Genetic stability of Schmallenberg virus *in vivo* during an epidemic, and *in vitro*, when passaged in the highly susceptible porcine SK-6 cell line

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A B S T R A C T

Schmallenberg virus (SBV), an arthropod-borne orthobunyavirus was first detected in 2011 in cattle suffering from diarrhea and fever. The most severe impact of an SBV infection is the induction of malformations in newborns and abortions. Between 2011 and 2013 SBV spread throughout Europe in an unprecedented epidemic wave. SBV contains a tripartite genome consisting of the three negative-sense RNA segments L, M, and S. The virus is usually isolated from clinical samples by inoculation of KC (insect) or BHK-21 (mammalian) cells. Several virus passages are required to allow adaptation of SBV to cells *in vitro*. In the present study, the porcine SK-6 cell line was used for isolation and passaging of SBV. SK-6 cells proved to be more sensitive to SBV infection and allowed to produce higher titers more rapidly as in BHK-21 cells after just one passage. No adaptation was required. In order to determine the *in vivo* genetic stability of SBV during an epidemic spread of the virus the nucleotide sequence of the genome from seven SBV field isolates collected in summer 2012 in Switzerland was determined and compared to other SBV sequences available in GenBank. A total of 101 mutations, mostly transitions randomly dispersed along the L and M segment were found when the Swiss isolates were compared to the first SBV isolated late 2011 in Germany. However, when these mutations were studied in detail, a previously described hypervariable region in the M segment was identified. The S segment was completely conserved among all sequenced SBV isolates. To assess the *in vitro* genetic stability of SBV, three isolates were passage 10 times in SK-6 cells and sequenced before and after passaging. Between two and five nt exchanges per genome were found. This low *in vitro* mutation rate further demonstrates the suitability of SK-6 cells for SBV propagation.

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1. Introduction

Schmallenberg virus (SBV) was named after the community in Germany where it was first isolated in late 2011 from a cow showing diarrhea, fever, and a drop in milk production (Hoffmann et al., 2012). Since then SBV has swept across Europe in an unprecedentedly fast epidemic. By summer 2013 the ruminant population of almost all European countries was infected by SBV, and the virus continued to spread eastwards, reaching as far as the Baltic's and Greece (ProMED-mail-reported SBV case on Sept. 23rd, 2013). Despite the high seroprevalence in ruminants in Europe, new SBV cases have been detected in Germany and The Netherlands in 2014 (ProMEDmail no. 20141121.2978286 from November 13th). The origin of the SBV causing the first outbreak in Germany is still not known. In a very recent report (Blomström et al., 2014) the

presence of SBV antibodies was described in cattle, sheep and goats in Mozambique, Africa, with a seroprevalence between 47 and 100%. Although these findings have to be confirmed, they represent the first indication from where SBV could have been introduced into Europe.

Cattle infected with SBV show various degrees of clinical symptoms such as diarrhea, fever, drop in milk production. These symptoms usually last only a few days before the animals recover completely (Beer et al., 2013; Doceul et al., 2013). Adult sheep and goats usually exhibit few clinical signs. However, the most serious impact of SBV is the infection of the fetus in pregnant animals, resulting in abortion or death of newborn calves, lambs and kids (Garigliany et al., 2012). This is often accompanied by extensive malformations of the limbs and in the brain (Hahn et al., 2012). Although domestic and wild ruminants represent the main mammalian host species, the virus can also cause clinical disease in dogs (Sailleau et al., 2013). Although SBV-specific antibodies in wild boars have been reported (Desmecht et al., 2013), a recent study showed that pigs are inefficient hosts for SBV infection and transmission (Poskin et al., 2014).

SBV is transmitted by insect vectors. It has been shown that several *Culicoides* spp. midges function as main insect vector for SBV transmission (Elbers et al., 2013). Furthermore, it is speculated that also certain mosquito species (*Culex* spp., *Aedes* spp.) which play a role in the transmission of other *Bunyaviridae* such as the Akabane virus that is closely related to SBV could also be capable to transmit SBV (Garigliany et al., 2012). High amounts of SBV RNA found in *Culicoides* indicate that SBV actively replicates during its migration from the midgut to the saliva glands (Rasmussen et al., 2012).

SBV is a new member of the family *Bunyaviridae* that comprises many other arthropod-born viruses. Whereas initial genome sequence data suggested that SBV is a reassortant between Sathuperi and Shamonda virus (Hoffmann et al., 2012), belonging to the Simbu serogroup, phylogenetic analysis using more extensive sequence data now indicates that SBV is most closely related to the Sathuperi virus species (Doceul et al., 2013). For viral RNA detection several RT-qPCR protocols have been published, targeting either the S segment (Bilk et al., 2013) or the L segment (De Regge et al., 2012) of the viral genome.

Several mammalian cell lines such as baby hamster cells (BHK-21), monkey kidney cells (VERO), as well as insect cells (KC) have been described for the isolation and propagation of SBV from viremic serum in cell culture, albeit resulting in low virus titers upon the initial passage (Wernike et al., 2012) and therefore requiring at least a few passages before rapid cytopathic effect (CPE) appears and virus titers rise to $>10^5$ $TCID_{50}$/mL. However, such an adaptation to cell lines usually represents a bottleneck for infection, increasing the chance that the viral genome acquires mutations. Therefore, a cell culture system that allows efficient virus isolation, *i.e.* leading to high virus titers and rapid appearance of CPE in the first passage without the need for serial passaging, is highly desirable.

The porcine kidney cell line SK-6 is widely used for the propagation of many different viruses (Kasza et al., 1972). We have shown that these cells do not produce type I interferon upon stimulation with double-stranded RNA (Ruggli et al., 2003). SK-6 cells have also been shown to maintain the genetic stability of classical swine fever virus for >90 passages (Vanderhallen et al., 1999).

Here we demonstrate that SK-6 cells are highly suitable for SBV isolation and propagation by exhibiting a rapid CPE (within 24–48 h) and yielding high virus titers upon inoculation of the cells with viremic serum. Serial passaging of SBV kept the viral genome virtually unchanged, further supporting the suitability of SK-6 cells.

In addition, nucleotide (nt) sequence comparison of the genome from several SBV isolates collected in Switzerland and other strains mainly from Germany and Belgium demonstrated that SBV evolves rather slowly despite its very rapid spread during the epidemic 2011–2013, and the few identified mutations among the Swiss isolates did not correlate with the geographic or temporal origin of the virus.

2. Materials and methods

2.1. Cells and viruses

The swine kidney cell line SK-6 was kindly provided by M. Pensaert, Faculty of Veterinary Medicine, Gent, Belgium. BHK-21 were obtained from ATCC (no. CCL-10).

2.1.1. High titer viremic sera used for passaging and genetic studies

Seven different SBV isolates with high amounts of viral RNA collected from clinical SBV infection in cattle throughout Switzerland during the epidemic wave of SBV over a time period of four weeks (Table 1) were included in the present study (sample numbers no. 79.4, no. 91.1, no. 96.1, no. 100.3, no. 102.2, no. 175.2, no. 200.2); the latter two samples were collected one week apart from two different animals from the same farm in order to determine the in-herd genetic variability of SBV.

