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Original Scientific Article

FIRST DETECTION OF SCHMALLENBERG VIRUS INFECTIONS IN SLOVENIA, 2012

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Summary: In late summer 2011, Germany and the Netherlands reported the first cases of acute infection in cattle caused by a novel *Orthobunyavirus*, named the Schmallenberg virus (SBV). The first malformations due to SBV were observed in December 2011 in the Netherlands, Belgium and Germany. SBV was first identified in Slovenia in a flock of 23 sheep where nine aborted foetuses with malformations were found on a farm. Viral nucleic acid of SBV was detected by real-time polymerase chain reaction (RT-qPCR) from the brain and spleen samples with a protocol developed by the Friedrich-Loeffler Institute (FLI), Germany. Between January and April 2013 a total of 77 malformed calves were tested and 25 calves identified as SBV positive by RT-qPCR. The majority of malformed animals had one or more of the following pathological lesions: arthrogryposis, brachygnathia, torticollis, scoliosis, hydranencephaly and brain and spinal cord malformations. Additionally, two archive samples collected in September 2012 were identified as SBV positive, confirming that SBV infection was already present in Slovenia in 2012. The sequencing analysis of the partial L-segment confirmed that the strain detected in Slovenia was 100% identical to the Schmallenberg virus isolate Germany (JX853179), identified in 2011. SBV-positive herds have been located throughout Slovenia.

Key words: first report; Schmallenberg virus; diagnosis; Slovenia

Introduction

The Schmallenberg virus (SBV) is the name of a new virus that recently emerged in Europe (1). SBV has a high homology to the genus *Orthobunyavirus* of the Simbu serogroup (1, 2). It was first detected in late summer 2011 in Germany and the Netherlands in cattle exhibiting milk drop, fever and diarrhoea. Later, the infection was associated with congenital malformations in lambs, calves and goat kids, as a result of infection from the mother during the vulnerable early stages of gestation (3, 4). The results of full-genome and serologic investigations indicated that SBV belongs to the species Sathuperi virus and is a possible ancestor of the reassortant Shamonda virus (5).

Since the first report, SBV has continued to spread to neighbouring countries and, after more than a year, has become endemic in north-western parts of Europe (1, 3, 6, 7). Data from the Netherlands and Belgium indicate that the virus spreads very rapidly, reaching a seroprevalence of

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80-90% within a few months (2, 4, 8). The collected data from 15 European countries covering 6,000 infected herds become available at the end of 2012 (6). After detection of SBV infection in eight countries (Belgium, France, Italy, Germany, the Netherlands, Luxembourg, Spain and the United Kingdom) at the beginning of 2012, SBV infections were identified in Denmark, Finland, Poland, Sweden, Switzerland, Ireland, Norway, Estonia and Austria in summer and autumn 2012 (6). Transmission of SBV into Polish and Estonian cattle herds provide evidence of recent spreading of infection to the eastern part of Europe (7, 9). Infection of a herd is confirmed by virus detection or detection of SBV antibodies (1, 10).

The detection of SBV in *Culicoides* (*C. obsoletus complex*, *C. dewulfi* and *C. chiopterus*) during the summer and early autumn in Belgium, Denmark and Poland by real-time polymerase chain reaction (RT-qPCR) strongly indicates that these species are relevant vectors for SBV and are probably involved in the rapid transmission of the disease (9, 11, 12). Comparative analysis of different organs and tissues suggests that the cerebrum, cerebellum and brain stem are the most appropriate tissues for SBV detection by RT-qPCR (13, 14). RT-qPCR for the detection of the SBV genome in the L-segment was developed and provided by the Friedrich-Loeffler Institute (FLI), Germany. This method was implemented at the National Veterinary Institute in Ljubljana in Slovenia in the beginning of 2012. The aims of this study are to provide the results of SBV monitoring from clinical samples of malformed foetuses in Slovenia, and to present the regional distribution of identified SBV-positive herds.

