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Schmallenberg virus affects T-bet, Gata3, RoRγt, Foxp3 and Eomes in mice brain

E. AKSOY, A. K. AZKUR*

Department of Virology, Faculty of Veterinary Medicine, Kirikkale University, 71450 Yahsihan, Kirikkale, Turkey

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Summary. – Schmallenberg virus (SBV), a neurotropic member of the genus *Orthobunyavirus*, infects ruminants and causes neurological lesions and fetal malformations including cerebellar hypoplasia, hydranencephaly, and porencephaly. The aim of this study is to establish intracerebral (i.c.) infection of SBV in newborn BALB/c mice and to investigate some of the transcription factors in brain. For this aim, brain samples of newborn BALB/c mice which were infected with SBV i.c. were analyzed by plaque titration and real-time RT-PCR for T-bet, Gata3, RoRγt, Foxp3 and Eomes mRNA levels. Study results showed that SBV can replicate in BALB/c mice brain and cause death of newborn mice with generation of infectious viral particles. Analyses of transcription factor mRNA levels indicated up-regulation of T-bet, Gata3, RoRγt, Foxp3 and down-regulation of Eomes. In this report, we introduce preliminary data of T cell transcription factors affected by SBV infection of BALB/c mice.

Keywords: Eomes; Foxp3; Gata3; RoRγt; Schmallenberg virus; T-bet

Introduction

Schmallenberg virus (SBV), first reported in Germany in 2011, is a member of Simbu serogroup of the *Orthobunyavirus* genus in the *Peribunyaviridae* family and is transmitted by *Culicoides* spp. or by semen of infected animals. SBV has a tripartite negative-sense RNA genome which encodes four structural and two non-structural proteins (Hoffmann *et al.,* 2012; Doceul *et al.,* 2013). SBV infection is reported in cattle, sheep, goat, Anatolian water buffalo, and wild ruminants (Azkur *et al.,* 2013a; Doceul *et al.,* 2013). SBV causes a range of clinical symptoms including decrease in milk yield, fever, diarrhea, abortions, and congenital malformations in central nervous system (Hoffmann *et al.,* 2012; Herder *et al.,* 2012).

Viral infection induces $CD4^+$ T cell response in hosts with contribution of different T cell subsets which undergo division and differentiation controlled by unique transcription factors, such as T-bet (T-box transcripton factor), Gata3 (GATA binding protein 3), RoRγt (retinoic-acid-receptor-related orphan nuclear receptor γt), Foxp3 (Forkhead box protein 3) and Eomes (Eomesodermin) (Swain *et al.,* 2012). T-bet and Gata3 are necessary for generation and differentiation of Th1 and Th2 cells, respectively (Swain *et al.,* 2012; Sato *et al.,* 2017). RoRγt has crucial function in differentiation of Th17 while Foxp3 is the master transcription factor of regulatory T cells (Treg) which maintain immunotolerance and play role in controlling viral immunological lesions, such as in herpes simplex virus infection (Suvas *et al.,* 2004). Eomes plays role in cytolytic $CD4^+$ T cell development, and natural killer (NK) maturation (Swain *et al.,* 2012). There is paucity of information on whether transcription factors of these immune cells are increased or decreased upon SBV infection.

NIH-Swiss mice had been used for pathogenicity, pathogenesis and tropism of SBV (Varela *et al.,* 2013, 2016), while $IFNAR^{-/-}$ mice, on C57BL/6 or 129/SV genetic backgrounds, had been used for vaccine development studies, pathogenicity and pathogenesis experiments to date (Kraatz *et al.,* 2015;

*Corresponding author. E-mail: azkurkursat@hotmail.com, kursatazkur@kku.edu.tr; phone: +90318-357-4242/6487.

