

Circulation of Schmallenberg virus in Turkey, 2013

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Abstract: Schmallenberg virus (SBV) infection emerged in European domestic and wild ruminants in 2011. There is very limited information about the characterization of SBV isolates and the epidemiology of its infections in the rest of world, except for in European countries. We investigated the circulation of SBV in cattle herds in Central Anatolia, Turkey, in 2013. A total of 180 whole-blood samples were analyzed using real-time RT-PCR. The presence of SBV RNA was detected in 6 (3.3%) samples. For phylogenetic analysis and confirmation of real-time RT-PCR results, the S gene segment was amplified, sequenced, and compared to other segments. In addition, SBV-specific antibodies were detected in 87 (24.1%) of 360 sera using a virus neutralization test. In the S gene sequence analysis of four randomly selected samples, 98%–99% nucleotide identity was observed between our strains and SBVs isolated in European countries between 2011 and 2013. The results of this study indicate that SBV was in Turkey in 2013. Furthermore, the sequencing results suggest that it could be the same virus that is in European countries.

Key words: Circulation, RT-PCR, Schmallenberg virus, Turkey

1. Introduction

The Schmallenberg virus (SBV) is a negative-sense single-stranded RNA virus in the Simbu serogroup of the genus *Orthobunyavirus* in the family *Bunyaviridae*. It is transmitted by hematophagous insect vectors (1-3) and is associated with reduced milk yield, inappetence, and diarrhea in adult ruminants as well as malformations in aborted ruminants (4,5). Antibodies to SBV and/or its genome were detected in domestic and wild animals in many countries including Turkey (1,6-13). To diagnose SBV infection, various ELISAs and real-time RT-PCR assays were routinely used in many laboratories in Europe (7).

To detect SBV antibodies, ELISAs and virus neutralization tests (VNTs) have been used by a number of researchers (10,14,15). The SBV ELISA has become commercially available (16), but the VNT is generally accepted as the gold standard to test for SBV serology (14). It has also been reported that the VNT seems to be more sensitive in detecting SBV antibodies than several in-house and commercial ELISA assays (15).

There is a very limited number of reports on the epidemiology of SBV in any part of the world except European countries (11,13). Recently, the seroprevalence of SBV was reported in Africa (17). Azkur et al. previously reported the seroprevalence of SBV as 24.5% in Turkey with an ELISA test (11). Presence of SBV was also described using RT-PCR and sequencing in abortion cases from cattle, but only 241 bp of the S segment of SBV were identified. (13). Therefore, further studies are needed to identify and better characterize the circulation of SBV in non-European countries such as Turkey. We aimed to contribute to a better understanding of the epidemiology of SBV outside European countries. For this purpose, blood samples were collected from cattle during the spring and summer seasons when possible vectors of SBV could be present. The presence of SBV and characterization of the SBV S gene segment were examined. SBV-specific antibodies were detected using VNTs to obtain additional epidemiological data in addition to real-time RT-PCR and sequencing.

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2. Materials and methods

2.1. Samples

Blood samples were collected from cattle of 2–5 years old that had abortions or fertility problems from nine different flocks in Kırıkkale Province (39°50'30"N, 33°30'50"E; altitude 714 m) between June and August in Turkey in 2013. This study was approved by the committee on animal research and ethics of Kırıkkale University on 08/12/2011 with approval number 11/225. A total of 360 serum samples and 180 whole-blood samples were collected in tubes without any anticoagulant and in tubes containing EDTA, respectively. The tubes were centrifuged at 3000 rpm for 10 min. All serum samples were stored in tubes at –86 °C until analyses.