Isolate no. BH80/11-4 from the first SBV outbreak in Germany was kindly provided by Martin Beer, FLI, Germany as cell culture supernatant (one passage in KC cells followed by one passage in BHK-21 cells). The nt sequence of the complete genome of this virus was taken from GenBank (accession numbers HE649912, HE649913, HE649914 for L, M, and S segment, respectively). The German isolate was further passaged in our laboratory once on KC cells, three times on BHK-21 cells and finally once in SK-6 cells. The Swiss SBV isolates were isolated directly in SK-6 cells, using serum from viremic cattle. In order to compare the sequence data from the Swiss isolates with other SBV strains, 32 full-length M (16 from Belgium, 14 from Germany, and 2 from the Netherlands) and 17 full-length L (9 from Germany, 6 from Belgium and 2 from the Netherlands) sequences were retrieved from GenBank (for accession numbers, see Fig. 5)

2.1.2. Low titer viremic sera used for comparison of susceptibility of SK-6 versus BHK-21 cells

Five additional viremic sera with low amounts of viral RNA (no. 91.1, no. 91.2, no. 96.2, no. 96.3, no. 109.1) (Table 3B) were used for the comparison of the susceptibility of SK-6 and BHK-21 cells.

Table 1

Geographic origin and isolation date of the eight SBV isolates characterized in the present study. An overview of the samples subjected to complete sequencing of the coding region of the L, M, and S genome segments, respectively, is provided. GenBank accession numbers referring to the original isolates (*i.e.* viremic field sera, no cell culture passaging) are also listed. The sequence of the S segment was not deposited since it was identical in all eight viruses studied.

SBV isolate no.	Isolation date	Geographic Origin			GenBank accession numbers segment		P10[**] sequencing
		Community	Canton	Country	L	M	
B80/11-4[*]	11/2011	Schmallenberg	NRW	Germany	HE649912	HE649913	No
79.4	20/07/2012	Mühleberg	BE	Switzerland	KM047416	KM047423	No
91.1	24/07/2012	Sigigen	LU	Switzerland	KM047417	KM047424	No
96.1	26/07/2012	Courcelon	JU	Switzerland	KM047418	KM047425	Yes
100.3	26/07/2012	Curio	TI	Switzerland	KM047419	KM047426	Yes
102.2	27/07/2012	Niederried	BE	Switzerland	KM047420	KM047427	No
175.2[***]	13/08/2012	Bendern	na	Liechtenstein	KM047421	KM047428	Yes
200.2[***]	17/08/2012	Bendern	na	Liechtenstein	KM047422	KM047429	No

na: Not applicable.
[*] Reference strain (cell culture supernatant from German SBV isolate B80/11-4).
[**] P10 sequences were not submitted to GenBank.
[***] Two subsequent cases from the same cattle holding to determine the in-herd heterogeneity of SBV.

2.2. Virus isolation and titration

SK-6 cells were inoculated with either infectious serum diluted 1:10 in EMEM (Gibco, LuBio Science GmbH, Luzern, Switzerland) with 2% horse serum (Gibco, LuBio Science GmbH, Luzern, Switzerland) or undiluted cell culture supernatant. When a distinct CPE appeared cell cultures were frozen at $-70\,°C$ and thawed three times. Supernatants were clarified by low-speed centrifugation, stored in aliquots at $-70\,°C$, and used for titration and further passaging. Titers expressed as $TCID_{50}/mL$ in SK-6 cells were determined for virus stocks obtained during the serial passaging by three independent titrations. In order to compare the permissiveness of SK-6 *versus* BHK-21 cells, supernatants of five additional SBV viremic field sera (see above) were passaged once in both cell lines, followed by titration both in SK-6 and BHK-21 cells. Furthermore, the amount of viral RNA was quantified based on three independent assays of an S segment-specific RT-qPCR (Bilk et al., 2013) each run in triplicates.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from $250\,\mu L$ serum or cell culture supernatant with Trizol (Invitrogen, LuBio Science GmbH, Luzern, Switzerland) and dissolved in $50\,\mu L$ water. Reverse transcription was initiated with primers SBV_L1_F, SBV_M1_F und SBV_S1_F, respectively, for the cDNA synthesis of the L, M, and S segment (Table 2). Eight microliter RNA was mixed with $1\,\mu L$ primer ($2\,\mu M$) and denatured at $65\,°C$ for 5 min. Reverse transcription was carried out with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Lubio GmbH, Luzern, Switzerland) following the manufacturer's protocol.

2.4. PCR and sequencing of the L, M, and S segment, and sequence analysis

cDNA was amplified with the Platinum Taq DNA Polymerase kit (Invitrogen, Lubio GmbH, Luzern, Switzerland), following the manufacturer's protocol, and using $8\,\mu L$ cDNA in a $50\,\mu L$ reaction volume. PCR was run on an Eppendorf MasterCycler (Vaudaux-Eppendorf, Schönenbuch, Switzerland) using the following cycling profile: 1 cycle $95\,°C/30\,s$, then 30 cycles [$95\,°C/30\,s$, $55\,°C/30\,s$, $68\,°C/8\,min$], followed by 1 cycle of $68\,°C/20\,min$. After PCR, the nt sequence of the complete open reading frame (ORF) of the L, M, and S segment, respectively, was determined in both directions by direct sequencing of agarose gel-purified PCR products obtained from the seven Swiss SBV isolates and from the German sample B80/11-4 (Table 1) with the same terminal primers (Table 2) that had been used previously for PCR, and a series of internal forward and reverse primers, which were designed manually, each covering a distance of approximately 500 nt (for primer sequences, see Supplementary Table 1). Sequencing

Table 2

Primers used for cDNA synthesis and full-length PCR of the coding region of the viral RNA segments L, M, and S. Primers were designed based on the genome of SBV isolate B80/11-4 (GenBank accession numbers HE649912, HE649913, HE649914 for L, M, and S segment, respectively), encompassing the termini and extending into the interior of the respective gene. The length of the primers were calculated by the G/C-A/T rule aiming at a melting temperature of 62–$64\,°C$. Numbers in the primer names refer to the respective nt positions.

SBV RNA segment	Primer orientation	Primer name	Primer sequence ($5' \rightarrow 3'$)
L	Forward	SBV_L1_F	CTAATTACAATCACTATGGAGAC
	Reverse	SBV_L6864_R	GTGCCCCTAATTACATGAAACT
M	Forward	SBV_M1_F	CCACAATCAAAATGCTTCTCAAC
	Reverse	SBV_M4398_R	TATTGTCAGTCTCCAATAGAAAG
S	Forward	SBV_S1_F	CTCCACTATTAACTACAGAAATAT
	Reverse	SBV_S830_R	AGTAGTGTTCTCCACTTATTAAC

Table 3

Comparison of SBV replication efficiency. Infectious sera from Swiss SBV cases were passaged once. (A) comparison of titers and viral RNA before and after passaging in SK-6 cells of those samples that were used for complete genome sequencing and serial passaging (one titration only). (B) Comparison of replication efficiency of SK-6 *versus* BHK-21 cells determined by passaging five additional SBV viremic sera once. The progeny virus titer of these samples was determined by three independent titrations on both cell lines; viral RNA was quantified by three independent RT-qPCRs as well.

