Japanese Journal of Veterinary Research 72(2): 65–70, 2024

JJVR

SHORT COMMUNICATION Experimental Research

Mouse oligoadenylate synthetase 1b inhibits West Nile virus replication by acting through the stem-loop 2 in the 3′-untranslated region

Keisuke Sato[1)], Teppei Nakamura[1)], Masami Morimatsu[1)] and Takashi Agui[1,*)]

[1)] Laboratory of Laboratory Animal Science and Medicine, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

Received for publication, March 10, 2024; accepted, August 1, 2024

Abstract

Oligoadenylate synthetase (OAS) plays a role in intracellular viral defense. Mouse *Oas1b* is responsible for the susceptibility to flaviviruses. The flavivirus replication inhibitory effect of Oas1b is independent of its enzymatic activity; however, its mechanism remains unclear, though mouse Oas1b possibly targets the 3′-stem-loops (SLs). We measured the effect of Oas1b by removing and overexpressing SLs in the 3′-untranslated regions (UTRs) of the West Nile virus (WNV) replicon. Both SL2 deletion and SL2 RNA overexpression in the 3′-UTR of the WNV replicon resulted in the loss of Oas1b-mediated replication inhibition. These results revealed that WNV SL2 is essential for Oas1b-mediated replication inhibition.

Key Words: Inhibition of virus replication, Oligoadenylate synthetase, West Nile virus

Oligoadenylate synthetase (OAS), which plays a role in intracellular viral defense, is conserved in many animal species and activated by the recognition of double-stranded RNA that enters the cell and polymerizes ATP to synthesize oligoadenylates[10,24)]. Oligoadenylates dimerize RNase L into its active form, resulting in random cleavage of single-stranded RNA[11)]. RNA-sensing proteins, such as MDA5 and RIG-I, recognized fragmented RNA and induces interferon (IFN) production. Because *OAS* is an interferon-stimulated gene (ISG), the OAS/RNase L pathway is further amplified. Thus, OAS plays a role as both sensor and enhancer of viral defense in cells.

Most laboratory mice are susceptible to West Nile virus (WNV) infection, whereas wild mice are resistant. Mouse strains derived from wild mouse such as MSM/Ms, are also resistant. Positional cloning has identified that *Oas1b* is responsible for the susceptibility or resistance[14,15,19)]. The mouse Oas family consists of 12 members, *Oas1a–h*, *Oas2*, *Oas3*, *OasL1*, and *OasL2*, but only *Oas1b* is responsible for the susceptibility or resistance to WNV[6,8,20)]. Oas1b sequence comparison has revealed that truncated Oas1b is translated in most laboratory mice due to a nonsense mutation in the fourth exon. Functional analysis of Oas1b showed that it cannot bind to double-stranded RNA and has no oligoadenylate synthetase activity[5,9)]. Furthermore, RNase L-knockout mice are resistant to WNV infection, suggesting that the resistance by Oas1b does not involve the OAS/

* Corresponding author: Takashi Agui
Laboratory of Laboratory Animal Science and Medicine, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan
E-mail address: agui@vetmed.hokudai.ac.jp
doi: 10.57494/jjvr.72.2_65