Abbreviations: dpi = days post-infection; Eomes = eomesodermin; Foxp3 = forkhead box protein 3; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; Gata3 = GATA binding protein 3; i.c. = intracebral(ly); NK = natural killer cells; RoRγt = retinoic acid receptor-related orphan receptor-γt; SBV = Schmallenberg virus; T-bet = T-box expressed in T cells; Th = helper T cell; Treg = regulatory T cell

Table 1. Primer sets used for amplification of SBV S and M segments and mouse *GAPDH*, *T-bet*, *Gata3*, *RoRγt*, *Foxp3* and *Eomes* genes

Gene	Primer sequence (5'→3')	Product	Reference
SBV S	F: TCAGATTGTCATGCCCCTTGC R: TTCGGCCCCAGGTGCAAATC	89 bp	Bilk *et al.,* 2012
SBV M	F: GCACTCCCACTTAAGGAAGG R: GCCAACCGATCTCCTGATTT	165 bp	Present study
GAPDH	F: CATCCTGCACCACCAACTGCTTAG R: GCCTGCTTCACCACCTTCTTGATG	430 bp	Azkur *et al.,* 2005
T-bet	F: CATGCCAGGGAACCGCTTAT R: TTGGAAGCCCCCTTGTTGTT	117 bp	Present study
Gata3	F: AGCTGTCTGCGAACACTGAG R: CCGATCACCTGAGTAGCAAGG	204 bp	Present study
RoRγt	F: CAGAGGAAGTGTCAGAGGCT R: TGCAAATGTGAAGTGCCAGC	198 bp	Present study
Foxp3	F: GGCCCTTCTCCAGGACAGA R: GCTGATCATGGCTGGGTTGT	112 bp	Suvas *et al.,* 2004
Eomes	F: GGAAGTGACAGAGGACGGTG R: AGCCGTGTACATGGAATCGT	184 bp	Present study

Varela *et al.,* 2013, 2016; Wernike *et al.,* 2012). BALB/c mice infection was only used for monoclonal antibody production against SBV (Zhang *et al.,* 2013). There is no infection model established in BALB/c mice for intracerebral SBV infection, and no data about titer of infectious viral particles of SBV generated in mice brain determined by plaque titration. In this study intracerebral SBV infection of newborn BALB/c mice was investigated and expression analyses of T-bet, Gata3, RoRγt, Foxp3 and Eomes transcription factors were carried out to understand the effects of SBV on immune cell response following intracerebral infection.

Materials and Methods

Cell line and virus. Vero cell line (ATCC® CCL-81) was maintained in Dulbecco's modified Eagle's medium (DMEM, Capricorn Scientific, Germany) with 10% fetal bovine serum in incubator at 37°C with 5% CO_2. Schmallenberg virus was kindly provided by Dr. Wim H. M. van der Poel (Wageningen University, Netherlands) and was propagated in Vero cells.

Plaque titration assay. Plaque titration of SBV from infected brain samples obtained from mice was performed in Vero cell line. Briefly, Vero cells were seeded in 24-well cell culture plates and cells were infected with SBV or brain sample dilutions. After 3 h incubation of virus, medium was removed and 1:1 mixture of 2× DMEM and 2% carboxymethyl cellulose (CMC) were dispersed to all wells. Following 6 days of incubation, cells were fixed by 10% neutral buffered formalin (Sigma, USA) and stained with crystal violet (Aksoy and Azkur, 2019).

Mice experiments. BALB/c mice provided by Kobay Experimental Animal Laboratory (Ankara, Turkey) were maintained in Faculty of Veterinary Medicine in Kirikkale University with ethical approval number 16/79 from Kirikkale University Local Ethics Committee for Animal Experiments. Anesthesia for infection of newborn mice was accomplished by putting each newborn mouse on crushed ice covered with aluminum foil for 2–3 min and each mouse was observed for skin color change to slightly blue. Twelve mice were inoculated with SBV and control mouse inoculated with sterile PBS intracerebrally (i.c.) with insulin syringe and 31G needle (Becton Dickinson, USA). Seven days old newborn BALB/c mice were infected i.c. with SBV and were euthanized at 1, 2, 3, and 4 days post-infection (dpi). Brain samples of infected and control mice were collected, homogenized and kept at -80°C until further use.