2.2. Titration and neutralization

African green monkey kidney (Vero) cells were propagated in minimum Dulbecco essential medium supplemented with 10% fetal bovine serum. To prepare SBV (provided by the Central Veterinary Institute of Wageningen University and Research Centre) stocks, monolayer Vero cells were infected and incubated at 37 °C in 5% CO₂ until the appearance of cytopathic effects (CPEs) (4 to 5 days after infection). Culture supernatants were collected and stored at –80 °C. The virus titer was determined with the Reed and Muench methods. Briefly, a ten-fold serial dilution of the virus was made and Vero cells (3 × 10⁵ cells/mL (50 µL)) were infected with 100 µL of each virus dilution in 96-well plates. Cell monolayers were examined with an inverted microscope during 5 days after seeding of cells to detect CPEs. Cell layers with CPEs were scored as positive. The inoculation stock used in this study was titrated at 3.8 × 10⁴ TCID₅₀/mL. The SBV VNT was performed as described elsewhere (10). Shortly, sera were inactivated for 30 min at 56 °C and serially diluted (1/4 to 1/512). Each 50 µL of heat inactivated test serum samples was mixed with an equal volume of SBV (500 TCID₅₀ of SBV). The mixtures were incubated for 1 h at 37 °C in an incubator for serum neutralization, and 3 × 10⁵ cells/mL (50 µL) Vero cells were added into all wells. Five days after infection, the SBV neutralization was examined with an inverted microscope, and CPEs were scored as positive and titers were calculated. There were no CPEs in control cells and all virus controls showed CPEs.

2.3. RNA extraction and real-time RT-PCR

RNA was isolated from the whole-blood samples by using a commercial nucleic acid extraction kit (High Pure Viral Nucleic Acid Kit) and following the procedures recommended by the manufacturer (Roche Co., Mannheim, Germany). In real-time RT-PCR, the primers (sense primer: 5'-TCA GAT TGT CAT GCC CCT TGC-3' and anti-sense: 5'-TTC GGC CCC AGG TGC AAA TC-3') and the probe (5'-6-FAM- TTA AGG GAT GCA CCT

GGG CCG ATG GT-BHQ1-3') were used (18). RT-PCR methods were performed as recommended by the institute where this was developed (Friedrich-Loeffler-Institute, Greifswald-Insel Riems, Germany) The real-time RT-PCR amplifications were performed using the AgPath-IDTM One-Step RT-PCR Kit (Applied-Biosystems, İstanbul, Turkey) in the Roche Real-Time Cyclor 2.0 (Roche Co., Penzberg, Germany). The amplification was carried out under the following conditions: 1 cycle at 48 °C for 15 min, 95 °C for 10 min, and 40 cycles at 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 30 s. All real-time RT-PCR positive samples were tested in duplicate. Furthermore, all of the SBV RNA-positive samples detected with real-time RT-PCR were tested for the presence of the Akabane virus as described previously (19). In real-time RT-PCR, nuclease-free water and the beta-actin gene were used as the negative control and internal control, respectively.

2.4. Sequencing and phylogenetic analysis

The SBV S gene region was amplified with RT-PCR from the SBV-positive samples using two different primer sets and a one-step RT-PCR kit (QIAGEN OneStep RT-PCR Kit; Valencia, CA, USA) for sequencing, phylogenetic analysis, and confirmation of real-time RT-PCR results. The one-step RT-PCR reaction was carried out with 50 µL total volume containing 10 µL of 5X RT-PCR buffer, 400 µM of each dNTP, 2 µL of RT-PCR enzyme, 400 nM of each of the primers (for amplification of 474-bp-long F1 primer: 5'-AGTAGTGAACCTCCAC-3' and R1 primer: 5'-GCCCCAGGTGCAAAT-3' and for amplification of 443-bp-long F2: TGTCATGCCCTTGC and R2'-AGTAGTGTCTCCAC-3'), 1 µL of 1.5 mM MgCl₂, 3 µL of template RNA, and 30 µL of RNase-free water. The one-step RT-PCR products were purified using the QIAquick gel purification kit (QIAGEN, Hilden, Germany) and sequenced in both directions with the forward and reverse primers. The gene sequences were aligned (839 bp long) using the ClustalW program and then compared to the sequences of SBV obtained from the GenBank database.