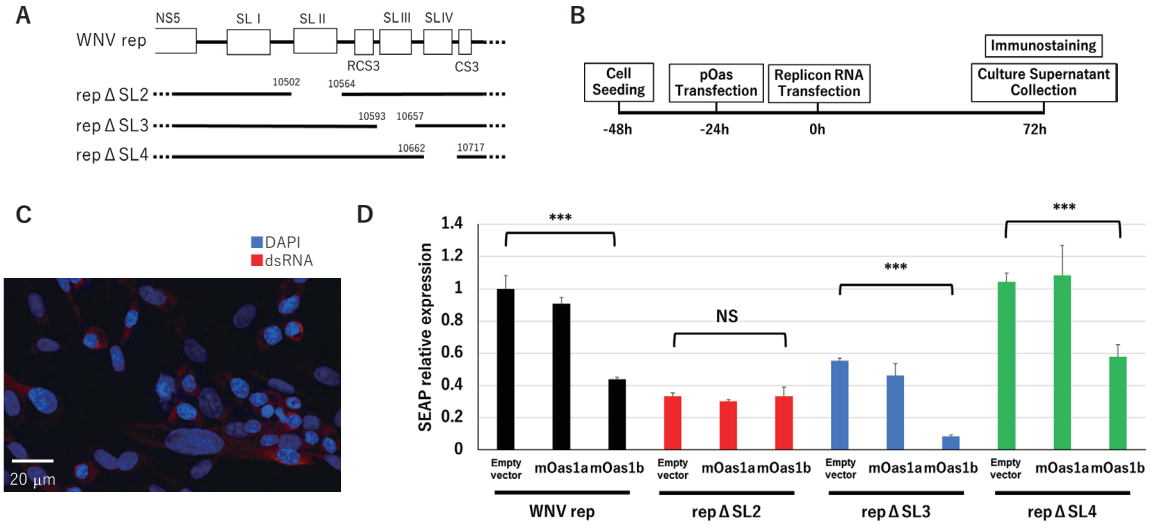

Fig. 1. Measurement of the inhibitory effect of mOas1b on WNV replicon replication using WNV replicon lacking the sequence constituting the SL of the 3'-UTR of the WNV replicon. A, The structure of the 3'-UTR of the viral replicon with deleted SL2, SL3, and SL4. The numbers indicate RNA base pairs in the WNV NY99 genome. mOas1b: mouse oligoadenylate synthetase 1b, WNV: West Nile virus, NS: non-structural protein, SL: stem-loop structure, UTR: untranslated regions, RCS: repeated conserved sequence, CS: conserved sequence[13,21]. B, Timeline of experiments using cultured cells to measure the inhibitory effect of mOas1b. pOas indicates pCAG–mOas1a–IRES–EGFP, pCAG–mOas1b–IRES–EGFP, or the empty vector pCAG–IRES–EGFP as negative control. C, immunostaining BHK cells transfected with WNVrep ΔSL2 RNA. Blue area indicates DAPI staining. DsRNA was detected by anti-dsRNA antibody (red). D, Relative amounts of SEAP, a reporter protein transcribed from WNV replicons in the supernatant, are shown. SEAP: Secreted alkaline phosphatase. Error bars indicate standard errors. *** indicates statistically significant ($P < 0.001$) compared to empty vector. Error bars indicate standard errors. ** and *** indicate $P < 0.01$ and $P < 0.001$, respectively.

RNase L pathway, although the mechanism has not yet been clarified[12].

The untranslated region (UTR) of flaviviruses is conserved among multiple viral species and involved in viral replication and virulence. Both 5'- and 3′-UTR ends are essential for forming circular structures during viral genomic RNA synthesis[27]. The 3′-UTR contains sequences that constitute multiple stem–loops (SLs), which create a region that is resistant to RNA degradation by exoribonuclease 1 and accumulate in the cell as RNA fragments[2,3]. The accumulated RNA is called subgenomic flavivirus RNA (sfRNA) and affects virulence; however, the details have not been clarified[22].

OAS binds to double-stranded RNA to induce enzymatic activity. Furthermore, it is activated upon binding to single-stranded RNA, which temporarily forms double-stranded structures[5]. For example, the OAS/RNase L pathway is activated by single-stranded RNA viruses such as SARS-CoV-2 and Sindbis virus, which have sequences that form SL structures within the genomic RNA[7,18,25]. According to a previous report, Oas1b-mediated flavivirus replication inhibition was lost in a tick-borne encephalitis virus (TBEV) strain partially lacking the 3′-UTR[28]. These facts suggest that Oas1b binding to 3′-UTR SLs is necessary for the anti-flavivirus activity of Oas1b. In this study, we investigated the role of WNV 3′-UTR SLs in mouse Oas1b (mOas1b)-mediated WNV replication inhibition to clarify the viral factor that Oas1b act through.

Since the WNV 3′-UTR has a secondary structure, we attempted to confirm whether mOas1b inhibits the replication of WNV replicon lacking each SL. We deleted the SL2, SL3, and SL4 sequences of the WNV replicon based on the previously reported SL structures in flavivirus 3′-UTRs (Fig. 1A)[4,22,23]. We cloned and joined the sequences, excluding the RNA sequences constituting each SL structure, using inverted PCR based on the WNV NY99 strain replicons harboring the secreted alkaline phosphatase