Materials and methods

In total, five malformed lambs and 77 malformed calves originating from 73 different herds were included in the study. Aborted and/or stillborn calves and lambs showing one or more of the malformations (arthrogryposis, torticollis, scoliosis, brachygnathia, hydranencephaly and hypoplasia of the cerebrum) and therefore considered as SBV suspected, were collected from January to April 2013 by famers and veterinarians. Suspect cases were sent to pathology for necropsy at the National Veterinary Institute. Samples were collected and tested in the frame of official surveillance activities for SBV. Additionally, nine aborted and/or stillborn calves (non-malformed) stored as archive tissue samples in laboratory (collected in 2012) and 135 randomly selected serum samples obtained from clinically healthy cattle, collected between August and November 2012 were tested for SBV by RT-qPCR. From the 82 malformed animals showing arthrogryposis-hydranencephaly syndrome and nine archive, non-malformed animals, tissue samples of the spleen and brain (cerebral cortex, brain stem and cerebellum) were collected and tested by RT-qPCR.

In the laboratory for virology, a total of 2 g of different tissues (spleen, cerebral cortex, brain stem and cerebellum) were homogenized as a pool in 10 ml of RPMI-1640 (Gibco, Invitrogen Corporation, USA) with a homogenizer (IUL Instruments, Germany). After homogenization, the suspension samples were centrifuged for 15 minutes at 3,000rpm × g. Tissue supernatants were recovered and used for the extraction of total RNA. Total RNA was extracted from 140 µl of tissue supernatant/serum samples using the QIAamp® Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The previously designed primers and probes and protocol for the detection of the SBV genome (L-segment) by RT-qPCR were kindly provided by FLI, Germany (2). The forward primer sequence SBV-L1-11F was 5'-TTGCCGTTTGATTTTGAAGTTGTG-3' and the reverse primer sequence SBV-L1-155R was 5'-TCAGGGATCGCAAATTAAAGAACC-3'. The TaqMan probe sequence SBV-L1-36 (5'-TCATCCGTGCTGACCCTCTGCGAG-3') was labelled with the fluorescent reporter dye FAM (6-carboxyfluorescein) at the 5' end and with the black hole quencher dye (BHQ1) at the 3' end. The protocol was applied and adapted to our laboratory conditions as follows. Amplification was carried out in a single step with Superscript™ III Platinum® One–Step qRT-PCR kit with ROX (Invitrogen, USA). Briefly, the reaction was performed in 20 µl total volume, consisting of 1.5 µl water, 10 µl of 2 x reaction mix with ROX, 0.5 µl of 50 mM Mg SO_4, 1 µl of each primer with concentration 20 µM, 0.5 µl of 5 µM probe and 5 µl of extracted RNA. The protocol was optimized using a series of ten-fold RNA dilutions. RT-qPCR was performed on the Mx3005P system (Stratagene, USA) with the program including a reverse transcription step at 55 °C for ten minutes, followed by a denaturation step at 95 °C for ten minutes, and

45 cycles of 95 °C for 15 seconds, 56 °C for 20 seconds and 72 °C for 30 seconds. To ensure the accurate performance of the real-time PCR test, at least one positive control (SBV RNA provided by FLI, Germany; dilution of standard with a cycle threshold [Ct] of approximately 30) and one negative control (tissue sample of bovine origin from year 2010, free of SBV RNA) were included in each run.

The positive samples from the first three SBV-positive herds were directly sequenced from RT-PCR products in both directions using the Macrogen sequencing service (Macrogen, The Netherlands) and the RT-qPCR amplification primers to confirm the specificity of the RT-PCR assay. For each sample, 97 nucleotide long sequences of segment-L were aligned with the published data using BLAST (available at http://www.ncbi.nlm.-nih.gov/) at the National Centre for Biotechnology Information (NCBI).

