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Short hairpin RNA targeting NP mRNA inhibiting Newcastle disease virus production and other viral structural mRNA transcription

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Abstract Newcastle disease virus (NDV), formally recognized as avian paramyxovirus 1 (APMV-1), is the etiological agent of Newcastle disease (ND), an affliction which can cause severe losses in the poultry industry. Better understanding of the molecular basis of viral structural genes involved with production should contribute significantly toward the development of improved prophylactic and therapeutic reagents to control the infection. Here we show that a short hairpin RNA (shRNA) eukaryotic expression vector targeting nucleocapsid (NP) gene of NDV can potently inhibit NDV production in both primary cells and embryonated chicken eggs. Moreover, shRNA specific for NP abolished the accumulation of not only the corresponding mRNA but also P, HN, F, M gene mRNA. The findings reveal that newly synthesized NP mRNA is essential for NDV transcription and replication, and provide a basis for the development of shRNAs as a prophylaxis and therapy for NDV infection in poultry.

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Introduction

Newcastle disease virus (NDV) is a member of the Paramyxoviridae family, genus Avulavirus, and a pathogen of Newcastle disease (ND) [1]. ND is known as one of the most serious avian diseases with a worldwide distribution that can cause severe economic losses in the poultry industry [1, 2]. The virus possesses a non-segmented negative strand RNA genome of 15186 nucleotides [3] that encodes six main structural proteins: nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin–neuraminidase (HN), and large protein (L). The NDV genes are arranged on the genomic RNA in the order of 3′-NP-P-M-F-HN-L-5′ [4]. The transcription process for NDV is similar to that for other non-segmented negative-sense RNA viruses [2]. The genomic RNA is completely covered by the nucleocapsid protein (NP) and in addition contains several copies of the large protein (L) and phosphoprotein (P), which together constitute the viral RNA-dependent RNA polymerase (RdRp). Apart from genomic RNA encapsidation which requires the interactions between NP–NP itself and NP-RNA, the NP protein must also interact specifically with the P and L proteins to form the ribonucleoprotein complex (RNP), which serves as a template for RNA synthesis [5–11]. RdRp is the active template for transcription and replication of the viral genome. Early in infection, transcription results in the synthesis of a short non-translated leader RNA and six mono-cistronic subgenomic mRNAs which are translated to produce the viral

proteins. The viral mRNAs are generated by a sequential and discontinuous RNA synthesis mechanism involving conserved cis-acting transcription start and stop signals. Later in infection, when viral proteins accumulate, RNA synthesis switches from transcription to replication. This switch is mediated by the binding of NP to the nascent leader RNA [2]. Newly synthesized NP molecules are sequentially added to the growing RNA chain. As a result of binding of NP, RdRp ignores the transcription start and stop signals, resulting in the synthesis of full-length antigenomic RNA. The antigenomic RNA, in turn, serves as a template for the synthesis of full-length genomic RNA [2]. The detailed mechanisms of the transcription and replication cellular events for any of the negative-stranded RNA viruses are yet to be fully revealed [7]. In view of this, further comprehensive understanding of the relation between NP gene and proliferation as well as other structural genes of NDV are fundamental toward a better understanding of these transcription and replication processes.

Here we constructed a single short hairpin RNA (shRNA) plasmid expression system targeting NP gene and identified that a shRNA can potently inhibited NDV production in both primary cells and embryonated chicken eggs. We show that the inhibition by the most potent shRNA is a result of both sequence-specific interference with the target mRNA accumulation and broad inhibition transcription of other viral structural genes.

Materials and methods

shRNAs

Based on several principles presented by K. U. Tei et al. [12], three highly conserved regions were selected as the candidate targets by analyzing full-length sequence of NP mRNA from 15 NDV isolations available in GenBank. These target sites are located in 183–202 bp, 191–210 bp, and 233–252 bp, respectively. To design a DNA template expressing a single shRNA, a 19 bp sense strand was connected to the anti-sense strand by using nine non-complementary nucleotide acids to form a stem-loop. The DNA templates were then inserted into pGenesil-3 plasmid with a U6 promoter system and reporter gene red-fluorescent protein (RFP). The recombinant plasmids were named as ndv1, ndv2, and ndv3, respectively. The G + C content of shRNAs were 52.64, 52.64, and 42.11%, respectively. A plasmid vector HK which contained unrelated sequence was constructed as a negative. All shRNAs expression plasmids were constructed by Genesil Biotechnology Co., Ltd (Wuhan, China).