RNA isolation and reverse transcription. RNA samples were isolated from cells using High pure RNA kit (Roche, Germany) according to the manufacturer´s instructions. Reverse transcription of RNA samples was performed as described elsewhere (Azkur *et al.,* 2013b).

Real-time PCR. Real-time PCR amplification was performed with SYBR Green master mix (Roche, Germany) using primers specific for SBV S and M segments, T-bet, Gata3, RoRγt, Foxp3, Eomes transcription factors and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All primer sets used in present study are listed in Table 1 and real-time PCR amplification profiles and the formula are provided in Supplementary Table S1.

Statistical analyses. Real-time PCR data of transcription factors were analyzed with ANOVA test in IBM SPSS Statistics 21 software. Results were considered as statistically significant at $p < 0.05$. Graphics were created with GraphPad Prism software and Microsoft Paint.

Results and Discussion

SBV was discovered as an agent that causes aborts, stillbirths and fetal malformations and its genome is detected in

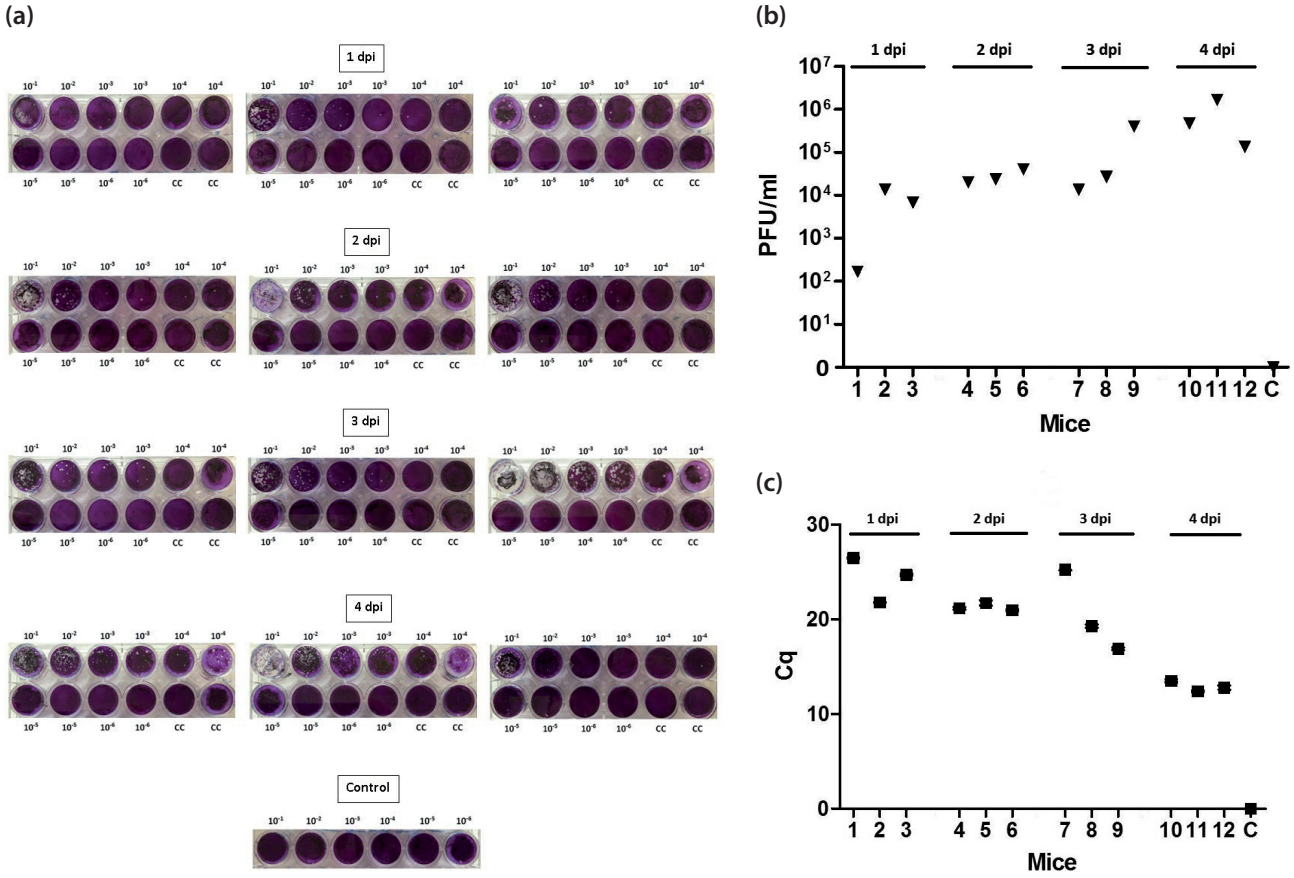

Fig. 1

Plaque titration assay and real-time PCR of mice samples infected with SBV

(a) Plaque titration assay of samples from infected and control mice. Plaques were visualized by staining with crystal violet. CC – cell control. **(b)** Plaque titers of SBV from brain samples of mice. C – control mouse. Each mouse brain sample was titrated and PFU/ml calculated with formula of PFU/ml = (average number of plaques) / (dilution factor) × (volume of inoculum in ml). **(c)** Real-time PCR results of SBV S segment from brain samples of mice. C – control mouse.