Results

Various degrees of deformities were observed in the five lambs and 77 calves at necropsy, namely arthrogryposis, torticollis, scoliosis, brachygnathia, hydranencephaly and hypoplasia of the cerebrum, cerebellum and spinal cord (Table 1, Figure 2-8). The first clinical case of SBV infections, confirmed by laboratory diagnosis in Slovenia, was identified on January 9, 2013 from a flock with 23 sheep, where nine aborted foetuses were found. Five foetuses were sent for necropsy and two (O-1a/2013 and O-1b/2013) were detected as SBV positive by RT-qPCR. By the end of April 2013, an additional 25 bovine herds were identified as SBV positive (Table 1). The distribution of positive herds on the map of Slovenia revealed that SBV infection was already present throughout the country (Figure 1). The detection of the highest number of positive herds in the central area is the result of intensive sampling in this region. RT-qPCR results showed that 27 (32.9%) of 82 examined malformed animals were interpreted as SBV-genome positive according to detected Ct values. Despite observed congenital malformations, 56 out of 82 tested malformed animals were identified as SBV negative.

Out of 144 tested archive samples (nine tissue samples of aborted and/or stillbirth calves and 135 serum samples), two samples (from herds B-1/2012 and B-2/2012) were identified as SBV positive. Both of these SBV-positive samples were collected on September 13, 2012, thus confirming the presence of infection already in 2012. The first SBV-positive sample from herd B-1/2012 originated from a foetus aborted in the fourth month of gestation; the farm was located in the municipality of Žiri, approximately 50 km north-west of Ljubljana (Figure 1). The detected Ct value in this foetus was 22.98, which was the lowest detected Ct value among the positive samples in this study. The second SBV-positive sample from

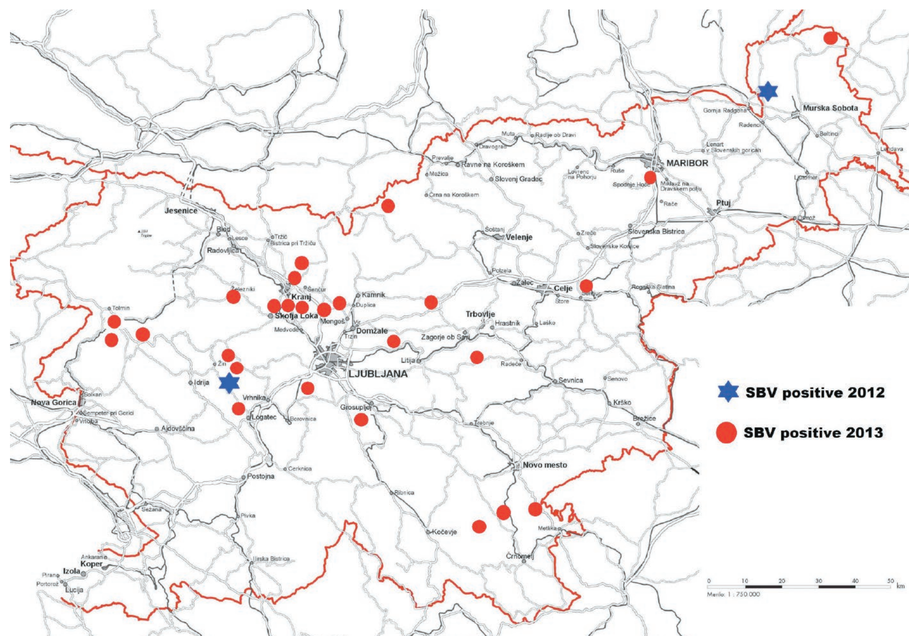

Figure 1: Location of 28 herds with SBV-genome positive results identified in Slovenia by end of April 2013 (blue stars for positive herds [B-1/2012 and B-2/2012] detected in September 2012, red dots for herds detected in 2013

Table 1: Total number of SBV-positive animals detected by RT-qPCR from September 2012 to end of April 2013 (samples, date of collection, municipality name, detected Ct value and description of observed malformations during necropsy)