Virus and assays

NDV F48E9, genotype IX, was provided by China National Supervision Institute of Veterinary Medicine. The viruses were grown in the allantoic cavity of 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs (Medical Apparatus Management Factory of Nanjing, Nanjing, China) at 37°C. Allantoic fluid was harvested 48 h after virus inoculation and stored at −80°C. Virus titer was measured by hemagglutination assay in V-bottom 96-well plates. Serial two-fold dilutions of virus samples were mixed with an equal volume of a 1% suspension (vol/vol) of chicken erythrocytes and incubated at room temperature for 30 min. Wells containing an adherent, homogeneous layer of erythrocytes were scored as positive [13]. For egg lethal doses ELD50 assays, serial 10-fold dilutions of virus samples were injected into the allantoic cavity of a 10-day-old embryonated chicken egg. After 72 h infection, live and dead eggs were recorded and statistically analyzed by Reed-Muench's method.

Cell culture, plasmid delivery, and virus infection

Chicken embyro fibroblasts (CEF) were prepared essentially as described [14] from SPF 10-day-old embryonated eggs. CEF cells were grown in DMEM containing 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C 5% CO_2. For plasmid introduction, logarithmic-phase CEF cells were trypsinized, washed, and grown in a 48-well culture plate at 5×10^4 cells per well until 80% confluent. Plasmids were mixed with liposome transfection reagent (silent-fect, BioRad corporation) gently at a ratio of 1:3 (μg:μl) and incubated at room temperature for 30 min. The mixture containing 1 μg plasmids and 200 μl antibiotics-free and serum-free DMEM medium (Gibco/BRL corporation, USA) was added to each well and incubated at 37°C 5% CO_2 for 1.5 h. The culture medium was then replaced with DMEM containing 3% FCS and cultured for 4 h. The culture medium was then removed and 100 μl F48E9 viruses (10^5 ELD50) were added to each well. After incubation for 1 h at 37°C, inoculums was removed and 200 μl DMEM supplemented with 3% FCS was added into each well and the cells were cultured at 37°C CO_2. At different times after infection, supernatants were harvested from infected cultures and the virus titer was determined. As a negative control, HK was similarly introduced into CEF cells, followed by virus infection. Virus titer was assayed as above triplicates. All the data were statistical analyzed.

Virus and shRNA inoculation in chicken embryos

A graded amount of ndv1 (1, 2, 3 μg for each inoculation) was injected into the allantoic cavity of a 10-day-old embryonated chicken egg. The mixture of plasmids and liposome transfection reagent was prepared as above, combined with 100 μl F48E9 virus (10^5 ELD50), and then immediately injected. The eggs were incubated at 37°C for 17 h and allantoic fluid was harvested to measure virus titer. As a negative control, HK (3 μg) was treated similarly as above triplicates. All the data were statistical analyzed.

RNA extraction, reverse transcription (RT), and real-time PCR

Infected CEF cells with virus F48E9 were harvested at selected time points after infection. RNA was isolated using Trizol reagent (TaKaRa Biotechnology Co., Ltd., Japan) according to the manufacturer's protocol. RT was carried out by using an RNA PCR Kit (AMV) Ver.3.0 (TaKaRa Biotechnology Co., Ltd., Japan) in a 20-μl reaction mixture, containing Oligo(dT)18 primers of RT reaction mixture, at 30°C, 10 min; 42°C, 15 min; 99°C, 5 min; 5°C, 5 min, and was then used for real-time PCR in a 20-μl reaction mixture, containing 10 μl of SYBR Premix Ex TaqTM, 0.4 μl of ROX Reference Dye, 0.5 μl each of forward and reverse primer (10 μmol/l), and 2 μl of cDNA. The gene-specific primers designed in this report and optimal conditions of real-time PCR are shown in Table 1. Before the PCR, the mixture was incubated at 95°C for 2 min. The levels of PCR products were monitored with 7300 real-time PCR system (Applied Biosystems Co., Ltd, USA). The duplicate cycle times were averaged and normalized to the cycle time of β-actin. The relative changes in mRNA expression of NP, P, M, F, HN, L gene were calculated using $2^{-\triangle\triangle Ct}$ method [15].