A

Sample no.	Infectious virus (log $TCID_{50}$/mL)		Viral RNA ($50 - C_q$)	
	Viremic serum	1 Passage in SK-6 cells	Viremic serum	1 Passage in SK-6 cells
B80/11-4[*]	4.9	5.6	31.5	37.4
79.4	3.9	4.7	25.9	39.5
91.1	na	na	30.6	27.3
96.1	3.5	5.7	27.5	34.5
100.3	2.5	5.1	28.4	30.5
102.2	na	na	20.6	16.8
175.2[**]	2.5	5.1	30.2	33.4
200.2[**]	3.5	4.9	27.9	30.2

B

Sample no.	Infectious virus (log $TCID_{50}$/mL)						Viral RNA ($50 - C_q$)		
	Viremic serum		1 Passage in SK-6 cells		1 Passage in BHK-21 cells		Viremic serum	1 Passage in SK-6 cells	1 Passage in BHK-21 cells
	SK-6[*]	BHK-21[*]	SK-6[*]	BHK-21[*]	SK-6[*]	BHK-21[*]			
91.1	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	27.0 ± 0.5	16.7 ± 0.5	no C_q
91.2	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	26.4 ± 0.2	13.4 ± 1.2	no C_q
96.2	2.6 ± 0.2	<1.0	3.0 ± 0.2	<1.0	<1.0	<1.0	30.3 ± 0.5	29.8 ± 0.2	7.4 ± 1.4
96.3	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	30.1 ± 0.5	16.2 ± 1.6	no C_q
109.1	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	25.8 ± 0.5	no C_q	no C_q

na: No infectious virus detected.

[*] Previously passaged once in KC and once in BHK-21 cells, obtained from FLI, Riems, Germany.

[**] Samples taken from two individual animals from the same cattle herd.

[*] Cells used for titration, mean value and standard deviation from 3 independent titrations.

was performed with the Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Zug, Switzerland), and analyzed on an Applied Biosystems 3130 Genetic Analyzer (Life Technologies). The MEGA version 6 software (Tamura et al., 2013) was used for sequence alignments and maximum likelihood dendrogram construction with bootstrapping (500 replicates). Similarity plots to visualize the distribution of mutations were computed with the Simplot software (Lole et al., 1999), using a scanning window of 100 nt and a sliding distance of 10 nt. Sequences from the seven Swiss SBV isolates determined in the present study were submitted to GenBank (Table 1).

2.5. Replication kinetics of SBV in SK-6 cells

Replication kinetics was determined for the two Swiss SBV isolates no. 96.1 and no. 175.2 (passage 4 in SK-6 cells each). Confluent monolayers in 12.5 cm² cell culture flasks (12 flasks each for the two viruses) were inoculated at a multiplicity of infection (m.o.i.) of 0.1, followed by virus adsorption for 1 h at 4 °C. Cells were then incubated at 37 °C. At 0, 3, 6, 9, 12, 15, 18, 24, 30, 36, 48, and 72 h post inoculation (p.i.) one flask from both viruses was frozen at −70 °C until the end of the experiment. Finally, all collected samples were thawed, clarified by low-speed centrifugation and the resulting supernatants were titrated by cell culture and RT-qPCR as described above.

2.6. Serial passaging of SBV in SK-6 cells to assess its genetic stability

In order to monitor a potential adaptation process of SBV in SK-6 cells, and to determine the genetic stability of SBV *in vitro* the Swiss isolates no. 96.1, no. 100.3 and no. 175.2 were each passaged 10 times at an m.o.i. of 1 until a distinct CPE appeared. Virus titer and amount of viral RNA from each virus passage were determined by three-fold titrations (both infectious virus and amount of viral RNA). Virus from the tenth passage was subjected to complete L, M, and S ORF sequencing, respectively.

3. Results

3.1. SK-6 cells are highly permissive to SBV infection and support instant high titer virus replication

3.1.1. High titer sera

Whereas the KC- and BHK-21 cells inoculated with high titer viremic sera (Table 3A) showed only a discrete CPE at the end of the observation period (5 days p.i.), yielded low titers and required several passages until CPE appeared readily (data not shown), SK-6 cells in contrast showed an extensive CPE after 24–48 h p.i. in most of the infectious serum-inoculated as well as in previously BHK-21 passaged virus (Table 3A). The observed increase of the infectious virus titer after a single SK-6 cell passage was in agreement with an increase of the amount of viral RNA (*i.e.* a decrease of

quantification cycle (C_q) values). Despite of revealing clearly positive RT-qPCR scores, samples no. 91.1 and no. 102.2 did neither show CPE upon the initial SK-6 passage nor after three additional passages (data not shown). The high susceptibility of SK-6 cells also allowed a swift virus titration of cell culture supernatants as well as viremic serum.

3.1.2. Low titer sera

To compare the permissiveness of the two cell lines, low titer SBV-viremic sera (Table 3B) were passaged once in parallel in SK-6 and BHK-21 cells, followed by three-fold titration, again in both cell lines. In all but one serum, no infectious virus could be detected in the viremic sera anymore, and no infectious virus could be recovered in either cell line. The loss of infectivity was confirmed by rising C_q values representing the dilution effect of the inoculum. However, in sample no. 96.2 infectious SBV was detected in the field serum, leading to a CPE 72 h p.i. When this sample was passaged once in both cell lines, followed by titration again in both cell lines, the virus could only be detected in the passage one in SK-6 cells titrated in SK-6. The replication of SBV in this sample was confirmed by the RT-qPCR result, *i.e.* being the only sample in which an increase of the $50 − C_q$ value was observed. These results clearly demonstrate the superior susceptibility of SK-6 compared to BHK-21 cells.

3.2. SBV does not go through a phenotypic adaptation process and remains genetically stable during serial passaging in SK-6 cells

SBV samples no. 96.1, no. 100.3, and no. 175.2 were serially passaged 10 times in SK-6 cells in order to detect a potential phenotypic adaptation and to determine the genetic stability of SBV *in vitro*. As shown in Fig. 1A, random titer fluctuations between passage number and virus isolate were observed. Levels of viral RNA determined for the 10 virus passages remained roughly stable within a narrow C_q range (Fig. 1B). This suggests that SBV is able to replicate to its full potential in SK-6 cells as early as during the first passage.

To study the evolution of SBV *in vitro*, the complete coding regions of the L, M, and S genome segments, respectively, of isolates no. 96.1, no. 100.3 and no. 175.2 were sequenced twice, (i) SBV present in infectious serum (*i.e.* before any cell culture passaging), and (ii) after 10 passages of the viruses in SK-6 cells. As shown in Table 4, only 2, 5, and 3 nt exchanges in SBV isolates no. 96.1, no. 100.3 and no. 175.2, respectively, were found among a total number of 11679 nt corresponding to the fused L, M, and S ORF sequence. This resulted in an nt identity level of ≥99.957%. In order to assess these data, the genome of the two Belgian SBV isolates "Na-1" and "Na-2" (GenBank accession numbers see Fig. 5) was also compared before and after 10-fold passaging in BHK-21. Four mutations were found for "Na-1" and 27 for "Na-2", respectively.

3.3. Replication kinetics of SBV in SK-6 cells

Growth curves were established for the two SBV isolates no. 96.1 and no. 175.2 in SK-6 cells by monitoring infectious progeny production by virus titration and synthesis of viral RNA by RT-qPCR. Typical and virtually identical growth curves were obtained for both isolates (Fig. 2). Virus replication started almost instantly after shifting the incubation temperature of the cells from 4 °C to 37 °C; hence a lag phase was not observed. There was a quick and steep increase of both viral RNA and virus replication, infectious progeny virus was produced simultaneously to viral RNA replication. Virus replication peaked already at 18 h p.i. yielding titers close to 10^6 TCID$_{50}$/mL. Viral RNA levels continued to rise as late as 36 h p.i. and stayed on a plateau until the end of the experiment at 72 h p.i. Infectious virus titers slightly decreased about one log until 72 h p.i.