Herd number	Date of sampling	Municipality	Ct value	ART	BRA	TOR	SCO	HYD	HYP
B-1/2012	*13 September 2012*	*Žiri*	*22.98*						
B-2/2012	*13 September 2012*	*Murska Sobota*	*29.60*						
O-1a/2013	04 January 2013	Semič	26.48	X	X	X			X
O-1b/2013	04 January 2013	Semič	27.07		X	X			X
B-2/2013	30 January 2013	Kamnik	36.67	X	X	X	X		X
B-3/2013	19 February 2013	Komenda	34.23	X		X	X		X
B-4/2013	22 February 2013	Gorenja vas-Poljane	35.31	X		X	X	X	
B-5/2013	04 March 2013	Dol pri Ljubljani	38.58	X	X		X		X
B-6/2013	03 March 2013	Železniki	37.95	X		X			
B-7/2013	07 March 2013	Logatec	33.93	X	X	X	X		
B-8/2013	07 March 2013	Žiri	39.03	X			X		
B-9/2013	05 March 2013	Kranj	29.64	X		X	X	X	X
B-10/2013	13 March 2013	Kranj	37.33	X			X		X
B-11/2013	18 March 2013	Hodoš	37.10			X			
B-12/2013	19 March 2013	Tolmin	34.50	X		X			
B-13/2013	19 March 2013	Nova Gorica	34.89	X		X	X		X
B-14/2013	18 March 2013	Šentjur pri Celju	36.55	X			X		X
B-15/2013	18 March 2013	Preddvor	37.53	X		X	X		X
B-16/2013	26 March 2013	Vodice	29.80	X	X	X	X	X	
B-17/2013	22 March 2013	Semič	35.45		X	X			X
B-18/2013	21 March 2013	Kanal	33.10	X		X			X
B-19/2013	02 April 2013	Kočevje	36.80	X	X		X		
B-20/2013	02 April 2013	Kranj	37.16	X		X	X		
B-21/2013	02 April 2013	Kranj	42,43	X		X	X		X
B-22/2013	02 April 2013	Litija	34.88	X		X			X
B-23/2013	02 April 2013	Brezovica	36.88	X			X	X	X
B-24/2013	05 April 2013	Ivančna Gorica	35.48	X	X	X		X	X
B-25/2013	21 March 2013	Solčava	37.79	X		X	X		
B-26/2013	08 April 2013	Hoče-Slivnica	37.88	X		X	X	X	X

Abbreviations: (herd numbers) B: bovine herd, O: ovine herd; ART: arthrogryposis; BRA: brachygnathia; TOR: torticollis; SCO: scoliosis; HYD: hydranencephaly/porencephaly; HYP: cerebellar hypoplasia

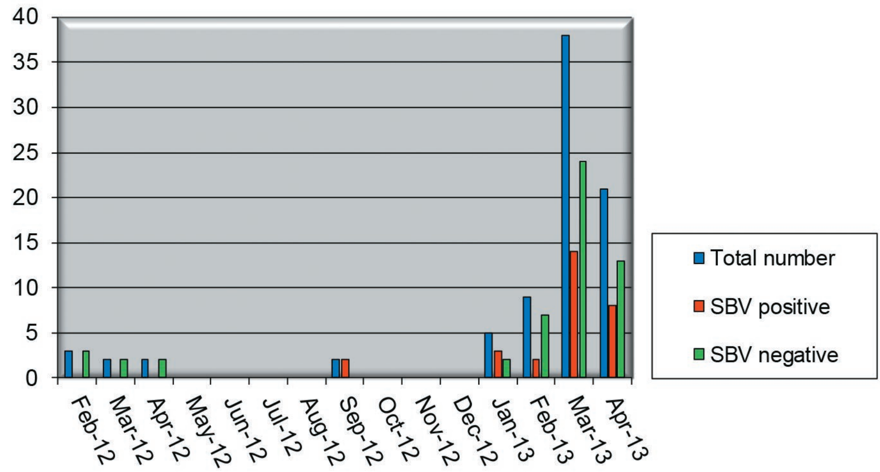

Figure 2: Number of tested malformed lambs and calves in Slovenia February 2012 to end of April 2013 (SBV-positive and -negative sample numbers by RT-qPCR, monthly