Results

Design of shRNAs specific for NDV

Besides having no more than one mismatch in the 21 nucleotides among different strains, the shRNAs designed in this study did not share identity with any known human and animal gene. All the shRNAs tested were sequenced and shown in Table 2.

Inhibition of NDV production in CEF cells

As shown in Table 3, in mock transfection (HK), virus titers in the infected cultures increased over time, reaching peak values between 40 and 48 h. Transfection of shRNAs specific for NP gene of NDV generated two types of results. First, ndv2 and ndv3 had no discernable effect on the virus titer ($P > 0.05$), indicating that they were not effective in interfering with NDV production in CEF cells. Second, ndv1 significantly inhibited virus production ($P < 0.01$). When ndv1 was used, inhibition was so

Table 2 Information of shRNAs target to NP mRNA

shRNAs	Sequence	G + C (%)
ndv1	5′-CACAGGGCGGATCAAACAA-3′	52.64
ndv2	5′-GGATCAAACAAGCCGCAAG-3′	52.64
ndv3	5′-GAGACCCAATTCTTGGATT-3′	42.11

Table 1 Primers and reacting conditions for amplification of NDV structural gene mRNAs by real-time RT-PCR

Target gene	Primers	Sequence	Reacting conditions
NP	np1 (forward)	5′-CCCAACGAGTGTCTGAGGAG-3′	94°C 30 s, 62°C 30 s, 72°C, 40 s, 40 cycles
	np2 (reverse)	5′-CTACCGCTCTCATAAGGTCCAA-3′	
P	p1 (forward)	5′-GATGCGGTCTGAAATCCAACA-3′	94°C 15 s, 56°C 30 s, 72°C 30 s, 40 cycles
	p2 (reverse)	5′-GCGACTGCCCGTAGATCACT-3′	
M	m1 (forward)	5′-CTTGGACCCTCTGTGCTCGT-3′	94°C 15 s, 58°C 30 s, 72°C 30 s, 40 cycles
	m2 (reverse)	5′-GTGCCCGCTTGGATAATGAC-3′	
F	f1 (forward)	5′-TGAATTCTTGATGGCAGGCCTCTTGC-3′	94°C 30 s, 58°C 30 s, 72°C 40 s, 40 cycles
	f2 (reverse)	5′-GCTTATTGCTACACTGCCG-3′	
HN	hn1 (forward)	5′-ACCAGCGAGGGTCATCATAC-3′	94°C 15 s, 58°C 30 s, 72°C 30 s, 40 cycles
	hn2 (reverse)	5′-AACACACGAGTTAGGGCATCT-3′	
L	l1 (forward)	5′-ACAACCTGCGGCGTTTTTAC-3′	94°C 15 s, 55°C 30 s, 72°C 30 s, 40 cycles
	l2 (reverse)	5′-TGGCTCCACTTCCTTCTGCT-3′	
β-actin	β-actin 1 (forward)	5′-CTGTGCCCATCTATGAAGGCTA-3′	94°C 30 s, 60°C 30 s, 72°C 40 s, 40 cycles
	β-actin 2 (reverse)	5′-ATTTCTCTCTCGGCTGTGGTG-3′	

Table 3 Effects of shRNAs on NDV production in CEF cells (titer in HA units)

shRNA	Infection time (h)				
	16	24	32	40	48
HK	0	1.33 ± 1.15	4.00 ± 0	8.00 ± 0	8.00 ± 0
ndv1	0	0	0	0	0
ndv2	0	1.33 ± 1.15	3.33 ± 1.15	6.67 ± 1.89	8.00 ± 0
ndv3	0	0.67 ± 1.15	4.00 ± 0	5.33 ± 2.31	6.67 ± 1.89

Table 4 Effects of shRNAs on NDV production in embryonated chicken eggs (titer in HA units)

shRNAs	Dosage (μg)		
	1	2	3
ndv1	64.00 ± 0	32.0 ± 0	6.7 ± 8.3
ndv2	53.3 ± 18.5	64.0 ± 0	32.0 ± 0
ndv3	64.0 ± 0	53.3 ± 18.5	53.3 ± 18.5
HK	53.3 ± 18.5	64.0 ± 0	64.0 ± 0

pronounced that culture supernatants lacked detectable HA activity within 48 h (Table 3).

