MicroRNA miR-214 inhibits snakehead vesiculovirus replication by targeting the coding regions of viral N and P

Chi Zhang, Lizhu Yi, Shuangshuang Feng, Xueqin Liu, Jianguo Su, Li Lin and Jiagang Tu

Abstract
Snakehead vesiculovirus (SHVV), a new member of the family Rhabdoviridae, has caused enormous economic losses in snakehead fish culture during the past years in China; however, little is known about the molecular mechanisms of its pathogenicity. MicroRNAs (miRNAs) are small non-coding RNAs that play important roles in virus infection. In this study, we identified that SHVV infection downregulated miR-214 in striped snakehead (SSN-1) cells in a time- and dose-dependent manner. Notably, transfecting SSN-1 cells with miR-214 mimic significantly inhibited SHVV replication, whereas miR-214 inhibitor promoted it, suggesting that miR-214 acted as a negative regulator of SHVV replication. Our study further demonstrated that N and P of SHVV were the target genes of miR-214. Over-expression of P, but not N, inhibited IFN-α production in SHVV-infected cells, which could be restored by over-expression of miR-214. Taken together, these results suggest that miR-214 is downregulated during SHVV infection, and the downregulated miR-214 in turn increased N and P expression and decreased IFN-α production, thus facilitating SHVV replication. This study provides a better understanding of the molecular mechanisms on the pathogenesis of SHVV and a potential antiviral strategy against SHVV infection.

INTRODUCTION
In 2014, snakehead vesiculovirus (SHVV) was isolated from diseased hybrid snakehead fish at a farm in the Guangdong province, China, and has caused enormous economic losses in snakehead fish culture [1]. The genome sequencing and phylogenetical analysis of SHVV have revealed that SHVV shared 94% nucleotide sequence identity with Siniperca chuatsi rhabdovirus (SCRV) and was phylogenetically located in the same group with SCRV and Monopterus albus rhabdovirus (MARV) [1]. Belonging to the genus Perhabdovirus, family Rhabdoviridae [2], SHVV possesses an unsegmented negative-sense ~11kb RNA genome that encodes five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA-dependent RNA polymerase protein (L) [1].

MicroRNAs (miRNAs) are small (~22 nt) non-coding RNAs that have been identified as post-transcriptional regulators of gene expression [3]. Produced by most multicellular organisms and some viruses [4–11], miRNAs degrade and/or suppress translation of target mRNAs through base pairing between the ‘seed sequences’ (nucleotides 2–8 at the 5’ end) of miRNAs and the target transcripts [12–16]. Typically, miRNAs bind to 3’ untranslated regions (UTRs) of target genes [5, 11, 13, 16–20], but growing evidence suggests that miRNAs can also bind to 5’ UTRs and coding regions of target genes [6, 21–24]. In addition, to be involved in various biological processes in eukaryotes, including cellular proliferation, development, differentiation, metabolism, oncogenesis and apoptosis [25–30], miRNAs also play important roles in the regulation of virus replication by targeting viral genes or host genes associated with virus replication [5, 6, 11, 12, 14, 16–18]. Moreover, some viruses even take advantage of host miRNAs to facilitate their replication [5, 18, 31, 32]. Therefore, comprehensive investigation of miRNAs’ role in virus infection will contribute to a better understanding of virus–host interactions and virus pathogenesis. Although miRNAs have been widely studied in human and other mammalian virus infections, their roles in fish virus infection have seldom been extensively investigated.

Our previous study revealed that many miRNAs were altered in striped snakehead (SSN-1) cells upon SHVV infection, suggesting that they might be involved in the pathogenesis of
SHVV [33]. In this study, we found that miR-214 was downregulated in SSN-1 cells by SHVV infection and could significantly inhibit SHVV replication. Further investigations revealed that the P gene of SHVV, the target gene of miR-214, could inhibit IFN-α production during SHVV infection, which could be restored by over-expression of miR-214. In conclusion, these results reveal a regulatory loop that miR-214 is downregulated during SHVV infection, and the downregulated miR-214 in turn facilitate SHVV replication by increasing N and P expression and decreasing IFN-α production. These data not only provide new insights into SHVV–host interactions but also suggest potential antiviral strategy against SHVV infection in the future.