brain samples of infected ruminants (Bilk *et al.,* 2012; Herder *et al.,* 2012). Because central nervous system pathology experiments are complicated to study in ruminants, experimental mice models for SBV infection and pathogenesis have been developed in IFNAR$^{-/-}$ and NIH-Swiss mice (Kraatz *et al.,* 2015; Varela *et al.,* 2013, 2016; Wernike *et al.,* 2012). In present study, BALB/c mice susceptibility to SBV infection and SBV replication in mice brain were investigated. As it is known, viral virulence is affected by features of both host and the virus, and results of experimental studies on mice can depend on mouse strain, age, immunocompetence, viral isolate, titer of virus and more (Henderson *et al.,* 2015). In the present study, in first experiment, 2–5 days old BALB/c newborns were infected via i.c. route with SBV, however, they did not survive. In the second experiment, 7 days old suckling BALB/c mice were tested. We have found that 2–5 days old suckling BALB/c mice were more susceptible to i.c. inoculation of SBV than 7 days old mice. Seven days old mice survived for 4 days after i.c. SBV infection. Therefore, ideal SBV infection model was established in 7 days old BALB/c mice. Varela *et al.* (2016) used NIH-Swiss strain and 5 and 11 days old age groups, infected with SBV serially passaged in CPT-Tert cell line, whereas different SBV strains propagated on Vero cells were inoculated to 2 to 7 days old BALB/c strain in the present study. In the present study 2 days old BALB/c mice died after 1 day post infection, whereas 2 days old NIH-Swiss mice died at 8 dpi (Varela *et al.,* 2013). Early death and shortened survival period of mice can be explained by infection of different mice strains, inoculation with different SBV isolates and titer of the virus. SBV used in present study efficiently grew and was passaged in Vero cell line, while Varela *et al.* (2016) used CPT-Tert cell line for SBV propagation. The cell line selection could influence the virulence and possible mutations on SBV genome that