3.4. Genetic stability of SBV in vivo

RNA viruses in general evolve rapidly during the spread of the virus in an epidemic wave (Steinhauer and Holland, 1987). Since SBV is an RNA virus we decided to characterize the genome of seven Swiss SBV isolate that were isolated in different regions of Switzerland (see Supplementary Fig. 1) over a 4 week period. Samples no. 175.2 and no. 200.2 originated from the same cattle farm (but from different animals). The genome of these two SBV isolates was sequenced to determine the in-herd genetic stability. Surprisingly, these two SBV were quite different, showing 12 nt exchanges. This indicates that different SBV strains can be present simultaneously in an individual cattle herd.

The frequency of nt heterogeneity was not evenly distributed along the M and the L segment. As shown by Simplot analysis a distinctly higher degree of nt sequence heterogeneity was found between position 2100 and 2300 and toward the 3′ end in the M segment (Fig. 3A) as well as in both termini and around position 2000 in the L segment (Fig. 3B).

3.5. Detailed analysis of mutations

Mutations found in the genome of the seven Swiss SBV isolates were studied in detail, (i) the sequences of the animal-derived samples were compared to the German reference sequence and among themselves (Fig. 4A), and (ii) the sequences of the three isolates no. 96.1, no. 100.3 and no. 175.2 that had been passaged 10 times in SK-6 cells (Fig. 4B), were compared to their ancestors (*i.e.* the same SBV isolate from infectious serum, before any cell culture propagation).

Surprisingly, the S segment did not reveal any mutation among all analyzed viruses before and after passaging (in BHK-21 and KC cells as well as after 10 passages in SK-6 cells) and was identical to the German reference sequence (accession number HE649914, data not shown). A total of 101 point mutations were recorded in the L and the M segment, of which the vast majority were transitions, namely 29/42 for the L segment, and 55/59 for the M segment. The mutations were concentrated in 14 and 22 genome positions of the L and the M segment, respectively, with a frequency of one to seven viruses. Strikingly, only one type of transversion, namely T → A,
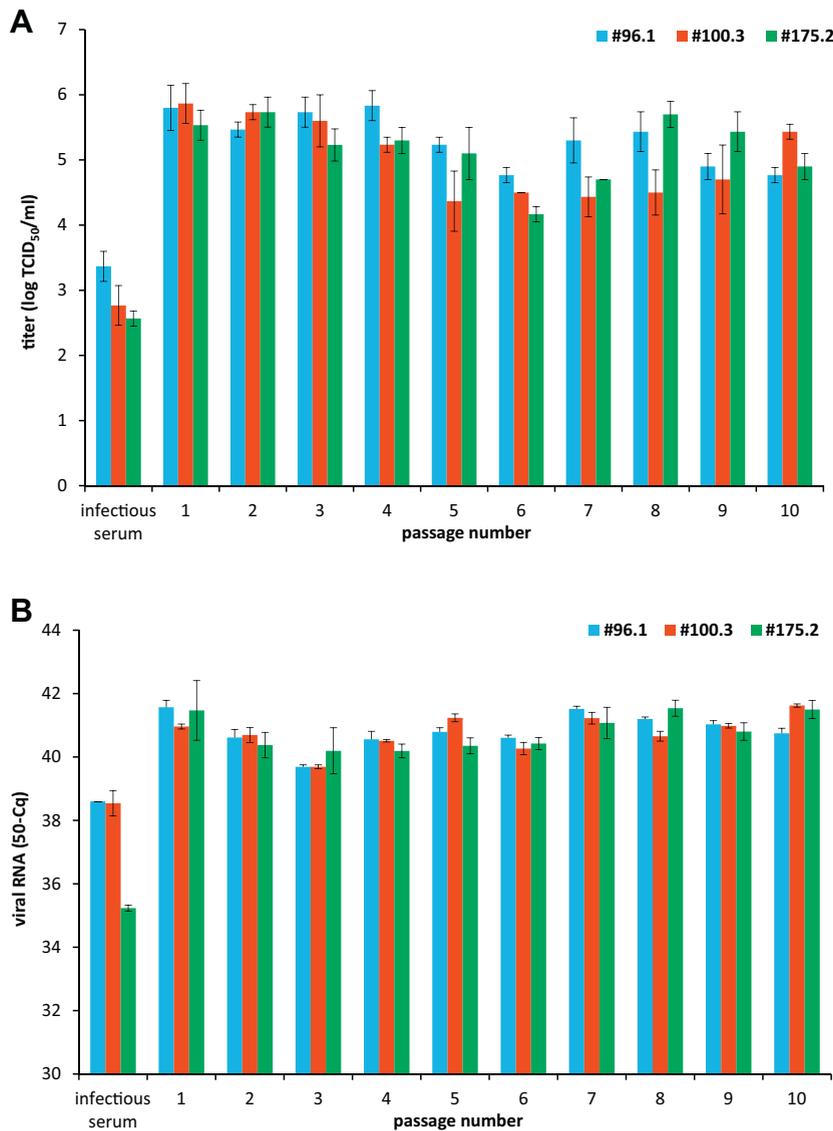
Fig. 1. Serial passaging of SBV in SK-6 cells. Virus replication was monitored by three-fold independent infectious virus titration (A) and viral RNA detection by an S segment–targeting RT-qPCR (B). Each of the three independent RT-qPCR reactions was run in triplicates, resulting in 9 C_q values per sample. RT-qPCR results are displayed as $50 - C_q$ to allow a better comparison of the data. Error bars represent the standard deviation from $n = 3$ (infectivity) or $n = 9$ (RT-qPCR), respectively.

was found in the L segment in 13 instances, whereas in the M segment merely 3 transversions of mixed types were identified. All the mutations found in the L segment and 49/59 (in 18/22 positions) in the M segment were silent, leaving the amino acid (aa) sequence unchanged. Interestingly, the seven aa exchanges found in the M segment were all located in the central hypervariable region (Fischer et al., 2013; Coupeau et al., 2013) and in the 3′ terminal area. Three out of seven aa exchanges were Glu–Lys. When the L and M sequence of individual SBV isolates was compared pairwise, the number of mutations ranged from 2 to 14 differences (mean = 10 ± 3) between the Swiss isolates. Slightly higher values were obtained when the Swiss sequences were compared to the German reference strain B80/11-4, ranging from 9 to 17 nt exchanges (mean = 14 ± 3).

Interestingly, the two SBV isolates no. 175.2 and no. 200.2 that originated from the same cattle heard and were collected within four days revealed a 12 nt difference.