RESULTS

SHVV infection downregulated miR-214 expression

Our previous small RNA deep sequencing has revealed that a panel of miRNAs was altered in SSN-1 cells upon SHVV infection [33]. Among them, miR-214 has previously been identified as an antiviral miRNA [34], it thereby was selected for further study. To reveal the role of miR-214 in SHVV infection, SSN-1 cells were infected with SHVV at a multiplicity of infection (m.o.i.) of 1 and harvested at various time points post of infection (p.o.i.). The levels of viral G mRNA and miR-214 were determined by quantitative real-time PCR (qRT-PCR). We found that G mRNA increased significantly at 12 and 24 h p.o.i., indicating that SHVV replicated efficiently in SSN-1 cells (Fig. 1a). In contrast, the miR-214 expression in SHVV-infected cells was reduced significantly at 12 and 24 h p.o.i. (Fig. 1b), indicating that miR-214 was downregulated along with SHVV replication. Furthermore, the level of miR-214 was measured in SSN-1 cells infected with different doses of SHVV. The results showed that miR-214 was downregulated by SHVV infection in a dose-dependent manner (Fig. 1c). These data suggested that SHVV infection downregulated miR-214 expression in SSN-1 cells.

miR-214 inhibits SHVV replication

To assess the effect of miR-214 on SHVV replication, SSN-1 cells were transfected with the synthetic miR-214 mimic or inhibitor to over-express or suppress the cellular miR-214, followed by SHVV infection. As shown in Fig. 2a, transfection of miR-214 mimic increased, whereas miR-214 inhibitor decreased, the cellular miR-214 level about 300 and 200 times, respectively, suggesting that the synthetic miR-214 mimic and inhibitor could significantly alter cellular miR-214 expression. The effect of miR-214 on SHVV replication was determined via measuring virus titres in the supernatant by TCID_{50}. The results showed that over-expression of miR-214 decreased, whereas suppressed-expression of miR-214 increased, viral titres in the supernatant (Fig. 2b), suggesting that miR-214 could inhibit SHVV replication in SSN-1 cells.

N and P of SHVV are target genes of miR-214

Target gene prediction software Miranda was used to determine the viral genes targeted by miR-214 and identified that the coding regions of N and P possessed the target sequences of miR-214 [33]. To verify whether N and P are indeed target genes of miR-214, dual-luciferase reporter plasmids pmirGLO-N, pmirGLO-P, pmirGLO-N-MUT and pmirGLO-P-MUT, containing putative miR-214 binding sequences or miR-214 seed-region-mutated sequences from N or P, were transfected into SSN-1 cells with miR-214 mimic or inhibitor (Fig. 3a). We found that transfection of miR-214 mimic reduced, while miR-214 inhibitor increased, the luciferase activity of plasmids containing wild-type target sequences (Fig. 3b, c). However, the luciferase activity was not significantly altered when miR-214 mimic or inhibitor was co-transfected with plasmids harbouring miR-214 seed-region-mutated sequences (Fig. 3b, c). These data suggested that N and P of SHVV are target genes of miR-214.

![Fig. 1. SHVV infection downregulated miR-214 expression in SSN-1 cells. SSN-1 cells were infected with SHVV (m.o.i.=1.0) or equal volume of PBS, and harvested at various time points. (a) The G mRNA level in SSN-1 cells was measured using qRT-PCR. β-actin was used as the internal control. (b) The expression of miR-214 in SSN-1 cells was analysed using qRT-PCR. 5S rRNA was used as the internal control. (c) SSN-1 cells were infected with a different dose of SHVV, and harvested at 24 h p.o.i. The expression of miR-214 in SSN-1 cells was analysed using qRT-PCR. 5S rRNA was used as the internal control. (a) (b) (c)](image-url)
Effect of miR-214 on the expression of N and P