Figure 3: SBV-positive calf with severely deformed limbs (*arthrogryposis)*

Figure 4: SBV-positive lamb with shortened lower jaw (*brachygnathia*)

Figure 5: SBV-positive calf with twisted neck (*torticollis*) and arthrogryposis of the limbs

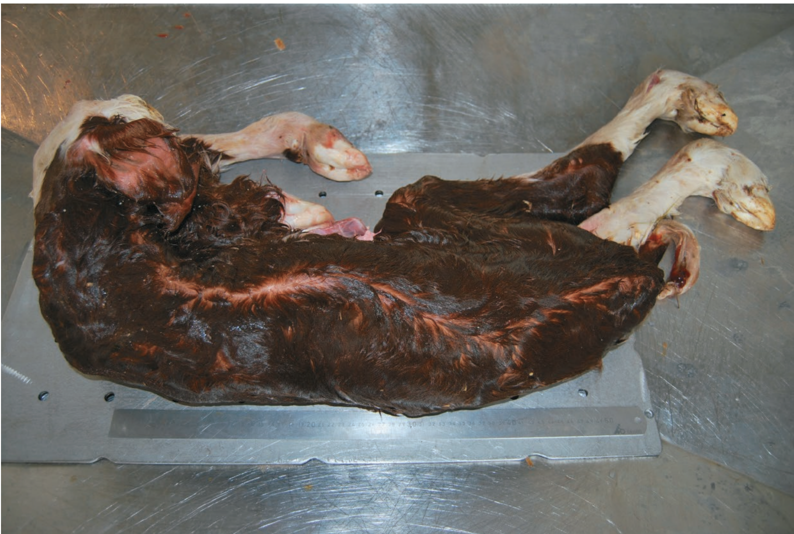

Figure 6: SBV-positive calf with lateral deviation of the spine (*scoliosis*), arthrogryposis and mild torticollis

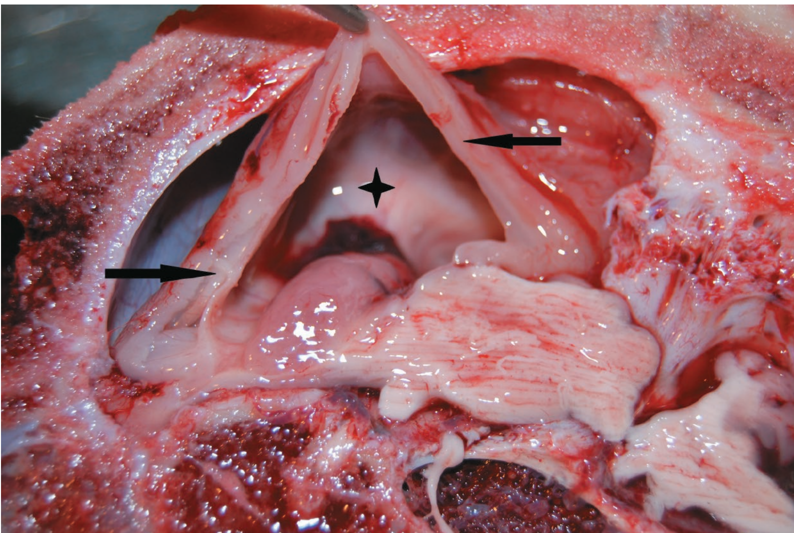

Figure 7: SBV-positive calf brain section; parenchyma of the cerebral hemisphere is very thin (arrows) and almost completely replaced with a fluid filled cyst (+) (*hydranencephaly*).