Sequence alignments (data not shown) and Maximum Likelihood Trees of the L and the M segment including the SK-6-passaged viruses no. 96.1, no. 100.3 and no. 175.2 were constructed to study the genomic relationship of the SBV isolates. As shown in Fig. 5A (M segment) and Fig. 5B (L segment) the Swiss isolates formed their own cluster, but encompassed also some of the German isolates. Interestingly, SBV no. 175.2 (both before and after 10 SK-6 passages) was widely separated from the remaining isolates, forming its own subbranch in the dendrogram. Notably, all three viruses that had been passaged 10 times in SK-6 cells were placed next to each other and at a shorter

Table 4

Mutations identified through comparative nt sequencing of the three SBV isolates that were passaged 10 times in SK-6 cells. Complete L, M and S ORF sequences were determined from SBV before and after 10 consecutive passages. No mutations were found in the S ORF.

SBV isolate no.	Genome segment	nt Change	aa Change	Type of aa change[*] (C/NC)
96.1	L	A6518C	Asn → Thr	C
	M	C1813T	Thr → Ile	NC
	M	C3413T	Silent	na
100.3	L	C202T	Silent	na
	L	G4278A	Silent	na
	L	A4719G	Silent	na
	M	G1505A	Silent	na
	M	G1605A	Glu → Lys	NC
175.2	M	T2051C	Silent	na
	M	G2079A	Glu → Lys	NC

[*] C, conservative aa change; NC, non-conservative aa change; na, not applicable.
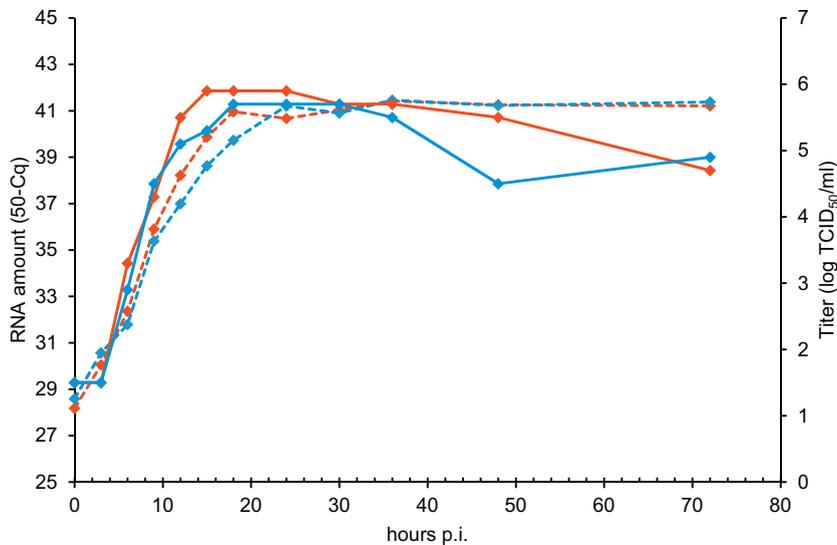
Fig. 2. Replication kinetics of SBV isolates no. 96.1 (red) and no. 175.2 (blue) previously passaged four times in SK-6 cells. Cells were infected with an m.o.i. of 0.1. Samples were taken at the indicated time points. Infectivity titers (solid lines) and amount of viral RNA (dotted lines) were determined each by titration in SK-6 cells and RT-qPCR, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

distance than the two Belgian SBV "Na-1" and "Na-2" that were sequenced before and after 10 BHK-21 passages.

4. Discussion

Usually, SBV is isolated from clinical samples by inoculating KC (insect) or BHK-21 (mammalian) cell cultures. Several passages are required to adapt the virus to the host cells. Coupeau et al. (2013) showed that – depending on the SBV isolate – 10 to 15 BHK-21 passages were required to obtain maximal titers. In the present study, the porcine SK-6 cell line was used to isolate SBV from sera collected during the peak of the SBV epidemic in Switzerland from viremic, clinically affected cattle (Table 1, Supplementary Fig. 1). Compared to BHK-21 cells, SBV isolates replicated in SK-6 cell in a more efficient way; peak titers were obtained already upon the first SK-6 cell passage. This was shown both by determining the infectivity titers (Fig. 1A) as well as by quantifying the viral RNA by an S segment-specific RT-qPCR (Fig. 1B).

Cross-titration experiments of SBV-infected SK-6 and BHK-21 cells (Table 3B) yielded infectious virus titers only for SK-6 cells, confirming the high permissiveness of this cell line for SBV propagation. The susceptibility of the SK-6 cell line for SBV infection and propagation was discovered by chance and was unexpected, since a recent study (Poskin et al., 2014) demonstrated that SBV replicates in pigs in an inefficient way, merely leading to a seroconversion in some animals, but no virus could be detected in any clinical sample nor was SBV transmitted to contact animals. This lacking susceptibility of pigs to SBV infection was surprising considering that other members of the Bunyaviridae such as Akabane virus are able to infect pigs in the field (Huang et al., 2003). On the other hand, Kasza et al. (1972) reported that several porcine viruses such as swine influenza, vesicular stomatitis and encephalomyelitis virus could not be propagated in SK-6 cells. However, since SK-6 cells were probably extensively passaged in the past 40 years, it is likely that the phenotype of this cell line has changed. Probably the most significant alteration was the
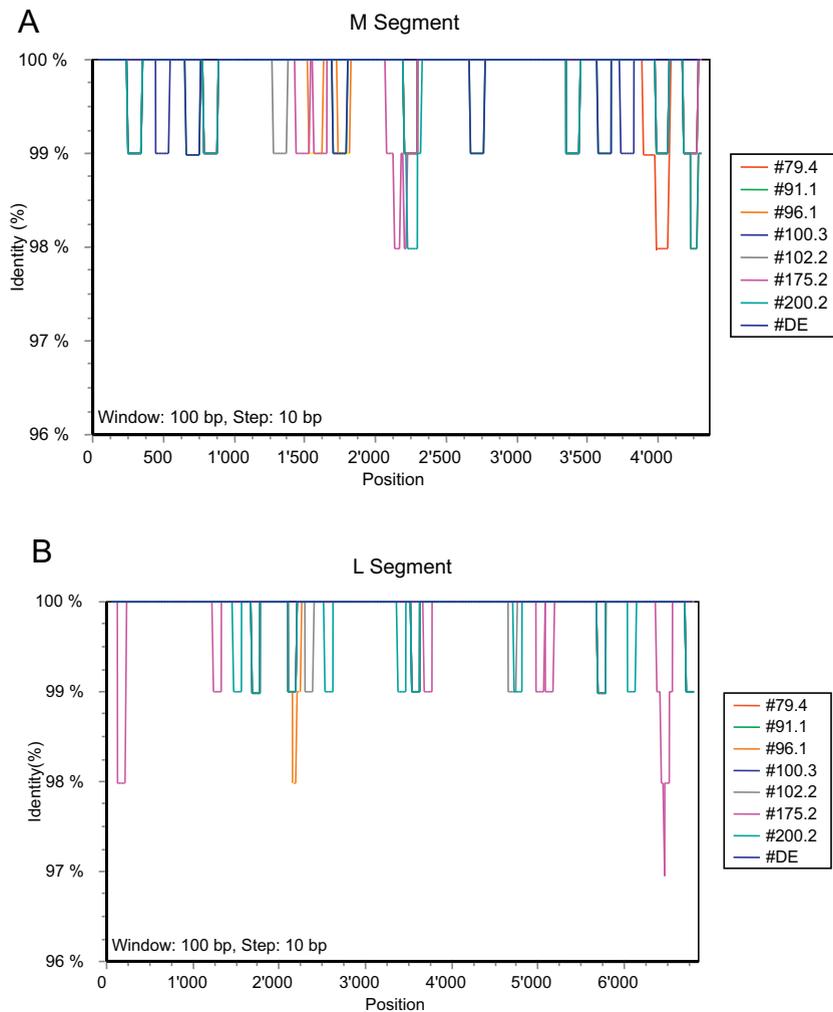
Fig. 3. Nucleotide similarity plots constructed by Symplot software based on the alignment of the seven Swiss and the German reference SBV sequence. (A) M segment, showing the previously described central hypervariable area as well as an additional, 3′ terminally located increased nt variability. (B), L segment: no distinct mutation cluster area can be identified.