Our previous study reported that miR-214 reduced the mRNA and protein levels of N and P in SHVV-infected SSN-1 cells [33]. To further validate the effect of miR-214 on the expression of N and P, SSN-1 cells were co-transfected with miR-214 mimic or inhibitor, together with plasmids expressing the ORF of N or P. The results showed that over-expression of miR-214 significantly reduced, whereas suppressed-expression of miR-214 increased, the mRNA and protein levels of N and P in SSN-1 cells (Fig. 4). These data suggested that miR-214 could inhibit the expression of N and P, which might result in the inhibition of SHVV replication.

miR-214 can restore the inhibiton of P protein on IFN-α production

Previous reports have revealed that N and P genes of another fish rhabdovirus SVCV could inhibit type I IFN production [35, 36]. In this study, we investigated whether N and P of SHVV could inhibit type I IFN production. As shown in Fig. 5(a), P but not N of SHVV significantly inhibited SHVV-induced IFN-α production. Furthermore, we investigated the effect of P on SHVV replication. As expected, over-expression of P significantly increased SHVV replication in a dose-dependent manner (Fig. 5b, c). To illustrate whether miR-214 could affect P-mediated inhibition of IFN-α production, we first estimated IFN-α production in SSN-1 cells induced by over-expression of the N-terminal caspase activation and recruitment domain (CARD) of retinoic acid-inducible gene I (RIG-I<sub>N</sub>). The results showed that over-expression of RIG-I<sub>N</sub> significantly increased IFN-α production (Fig. 5d). However, when co-transfected with pCDNA3.1-P, the level of IFN-α production was decreased (Fig. 5d), suggesting that P protein of SHVV could inhibit RIG-I signalling-induced IFN-α production. Furthermore, we investigated whether P-mediated
inhibition of IFN-α production could be restored by miR-214. As shown in Fig. 5(d), over-expression of miR-214 significantly restored the inhibition of P on IFN-α production in a dose-dependent manner. Taken together, these data suggested that P of SHVV could inhibit SHVV- or RIG-I signalling-induced IFN-α production, which could be restored by over-expression of miR-214.

DISCUSSION
As post-transcriptional regulators of gene expression, miRNAs play important roles in virus infection. Growing evidence has revealed that virus infection altered host miRNA expression, and in turn, the altered miRNAs affected virus replication [4, 9, 17–19, 32, 37–45]. Some viruses even utilized the regulatory roles of miRNAs to facilitate their replication by altering host miRNA expression [4, 18, 19, 32, 38, 40–42, 45, 46]. For example, Japanese encephalitis virus (JEV) infection upregulated miR-301a, and in turn, the upregulated miR-301a inhibited type I IFN production by reducing the abundances of the transcription factor interferon regulatory factor 1 (IRF1) and the signalling protein suppressor of cytokine signalling 5 (SOCS5), thus promoting JEV replication [17]. As a newly isolated fish rhabdovirus that has caused great economical losses in snakehead fish culture in China [1], SHVV has been reported to alter many miRNAs in SSN-1 cells [33]. However, little is known about the roles of these miRNAs in SHVV infection.

SHVV infection has been reported to downregulate the expression of miR-214 [33], which has been widely studied in cancer and identified as an important regulator of cancer development [47–55]. In virus infection, miR-214 has been reported to be downregulated in both murine- and human-cytomegalovirus-infected cells, and over-expression of miR-214 inhibited the replication of both viruses, suggesting virus-induced downregulation of miR-214 was beneficial for virus replication [34]. In this study, we focused on the regulatory role of miR-214 in SHVV infection and found that miR-214 was downregulated upon SHVV infection and found that miR-214 was downregulated upon SHVV infection in a time- and dose-dependent manner (Fig. 1), and miR-214 inhibited SHVV replication (Fig. 2). Our data as well as previous study suggested that miR-214 was an antiviral miRNA [34], and viruses needed a cellular circumstance with low miR-214 level for their replication.