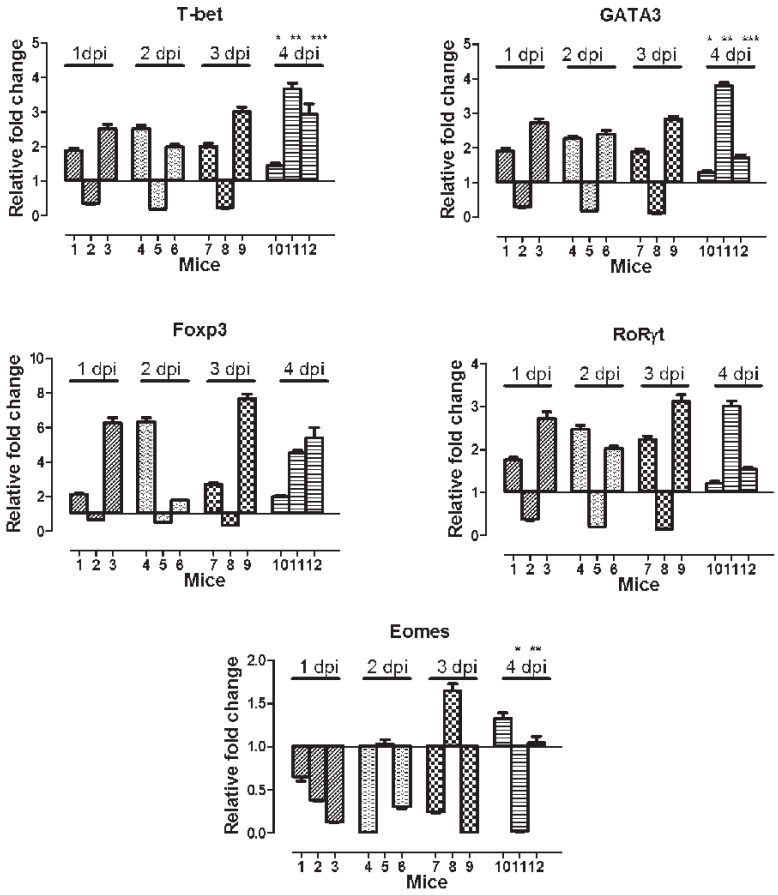

Fig. 2

mRNA relative fold changes of transcription factors T-bet, Gata3, RoRγt, Foxp3 and Eomes

All relative fold changes were normalized to GAPDH reference gene and calculated with $2^{-\Delta\Delta Cq}$ formulation (Schmittgen and Livak 2008). *indicates statistical significance between 1 dpi and 4 dpi groups; **indicates statistical significance between 2 dpi and 4 dpi groups; ***indicates statistical significance between 3 dpi and 4 dpi groups (p <0.05).

increase or decrease the pathology in mice brain. Varela *et al.* (2016) investigated mutations on SBV segments but we did not analyse SBV genome sequence for any changes that affect the virulence. The present study shows that SBV which is propagated in Vero cell line can replicate and generate infectious viral particles in mouse brain.

Standard techniques for detection of infectious viral particles are viral titration or plaque titration assays. PCR or immunohistochemistry which are used in previous studies for SBV (Varela *et al.,* 2013, 2016; Wernike *et al.,* 2012) are not suitable for detection of infectious viral particles. It is known that infectious viral particles can only be detected by viral titration assays, not by PCR assays which can detect only number of viral genomes. Thus, in the present study, to address whether SBV replicates and generates infectious particles, plaque titration was performed with brain samples of newborn mice at 1, 2, 3, and 4 dpi and control mouse brain samples. Mice infected with SBV were positive on plaque formation with different titers except for control mouse which showed no plaque (Fig. 1a). Plaque titers of SBV obtained from mice are shown in Fig. 1b. Plaque titration results showed that SBV replicates in mice brain. Viral titer was gradually increased upon infection and the highest titer of 1.6×10^6 PFU/ml at 4 dpi was observed (Fig. 1b). Tauscher *et al.* (2017) reported that infectious virus was isolated from IFNAR$^{-/-}$ mice, however without titer determination for SBV. Because there is no data about production of infectious virus particles and viral titer in SBV infected BALB/c mice, in this study, we have demonstrated that newborn BALB/c mice are susceptible to SBV infection and SBV generates infectious viral particles in BALB/c mice brain which could be detected by plaque titration.

To confirm SBV genome presence in mice brain, samples were analyzed with RT-PCR (Supplementary Fig. S1) and

real-time PCR for SBV S (Fig. 1c, Supplementary Fig. S2) and M segments (data not shown). Compatible with the results from plaque titration, all SBV infected mice brain samples were positive for both S and M segments in real-time PCR. Mock infected mice samples were negative in real-time PCR for both SBV S and M segments. To date, SBV viral genome equivalents in brain samples were quantified by qRT-PCR (Varela *et al.,* 2016), but not with plaque assay, as shown in the present study. We have found that the SBV genome content in the brain increased very significantly over a 3 days period (Fig. 1c). Similar result achieved by Varela *et al.* (2016) is compatible with the results of the present study showing gradually increasing levels of both viral genome and infectious viral particles. Unlike in the present study, Varela *et al.* (2016) has not tested infectious viral particles that were generated *in vivo* in mice brain.

