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Short communication

Inhibition of avian metapneumovirus (AMPV) replication by RNA interference targeting nucleoprotein gene (N) in cultured cells

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Abstract

Avian metapneumovirus (AMPV) is the primary causative agent of severe rhinotracheitis in turkeys. It is associated with swollen head syndrome in chickens and is the source of significant economic losses to animal food production. In this study, we designed specific short interfering RNA (siRNA) targeting the AMPV nucleoprotein (N) and fusion (F) genes. Three days post-virus infection, virus titration, real time RT-PCR, and RT-PCR assays were performed to verify the effect of siRNA in AMPV replication. A marked decrease in virus titers from transfected CER cells treated with siRNA/N was observed. Also, the production of N, F, and G mRNAs in AMPV was decreased. Results indicate that N-specific siRNA can inhibit virus replication. In future studies, a combination of siRNAs targeting the RNA polymerase complex may be used as a tool to study AMPV replication and/or antiviral therapy.

Keywords: Avian metapneumovirus; RNA interference; Short interfering RNA; Inhibition; Real time PCR

RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing process initiated by double-stranded RNA (dsRNA). This phenomenon was first observed in the nematode Caenorhabditis elegans (Fire et al., 1998) and then in other organisms, such as plants, invertebrates (Drosophila), and vertebrates (Bitko et al., 2005; Buckingham, 2006; Ronemus et al., 2006). The RNAi pathway is triggered by a longer dsRNA which is cleaved in 21–25 nucleotides (nt) fragments (siRNAs) by RNAse III-like protein DICER. The siRNAs are subsequently incorporated into the RNA-induced silencing complex (RISC), which recognizes and cleaves the target messenger RNA (mRNA) (Elbashir et al., 2001a). Previous studies showed that many viruses can be inhibited by siRNA, such as foot and mouth disease virus (Chen et al., 2006; Grubman and de los Santos, 2005), poliovirus (Gitlin et al., 2005), SARS (Li et al., 2005; Wang et al., 2004), influenza virus (Ge et al., 2003, 2004a,b), human respiratory syncytial virus (Bitko and Barik, 2001; Bitko et al., 2005), and avian metapneumovirus (Munir et al., 2006).

AMPV belongs to the Paramyxoviridae family, Pneumovirinae subfamily, within the genus Metapneumovirus (Cook, 2000; Easton et al., 2004; Njenga et al., 2003). It was first reported in the late 1970s in South Africa (Buys and du Preez, 1980) and subsequently in France and in the UK (Giraud et al., 1986; McDougall and Cook, 1986). There are four subtypes of AMPV (A–D) throughout the world (Bäyon-Auboyer et al., 2000; Dar et al., 2001; Juhasz and Easton, 1994; Toquin et al., 2000). In Brazil, only AMPV subtype A (AMPV/A) was detected so far (D'Arce et al., 2005; Dani et al., 1999).

Metapneumoviruses contain a non-segmented, negative-sense RNA with an approximately 13,000-nt long genome. The AMPV genome consists of eight viral genes arranged as follows: '3–N–P–M–F–M2–SH–G–L–5'. Its RNA genome, in association with nucleoprotein (N), large protein (L), and phosphoprotein (P) are often referred to as RNA polymerase complex (Easton et al., 2004). The N protein intimately wrapped with the viral RNA genome forms a nucleoprotein complex (N-RNA) and becomes resistant to RNases. N-RNA serves also as biological template, because RNA polymerase complex does not recognize pure deproteinized genomic RNA (Barik, 2004). RNA polymerase complex is responsible for the synthesis of all viral RNA, including mRNA, replicative intermediates, and the progeny