In addition to binding to the host factors required for virus replication, miRNAs can also regulate virus infection by binding directly to viral genes [16, 22, 43, 56–58]. For example, miR-965 inhibited white spot syndrome virus (WSSV) infection by targeting the viral wsv240 gene [43]. Target gene prediction of miR-214 has revealed that N and P genes of SHVV were the potential target genes of miR-214 [33]. In this study, a dual-luciferase reporter assay confirmed that the N and P were the target genes of miR-214 (Fig. 3). Moreover, we investigated the effect of miR-214 on N and P expression by transfecting miR-214 mimic or inhibitor.
MicroRNAs regulated gene expression through base pairing between their 'seed sequences' (nucleotides 2–8 in the 5' end) and the target transcripts [13, 16]. In this study, we found that the suppression of miR-214 on P protein expression was much stronger than on N protein even though both N and P possessed the complementary sequences of miR-214 seed-region sequences (Figs 3 and 4). Gerresheim et al. suggested that the surrounding sequences of the binding sites might affect the accessibility of miRNAs to the targets [22]. Therefore, different sequences up- and downstream of miR-214 binding sites in N and P might lead to different RNA secondary structures and result in different accessibility for miR-214, thus causing different inhibitory effects on N and P expression.

Previous reports have revealed that N and P genes of SVCV could inhibit SVCV-induced type I IFN production [35, 36]. Our study revealed that P protein of SHVV suppressed SHVV-induced IFN-α production (Fig. 5a). Actually, the P protein of the rabies virus has been extensively proved to suppress type I IFN production [59–61]. Here, we investigated whether miR-214 could restore the inhibitory effects of P protein on RIG-I signalling-induced IFN-α production instead of SHVV-induced IFN-α production for two reasons. First, our transcriptomic sequencing of SHVV-infected SSN-1 cells has revealed that SHVV-infection activated the RIG-I signalling and upregulated the IFN-α production (data not shown). Second, except for binding to viral P protein, miR-214 might also bind to host factors to inhibit SHVV replication, thus producing a lower level of IFN-α, which thereby interfered our study.

In summary, our study demonstrated that miR-214 was downregulated in response to SHVV infection in SSN-1 cells. The results showed that miR-214 reduced the mRNA and protein levels of N and P (Fig. 4), which might lead to the inhibition of SHVV replication. In addition to directly targeting viral N and P, miR-214 might also inhibit SHVV replication via targeting the host factors required for SHVV replication, which needs to be investigated in the future.

Moreover, we found that the suppression of miR-214 on P protein expression was much stronger than on N protein even through both N and P possessed the complementary sequences of miR-214 seed-region sequences (Figs 3 and 4). Gerresheim et al. suggested that the surrounding sequences of the binding sites might affect the accessibility of miRNAs to the targets [22]. Therefore, different sequences up- and downstream of miR-214 binding sites in N and P might lead to different RNA secondary structures and result in different accesses for miR-214, thus causing different inhibitory effects on N and P expression.

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cells, in turn, the downregulated miR-214 increased viral N and P expression and decreased IFN-α production, thus promoting SHVV replication. These findings may have important implications for the understanding of the molecular mechanisms on virus pathogenesis and provide antiviral strategy against SHVV infection.

METHODS

Cells and viruses

SSN-1 cells (kindly provided by Hong Liu from Shenzhen Animal and Plant Inspection and Quarantine Technology Center) were maintained at 25°C in minimum essential medium (MEM) (HyClone, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, New Zealand), penicillin (100 µg ml⁻¹), and streptomycin (100 µg ml⁻¹). SHVV was isolated from diseased hybrid snakehead fish at a farm in the Guangdong province, China, and stored at −80°C.