loss of the ability to induce type I interferon upon stimulation with double-stranded RNA (Ruggli et al., 2005). It is likely that this change led to an increase in the susceptibility range, including non-porcine viruses, making it unpredictable which viruses are able to replicate in SK-6 cells. No progeny virus could be isolated from the two samples no. 91.1 and no. 102.2 even after several-fold blind passaging in SK-6 and BHK-21 cells (Table 3A). However, they contained similar amounts of viral RNA (concluded from the similar C_q values) as the infectious samples. These samples did not contain detectable levels of infectious SBV, and the observed increase of C_q values represented a dilution effect upon passaging. Unfavorable storage conditions may have inactivated the virus present in these two samples. Nevertheless, the sequence of all three RNA segments could be determined using RNA from the clinical samples. All three isolates used for the serial passaging study rendered similar results (Fig. 1A and B). The appearance of CPE as early as 24 h p.i. and the high titers obtained upon the first passage make it unlikely that

the virus required an adaptation phase. The few mutations after 10-fold SK-6 passaging (Fig. 4B) together with the high titer obtained after one cell culture passage in SK-6 cells argues against a selection pressure of the replicating virus. Growth kinetics with SBV from the fourth passage in SK-6 cells at an m.o.i. of 0.1 also demonstrated the efficient SBV replication in SK-6 cells (Fig. 2). A steep increase of infectious virus and viral RNA synthesis was already seen at 4–6 h p.i. Plateaus for infectious progeny virus and viral RNA production were reached as early as 15–18 h p.i. S segment RNA copy numbers remained stable thereafter demonstrating a high thermostability of SBV RNA at 37 °C, whereas a one log decrease of progeny virus was seen at the end of the experiment at 72 h p.i. This growth curve sharply contrasted with earlier data using BHK-21-adapted virus and several other cell lines with revealed much slower replication kinetics irrespective of the cell line used (Varela et al., 2013). For performing in vivo pathogenesis studies, SBV viremic serum should preferentially be used as inoculum, since BHK-21-adapted virus

A

segment (length)	position	BH80/11-4 GenBank Ref base	triplet	BH80/11-4 reference strain* base	triplet	#79.4 base	triplet	#91.1 base	triplet	#96.1 base	triplet	#100.3 base	triplet	#102.2 base	triplet	#175.2 base	triplet	#200.2 base	triplet	aa change
L (6864 nt)	1515	C	TAC	C	TAC	T	TAT	C	TAC	C	TAC	C	TAC	C	TAC	C	TAC	T	TAT	silent
	1728	T	GGT	T	GGT	A	GGA	A	GGA	T	GGT	A	GGA	A	GGA	A	GGA	A	GGA	silent
	2154	T	ACT	T	ACT	C	ACC	C	ACC	C	ACC	C	ACC	C	ACC	C	ACC	C	ACC	silent
	2205	C	GGC	C	GGC	C	GGC	C	GGC	T	GGT	C	GGC	C	GGC	C	GGC	C	GGC	silent
	2346	C	ATC	C	ATC	C	ATC	C	ATC	C	ATC	C	ATC	T	ATT	C	ATC	C	ATC	silent
	2574	A	ACA	A	ACA	A	ACA	A	ACA	A	ACA	A	ACA	A	ACA	A	ACA	G	ACG	silent
	3411	C	CTC	C	CTC	C	CTC	C	CTC	C	CTC	C	CTC	C	CTC	C	CTC	T	CTT	silent
	3579	T	ATT	T	ATT	C	ATC	C	ATC	T	ATT	C	ATC	C	ATC	C	ATC	C	ATC	silent
	4707	C	AAC	C	AAC	C	AAC	C	AAC	C	AAC	C	AAC	T	AAT	C	AAC	C	AAC	silent
	4764	G	GAG	G	GAG	A	GAA	G	GAG	G	GAG	G	GAG	G	GAG	G	GAG	A	GAA	silent
	5275	C	CTA	C	CTA	T	TTA	C	CTA	C	CTA	C	CTA	C	CTA	C	CTA	C	CTA	silent
	5736	T	CCT	T	CCT	A	CCA	A	CCA	A	CCA	A	CCA	A	CCA	A	CCA	A	CCA	silent
	6087	C	AAC	C	AAC	C	AAC	C	AAC	C	AAC	C	AAC	C	AAC	C	AAC	T	AAT	silent
	6765	G	AAG	G	AAG	A	AAA	A	AAA	G	AAG	A	AAA	A	AAA	A	AAA	A	AAA	silent
M (4363 nt)	296	C	CAC	C	CAC	T	CAT	T	CAT	C	CAC	T	CAT	T	CAT	C	CAC	C	CAT	silent
	500	C	AAC	C	AAC	C	AAC	C	AAC	C	AAC	T	AAT	C	AAC	C	AAC	C	AAC	silent
	701	G	ACG	G	ACG	G	ACG	T	ACT	G	ACG	T	ACT	G	ACG	G	ACG	G	ACG	silent
	836	G	TCG	G	TCG	A	TCA	A	TCA	A	TCA	A	TCA	A	TCA	A	TCA	A	TCA	silent
	1323	C	CTG	C	CTG	C	CTG	C	CTG	C	CTG	C	CTG	T	TTG	C	CTG	C	CTG	silent
	1490	T	AAT	T	AAT	T	AAT	T	AAT	T	AAT	T	AAT	T	AAT	C	AAC	T	AAT	silent
	1580	T	TGT	T	TGT	T	TGT	T	TGT	C	TGC	T	TGT	T	TGT	T	TGT	T	TGT	silent
	1604	C	TCC	C	TCC	C	TCC	C	TCC	C	TCC	C	TCC	C	TCC	T	TCT	C	TCC	silent
	1748	G	GTG	G	GTG	G	GTG	A	GTA	G	GTG	A	GTA	G	GTG	G	GTG	G	GTG	silent
	1773	A	ATA	A	ATA	A	ATA	A	ATA	G	GTA	A	ATA	A	ATA	A	ATA	A	ATA	Ile > Val
	2127	C	CAC	C	CAC	C	CAC	C	CAC	C	CAC	C	CAC	T	TAC	C	CAC	C	CAC	His > Tyr
	2171	G	CTG	G	CTG	G	CTG	G	CTG	G	CTG	G	CTG	G	CTG	A	CTA	G	CTG	silent
	2247	A	AAA	A	AAA	G	GAA	G	GAA	G	GAA	G	GAA	G	GAA	G	GAA	G	GAA	Lys > Glu
	2279	A	GGA	A	GGA	A	GGA	A	GGA	A	GGA	A	GGA	A	GGA	A	GGA	G	GGG	silent
	2711	G	CAG	G	CAG	G	CAG	G	CAG	A	CAA	G	CAG	A	CAA	G	CAG	G	CAG	silent
	3395	G	TCG	G	TCG	A	TCA	A	TCA	G	TCG	A	TCA	A	TCA	G	TCG	A	TCA	silent
	3620	T	GGT	T	GGT	T	GGT	C	GGC	T	GGT	C	GGC	T	GGT	T	GGT	T	GGT	silent
	3780	T	TCA	T	TCA	T	TCA	T	TCA	T	TCA	C	CCA	T	TCA	T	TCA	T	TCA	Ser > Pro
	3937	A	AAT	A	AAT	C	ACT	A	AAT	A	AAT	A	AAT	A	AAT	A	AAT	A	AAT	Asn > Thr
	4029	A	AAA	A	AAA	G	GAA	G	GAA	G	GAA	G	GAA	G	GAA	G	GAA	G	GAA	Lys > Glu
	4032	G	GAA	G	GAA	A	AAA	G	GAA	G	GAA	G	GAA	G	GAA	G	GAA	G	GAA	Glu > Lys
	4230	T	TTA	T	TTA	C	CTA	C	CTA	C	CTA	C	CTA	C	CTA	C	CTA	C	CTA	silent