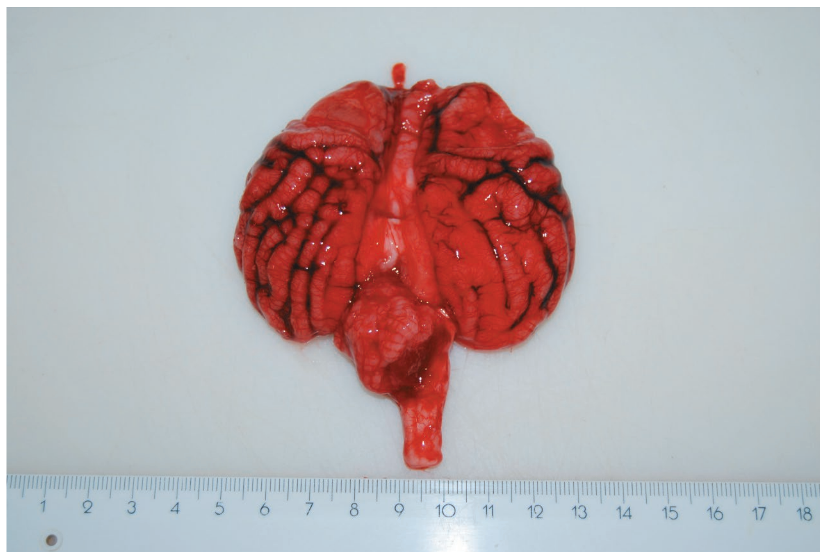

Figure 8: SBV-positive calf brain; greatly reduced size of cerebellum (*cerebellar hypoplasia*)

herd B-2/2012 was identified from a three-month old healthy calf located in a small village near the Austrian border in the municipality of Cankova, near Murska Sobota, approximately 220 km east from the location of the first SBV-positive herd (Figure 1). This observation confirmed that acute infection with SBV was already present in two separate locations in Slovenia in September 2012. The sequencing results confirmed that the detected strain in Slovenia shows a 100% identity in 97 determined nucleotides (partial L-segment, genome position 403-500) with the strain named Schmallenberg virus isolate Germany (GenBank Acc. No. JX853179), identified in Germany in 2011.

Discussion

This study demonstrates the detection of the SBV genome from clinically affected ruminants in Slovenia for the first time. The first official detection of the SBV genome in two malformed lambs was on January 9, 2013 from an ovine flock located in Semič near the Croatian border in the southern part of Slovenia. Later, SBV was detected on 27 additional cattle farms throughout Slovenia. Malformations found in detected SBV-positive cases were similar to those described in lambs and calves in other European countries (Germany, France, and Belgium), and the causative agent was the same.

The rapid and wide expansion of SBV is probably the result of transmission by vectors in the southern direction during the summer months since the first SBV antibodies were detected in September 2012 in neighbouring country Austria (15). The preliminary study results for SBV antibodies showed that Slovenian cattle had been naïve until August 2012, but on August 29, 2012, the first SBV-antibody-positive herd was identified by enzyme-linked immunosorbent assay (ELISA) test (16). Previous reports showed that acute infection with SBV in adult animals causes mild or unspecific clinical signs in ruminants (1, 3). This explains why the first stage of the SBV infection in 2012 was not recognised by our farmers and veterinarians. However, with retrospective detection of the SBV genome from two archive samples collected in September 2012, it is confirmed here that infection started in Slovenia almost at the same time as it was detected in Austria. From January to the end of April 2013, numerous cases of devastating malformations in new-borns were observed and an increased number of samples from different locations were detected positive by RT-qPCR (Figure 1).