Inhibition of NDV production in embryonated chicken eggs

As expected, coinjection of virus plus HK did not affect the virus titer. The virus titers increased along with the infection time. Coinjection of ndv1 reduced virus titers, whereas ndv2 and ndv3 were not effective in chicken embryos (Table 4). To determine the potency of shRNAs, a graded amount of ndv1, ndv2, and ndv3 were coinjected with virus into embryonated chicken eggs. As the amount of shRNA decreased, virus titer increased in the allantoic fluid. However, 1 μg ndv1 was ineffective. Thus, ndv1 also effectively interferes with NDV production in embryonated chicken eggs.

ndv1 inhibits accumulation of other structural genes except L gene of NDV

We examined the effect of ndv1 shRNA on the accumulation of NP-specific mRNA, P-specific mRNA, M-specific mRNA, F-specific mRNA, L-specific mRNA, and HN-specific mRNA by RT, followed by real-time PCR. At 3, 5, 8, and 12 h after infection, in the presence of ndv1, the amount of NP mRNA was inhibited strongly. Moreover, ndv1 exhibited a global inhibitory effect on other viral genes, excluding L gene. As shown in Fig. 1, in the ndv1-treated cells, the accumulation of P-, M-, F-, and HN-specific mRNA was also inhibited.

Discussion

Barik S. [16] proved that small interfering RNAs (siRNAs) against specific mRNAs of non-segmented negative-stranded RNA (NNR) could abrogate expression of the corresponding viral proteins, and generated the predicted viral phenotypes. Knockdown was demonstrated across different genera: respiratory syncytial virus (RSV), a pneumovirus [16]; vesicular stomatitis virus (VSV), a rhabdovirus [17]; and human parainfluenza virus (HPIV), a paramyxovirus [16]. The results provided the first proof-of-concept that double-stranded short interfering RNA could act as potent and specific antiviral agents [16]. The targeted genes could have a wide range of functions, thus documenting the versatility of the technique. siRNAs offered complementary advantages over traditional mutational analyses that were difficult to perform in NNR viruses, and were also an important new tool to dissect host-virus interactive pathways. The interference effect of siRNAs could be affected by several factors such as the GC content, the secondary structure of the target sequence, and properties of the terminal nucleotides of siRNAs. Ui-Tei K. et al. [12] introduced several principles to design efficient siRNA sequences: (1) A/U at the 5′ end of the antisense strand; (2) G/C at the 5′ end of the sense strand; (3) at least five A/U residues in the 5′ terminal one-third of the antisense strand; and (4) the absence of any GC stretch of more than 9 nt in length. To design the shRNA molecules, we analyzed full-length mRNA sequences of NP gene from 15 NDV isolations available in the GenBank and divided these sequences into two clusters, FJ-1-85 (partial cds. ACCESSION, AF512535, VERSION, AF512535.1, GI:21314540 CDS,1…373) and JS-5-01 (partial cds. ACCESSION, AF51 2534 VERSION AF512534.1 GI:21314538, CDS,1…373). The FJ-1-85 and JS-5-01 have highly homologous sequences between 1 and 373 bp. Three shRNAs targeted to NDV NP gene were designed based on the sequence in these regions. The results in this report showed that only the shRNA targeting at the region between 183 and 201 bp of NP gene could effectively inhibit the expression of the target gene, while the other two shRNAs had no obvious interference effect. These suggested that further screening experiments were needed to identify the effect of designed shRNAs.

In this report we showed that (1) shRNA could potently inhibit NDV production and (2) NP-specific shRNA could exert their inhibitory effect by interfering with the accumulation of not only NP-specific mRNA, but also other viral structural genes. These findings have significant implications for the use of shRNA for prophylaxis and therapy of NDV infection, and for the mechanisms underlying NDV transcription and replication. RNAi technology has been used successfully in numerous applications over the past 10 years. A large number of studies

