBI gene bank using BLASTN algorithm analysis (32).

The multiple sequence alignment was done in program Mega X, using ClustalW and MUSCLE algorithms for maximum likelihood (ML) analyses (33). After the alignment, sequences were trimmed to the size of the 155 nt sequence obtained from Phlebotomine sandflies. Maximum Likelihood phylogeny analysis was done using the Bootstrap method with 1000 replications and Tamura-Nei model (34) with uniform rates among sites in the MEGA X software. The phylogeny was also estimated using the Neighbor-Joining algorithm, Maximum Composite Likelihood method (35) and 1000 bootstrap replications.

RESULTS

Phlebotomus spp. were trapped in Herzegovina (Stolac, Drežnica, Blatnica, Ljubuški and Mostar) as well as in small towns Živinice and Lukavac in northeast Bosnia. A total of 751 phlebotomine sandflies were collected and morphologically identified. Based on the location, sandflies were divided into 30 pools, consisting of 3 specimens only to the maximum of 46 sandflies specimens.

After the amplification with specific Phleboviruses or TOSV primers, none of the thirty pooled samples gave a positive result, while fragments of the expected size were obtained for all PCR positive controls.

The amplification with the outer primers specific for Flaviviruses generated a large number of non-specific fragments in tested samples. After the hemi-nested PCR, a fragment of the expected size (210 bp) was detected in one pooled sample from Drežnica and in PCR positive control. The purified ~210 bp fragment was sequenced, resul-

ting in a 155 nt sequence (GenBank accession no. MN090154) (36).

Since we were not able to culture the detected unknown virus, basic local alignment analysis and phylogenetic analysis were conducted on the obtained 155 nt long sequence of the pooled sample that is provisionally named as "Drežnica". The nucleotide sequence of the sample Drežnica showed a maximum of 70-80% sequence identity in 70-88% (110-137 nucleotides) of the query coverage, mostly with several sequences of *Anopheles*, *Sabethes*, *Calbertado* and *Culex* flaviviruses.

For the phylogenetic analysis, nucleotide sequence of Drežnica was compared to a total of 18 nucleotide sequences (16 flavivirus sequences with the highest sequence identity and two flaviviruses sequence isolated from phlebotomine sandflies (GenBank: FJ817075 and HM563684). Maximum likelihood phylogenetic analysis of chosen sequences showed that new virus Drežnica clusters together with flavivirus (GenBank: JF707857) isolated from *Culiseta annulata* mosquitos (Figure 1), however, two sequences have only 73% nucleotide identity in 137 nt long alignment. The same sequence identity (73%) in 127 nt long alignment was observed with Flavivirus RNA isolated from Portuguese sandflies (GenBank: HM563684). In comparison, there was only 71% of sequence identity between 106 compared nucleotides of *Drežnica* sequence and Phlebotomus flavivirus from Algeria (GenBank: FJ817075).

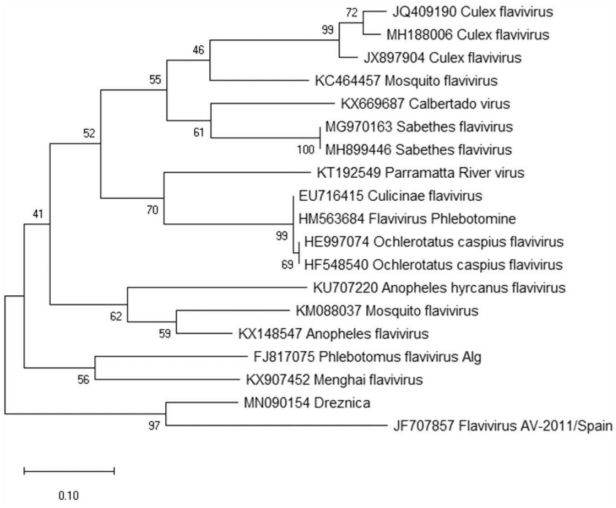

Figure 1. Phylogenetic tree based on 155 nucleotide sequence of MN090154 *Drežnica* flavivirus strain. The Maximum likelihood phylogenetic analysis is based on a total of 155 nucleotides fragment of 19 sequences. The tree with the highest log likelihood was displayed by using the program MegaX, Tamura-Nei model with 1000 bootstrap replicates and Maximum Composite Likelihood (MCL) approach. The tree branch lengths measured in the number of substitutions per site. GenBank accession numbers for used sequences: MN090154 Drežnica, EU716415, FJ817075, HE997074, HF548540, JF707857, JQ409190, JX897904, KC464457, KM088037, KT192549, KU707220, KX148547, KX669687, KX907452, MG970163, MH188006, MH899446, and HM563684.