Table 1. Primers used for constructing WNVrep lacking SLs and cloning SL2

Name	Direction	Primer sequence
WNVrepΔSL2	Reverse	CAGTCCTCCTGGGGTTCTCCTCAAAATTTTCTTAACTA
WNVrepΔSL2	Forward	ACCCCAGGAGGACTGGGTGAAC
WNVrepΔSL3	Reverse	TCTGACATTGGGCTTGGCTTTGTTCACCCAGTC
WNVrepΔSL3	Forward	AAGCCCAATGTCAGACCACG
WNVrepΔSL4	Reverse	GGGCTTTGAAGTTACAACAT
WNVrepΔSL4	Forward	GTAACTTCAAAGCCCTGCCCCAGGAGGACTGG
SL2- *Cla* I	Reverse	AGTCCATCGATAAAAAATTGAGTCGCAGGCAGCAC
*Bam*H I-SL2	Forward	GTAACGGGATCGAAAGTCAGGCCGGGAAG

(SEAP) reporter gene instead of the viral structural genes[16]. The WNV replicon RNAs were propagated using the mMESSAGE mMACHINE® Kit (Thermo Fisher Scientific, Waltham, MA, USA) as previously reported[16]. pCAG–mOas1a–IRES–EGFP and pCAG–mOas1b–IRES–EGFP were used for OAS expression in the cells[21]. *mOas1a* and *mOas1b* were cloned from C57BL/6J and MSM/Ms strains, respectively. MSM/Ms mice express the full-length Oas1b protein, which cause resistance for flavivirus[17]. BHK-21 cells were transfected with 1.5 μg OAS expression plasmid DNA and 0.5 μg WNV replicon RNA by Lipofectamine 2000® (Thermo Fisher Scientific) according to the timeline shown in Fig. 1B. To confirm the influence of the WNV replicon lacking SL on replication, immunostaining was performed using an anti-dsRNA antibody (Sigma-Aldrich, St. Louis, MO, USA). The results showed that dsRNA was detected 72 h post-transfection, indicating that the viral RNA had replicated (Fig. 1C). The amounts of SEAP, a reporter protein synthesized by WNV replicon, in the culture supernatant at 72 h after transfection was measured by Great EscAPe™ SEAP Chemiluminescence Kit 2.0 (Takara Bio Inc., Kusatsu, Japan) and an Infinite M200 PRO plate reader (TECAN Japan Co., Ltd., Kanagawa, Japan) (Fig. 1B). Statistical comparisons were performed using Dunnett's test. As in previous reports[6], SEAP expression did not change in the Oas1a-expressing cells, but was suppressed in the mOas1b-expressing cells (Fig. 1D). However, SEAP expression was not observed in the cells transfected with ΔSL2 and ΔSL3 replicons. The same phenomenon was reported previously after deleting the same WNV region[22]. However, SEAP expression in the cells transfected with ΔSL2 remained unchanged in the presence of mOas1b. In the cells transfected with ΔSL3 and ΔSL4, SEAP expressions in the supernatant were suppressed by mOas1b as that in the cells transfected with original WNV replicons. These results suggested that mOas1b could not inhibit the replication of WNV replicons lacking SL2 in the 3′-UTR.

Since mOas1b did not inhibit replication of the ΔSL2-WNV replicon, SL2 was the predicted site of action of mOas1b. To confirm this possibility, we generated a plasmid expressing SL2 RNA from the WNV. SL2 (10502–1564 nt in WNV NY99) was amplified from the WNV replicon plasmid by PCR and inserted into the pSIN-hU6 plasmid.

We examined the effect of SL2 on the inhibition of replicon replication by mOas1b by co-transfecting with plasmids expressing SL2 RNA and mOas1b according to the timeline shown in Fig. 2A at concentrations of 0.25, 0.5, 1.0, and 2.0 μg/well. The pSIN–hU6 plasmid (1.0 μg/well) was used as a control for pSIN–SL2–hU6. We examined the effects of SL2 on WNV replicon replication in the absence of mOas1b. The result showed that SEAP expression did not change regardless of the SL2 concentrations (Fig. 2B). When SL2 was expressed in mOas1b-expressing cells, SEAP levels increased with increasing pSIN–SL2–hU6 concentrations (Fig. 2B), suggesting that excess SL2 interrupted the mOas1b-meidated inhibition of WNV replicon replication.