The phylogenetic analysis of nucleotide sequences of the S gene of SBV amplified by RT-PCR was performed by comparing the sequences of same gene region of SBV obtained from the GenBank database using the neighbor-joining method with MEGA 5 software.

3. Results

Using the VNT, SBV antibodies were detected in 87 (24.1%) of 360 serum samples. The VNT titers ranged from 1:4 to 1:512 as shown in Figure 1. The overall geometric mean of the neutralizing antibody titer was 59.5 TCID. In addition to SBV antibody positivity, 6 (3.3%) samples, collected from five unique flocks, out of 180 total blood samples tested positive for SBV RNA in real-time RT-PCR. The mean Cq values of the internal positive control

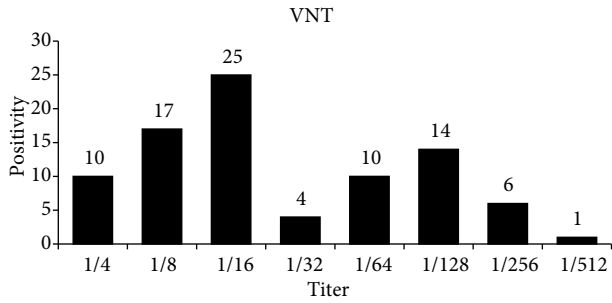


Figure 1. Schmallenberg virus antibody titers detected by virus neutralization test.

and SBV-positive samples were 23 and 27, respectively. Amplification products were not found in nuclease-free water. Real-time RT-PCR assays performed using primers for the Akabane virus showed no positive amplifications from SBV-positive samples.

Sequence analysis of the four RT-PCR products amplified with one-step RT-PCR revealed 100% homology at the nucleotide level. The nucleotide sequences obtained this study were similar, at between 98% and 99%, in the same gene region to SBVs obtained from the GenBank database. In the analysis, 99% homology was observed between our strains and SBV isolated in Germany in 2012 (sequence accession number JX853181). The S gene of an SBV-positive sample (2013/SBV-TR/Krkl.1), determined using RT-PCR, was submitted to GenBank (Accession Number KP279304). Phylogenetic analysis of this sample was performed using the neighbor-joining method of MEGA 5 software, confirming it was SBV (Figure 2). The genetic diversity and the relevance of specific mutations as potential explanations for the SBV-positive sample (2013/SBV-TR/Krkl.1) were examined by sequencing some members of the family *Bunyaviridae* (Figure 3). Our result showed that SBV S protein is highly conserved in the family SBV.

