JOURNAL OF
GENERAL VIROLOGY

SHORT COMMUNICATION
Poskin *et al*., *Journal of General Virology*
DOI 10.1099/jgv.0.000841

MICROBIOLOGY SOCIETY

Genetically stable infectious Schmallenberg virus persists in foetal envelopes of pregnant ewes

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Abstract

Schmallenberg virus (SBV) is a recently emerged vector-borne virus, inducing congenital defects in bovines, ovines and caprines. Here we have shown that infectious SBV is capable of persisting until the moment of birth in the foetal envelopes of ewes infected with SBV-infectious serum at day 45 (1/5 positive) and 60 (4/6 positive) of gestation. This persistence of at least 100 days is a new aspect of the SBV pathogenesis that could help to explain how SBV overwinters the cold season in temperate climate zones. Furthermore, sequencing of the M segment shows that the persisting virus in the foetal envelopes is genetically stable since only a few mutations compared to the inoculum were found. This supports the hypothesis that persisting virus could start the infection of new hosts. Finally, neutralization tests showed that infectious SBV present in the foetal envelopes at birth can be neutralized by the humoral immunity present in the infected ewes.

Schmallenberg virus (SBV) emerged in Europe in the summer of 2011 [1]. SBV belongs to the family *Bunyaviridae*, genus *Orthobunyavirus*, and its genome consists of three segments of single-stranded negative-sense RNA, which are named according to their size: small (S), medium (M) and large (L) [2].

It was first identified in adult cattle showing hyperthermia, drop of milk production and diarrhoea [1]. The virus is also responsible for congenital disease in calves, lambs and kid goats [3–5]. Malformation, abortion and stillbirth were the most frequently observed clinical symptoms, namely in lambs [6]. SBV is transmitted by small haematophagous insects called *Culicoides* [7].

SBV RNA has been detected in organs from experimentally infected adult sheep and cattle for a long time after inoculation [8–10], and SBV RNA was also identified in organs of calves and lambs that were aborted and stillborn under field conditions [11]. In a previously performed *in vivo* infection experiment (Ethical Committee approval number: 121017–01) at our laboratory, 11 ewes were inoculated with an SBV-infectious bovine serum (gift from Martin Beer of the Friedrich-Loeffler Institute containing 2×10^{3} $TCID_{50}$ ml^{-1} SBV) at day 45 (five ewes) and 60 (six ewes) of gestation and kept till the moment of lambing [12]. The ewes gave birth to 17 lambs. At the moment of birth, a total of 31 organs and fluids originating from the ewes (four samples), lambs (six samples) and foetal envelopes (21 samples) were found to be positive for SBV RNA in quantitative reverse transcription PCR (qRT-PCR) [12]. Here, we tested all of these 31 SBV qRT-PCR positive samples (Table 1) in virus isolation to evaluate whether the detected SBV RNA originates from infectious virus or only represents remaining non-infectious viral RNA.

After homogenization of organ samples with the Ultra-Turrax and filtration through 0.8 µm Millipore filters, SBV isolation was attempted on Vero cells grown in six-well plates. The inoculum was incubated with the cells for 2 h, washed away, replaced by 3 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 1000 IU penicillin ml^{-1}, 50 µg ml^{-1} gentamicin, 250 µg ml^{-1} Fungizone amphotericin B and 10 % foetal calf serum and incubated for 4 days. Two or three passages were performed. During each passage, the cell monolayer was analysed for the presence of lysis plaques under the light microscope and SBV isolation was confirmed by qRT-PCR, which was described before [12]. Virus isolation from amniotic fluids and from serum collected at the peak of viraemia was carried out as described above with the exception that those samples were directly added to the Vero cells without homogenization or filtration.

Received 13 December 2016; Accepted 20 May 2017
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Keywords: Schmallenberg virus; sheep; persistent infection; virus isolation; sequencing; overwintering.
Abbreviations: CI, Confidence interval; p.i., post inoculation; qRT-PCR, quantitative reverse transcription PCR; SBV, Schmallenberg virus; VNT, virus neutralization test.
The GenBank/EMBL/DDBJ accession numbers for the M segments of Schmallenberg virus sequenced in this article are KX034187 to KX034203.
One supplementary table is available with the online Supplementary Material.