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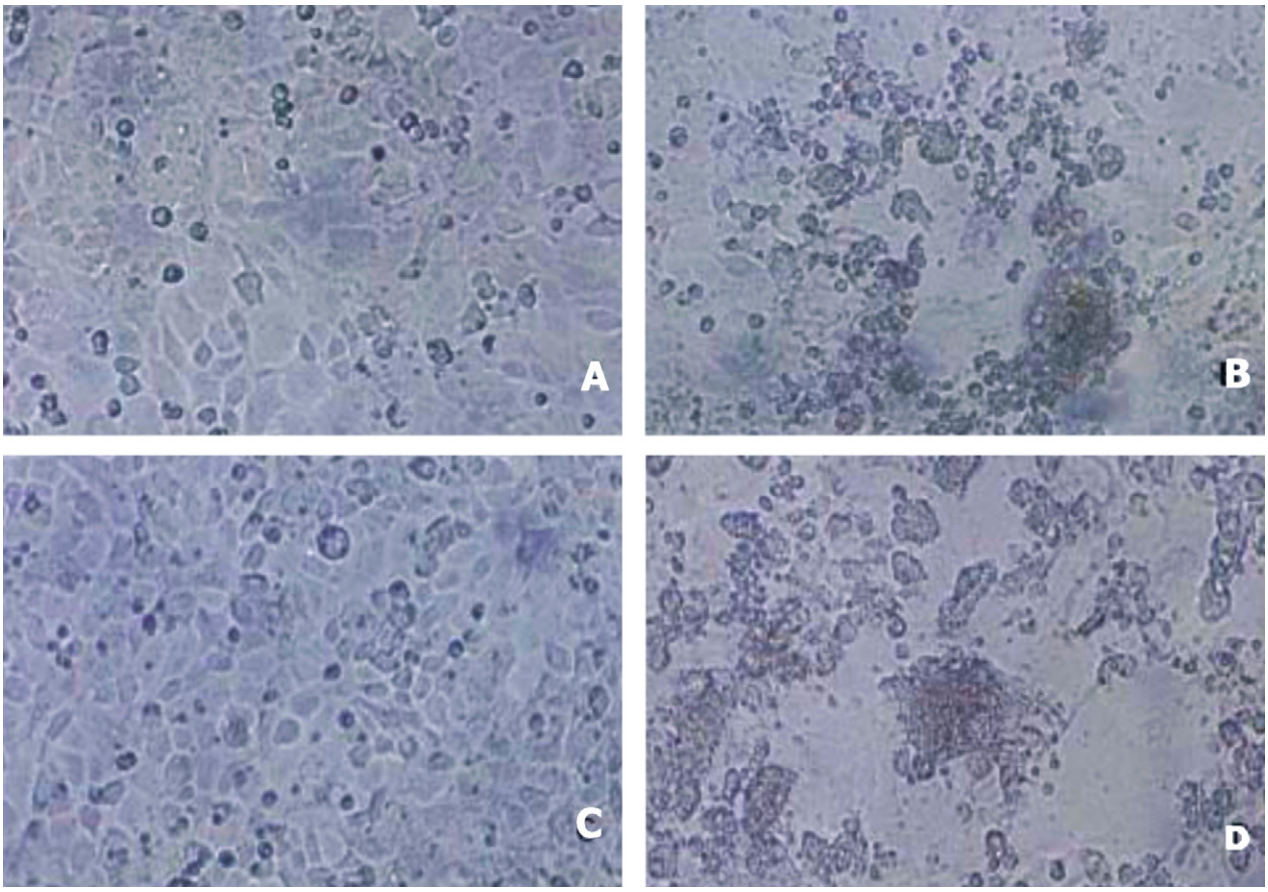


Fig. 1. CPE in transfected cells after 3 days (200×). CER cells were transfected with siRNAs at 75 nM. The transfected cells were infected after 6 h with AMPV subtype A: (A) negative control (without virus and siRNAs); (B) siRNA/GFP (control group); (C) siRNA/N; (D) siRNA/F.

RNA genomes (Easton et al., 2004). AMPV has two main surface glycoproteins in its membrane: the attachment (G) protein and the fusion (F) protein, which is responsible for fusion of viral and cell membranes (Walsh and Hruska, 1983). In the present study, we tested an siRNA sequence targeting the AMPV N and F genes to evaluate its inhibition effect in AMPV/A.

Based on previous research (Elbashir et al., 2001b), we designed 21-nt siRNA sequences with a G/C content of 30–70% and 2-nt 3′ overhangs. A leader sequence region of 5′ subgenomic AMPV N mRNA (GenBank accession number DQ175638) was targeted by siRNA/N [sense r(CCGGCGUGCCUCAAGGGUAUU)dTdT] and a leader sequence region of 5′ subgenomic AMPV F mRNA (GenBank accession number DQ175632) was targeted by siRNA/F [sense r(UGCCCUCCGGAACACAAAUGA)dTdT]. A negative control siRNA/GFP [sense, r(GACGGGAACUACAAGACACGU)dTdT] was designed, targeting the green fluorescent protein (GFP). The selected siRNA sequences were submitted to BLAST analysis, ensuring that only the intended gene was targeted. All RNA oligonucleotides were synthesized by IDT (Coralville, Iowa). CER (Chicken related embryo) cells line were cultured in Eagle's minimal essential medium (E-MEM) with 10% fetal bovine serum. CER cells were plated in 24-well plates (2×10^5 cells/well) for 1 day and transfected for 30 min with a total amount of 25, 50, 75, and 100 nM per well of siRNA duplexes (siRNA/N, siRNA/F and siRNA/GFP), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The transfected cells were infected after 6 h with AMPV/A (SHSBR/662/03 strain) at M.O.I. of 0.01 and examined 3 days later. Virus cytopathic effect (CPE) could be observed after 3 days incubation. Cells transfected with siRNA/N showed a significant decrease of virus