B

segment	position	BH80/11-4 reference sequence base	triplet	#96.1 P0 base	triplet	#96.1 P10 base	triplet	#100.3 P0 base	triplet	#100.3 P10 base	triplet	#175.2 P0 base	triplet	#175.2 P10 base	triplet	aa change
L (6864 nt)	202	C	CTG	C	CTG	C	CTG	C	CTG	T	TTG	C	CTG	C	CTG	silent
	4278	G	TCG	G	TCG	G	TCG	G	TCG	A	TCA	G	TCG	G	TCG	silent
	4719	A	TTA	A	TTA	A	TTA	A	TTA	G	TTG	A	TTA	A	TTA	silent
	6518	A	AAT	A	AAT	C	ACT	A	AAT	A	AAT	A	AAT	A	AAT	Asn > Thr
M (4363 nt)	1505	A	CCA	A	CCA	A	CCA	A	CCA	G	CCG	A	CCA	A	CCA	silent
	1605	G	GAA	G	GAA	G	GAA	G	GAA	A	AAA	G	GAA	G	GAA	Glu > Lys
	1813	C	ACC	C	ACC	T	ATC	C	ACC	C	ACC	C	ACC	C	ACC	Thr > Ile
	2051	T	GAT	T	GAT	T	GAT	T	GAT	T	GAT	T	GAT	C	GAC	silent
	2079	G	GAA	G	GAA	G	GAA	G	GAA	G	GAA	G	GAA	A	AAA	Glu > Lys
	3413	C	GGC	C	GGC	T	GGT	C	GGC	C	GGC	C	GGC	C	GGC	silent

Fig. 4. Overview of the mutations found in the L and M genome segment of the seven Swiss SBV isolates before cell culture passaging (A) and after 10 passages in SK-6 cells (B), compared to the BH80/11-4 Genbank reference sequences for L (HE649912) and M (HE649913). Nucleotides identical to the reference sequence are labeled in light green, mutations in light red. Amino acid changes are labeled in yellow (conservative exchange) or red (non-conservative exchange). The German reference virus BH80/11-4 that had undergone repeated KC/BHK-21/SK-6 cell culture passaging (see Section 2) is marked by an asterisk. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

titrated in calves yields lower titers than viremic serum (Wernike et al., 2012). However, SK-6 cell-derived virus could be an alternative inoculum, based on the rapid and efficient virus replication. Animal inoculation with passage 10 virus was not done in this study, but based on the few point mutations detected upon 10-fold SK-6 passaging, SBV passaged only a few times might be used as a standardized virus instead of viremic serum for in vivo pathogenesis studies.

The nucleotide sequence of the coding region for the three genome segments from seven Swiss and the German reference strain B80/11-4 were determined by Sanger
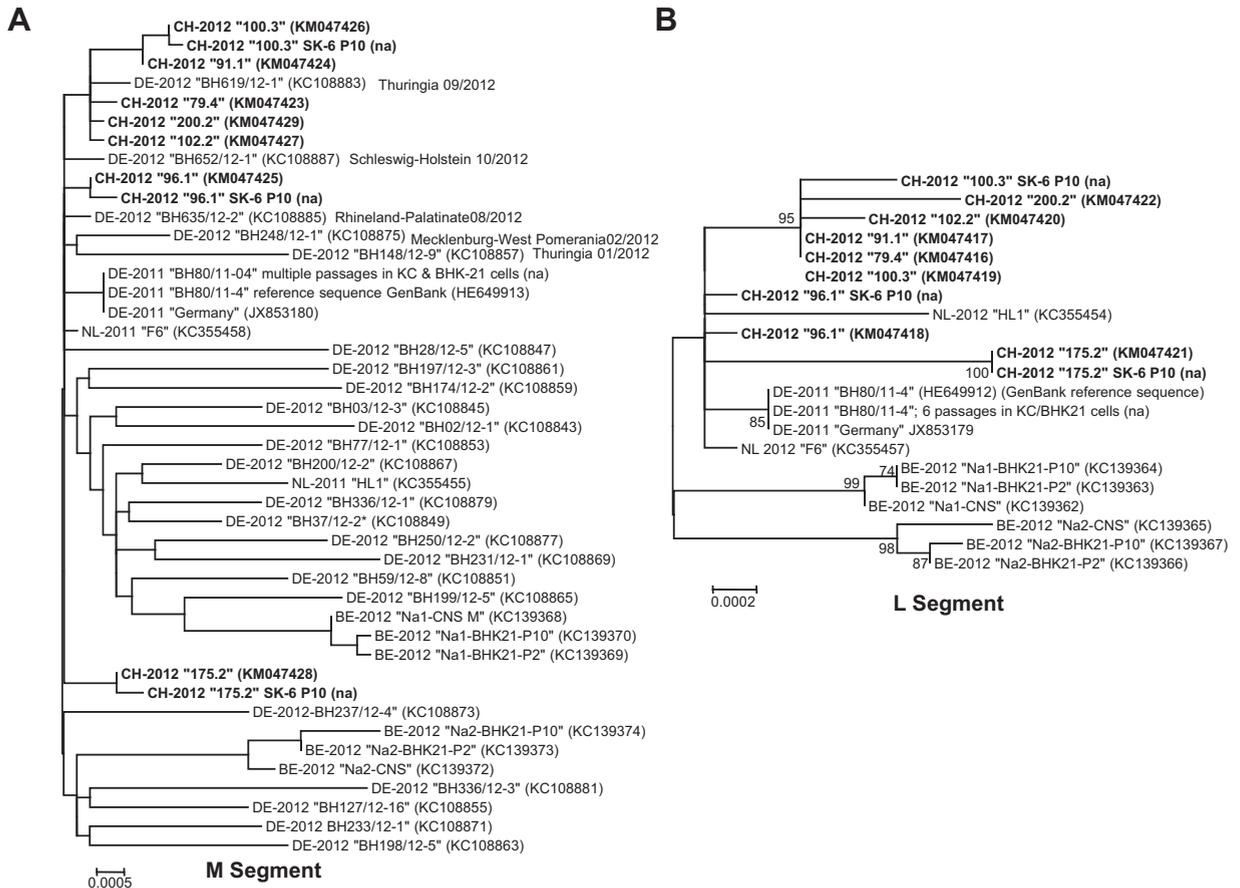
Fig. 5. Maximum likelihood trees showing the relationship of the Swiss SBV isolates characterized in this study, and GenBank-derived L and M sequences of SBV isolates from Germany, Belgium and The Netherlands. Swiss isolates are labeled in bold. The three serially SK-6 passaged Swiss and the two Belgian SBV isolates passaged in BHK-21 cells were either identical (L gene of isolate no. 175.2) or located in close proximity to their respective predecessor. Isolate designation is as follows: Country-year of isolation "#isolate name" (GenBank accession number, if available). For German SBV commingling with the majority of Swiss viruses in the M tree, isolation date (month/year) and location are also indicated in order to see if the German isolates that were most closely related to Swiss isolates were those being collected in the south of Germany.