Reagents and antibodies

The miR-214 mimic (double-stranded RNA oligonucleotides), miR-214 inhibitor (single-stranded chemically modified oligonucleotides), NC mimic and NC inhibitor were purchased from GenePharma (Shanghai, China). Their sequences were as follows: miR-214 mimic, 5¢-ACAGCAGGACAGACAGGCA-3¢ (forward) and 5¢-GCCUGUCUGCCUGCUUU-3¢ (reverse); miR-214 inhibitor, 5¢-CUGCCUGUCUGCCUGCUU-3¢; NC mimic, 5¢-UCUCGGCAACGGUUGACGUTT-3¢ (forward) and 5¢-ACGUGACAGUGCGAGAATT-3¢ (reverse); NC inhibitor, 5¢-CAGUACUUUUGUGAAGC-3¢. The antibodies against N or P protein of SHVV have been produced previously and stored in our laboratory [33]. The antibody against β-actin was purchased from Bioss Biotechnology (Beijing, China). The secondary antibody donkey anti-rabbit IgG antibody was purchased from Gene (Shanghai, China).
Table 1. Primers used in this study

<table>
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<tr>
<th>Primers*</th>
<th>Sequences (5’-3’)</th>
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<tr>
<td>SHVV-G-FW</td>
<td>ACACCAATGCAAGGAGCC</td>
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<tr>
<td>SHVV-G-BW</td>
<td>GCCTCGGTGGTATCCTAACCAAT</td>
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<tr>
<td>SHVV-N-FW</td>
<td>CGGCATCGGAAATACAGCAG</td>
</tr>
<tr>
<td>SHVV-N-BW</td>
<td>GTTACGCGCTGGGCACTTTTAA</td>
</tr>
<tr>
<td>SHVV-P-FW</td>
<td>ACACGTACCTCAGGACGTCGT</td>
</tr>
<tr>
<td>SHVV-P-BW</td>
<td>ACACGACATTCTGGCAAC</td>
</tr>
<tr>
<td>IFN-α-FW</td>
<td>TGTACCTCGGTCCTTTGCAAT</td>
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<td>IFN-α-BW</td>
<td>CCAAGCTGCACTGGGATG</td>
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<tr>
<td>β-actin-FW</td>
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<td>β-actin-BW</td>
<td>CCATCTGCTGGGCAAGT</td>
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<td>MIR-214-FW</td>
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<td>P-FW</td>
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<td>N-MUT-FW</td>
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<td>N-MUT-BW</td>
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<td>MiR-214-MUT-BW</td>
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<td>5SrRNA-FW</td>
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<tr>
<td>MiR-214-FW</td>
<td>CGGACAGCAGGCACAGACAGGCAGAAAA</td>
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</table>

*Primers with names starting with N- or P- were used to generate luciferase reporter plasmids. Primers with names starting with ORF- were used to generate plasmids expressing the ORF of the corresponding gene. Primers with names starting with RIG- were used to generate plasmids expressing the N-terminal CARD of RIG-I. Other primers were used for qRT-PCR.

Plasmids

Plasmids pCDNA3.1-N and pCDNA3.1-P, respectively expressing the ORF of N or P of SHVV, were constructed by cloning the PCR-amplified cDNAs of the N or P gene into vector pCDNA3.1 (+) with the primers listed in Table 1. Plasmid pCDNA3.1-RIG-Iα was constructed by cloning the N-terminal CARD of the RIG-I gene to the vector pCDNA3.1 (+) with the primers listed in Table 1. Luciferase reporter plasmids pmirGLO-N and pmirGLO-P were constructed by amplifying the miR-214 target sequences (~200 nt) in the coding regions of N and P genes and cloning into vector pmirGLO with the primers listed in Table 1. Plasmids pmirGLO-N-MUT and pmirGLO-P-MUT were generated by PCR-mediated mutations into plasmid pmirGLO-N or pmirGLO-P using the primers listed in Table 1.

Transfection

The NC mimic, NC inhibitor, miR-214 mimic, miR-214 inhibitor, or plasmids were incubated with TransIntr0TM EL Transfection Reagent (TransGen Biotech, China) in 500 µl Opti-MEM medium (Invitrogen, USA) for 30 min at room temperature. The incubated samples were then put onto the SNN-1 cells. After 6 h of transfection at 25 °C, the medium was replaced by 1 ml of MEM and continued incubation at 25 °C.