Infection route is one of the determinants of pathogenesis in experimental models. Previous studies showed that SBV genome copies had been detected in blood, brain, lungs, liver, spleen and small intestine after infection of adult IFNAR$^{-/-}$ mice with subcutaneous injection of SBV (Wernike *et al.,* 2012). But there was no information about presence of viral genome after i.c. infection of newborn BALB/c mice (Supplementary Fig. S1). In this study, only i.c. route of infection was carried out in newborn BALB/c mice and all samples but control were positive for SBV RNA by real-time RT-PCR in the brain (Fig. 1c).

In viral infections, T cell response contributes by many functions like viral clearance, immunopathogenesis, recovery and protection. Unique transcription factor sets direct the differentiation of these CD4$^+$ T cell subsets (Swain *et al.,* 2012). To investigate whether SBV infection in brain affects T-bet, Gata3, RoRγt, Foxp3, Eomes transcription factors, we have analyzed mRNA levels and relative fold changes of gene expression normalized against GAPDH reference gene. Expression analyses of transcription factors demonstrated upregulation of T-bet, Gata3, RoRγt and Foxp3 and downregulation of Eomes in SBV infected mice brains. T-bet and Gata3 expressions were gradually increased and reached maximum level at 4 dpi as statistically significant (p <0.05) in comparison to 1, 2 and 3 dpi. Foxp3 and RoRγt were upregulated, however, there was no statistically significant difference determined in expression levels between infection days. Eomes was downregulated and expression levels at 4 dpi statistically differ from 1 and 2 dpi but not from 3 dpi (Fig. 2). These results indicate, for the first time for SBV, that SBV infection can lead to an increase of transcription factors of Th1, Th2, Th17 and Treg cells. A study of Herder *et al.* (2013) showed that CD3-positive T cells were dominating inflammatory cell type in central nervous system tissues of SBV infected neonatal ruminants. The other cells detected in tissues were CD79α-positive B cells and CD68-positive microglia/macrophages (Herder *et al.,* 2013). In present study, we assume that T cell subsets infiltrate the central nervous system upon SBV infection, however, further analyses for immunophenotyping and activation of T cell subsets in SBV infection of mice should be investigated in future. There is also need to investigate whether SBV infection has effect on T cell transcription factors or whether transcription factors of T cell subsets regulate the SBV replication.

To our knowledge, this is the first report for SBV, to prove the presence of infectious viral particles and to determine its titers in mice brain. We have also, for the first time, provided first preliminary data about effects of SBV on some transcription factors of T cell subsets in mice brain. We have established new experimental model for SBV infection in BALB/c mice strain which could be used for further pathogenesis studies of SBV.

Supplementary information is available in the online version of the paper.

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Supplementary information

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E. AKSOY, A. K. AZKUR*

Department of Virology, Faculty of Veterinary Medicine, Kirikkale University, 71450 Yahsihan, Kirikkale, Turkey

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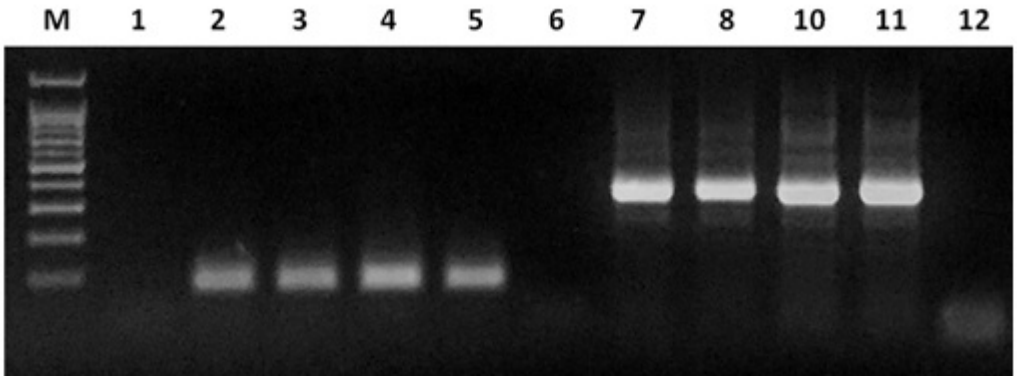