The obtained phylograms provided similar tree topologies using two different techniques. Phylogenetic analysis indicates that the novel sequence Drežnica isolated from Phlebotomus sandflies is more closely related to insects-only flaviviruses than to phleboviruses.

DISCUSSION

According to the European Centre for Disease Control (ECDC) until May 2018, four types of Phlebotomus sandflies were identified in B&H: *P. mascittii*, *P. neglectus*, *P. tobbi* and *P. papatasi*. It is known that the distribution of Phlebotomus sandflies is limited to areas that have a sufficient amount of moisture and temperature above 15·6 °C for at least three months of the year. Due to climate change, currently many regions of B&H have summer and autumn temperatures that are suitable for Phlebotomus survival. All previous studies on *Phlebotomus* sandflies in B&H were conducted only in the area of Herzegovina and this was the first study that confirmed the presence of *Phlebotomus* spp. in the regions of northeast Bosnia. The number of captured Phlebotomus specimens in those regions is a good indicator that sandflies are adapted to the Western Balkan climate, and probably could be found in other parts of B&H.

The main focus of this study was to isolate and identify Phlebovirus strains of the captured *Phlebotomus* sandflies. Using nested amplification strategy, we were not able to detect phlebovirus RNA in any of the 30 tested sandflies pools. However, 155 nt long sequence fragment of a flaviviral NS5 gene was detected in the pool of sandflies captured in Drežnica. According to the composition, the nucleotide sequence of the Drežnica sample showed the highest similarity with nucleotide sequences of several Anopheles and Sabethes flavivirus strains. Also, sequence alignment performed with the two flaviviruses RNA sequences isolated from *Phlebotomus* spp. showed only a medium level of identity (71-73% in 106 and 127 nt long coverage).

Although phlebotomine sandflies are common vectors of phleboviruses, in 2010 Moureau et al. reported for the first-time isolation of flavivirus RNA from *Phlebotomus perniciosus* (22). Authors reported that the nucleotide sequences of those novel Phlebotomine flaviviruses are genetically the most similar to nucleotide sequences of flaviviruses isolated from *Culex* mosquitoes. In the same year, another flavivirus RNA sequence isolated from phlebotomine sandflies captured in Portugal was submitted to GenBank. This study was not followed with a related publication, and details on *Phlebotomus* species, gender or place of capture are not available. Also, the novel flavivirus was for the first time isolated from New World's sandflies (*Psathyromyia abonnenci*, previously named *Lutzomyia abonnenci*) in Equador (37). Besides the presence of flaviviruses in Phlebotomus sandflies, different authors also reported the presence of rhabdoviruses and reoviruses (37-40). Those findings together with the results reported here suggest that the presence of flaviviruses, as well as the presence of other viruses in sandflies has not been studied comprehensibly. Zoonotic potential of different viruses transmitted by *Phlebotomus* spp. should be further investigated in the future.

Based on the results obtained, it seems feasible that we detected a formerly unknown flaviviral species in phlebotomes with the closest relation to other flaviviruses found in *Culiseta annulate* mosquitoes or *Phlebotomus perniciosus* sandflies across the Mediterranean basin, with so far unknown zoonotic pathology.

Although preliminary analysis conducted on 155 nt long virus sequence isolated from the pooled phlebotomine sample from Drežnica in Herzegovina, implies that we detected a new flavivirus or phlebotomine-related flavivirus, it is necessary to conduct a more comprehensive study. Future research should be directed towards isolation and culturing Drežnica virus in order to obtain longer or complete virus sequence that will provide more information for the virus phylogeny analysis. Clinical and epidemiological significance of isolated virus of the Drežnica sample is still unknown. As imperative, future research should also attempt to clarify the pathogenicity of the Drežnica virus.

In conclusion, it is necessary to investigate the biological distribution and taxonomic differentiation of *Phlebotomus* spp. across the whole of Bosnia and Herzegovina, and to indicate areas with the increased risk of contracting the disease transmitted by these insects.

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TRANSPARENCY DECLARATION

Conflicts of interest: None to declare.

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