SL2 plays a role in stacking RNA-degrading enzymes, thereby making them resistant to

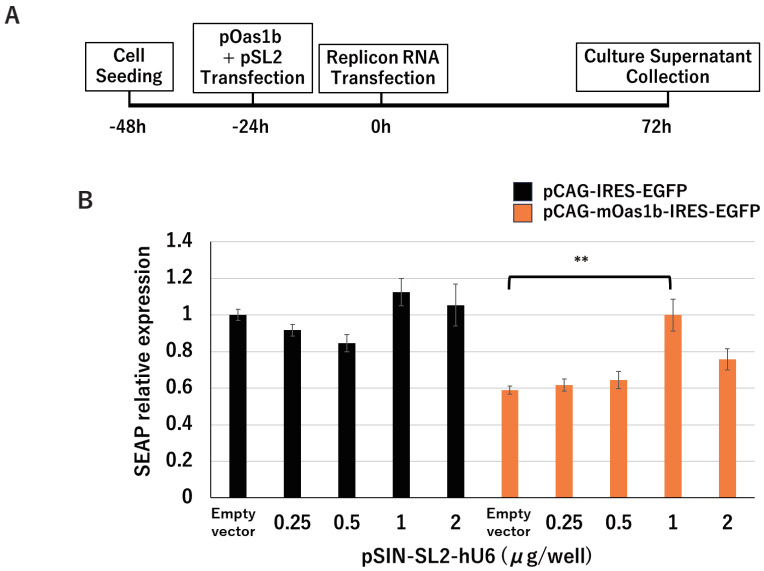

Fig. 2. Changes in the effects of mOas1b in the presence of excess SL2 structure. A, Timeline of experiments using cultured cells to measure the inhibitory effect of mOas1b. Cells were transfected with pCAG–mOas1b–IRES–EGFP and the empty vector pCAG–IRES–EGFP as a negative control. pSIN–SL2–hU6 encoding SL2 was transfected into cells at several concentrations simultaneously with pCAG-mOas1b-IRES-GFP. B, Luminescence levels indicating the amount of SEAP expression in the supernatant in SL2 expression. Bars shown in black and orange are data transfected with pCAG–IRES–EGFP and pCAG–mOas1b–IRES–EGFP, respectively. *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

degradation. Therefore, RNA on the 3′ side from SL2 of genomic RNA accumulate in cells as sfRNA[2,3]. sfRNA inhibits interferon induction and interferes with RNAi response and RNA degradation. Since sfRNA is closely associated with viral replication, the inhibition of sfRNA function by Oas1b may also inhibit replication. In this study, whether Oas1b acts through SL2 on genomic RNAs or sfRNA that accumulate intracellularly was not clear, and the mechanism of replication inhibition was not clarified. We hypothesized that mOas1b might bind to SL2 and have unknown functions in inhibiting genome replication via modifications, such as cleavage, degradation, and addition of some molecules. To clarify this, the role of SL2 in the presence of Oas1b should be further investigated.

mOas1b binds to the SL structure of sequence in the NS5 region of WNV, and its binding specificity differs from that of other OAS members[4]. Inhibition of flaviviral replication by mOas1b, as well as several avian OASs, is independent of RNase L[1,26]. Therefore, avian OASs might target SL2 as well as mOas1b. On the contrary, although the enzymatic activity of human and other mammalian OASs has been reported in detail, few reports have focused on their ability to inhibit flavivirus genome replication. Thus, identifying the SL2 of WNV as the site of action of mOas1b may be useful for clarifying the mechanism of the inhibitory effect of human and other mammalian OASs against flavivirus genome replication in future studies.

Although the SL2 sequences are partially different, the sequences important for the SL structure are conserved in many flaviviruses, such as Japanese encephalitis virus (JEV), Yellow fever virus (YFV), and TBEV (3reference)[21]. Since mOas1b can inhibit the replication of several flaviviruses, such as TBEV and JEV[12,28], mOas1b may target SL2 in other flaviviruses as well. However, because these experiments were conducted using WNV replicons, the same phenomenon should be confirmed in actual viruses.

In this study, we revealed that Oas1b prevents viral replication by acting through the SL2 of WNV replicon. We hope that the

mechanism underlying the inhibition of flaviviral genome replication by mOas1b will be elucidated in the future, thus establishing new therapeutics effective against many flaviviruses.

Acknowledgments

This work was supported by JST SPRING, Grant Number JPMJSP2119.