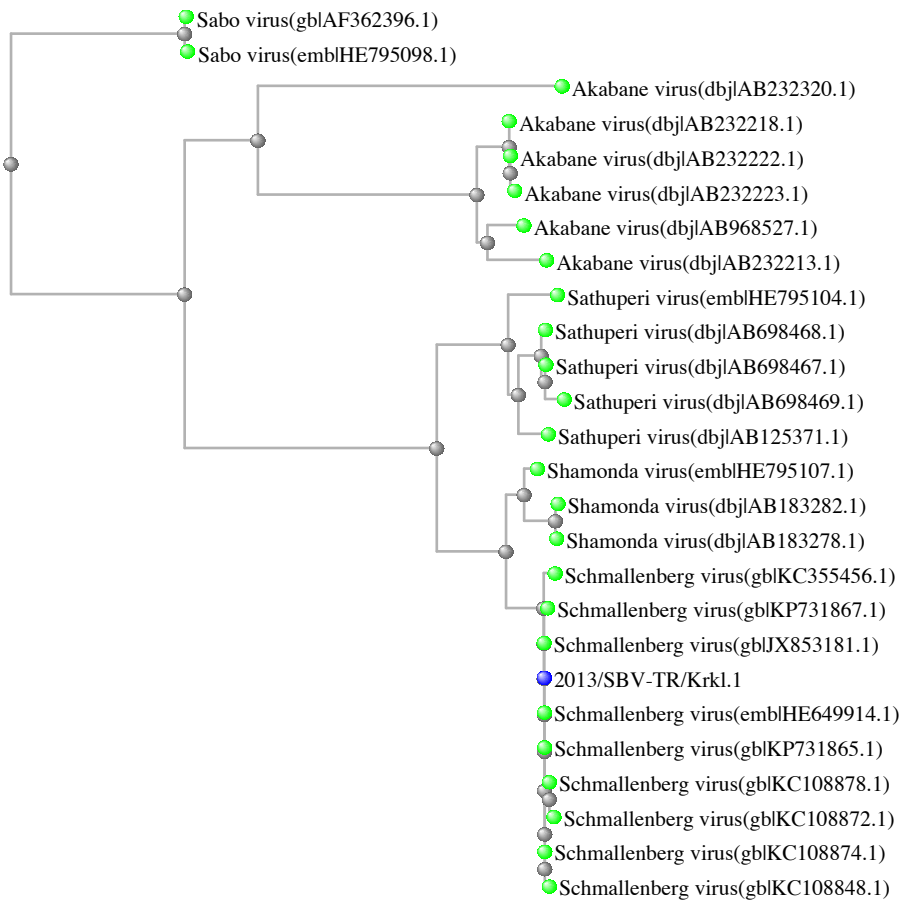


Figure 2. The phylogenetic tree constructed by neighbor-joining using the MEGA 5 program with comparison of the nucleotide sequences of the SBV S gene (GenBank Accession Number: KP279304 or 2013/SBV-TR/Krkl.1) amplified by RT-PCR in this study and the nucleotide sequences of same gene region of SBV viruses obtained from the GenBank database.