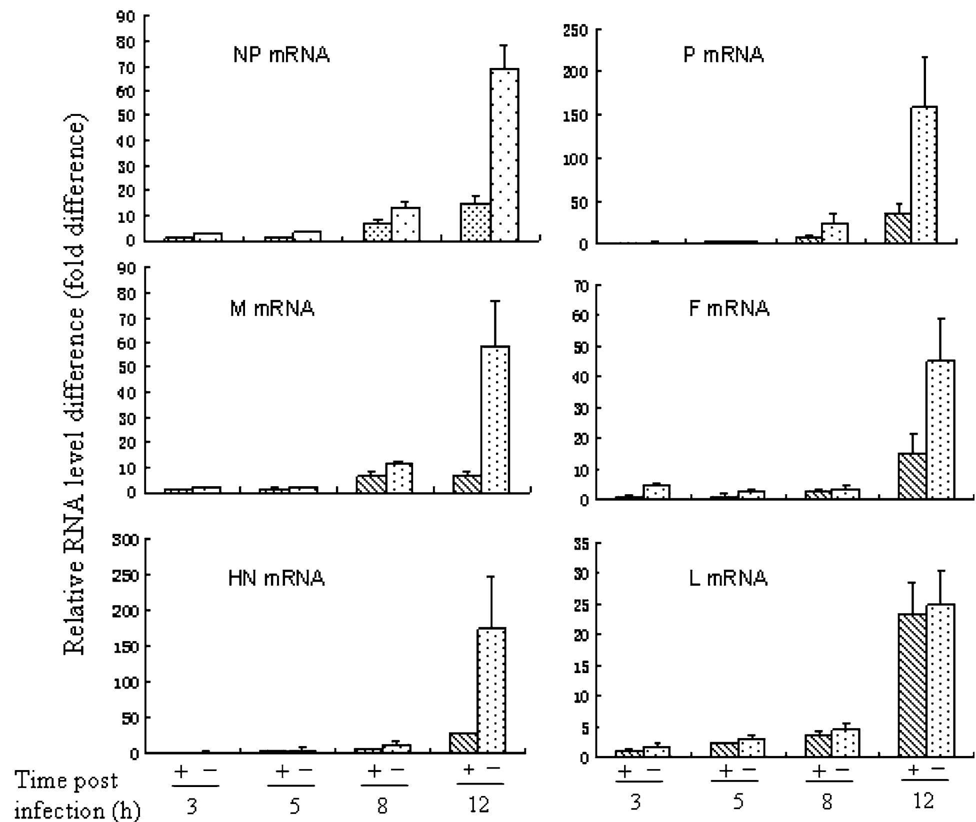

Fig. 1 NP-specific shRNA inhibits the accumulation of not only NP- but also P-, M-, F-, HN-, and L-specific mRNA. About 80% confluent CEF cells were transfected by ndv1 of 1 μg for each well, infected with 100 μg F48E9 (1×10^5 LD50), and harvested for mRNA isolation 3, 5, 8, and 12 h after infection. The levels of mRNA specific for NP, P, M, F, HN, and L were measured by real-time RT-PCR. The level of each structural mRNA species is normalized to the level of β-actin mRNA in the same sample. The relative levels of mRNAs are shown

have demonstrated that RNAi can effectively inhibit the production of various human and animal viruses in vivo and in vitro, including human immunodeficiency virus type 1 (HIV-1), hepatitis B virus (HBV), hepatitis C virus (HCV), SARS-associated coronavirus, swine fever virus (SFV), respiratory syncytial virus (RSV), influenza A virus (IAV), vesicular stomatitis virus (VSV), dengue fever virus (DFV), parainfluenza virus (PIV), poliovirus and avian infectious bronchitis virus (IBV), coxsackievirus (CBV) [16, 18–20]. We showed that shRNAs potently inhibited NDV production in both chicken primary cells and embryonated chicken eggs. These results suggested that the conserved sequence of NP transcription was an ideal target for RNAi to protect against NDV infection and provide a basis for further development of shRNAs for prophylaxis and therapy of NDV infection in poultry.

The RNA genome of NDV is tightly encapsidated by the NP protein. This ribonucleoprotein complex serves as the template for transcription and replication by viral RNA polymerase proteins, which are L and P proteins. The switch between replication and transcription is considered to be controlled by the presence of de novo NP protein in the family Paramyxoviridae [2]. We found, however, that NP- specific shRNA interfered with the accumulation of not only NP-specific mRNA, but also P-, M-, F-, and HN-specific mRNA. These findings revealed a critical role of newly synthesized NP mRNA in NDV transcription and replication. Knockdown NP mRNA presumably blocked the ribonucleoprotein accumulation additionally blocked the accumulation of other viral structural genes transcription. NDV RNA replication follows "the rule of six," that is, efficient replication occurs only if the genome size is a multiple of 6 nt [21]. Probably, in the presence of NP-specific shRNA, the newly transcribed NP mRNA is degraded; further viral transcription and replication are blocked, and new virion production are inhibited.