References

1) Chen S, Yang C, Zhang J, Wu Z, Wang M, Jia R, Zhu D, Liu M, Yang Q, Wu Y, Zhao X, Zhang S, Liu Y, Zhang L, Yu Y, You Y, Cheng A. Conserved active-site residues associated with OAS enzyme activity and ubiquitin-like domains are not required for the antiviral activity of goOASL protein against avian Tembusu virus. Viruses 10, 371, 2018. doi: 10.3390/v10070371.
2) Chen Y-S, Fan Y-H, Tien C-F, Yueh A, Chang R-Y. The conserved stem-loop II structure at the 3′ untranslated region of Japanese encephalitis virus genome is required for the formation of subgenomic flaviviral RNA. PLoS One 13, e0201250, 2018. doi: 10.1371/journal.pone.0201250.
3) Clarke BD, Roby JA, Slonchak A, Khromykh AA. Functional non-coding RNAs derived from the flavivirus 3′ untranslated region. Virus Res 206, 53–61, 2015. doi: 10.1016/j.virusres.2015.01.026.
4) Deo S, Patel TR, Chojnowski G, Koul A, Dzananovic E, McEleney K, Bujnicki J M, McKenna SA. Characterization of the termini of the West Nile virus genome and their interactions with the small isoform of the 2' 5'-oligoadenylate synthetase family. J Struct Biol 190, 236–249, 2015. doi: 10.1016/j.jsb.2015.04.005.
5) Elbahesh H, Jha BK, Silverman RH, Scherbik SV, Brinton MA. The Flv^{r}-encoded murine oligoadenylate synthetase 1b (Oas1b) suppresses 2-5A synthesis in intact cells. Virology 409, 262–270, 2011. doi: 10.1016/j.virol.2010.10.016.
6) Elkhateeb E, Tag-El-Din-Hassan HT, Sasaki N, Torigoe D, Morimatsu M, Agui T. The role of mouse 2',5'-oligoadenylate synthetase 1 paralogs. Infect Genet Evol 45, 393–401, 2016. doi: 10.1016/j.meegid.2016.09.018.
7) Feng Z, Ren X, Duren Z, Wang Y. Human genetic variants associated with COVID-19 severity are enriched in immune and epithelium regulatory networks. Phenomics 2, 389–403, 2022. doi: 10.1007/s43657-022-00066-x.
8) Graham JB, Thomas S, Swarts J, McMillan AA, Ferris MT, Suthar MS, Treuting PM, Ireton R, Gale M Jr, Lund JM. Genetic diversity in the collaborative cross model recapitulates human West Nile virus disease outcomes. mBio 6, e00493-15, 2015. doi: 10.1128/mBio.00493-15.
9) Kajaste-Rudnitski A, Mashimo T, Frenkiel M-P, Guénet J-L, Lucas M, Desprès P. The 2',5'-oligoadenylate synthetase 1b is a potent inhibitor of West Nile virus replication inside infected cells. J Biol Chem 281, 4624–4637, 2006. doi: 10.1074/jbc.M508649200.
10) Kristiansen H, Gad HH, Eskildsen-Larsen S, Despres P, Hartmann R. The oligoadenylate synthetase family: an ancient protein family with multiple antiviral activities. J Interferon Cytokine Res 31, 41–47, 2011. doi: 10.1089/jir.2010.0107.
11) Malathi K, Dong B, Gale M Jr, Silverman RH. Small self-RNA generated by RNase L amplifies antiviral innate immunity. Nature 448, 816–819, 2007. doi: 10.1038/nature06042.
12) Manet C, Roth C, Tawfik A, Cantaert T, Sakuntabhai A, Montagutelli X. Host genetic control of mosquito-borne Flavivirus infections. Mamm Genome 29, 384–407, 2018. doi: 10.1007/s00335-018-9775-2.
13) Markoff L. 5'- and 3′-noncoding regions in flavivirus RNA. Adv Virus Res 59, 177–228, 2003. doi: 10.1016/s0065-3527(03)59006-6.
14) Mashimo T, Glaser P, Lucas M, Simon-Chazottes D, Ceccaldi PE, Montagutelli X, Desprès P, Guénet J-L. Structural and

functional genomics and evolutionary relationships in the cluster of genes encoding murine 2',5'-oligoadenylate synthetases. Genomics 82, 537–552, 2003. doi: 10.1016/s0888-7543(03)00176-9.