sequencing. The resulting data were compared to other SBV sequences derived from GenBank, in particular from B80/11-4 that had been determined from viremic serum and was used as reference sequence for this study. For three of the Swiss SBV isolates the genome sequence before any cell culture propagation and after 10 passages in SK-6 cells was also determined to assess the mutation rate of SBV *in vitro*. All in all 101 individual point mutations were identified, which did not show an obvious distribution pattern but seemed to be randomly dispersed (Fig. 4A). Therefore, no correlation could be established between the number and position of mutations in the individual Swiss SBV isolates and the date and location of their origin. Nevertheless, the M gene sequences from six out of seven Swiss isolates mingled together with some isolates from various locations in Germany (Fig. 5A), making it unlikely that these German SBV isolates represented direct ancestors of the Swiss SBV. In the L segment tree (Fig. 5B) the Swiss SBV formed their own clade, and the NL isolate "HL-1" appeared as nearest neighbor. When the structure of the two trees was compared, similarities in the topology were found, *i.e.* those viruses from which both the

L and the M sequence was available from GenBank (*de novo* sequenced Swiss isolates and Belgian and Dutch isolates and German reference strain BH80/11-4) appeared in the similar location in the two trees. The only exception was Swiss isolate no. 175.2 which appeared at a clearly different position apart from the remaining Swiss isolates in both dendrograms. Paradoxically, this isolate originated from the same cattle herd as isolate no. 200.2 and the two viruses were collected only 4 days apart. This is an indication that the cattle herd in Liechtenstein got infected simultaneously by two clearly different viruses. Instead of a directional (temporal and geographical) evolution of SBV which is not supported by the data of this study, it is more likely that a pool of numerous slightly different virus strains representing a quasispecies population infected the Swiss ruminant population during the epidemic wave of SBV. Similarity plots of the L and the M segments (Fig. 3A and B) revealed a discrete accumulation of mutations in the central region and in the 3′ terminal area of the M segment. The accumulation of point mutations in the central region was in agreement with the hypervariable region described in previously published

studies (Coupeau et al., 2013; Wernike et al., 2012). All mutations found in the L segment and the majority of base exchanges in the M segment were silent. Interestingly, among the seven identified aa changes on the M segment, which were mostly non-conservative, three times a Lys–Glu substitution was found. Furthermore, the same aa mutation was also found twice in the M segment of the 10 times passaged viruses, albeit in different positions. Obviously this mutation is tolerated well by the virus, even though it represents an exchange of a basic by an acidic aa and *vice versa*.

When mutations (in M or L) of individual SBV isolates were compared pairwise (data not shown), higher numbers of mutations were found when the Swiss isolates were compared to the German reference strain. This might be due to ongoing evolution during the time between the isolation of the German strain in Nov 2011 and the Swiss isolates collected seven months later. Between isolates no. 91.1 and no. 100.3 only two nt differences were found (in the M segment, none in the L segment), suggesting that these two viruses were evolutionary closely related. However, the two viruses originated from two geographically >100 km separated cattle (Supplementary Fig. 1), and no epidemiological link (*e.g.* transport of animals) could be established. Finally, isolates no. 79.4, no. 91.1 and no. 100.3 that were identical (but different from the other isolates) in their L segment sequence were also placed next to each other in the M dendrogram. The two similar dendrograms (L, M) suggest a co-evolution for the L and M gene segment and there were no indications for reassortation events among the analyzed isolates.

In our study few mutations were detected both *in vitro* and *in vivo*. The low *in vivo* mutation rate argues against an efficient selection pressure during an epidemic. On the other hand, back-and-forth transmission of SBV between its mammalian host and insect vector might represent a bottleneck situation, which is likely to limit the number of tolerated mutations considerably and forcing the virus to conserve its genotype in an optimal state for both of its hosts (Jenkins et al., 2002). Generally RNA viruses show mutation rates of between 10^{-4} and 10^{-6} mutations per base per generation (Lauring et al., 2013). Translated into numbers for the SBV genome which is approximately 12 kb in length (made up by the three RNA segments L, M, and S), one would expect a mutation rate ranging from 0.01 to 1 mutation introduction during each RNA strand copying event. Although the majority of these mutations are likely to be deleterious for virus replication, the low number of nt exchanges suggests the presence of a proof-reading mechanism in SBV. Proof-reading mechanisms have been reported for several RNA viruses, *e.g.* coronaviruses (Denison et al., 2001).

The high transition *versus* transversion ratio observed in the third (wobble) position of the coding triplets helps to explain why most mutations did not cause an aa change. The uneven transition/transversion ratio between M and L segments might indicate a different RNA replication control mechanism. Furthermore, the low mutation frequency even of the M segment that encodes the viral glycoproteins Gn and Gc (Doceul et al., 2013) was unexpected. Gn and Gc are the most immunogenic

proteins of SBV. Under immunological pressure one could expect a much higher mutation rate when comparing field isolates of SBV. However, this was not the case in our study, suggesting that other, hitherto unknown mechanisms could be involved in maintaining the genetic stability of the virus even under immunological pressure. The internally located nucleoprotein, which interacts with the viral RNA and needs to maintain its secondary structure for this interaction, is protected from the humoral immune system. This might be a likely explanation why all S sequences analyzed in this study were identical. However, this finding is in contrast to the data from Fischer et al. (2013) who reported a considerable degree of S sequence heterogeneity among 23 analyzed SBV isolates.

As shown in Fig. 5, the three virus pairs (each unpassaged and ten passages in SK-6 cells) are all located close to each other. The two Belgian SBV isolates Na-1 and Na-2 that had been passaged 10 times in BHK-21 cells were also placed next to each other in the L and M dendrograms, albeit at a greater genetic distance than the SK-6-passaged viruses. An additional indication for the low mutation frequency *in vitro* was the fact that the German reference virus B80/11-4, which had been passaged twice in KC, four times in BHK-21 and once in SK-6 cells (see footnote in Table 1) was still identical to its original sequence derived from viremic serum.

In summary we present data demonstrating that porcine SK-6 cells are highly susceptible to SBV infection and allow a fast and efficient propagation of the virus with a low frequency of introducing mutations in the viral genome. We then analyzed and compared the genomes from *in vitro* and *in vivo* propagated SBV. Based on sequence alignment (not shown) followed by dendrogram construction we conclude that there is no evidence of a directional evolution during the course of the SBV epidemic, and the identified mutations might rather contribute to a quasispecies cloud.

Acknowledgement

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2015.01.010.

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