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Table 1. Study samples and result of virus isolation

In a previously described experimental infection study [12], 11 ewes were inoculated with SBV at day 45 or 60 of gestation. At the moment of birth, organs from all ewes and all 17 born lambs were collected, including different parts of the foetal envelopes. This table provides an overview of those samples from that experiment that are analysed in more detail in this study. It is the serum collected from the ewes at the peak of RNAaemia and all 31 tissues and fluids that tested positive for SBV in qRT-PCR at the moment of birth. Samples originate from ewes (E), lambs (L) or foetal envelopes (FE). Samples that were sequenced were given a code that is used throughout the manuscript. The result of the virus isolation (+, positive; –, negative) is indicated. Also the obtained isolates were given a code (starting with an 'I'), which is used throughout the manuscript.

Day of gestation at SBV inoculation	Ewe no.	Sample	Sample code	SBV isolation	Sample code of isolate
45	1	FE-Amniotic fluid		–	
	2	FE-Umbilical cord		–	
	2	FE-Placentome	2-Pl	–	
	2	FE-Amniotic fluid		–	
	2	L-Lung		–	
	2	FE-Umbilical cord	2-UC	+	I2-UC
	2	E-Serum (5 days p.i.)	2-Se_5	+	I2-Se_5
60	3	E-Ovary		–	
	3	FE-Intercotyledonary membranes		–	
	3	FE-Umbilical cord		–	
	3	FE-Placentome	3-Pl	+	I3-Pl
	3	E-Serum (4 days p.i.)	3-Se_4	+	I3-Se_4
	4	FE-Amniotic fluid		–	
	4	L-Spinal cord		–	
	4	L-Brainstem		–	
	5	E-Ovary		–	
	5	E-Spleen		–	
	5	L-Cartilage		–	
	5	L-Superficial cervical lymph node		–	
	5	L-Muscle		–	
	5	FE-Umbilical cord		–	
	5	FE-Placentome	5-Pl	–	
	5	FE-Amniotic fluid		–	
	5	E-Serum (5 days p.i.)	5-Se_5	+	I5-Se_5
	6	FE-Intercotyledonary membranes		–	
	7	FE-Amniotic fluid		–	
	8	FE-Placentome	8-Pl	–	
	8	FE-Intercotyledonary membranes	8-IM	+	I8-IM
	8	E-Serum (4 days p.i.)	8-Se_4	+	I8-Se_4
	9	FE-Intercotyledonary membranes		–	
	9	FE-Amniotic fluid		–	
	9	FE-Placentome	9-Pl	+	I9-Pl
	9	E-Serum (5 days p.i.)	9-Se_5	+	I9-Se_5
	10	E-Spleen		–	
	10	E-Serum (4 days p.i.)	10-Se_4	NT	
	11	FE-Umbilical cord		–	
	11	FE-Placentome	11-Pl	+	I11-Pl
	11	E-Serum (5 days p.i.)	11-Se_5	+	I11-Se_5

days p.i., days post inoculation; NT, not tested.

None of the four and six SBV RNA positive organs of ewes and lambs, respectively, was found to be positive in virus isolation (Table 1). This suggests that SBV RNA detected in maternal organs collected at 90 and 105 days post-inocualtion (p.i.) probably represents remaining non-infectious viral RNA. The incapability to isolate SBV from foetal tissues is in contrast with earlier successful isolations of SBV from brain material of newborn malformed lambs [13, 14] and can probably be explained by the small number of only low positive SBV RNA foetal tissues found in the current experiment.