Dual-luciferase reporter assay

SSN-1 cells were co-transfected with 30 pmol of NC mimic, miR-214 mimic, NC inhibitor, or miR-214 inhibitor, together with 500 ng of luciferase reporter plasmid pmirGLO-N, pmirGLO-P, pmirGLO-N-MUT, or pmirGLO-P-MUT using TransIntr0TM EL Transfection Reagent (TransGen Biotech, China). At 24 h post of transfection, the Renilla and firefly luciferase activities were measured using a Dual-Glo luciferase assay system (Promega, USA). Measurements were performed using a GloMax-Multi Jr Single Tube Multimode Reader (Promega, USA) according to the manufacturer’s protocol. The data were expressed as relative firefly luciferase activity normalized to Renilla luciferase activity.

Virus infection and titration

SSN-1 cells were incubated with SHVV at a m.o.i. of 1 or equal volume of PBS as the control. After 2 h adsorption at 25 °C, the inoculum was removed and the cells were washed twice with PBS followed by adding MEM medium with 5% FBS. At 24 h p.o.i., the supernatants were collected for virus titration by TCID₅₀. For the detection of viral miRNAs or host miRNAs by qRT-PCR with the primers listed in Table 1.

Quantitative RT-PCR of SHVV genes and miRNAs

Total RNAs were extracted from cells with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. For the detection of SHVV genes, 1 µg of RNA was mixed with 1 µl Oligo (dT), 4 µl 4 × gDNA wiper Mix, and RNase-free H₂O to a total volume of 16 µl. After incubated at 42 °C for 2 min, 4 µl 5 × select qRT supermix II was added and incubated at 50 °C for 15 min and 85 °C for 2 min. The quantitative PCR reactions were conducted in 20 µl volumes containing 10 µl AceQ qPCR SYBR Green Master mix, 1 µl cDNA template, 0.4 µl of forward primer, 0.4 µl of backward primer, and 8.2 µl ddH₂O with the following cycling conditions: 95 °C for 5 min, 45 cycles at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 15 s, and ended with a 95 °C at 5 °C s⁻¹ calefactive velocity to make the melt curve. Data were normalized to the level of β-actin in each sample using the 2⁻ΔΔCt method.

For the detection of miRNAs, an All-in-One miRNA qRT-PCR Detection System kit (GeneCopeia, China) was used. 2 µg of RNA was used to synthesize cDNA according to the
manufacturer’s instructions. The cDNA was then used to detect the level of miRNAs. The reaction mix composed of 2 µl cDNA, 10 µl All-in-One qPCR mixture, 2 µl forward primer as listed in Table 1 and 2 µl Universal Adaptor PCR primer (2 uM) as backward primer, and ddH2O to a total volume of 20 µl. The thermal cycling parameters were 10 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 20 s at 55 °C and 10 s at 72 °C. The expression level of miRNAs was calculated using the 2−ΔΔCt method after normalization to 55 rRNA.

**Western blotting**

Proteins were extracted from SSN-1 cells by cell lysis buffer, separated by SDS-PAGE gel and transferred onto a nitrocellulose membrane (Biosharp, China). Membranes were blocked with 5% skim milk in tri-buffered saline with tween 20 (TBST) at 4 °C overnight, followed by incubation with the primary antibody of SHVV protein (1 : 1000) or β-actin (1 : 1000) for 2 h at room temperature. The membranes were then washed three times with TBST and then incubated with IRDye 800CW conjugated donkey anti-rabbit antibody (1 : 10000) for 1 h at room temperature. The signal intensity was then determined using Odyssey CLx (LI-COR, USA).

**Statistical analysis**

All statistical analyses were performed using Graphpad Prism 5.0 (GraphPad Software, CA, USA). The statistical significance of the data was determined by Student’s t-test, and P<0.05 was considered statistically significant.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


**Zhang et al., Journal of General Virology 2017;98:1611–1619**