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References

1. D.J. Alexander, in *Diseases of poultry*, 10th edn., ed. by B.W. Calnek, H.J. Barnes, C.W. Beard, L.R. McDougald, Y.M. Saif (Iowa State University Press, Ames, IA, 1997), pp. 541–570
2. R.A. Lamb, G.D. Parks, Paramyxoviridae: the viruses and their replication, in *Fields virology*, 5th edn., ed. by D.M. Knipe, P.M. Howley (Lippincott Williams & Wilkins, Philadelphia, PA, 2007), pp. 1449–1496
3. R.J. Phillips, A.C.R. Samson, P.T. Emmerson, Arch. Virol. **143**(10), 1993–2002 (1998). doi:10.1007/s007050050435
4. K. Yusoff, W.S. Tan, Avian Pathol. **30**, 439–455 (2001). doi:10.1080/03079450120078626

5. M.G. Kurilla, H.O. Stone, J.D. Keene, Virology **145**(2), 203–212 (1985). doi:10.1016/0042-6822(85)90154-0
6. S.M. Horikami, J. Curran, D. Kolakofsky, S.A. Moyer, J. Virol. **66**(8), 4901–4908 (1992)
7. C.L. Kho, W.S. Tan, B.T. Tey, K. Yusoff, Arch. Virol. **149**(5), 997–1005 (2004). doi:10.1007/s00705-003-0273-8
8. H.D.P. Antua, L.W. McGinnes, M.E. Peeples, T.G. Morrison, J. Virol. **80**(22), 11062–11073 (2006). doi:10.1128/JVI.00726-06
9. S. Krishnamurthy, S.K. Samal, J. Gen. Virol. **79**, 2419–2424 (1998)
10. F. Jahanshiri, M. Eshaghi, K. Yusoff, Arch. Virol. **150**(3), 611–618 (2005). doi:10.1007/s00705-004-0439-z
11. B.S. Seal, J.M. Crawford, H.S. Sellers, D.P. Locke, D.J. King, Virus Res. **83**(1–2), 119–129 (2002). doi:10.1016/S0168-1702(01)00427-0
12. K.U. Tei, Y. Naito, F. Takahashi, T. Haraguchi, H.O. Hamazaki, A. Juni, R. Ueda, K. Saigo, Nucleic Acids Res. **32**(3), 936–948 (2004). doi:10.1093/nar/gkh247
13. W.H. Allan, C.N. Herbet, Arch. Gesamte Virusforsch. **25**, 330–336 (1968). doi:10.1007/BF01556561
14. M.B. Borges, E. Caride, A.V. Jabor, J.M.N. Malachias, M.S. Freire, A. Homma, R. Galler, Virus Genes **36**, 35–44 (2008). doi:10.1007/s11262-007-0173-1
15. K.J. Livak, T.D. Schmittgen, Methods **25**(4), 402–408 (2001). doi:10.1006/meth.2001.1262
16. S. Barik, Virus Res. **102**(1), 27–35 (2004). doi:10.1016/j.virusres.2004.01.012
17. V. Bitko, S. Barik, BMC Microbiol. **1**, 34 (2001)
18. N.J. Caplen, Y.J. Kim, J. Ahn, S.Y. Jeung, D.S. Kim, H.N. Na, Y.J. Cho, S.H. Yun, Y. Jee, E.S. Jeon, H. Lee, J.H. Nam, Virus Genes **36**, 141–146 (2008). doi:10.1007/s11262-007-0192-y
19. X.Y. Kong, W.D. Zhang, R.F. Lockey, A. Auais, G. Piedimonte, S.S. Mohapatra, Genet. Vaccines Ther. **5**(4), 1–8 (2007)
20. M.C. Saleh, R.R.P. Van, R. Andino, Virus Res. **102**(1), 11–17 (2004). doi:10.1016/j.virusres.2004.01.010
21. O.D. Leeuw, B. Peeters, J. Gen. Virol. **80**, 131–136 (1999)