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In contrast, five isolates were obtained from 21 samples belonging to the foetal envelopes (Table 1), showing the capacity of SBV to persist for at least 100 days in pregnant ewes. One isolate (I2-UC) was obtained from an umbilical cord (sample 2-UC) of a ewe inoculated at day 45 of gestation (sample collected at 105 days p.i.). In the group of ewes inoculated at day 60 of pregnancy (samples collected at 90 days p.i.), three SBV isolates (I3-Pl, I9-Pl, I11-Pl) were obtained from the placentome (samples 3-Pl, 9-Pl, 11Pl) of three ewes and one (I8-IM) from the intercotyledonary membranes (sample 8-IM) of another ewe (Table 1). Therefore, SBV isolation seems more successful from foetal envelopes of ewes inoculated with SBV at day 60 of pregnancy (4/6; 67 %) than on day 45 of pregnancy (1/5; 20 %), although this was not statistically significant (Fisher's exact test; P=0.24). Since this persistence in foetal envelopes occurred in the presence of a strong neutralizing humoral immune response [12], it further supports the role of the placenta as an immunosuppressed zone where different immune suppressive mechanisms prevent the foetus from being rejected by the maternal immune system. Several mechanisms have been described to contribute hereto: the placental barrier function, the absorption or blocking of noxious antibodies or the synthesis of non-specific systemic and local suppressor factor-like immunosuppressive proteins [15].

No infectious SBV could be recovered from the amniotic fluids. This seems to be in line with the observation that the mean SBV RNA copy number is a minimum of 1000-fold lower in amniotic fluids (3.06×10^5) than in umbilical cords (4.69×10^8), placentomes (4.83×10^9) and intercotyledonary membranes (1.44×10^9) (Kruskal–Wallis test; P=0.014). Although the incapability to isolate SBV from the amniotic fluid suggests that this externally shed fluid does not represent an important risk for virus transmission, we advocate that more research is performed regarding this subject before this is accepted as a general conclusion. Other studies have reported much higher viral RNA loads in amniotic fluids of field-aborted lambs than those found in our study [16].

SBV isolates (I2-Se5, I3-Se_4, I8-Se_4, I9-Se_5, I11-Se_5) were also obtained from the sera collected at the peak of RNAaemia (4 or 5 days p.i.) from the five ewes from which SBV was successfully isolated from the foetal envelopes (Table 1) and used in experiments described further on in this manuscript.

Previous studies have shown that the S and L segment of SBV strains found at different locations and moments in time are highly genetically stable [2, 14, 17–21]. More genetic variability has however been found in the M segment of the virus. This segment encodes a polyprotein precursor that is later cleaved into the non-structural (NSm) protein and the two envelope glycoproteins named Gn and Gc [22]. Several authors even identified a hypervariable region in the coding sequence of the putative Gc protein based on sequencing studies performed on organ and brain samples collected from aborted lambs and calves [2, 14, 17]. It was hypothesized that this hypervariable region might be important for the evasion of the host immunity [2].

Therefore, we next analysed whether the persisting virus in the foetal envelopes had mutated between the moment of inoculation and the moment of birth. We sequenced the M segment of (1) the inoculum, (2) viral RNA present in serum at the peak of RNaemia, (3) viral RNA present in placentomes at birth and (4) the virus isolates obtained from placentomes. Sequencing was performed using methods described in Fischer *et al.* [2] and using primers described in Coupeau *et al.* [17]. Sequences were submitted to GenBank and received accession numbers KX034187 to KX034203. The positions of the nucleotide (nt) and the predicted amino acid (aa) sequences were aligned with the complete M segment sequence published by Hoffmann and colleagues [1] (GenBank accession number HE649913.1).

Overall, SBV RNA present in serum collected at the peak of RNAaemia and in placentomes at the moment of birth showed a high sequence stability of the M segment. A mean homology of 99.92 % [95 % confidence interval (CI) =99.89 –99.95 %] with the inoculum was observed (Fig. 1 and Table S1, available in the online Supplementary Material). One to five nucleotide substitutions compared to the inoculum were found in SBV RNA present in the serum samples collected at the peak of RNAaemia from seven ewes. In three out of seven samples (3-Se_4, 10-Se_4, 11-Se_5) the corresponding aa sequence was completely homologous to the inoculum, while in three (2-Se_5, 5-Se_5, 9-Se_5) and one (8-Se_4) other serum samples, respectively, these nucleotide substitutions resulted in one and four aa substitutions. After persistence in the placentomes for between 90 and 105 days, between two and nine nucleotide substitutions compared to the inoculum were found in the M segment of SBV RNA present in the six placentome samples. In four out of six placentome samples (3-Pl, 8-Pl, 9-Pl, 11-Pl), these nucleotide substitutions did not result in any predicted aa sequence change compared to the inoculum.