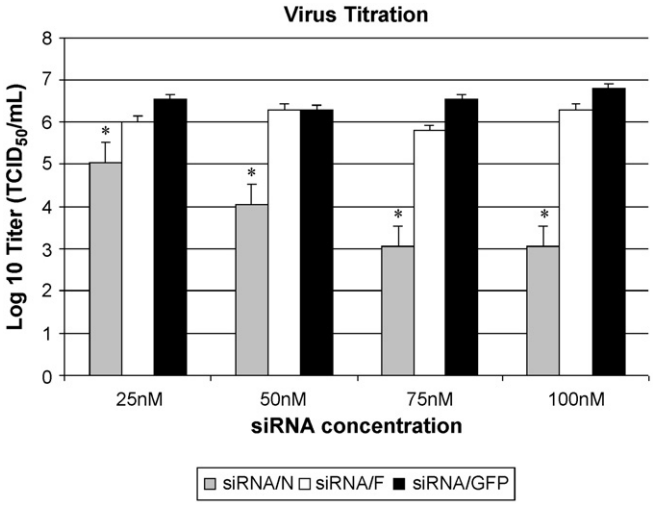
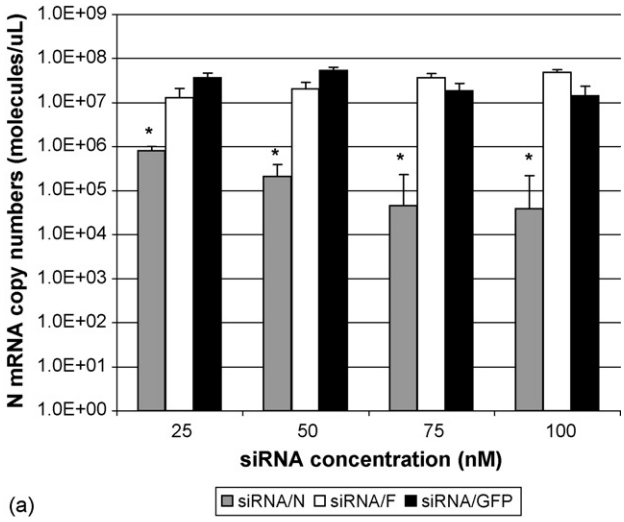


Fig. 2. Transfected cells monolayer were infected with AMPV after 6 h post-transfection. Supernatants were collected 3 days after infection and assayed for virus titration. $^{*}p < 0.05$ when compared to the control group.

CPE, whereas those transfected with other siRNAs showed syncytium formation, rounding of refringent cells and many detached cells at all concentration (Fig. 1). Culture supernatants were assayed by virus titration, real time RT-PCR, and RT-PCR to investigate AMPV inhibition by siRNA.

siRNA-transfected and virus-infected cultures were recovered and titrated by $TCID_{50}$ assay (Reed and Muench, 1938). Culture supernatants transfected with siRNA/N at 25, 50, 75, and 100 nM concentrations, presented reduction of AMPV titers by 96.8, 99.4, 99.9 and 99.9%, respectively, when compared to the siRNA/F and siRNA/GFP treated samples (Fig. 2).