15) Mashimo T, Lucas M, Simon-Chazottes D, Frenkiel M-P, Montagutelli X, Ceccaldi P-E, Deubel V, Guénet J-L, Desprès P. A nonsense mutation in the gene encoding 2'-5'-oligoadenylate synthetase/L1 isoform is associated with West Nile virus susceptibility in laboratory mice. Proc Natl Acad Sci U S A 99, 11311–11316, 2002. doi: 10.1073/pnas.172195399.
16) Moritoh K, Maeda A, Nishino T, Sasaki N, Agui T. Development and application of West Nile virus subgenomic replicon RNA expressing secreted alkaline phosphatase. J Vet Med Sci 73, 683–686, 2011. doi: 10.1292/jvms.10-0495.
17) Moritoh K, Yamauchi H, Asano A, Yoshii K, Kariwa H, Takashima I, Isoda N, Sakoda Y, Kida H, Sasaki N, Agui T. Generation of congenic mouse strains by introducing the virus-resistant genes, Mx1 and Oas1b, of feral mouse-derived inbred strain MSM/Ms into the common strain C57BL/6J. Jpn J Vet Res, 57, 89–99, 2009. doi: 10.14943/jjvr.57.2.89.
18) Nickens DG, Hardy RW. Structural and functional analyses of stem-loop 1 of the Sindbis virus genome. Virology 370, 158–172, 2008. doi: 10.1016/j.virol.2007.08.006.
19) Perelygin AA, Scherbik SV, Zhulin IB, Stockman BM, Li Y, Brinton MA. Positional cloning of the murine flavivirus resistance gene. Proc Natl Acad Sci U S A 99, 9322–9327, 2002. doi: 10.1073/pnas.142287799.
20) Perelygin AA, Zharkikh AA, Scherbik SV, Brinton MA. The mammalian 2'-5' oligoadenylate synthetase gene family: evidence for concerted evolution of paralogous Oas1 genes in Rodentia and Artiodactyla. J Mol Evol 63, 562–576, 2006. doi: 10.1007/s00239-006-0073-3.
21) Pijlman GP, Funk A, Kondratieva N, Leung J, Torres S, van der Aa L, Liu WJ, Palmenberg AC, Shi P-Y, Hall RA, Khromykh AA. A highly structured, nuclease-resistant, noncoding RNA produced by flaviviruses is required for pathogenicity. Cell Host Microbe 4, 579–591, 2008. doi: 10.1016/j.chom.2008.10.007.
22) Schnettler E, Sterken MG, Leung JY, Metz SW, Geertsema C, Goldbach RW, Vlak JM, Kohl A, Khromykh AA, Pijlman GP. Noncoding flavivirus RNA displays RNA interference suppressor activity in insect and Mammalian cells. J Virol 86, 13486–13500, 2012. doi: 10.1128/JVI.01104-12.
23) Schuessler A, Funk A, Lazear HM, Cooper DA, Torres S, Daffis S, Jha BK, Kumagai Y, Takeuchi O, Hertzog P, Silverman R, Akira S, Barton DJ, Diamond MS, Khromykh AA. West Nile virus noncoding subgenomic RNA contributes to viral evasion of the type I interferon-mediated antiviral response. J Virol 86, 5708–5718, 2012. doi: 10.1128/JVI.00207-12.
24) Schwartz SL, Conn GL. RNA regulation of the antiviral protein 2'-5'-oligoadenylate synthetase. Wiley Interdiscip Rev RNA 10, e1534, 2019. doi: 10.1002/wrna.1534.
25) Silverman RH. Viral encounters with 2',5'-oligoadenylate synthetase and RNase L during the interferon antiviral response. J Virol 81, 12720–12729, 2007. doi: 10.1128/JVI.01471-07.
26) Tag-El-Din-Hassan HT, Morimatsu M, Agui T. Functional analysis of duck, goose, and ostrich 2'-5'-oligoadenylate synthetase. Infect Genet Evol 62, 220–232, 2018. doi: 10.1016/j.meegid.2018.04.036.
27) Villordo SM, Gamarnik AV. Genome cyclization as strategy for flavivirus RNA replication. Virus Res 139, 230–239, 2009. doi: 10.1016/j.virusres.2008.07.016.
28) Yoshii K, Moritoh K, Nagata N, Yokozawa K, Sakai M, Sasaki N, Kariwa H, Agui T, Takashima I. Susceptibility to flavivirus-specific antiviral response of Oas1b affects the neurovirulence of the Far-Eastern subtype of tick-borne encephalitis virus. Arch Virol 158, 1039–1046, 2013. doi: 10.1007/s00705-012-1579-1.