Taken together, these sequencing results show that the persisting virus in foetal envelopes is highly stable since only a few mutations compared to the initial inoculum were found. Therefore, the previously reported high number of mutations found in the hypervariable region of the M segment of SBV isolates obtained from the brain tissue of malformed lambs [2, 17] are probably not acquired during the virus passage in the ewe or foetal envelopes. Our results suggest that it is more probable that those mutations might either be requisite to allow crossing of transplacental barriers and/or accumulate during the successive replication cycles in different foetal tissues before entering the brain of developing lambs. They might also be acquired during replication within the brain tissue of the lamb to evade the local immune pressure in that compartment. The genetic stability of the persisting virus is also in line with the results of Wernike *et al.* [20] who described a high genetic homology in all segments, including the M segment, between SBV strains present in field-collected blood samples after its first

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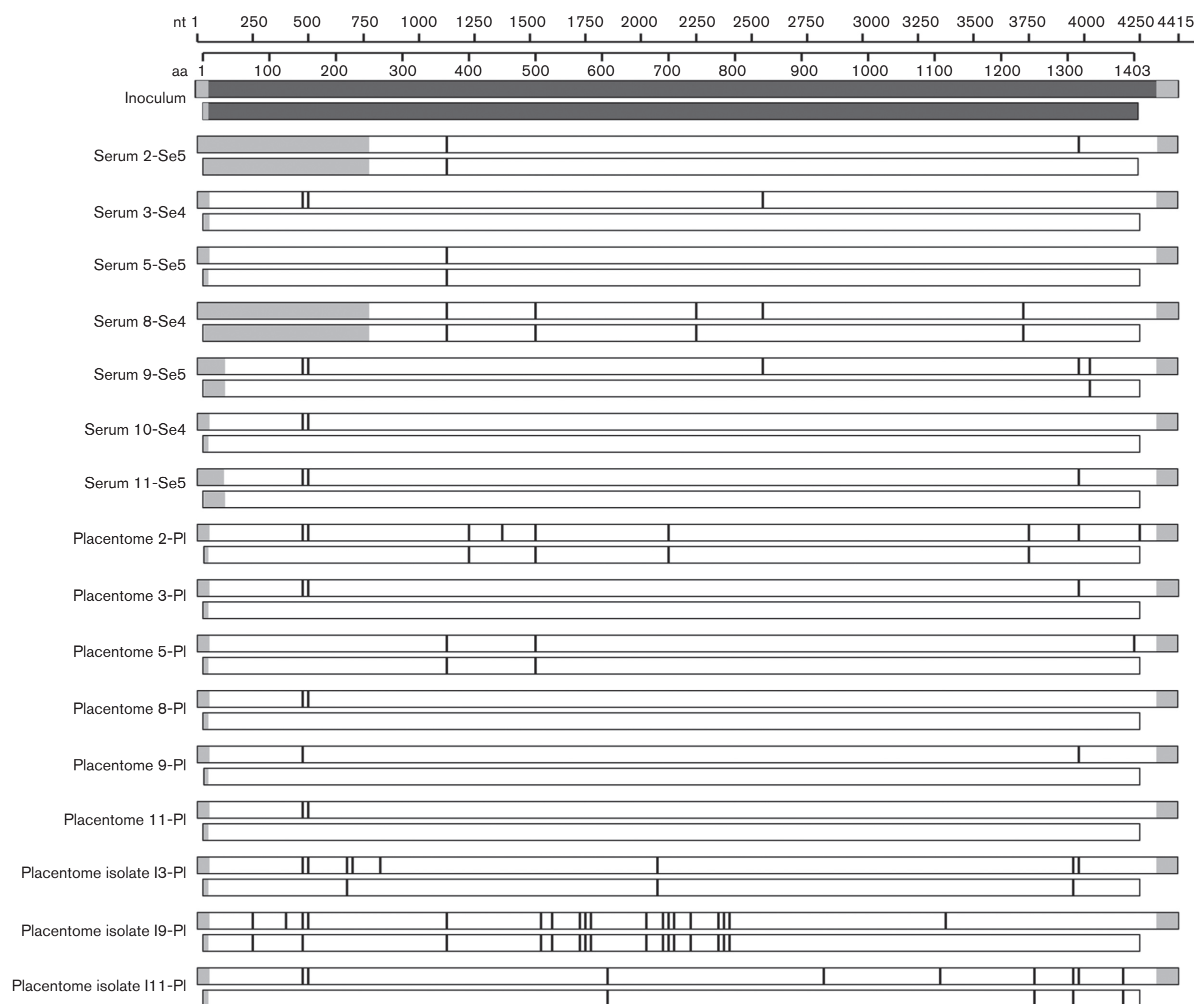