SBV-TR	1	-MSSQFIFEDVPQRNAATFNPEVGYVAFIGKYGQQLNFGVARVFFLNQKKAKMVLHHTAQPSVDLTFGGVKFTVNNHFP	79	
AGC93551	1	-MSSQFIFEDVPQRNAATFNPEVGYVAFIGRYGQQLNFGVARVFFLNQKKAKMVLHHTAQPSVDLTFGGVKFTVNNHFP	79	SBV
AGC93538	1	-MSSQFIFEDVPQRNAATFNPEVGYVAFIGKYGQQLNFGVARVFFLNQKKAKMVLHHTAQPSVDLTFGGVKFTVNNHFP	79	SBV
AAL78255	1	-MSSQFIFEDVPQRNAATFNPEGGYVAFIGKYGQQLNFGVAVVFFLNQKKAKMVLHHTAQPSVDLTFGGVKFTVNNHFP	79	Shamonda
AAL78233	1	-MSSQFIFEDVPQRNAATFNPEVGYVAFIGKYGQQLNFSVARVFFLNQKKAKMVLHHTAQPSVDLTFGGVKFTVNNHFP	79	Douglas
CCG93481	1	-MASQFIFDDVPQRNAATFNPDGYVAFISKYQQLNFTVARVFFLNQKKAKMVLHHTAQPSVDLTFGGVKFTVNNHFP	79	Sabo
AAL78239	1	-MASQFIFDDVPQRNAATFNPDGYVAFISKYQQLNFTVARVFFLNQKKAKMVLHHTAQPSVDLTFGGVKFTVNNHFP	79	Sabo
CCH15005	1	-MANQFIFQDVPQRNLATFNPEVGYVAFIAKHGAQLNFTDTRVFFLNQKKAKMVLHHTAQPSVDLTFGGIKFTLVNNHFP	79	Shuni
BAA24211	1	-MANQFIFNDVPQRNAATFNPDAGYVAFISKYQQLNFTVARVFFLNQKKAKMVLHHTQPSVDLTFAGVKFTVNNHFP	79	Akabane
BAG54894	1	-MANQFIFNDVPQRNAATFNPDAGYVAFISKYQQLNFTVARVFFLNQKKAKMVLHHTQPSVDLTFAGVKFTVNNHFP	79	Akabane
SBV-TR	80	QYVSNPVPDNAITLHRMSGYLARWIADTCKASVCLKAEASAQIVMPLAEVKGCTWADGYTMYLGFAPGAEMFLDAFDYFP	159	
AGC93551	80	QYVSNPVPDNAITLHRMSGYLARWIADTCKASVCLKAEASAQIVMPLAEVKGCTWADGYTMYLGFAPGAEMFLDAFDYFP	159	SBV
AGC93538	80	QYVSNPVPDNAITLHRMSGYLARWIADTCKANVLKLAESAQIVMPLAEVKGCTWADGYTMYLGFAPGAEMFLDAFDYFP	159	SBV
AAL78255	80	QYVSNPVPDNAITLHRMSGYLARWIADTCKASVCLKAEASAQIVMPLAEVKGCTWADGYTMYLGFAPGAEMFLDAFDYFP	159	Shamonda
AAL78233	80	QYVSNPVPDNAITLHRMSGYLARWVADTCKASVCLKAEASAQIVMPLAEVKGCTWADGYTMYLGFAPGAEMFLDAFDYFP	159	Douglas
CCG93481	80	QYIANPVPDTALTLHRMSGYLARWVAELCRGNQIKLAEAAATLIVMPLAEVKGCTWADGYTMYLGFAPGAEMFLETFEYFP	159	Sabo
AAL78239	80	QYIANPVPDTALTLHRMSGYLARWVAELCRGNQIKLAEAAATLIVMPLAEVKGCTWADGYTMYLGFAPGAEMFLETFEYFP	159	Sabo
CCH15005	80	QYTANPVPDTALTLHRLSGYLAKWVADQCKTNQIKLAEAMEKIVMPLAEVKGCTWTEGLTMYLGFAPGAEMFLETFEYFP	159	Shuni
BAA24211	80	QYTANPVSDTAFTLHRISGYLARWVAEQCKANQIKLAEAAATIVMPLAEVKGCTWSDGYAMYLGFPAPGAEMFLETFEYFP	159	Akabane
BAG54894	80	QYTANPVSDTAFTLHRISGYLARWVAEQCKANQIKLAEAAATIVMPLAEVKGCTWSDGYAMYLGFPAPGAEMFLETFEYFP	159	Akabane
SBV-TR	160	LVIEMHRVLKDNMDVNFMMKKVLRQRYGTMTAEWMTQKITEIKAAFNSVQGLAWAKSGFSPAARTFLQQFGINI	234	
AGC93551	160	LVIEMHRVLKDNMDVNFMMKKVLRQRYGTMTAEWMTQKITEIKAAFNSVQGLAWAKSGFSPAARTFLQQFGINI	233	SBV
AGC93538	160	LVIEMHRVLKDNMDVNFMMKKVLRQRYGTMTAEWMTQKITEIKAAFNSVQGLAWAKSGFSPAARTFLQQFGINI	233	SBV
AAL78255	160	LVIEMHRVLKDNMDVNFMMKKVLRQRYGTMTAEWMTQKIQEIKAAAFNSVQGLAWAKSGFSPAARTFLQQFGINI	233	Shamonda
AAL78233	160	LVIEMHRVLKDNMDVNFMMKKVLRQRYGTMTAEWMTQKITEIKAAFKFVGGQLAWAKSGFSPAARTFLQEFGINI	233	Douglas
CCG93481	160	LVIDMHRVLKDGMDVNFMRKVLQRQRYGTLTAEWMTTKIEAVRAAFNSVAQISWAKTGFSPAARAFANFGIQI	233	Sabo
AAL78239	160	LVIDMHRVLKDGMDVNFMRKVLQRQRYGTLTAEWMTTKIEAVRAAFNSVAQISWAKTGFSPAARAFANFGIQI	233	Sabo
CCH1505	160	LVIDMHRVLKDGMDVNFMRKVLQRQRYGTLTAEWMTQKIDAVRAAFNAVQGLSWAKSGFSPAARTFLAQFGINI	233	Shuni
BAA24211	160	LVIDMHRVIKDGMDVNFMRKVLQRQRYGQLTAEWMTSKLDAVKAAFNSVAQISWAKSGFSPAARAFLAQFGIQI	233	Akabane
BAG54894	160	LVIDMHRVIKDGMDVNFMRKVLQRQRYGQLTAEWMTSKLDAVKAAFSSVAQISWAKSGFSPAARAFLAQFGIQI	233	Akabane