The pathological findings made following detection of malformed foetuses and the rapid spread of the disease through Slovenia is very similar to the findings and spread reported in Germany, the Netherlands, Belgium and France in autumn 2011 and the beginning of 2012 (3, 8, 13). The detection of SBV outbreaks in new areas of Europe in 2012, such as Sweden, Norway, Finland, Estonia, Switzerland, Poland, Austria and now Slovenia, suggests the tendency of the

infection to spread quickly to new areas with a naïve population. Positive samples were identified in this study by the RT-qPCR method, with a protocol targeting the L-segment of SBV from a pool of samples consisting of the spleen and several parts of the brain, recommended as the most suitable material (13, 14). The identification of 32.9% SBV-positive samples from the tested malformed animals is similar to that of previous reports, while in Belgium, it was between 28% and 44% (4, 14), and in the Netherlands, from 9% to 70% (9, 13, 17). Our results support the previous observations that the presence of SBV RNA can be detected only in certain percentage of the SBV-suspected new-borns, because of SBV clearance after infecting the foetus (14).

Some authors have suggested using an additional tests for antibody detection in malformed animals, such as the virus neutralization test (VNT) or ELISA test to increase the reliability of SBV diagnosis (10, 17). The VNT showed that 95% of the malformed lambs were positive for SBV, although this percentage was lower for malformed calves (13). This observation was tested in our laboratory on 17 malformed calves (collected in April and May 2013) with RT-qPCR (tissue suspension) and ELISA (serum or thoracic fluid) and 15 (88.3%) of 17 calves were SBV-antibody-positive in ELISA test (ID Screen Schmallenberg Virus indirect ELISA, ID.vet Innovative Diagnostics, France), while only 7 (41.1%) of them were detected SBV-genome-positive by RT-qPCR (unpublished data). These preliminary results and the results from this study performed on malformed calves support previous observation detected in lambs (14) but in our case also, most of the malformed calves had developed SBV antibodies, thus providing confirmation of the SBV infection in an additional number of calves. This data is consistent with recent publication suggesting that neutralizing antibodies could play a role in the clearance of the virus from the foetus resulting in a subsequent inability to detect SBV by RT-qPCR. Nevertheless, the implementation of the new RT-qPCR assay in the laboratory based on L-segment will be necessary to increase the sensitivity for detection of SBV in samples (3, 9).

Slovenia is the first SBV-infected country near the Balkan area. With the ongoing spread of the infection we may expect that this new virus will soon be detected in other countries of southeast Europe.

Acknowledgments

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PRVI PRIMERI OKUŽB Z VIRUSOM SCHMALLENBERG V SLOVENIJI, 2012

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Povzetek: Konec poletja 2011 so iz Nemčije in Nizozemske poročali o prvih pojavih akutnih okužb goved povzročenih z virusom Schmallenberg (SBV) iz rodu *Orthobunyavirus*. Prve spačene zarodke zaradi SBV so opazili v decembru 2011 na Nizozemskem, v Belgiji in Nemčiji. V Sloveniji smo prvi primer okužbe z virusom SBV ugotovili v reji 23 ovac ob postavitvi suma na to bolezen po devetih zaporednih abortusih. Virusno nukleinsko kislino SBV smo v vzorcih dokazali iz možganov in vranice z metodo verižne reakcije s polimerazo v realnem času (RT-qPCR), ki jo je razvil Inštitut Friedrich-Loeffler iz Nemčije. Od januarja do aprila 2013 smo testirali 77 spačenih telet in pri 25 ugotovili prisotnost nukleinske kisline SBV z metodo RT-qPCR. Pri večini spačenih živalih smo ugotavljali eno ali več patoloških sprememb: artrogripozo, brahignatijo, tortikolis, skoliozo, hidranencefalijo ter nepravilen razvoj možganov in hrbtenjače. Dodatno smo tudi v dveh arhivskih vzorcih, odvzetih septembra 2012, ugotovili SBV, kar potrjuje prisotnost okužbe s SBV v Sloveniji že v septembru 2012. Z določanjem zaporedja nukleotidov v segmentu L smo potrdili, da je ugotovljeni sev v Sloveniji 100 % identičen z izolatom virusa Schmallenberg (JX853179), ki je bil ugotovljen v Nemčiji leta 2011. Pozitivne reje smo odkrili na celotnem območju Slovenije.

Ključne besede: prvo poročilo; virus Schmallenberg; diagnoza; Slovenija