Fig. 1. RNA and aa alignment of the SBV M segment. The RNA (upper bar associated with each sample) and corresponding aa (lower bar associated with each sample) sequences of the M segment of SBV present in the initial inoculum, in the serum at the peak of viremia, in placentomes at the moment of birth and in SBV isolates from placentomes were aligned and mutations compared to the sequence of the initial inoculum are indicated by vertical black lines. Nucleotide (nt) and aa positions are predicted based on the complete M segment sequence HE649913.1 [1]. No sequences were obtained for areas indicated by a light grey colour.

emergence in 2011 and its renewed circulation in 2014 [20]. This is probably caused by the fact that SBV needs to replicate in both insect and mammalian hosts, meaning that only mutations that do not impact its fitness in both hosts might be maintained. This phenomenon was described before for West Nile and dengue viruses, which exhibit a lower than predicted mutation rate [23–26].

Interestingly, all three isolates that were obtained from placentomes (I3-Pl, I9-Pl, I11-PL) originated from samples (3-Pl, 9-Pl, 11-Pl) containing SBV that had an aa sequence of the M segment that was 100 % homologous to the inoculum. Passaging the virus four times in Vero cells during the isolation process had, however, a variable influence on the sequence of the M segment. Two isolates acquired eight (I3-Pl) and nine (I11-Pl) nucleotide substitutions, respectively, leading to three and four aa substitutions. The third isolate (I9-Pl) was more severely affected and acquired 19 nucleotide substitutions, leading to 16 aa changes in the predicted protein sequence. In total, 14 of the 16 aa changes were located in the previously defined hypervariable region of the M segment. This finding agrees with a previous report showing that repeated passaging of SBV in BHK-21 cells leads to an accumulation of mutations in the hypervariable region of the M segment [17]. Hofmann *et al.* [19] reported that this might be overcome by performing SBV isolations in porcine SK-6 cells.

In a final experiment, it was evaluated whether the induced humoral immunity upon SBV infection was capable of neutralizing the infectious SBV that was recovered from the placenta at the moment of birth (being at 90 or 105 days after infection) in order to assess whether this persisting virus would be capable of escaping the neutralizing immune

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response upon renewed release in the circulation. In this respect, the capacity of serum collected at 35 days p.i. (samples 3-Se_{35}, 8-Se_{35}, 9-Se_{35}, 11-Se_{35}) and 90 days p.i. (samples 3-Se_{90}, 8-Se_{90}, 9-Se_{90}, 11-Se_{90}) to neutralize SBV isolated from the placentome (samples I3-Pl, I9-Pl and I11-Pl), intercotyledonary membranes (I8-IM) and serum at the peak of RNAaemia (I3-Se_4, I8-Se_4, I9-Se_5, I11-Se_5) from the corresponding animal was determined using a virus neutralization test (VNT). The VNT titres for the different combinations of serum (35 or 90 days p.i.) and SBV isolates (serum or organ) were determined in triplicate, as previously described [11].

The VNT titres measured in serum collected at 35 and 90 days p.i. against the SBV isolates obtained from serum at the peak of RNAaemia and from the foetal envelopes of four ewes were always positive ($\geq$4), suggesting that the infectious virus present in the foetal envelopes at birth would be neutralized when released in the bloodstream. The mean $\log_2$ titre for the VNT conducted with the serum isolates was 3.5 at 35 days p.i. and 4.7 at 90 days p.i. The mean $\log_2$ titre for the VNT conducted with the placentome isolates was 3.5 at 35 days p.i. and 4.8 at 90 days p.i. (Fig. 2). The use of a linear mixed model to estimate the mean effect of time on the SBV-specific antibody response showed that the VNT titres measured in serum collected at 90 days p.i. were significantly higher than those found in serum collected at 35 days p.i., and this against both serum (P=0.007) and placentome (P=0.004) isolates. This probably reflects the well-known affinity maturation of the humoral immune response [27].