Figure 3. Comparison of the 2013/SBV-TR S (Protein ID: 816206481) amino acid sequences from some other *Bunyaviridae* viruses with Basic BLAST software.

4. Discussion

In 2011, a previously unknown disease was reported in dairy cattle in European countries. The nonspecific symptoms were mainly decreased milk production, watery diarrhea, and high fever. The new virus was named the Schmallenberg virus after the village where the first positive samples were collected and tested using metagenomic analysis (1).

The ELISA seroprevalences of SBV in cattle, sheep, goats, and Anatolian water buffalo in Turkey are reported as 39.8%, 1.6%, 2.8%, and 1.5%, respectively (11). In this study, we used the VNT as a robust assay with higher specificity and sensitivity than ELISA (10). In the present study, we report that SBV seroprevalence is 24.1% in cattle in Central Anatolia, Turkey.

ELISA detects antibodies of the N protein, and VNT detects viral Gn and Gc proteins (16). In this study, a comparison of the two methods was not performed

because of limited resources. Applying a VNT that was previously used to test 1394 nulliparous animals in the Netherlands, 92% of the ewes, 96% of the cows, and 43% of the goats were found to have high seropositivity (20). We collected serum samples from farms that reported abortions or fertility problems and were suspected of SBV infection.

A pan-Simbu RT-PCR assay was the first broad tool available for screening RNA of Simbu serogroup viruses. It also allows the identification of related orthobunyaviruses in mammals (18). Reportedly, SBV RNA could be detected in sera and whole blood for approximately 1 week after sampling even though SBV does not replicate in bovine peripheral blood lymphocytes, but influences the lymphocyte homeostasis in blood (21).

Because of the 98% to 99% similarity between the S gene nucleotide sequences of SBVs obtained from our samples and SBV S genes obtained from the GenBank

database, we can conclude that the S gene is highly conserved. Furthermore, the sequencing results suggest that it may be the same virus that is in European countries. When 2013/SBV-TR (Protein ID: 816206481) was compared to other related proteins, the similarity of 2013/SBV-TR was 99%–100%, 98%, 97%, 81%–82%, and 80% to the Schmallerberg, Shamonda, Douglas, Sabo, Shuni, and Akabane viruses, respectively.

No acute clinical signs such as reduced milk yield or fever were detected in our SBV-positive animals, but abortion was reported in all of them. Our results show that SBV may be considered as an etiologic agent in abortion in cattle in Turkey. Education and informatory meetings should be organized for villagers and animal keepers about SBV infection and clinical outcomes to detect and diagnose SBV infections properly. There are some commercial SBV vaccines available and in use in Europe, but there is no vaccine for SBV in Turkey. There are approximately

56 million cattle, sheep, and goats in Turkey. According to Turkish Statistical Institute data 1.1 million cattle and 2 million sheep and goats have been imported from European countries to Turkey (Germany, the Netherlands, Austria, Italy, etc.), the United States, Australia, and Brazil in 2011–2014. These imported animals, which are mostly from European countries, could be the source of SBV infection in Turkey. However, more extensive studies will be required to solidly demonstrate the role of SBV in abortion.

Seropositivity against SBV and SBV detection in abortion cases found in Turkey might be explained by animal importation or the illegal animal trade. Our results indicate that SBV was in Turkey in 2013. Furthermore, our data suggest that the same virus may be in both European countries and Turkey. However, further studies performed with full genomic sequencing would provide more support for this theory.

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