Fig. S1

RT-PCR of Schmallenberg virus S segment from mice samples.
1-6: SBV S segment specific RT-PCR; 7-12: mouse GAPDH specific RT-PCR. Lines 1 and 7: mock infected mouse; Lines 2-4 and lines 8-11: SBV infected mice 4 dpi. Line 5: plasmid containing SBV S segment (positive control). Lines 6 and 12: no-template controls. M: DNA Marker (Bioron, Germany)

*Corresponding author. E-mail: azkurkursat@hotmail.com, kursatazkur@kku.edu.tr; phone: +90318-357-4242/6487.

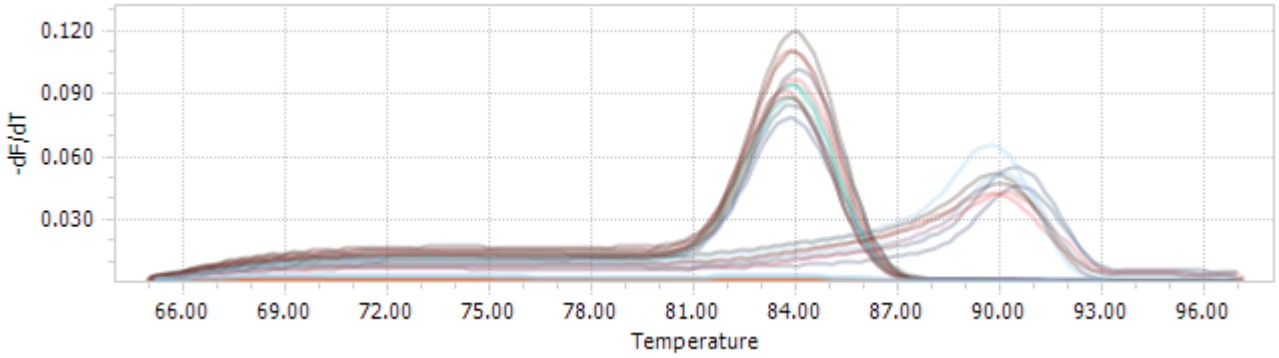

Fig. S2

Melting temperature (Tm) analysis of Schmallenberg virus S segment from mice samples
Tm values of S segment were 83.89°C and GAPDH was 90.05°C. Melting temperature results were evaluated with LightCycler 96 SW1.1 software.

Table S1. Real-time PCR amplification profiles for SBV S and M segments, mouse *GAPDH*, *T-bet*, *Gata3*, *RoRyt*, *Foxp3* and *Eomes* genes

Amplified genes	Amplification profile
SBV S SBV M	10 min at 95°C followed by 38 cycles of 10 s at 95°C, 10 s at 55°C, 10 s at 72°C, and melting
GAPDH *T-bet* *Gata3* *RoRyt* *Foxp3* *Eomes*	10 min at 95°C followed by 38 cycles of 10 s at 95°C, 10 s at 57°C, 10 s at 72°C, and melting

All real-time PCR assays were performed in LightCycler 96 real-time PCR instrument (Roche, Germany) and results were evaluated with LightCycler 96 SW1.1 software. Relative fold change of the transcription factors was calculated with $2^{-(\text{Cq gene of interest} - \text{Cq reference gene})\text{ infected sample} - (\text{Cq gene of interest} - \text{Cq reference gene})\text{ noninfected sample}}$ formulation (Schmittgen and Livak, 2008) with normalization against GAPDH reference gene.