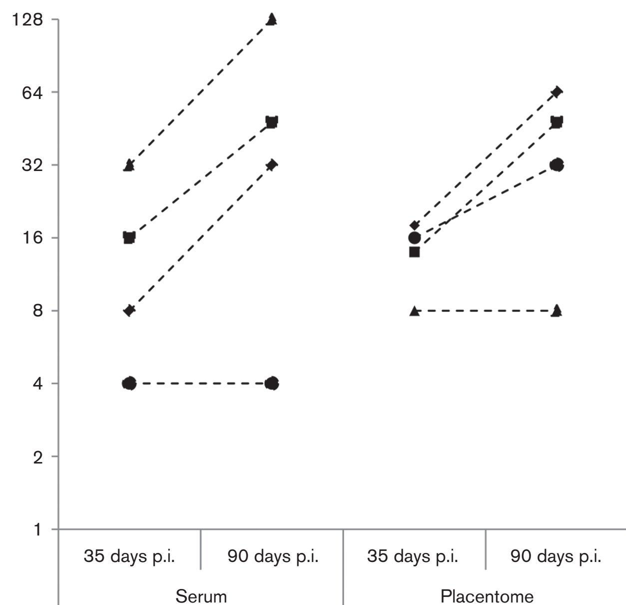

Fig. 2. Neutralizing capacity of serum collected at different time points after SBV inoculation of pregnant sheep. The neutralizing antibody titre of serum collected from four ewes at 35 and 90 days p.i. was determined against SBV isolates originating from those particular ewes. For each ewe, one isolate originated from the serum collected at the peak of viraemia and the other from the placentome collected at birth.

The most challenging question related to these results is whether the persisting virus in the placentomes could play a role in the transmission and overwintering of SBV. This will have to be evaluated in future studies, but several hypotheses can be formulated on how this persistent virus might perpetuate infection. A first hypothesis could be that infected placentas serve directly or indirectly as a food source for overwintering *Culicoides* larvae after being discarded on dung heaps, a preferred *Culicoides* larval habitat [28–30]. Another hypothesis could be that unprotected seronegative ewes could become infected after contact with infected placental material. Previous studies have, however, shown that oral inoculation of sheep and cattle with SBV-containing fluids was unsuccessful in transmitting infection under experimental conditions [9, 31], making this route of transmission rather unlikely. Disease transmission after uptake of infected placental or foetal material has already been reported for bluetongue virus, another *Culiciodes*-borne virus [32, 33], but more studies will be necessary to evaluate this for SBV. Other options for overwintering that cannot be excluded at this point are the potential role for immature or adult vectors, other than *Culicoides,* to transmit SBV after feeding on infected foetal envelopes, or the existence of viraemic newborn lambs that could serve as a food source for adult *Culicoides*.

Funding information
This study was financially supported by Federal Public Service Public Health and Safety of the Food Chain and Environment (RF12/6270) and the European Union as outlined in Council Decision 2012/349/EU concerning a financial contribution by the Union for studies on Schmallenberg virus. Funding agencies had no role in study design or manuscript preparation.

Acknowledgements
All the authors would like to thank Dennis Kozlowski, Virginie Colasse, Ikram Azzouz and Sarah Matuyia for their excellent technical assistance in this work.

Conflicts of interest
The authors declare that there are no conflicts of interest. Y. V. d. S. is currently employed with the European Food Safety Authority (EFSA) in the BIOCONTAM Unit that provides scientific and administrative support to EFSA's scientific activities in the area of Microbial Risk Assessment. The positions and opinions present in this article are those of the authors alone and are not intended to represent the views or scientific works of EFSA.

Ethical statement
This study uses material collected during an experimental animal infection study that was previously published (Martinelle et al. [12]). That study was approved by the Joined Ethical Committee of CODA-CERVA and the Institute of Public Health Belgium (Ethical Committee approval number: 121017–01).

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