Real time RT-PCR was employed to determine N mRNA and F mRNA reduction. RNA was isolated from infected cells using Trizol (Invitrogen, Carlsbad, CA), and first-strand cDNA was made using Superscript III Rnase H-reverse transcriptase kit (Invitrogen, Carlsbad, CA). Real time PCR amplification was performed using Quantitec Probe PCR kit (Qiagen, Hilden, Germany), with final concentrations of 900 nM for each primer, and 300 nM for the Taqman



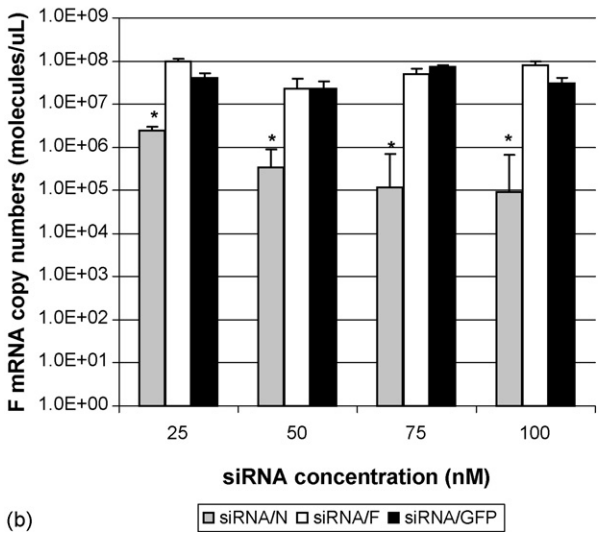


Fig. 3. Attenuation of AMPV mRNA copy numbers by siRNAs in N mRNA (a) and F mRNA (b). $^{*}p < 0.05$ when compared to the control group.

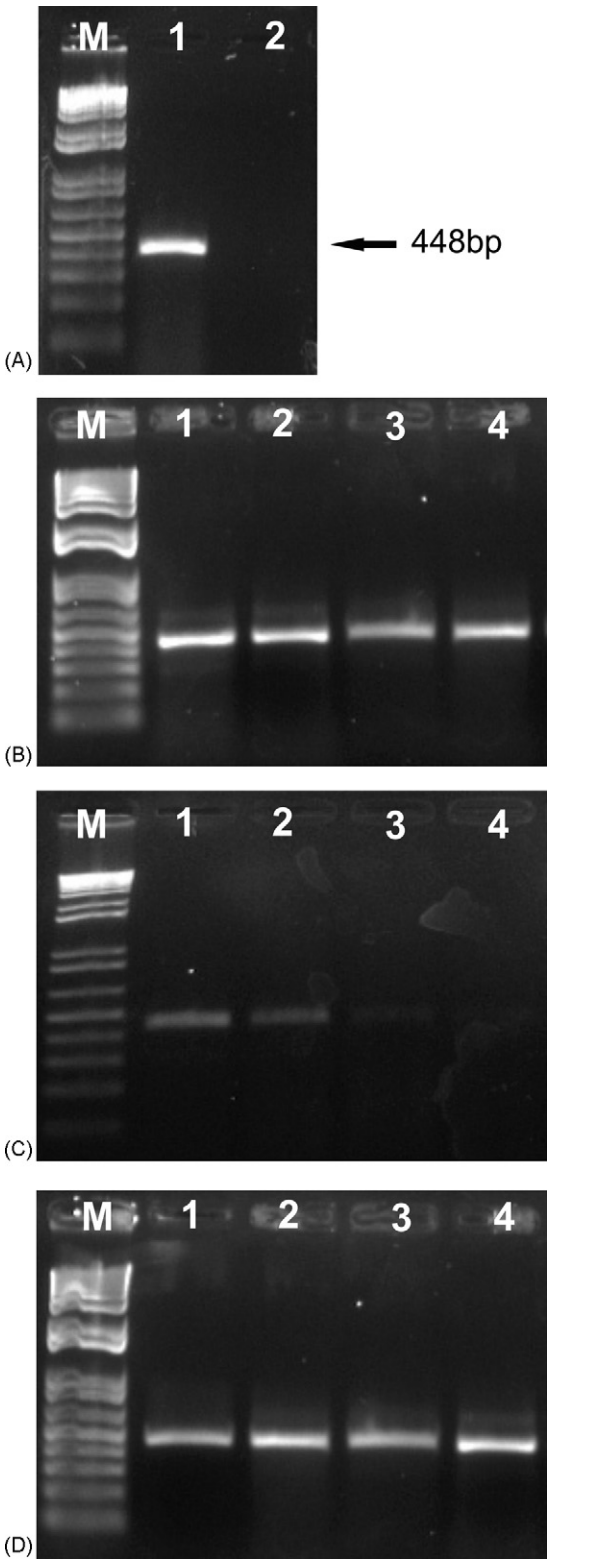


Fig. 4. Effect of different siRNAs on G mRNA production (448 bp) in the culture supernatants from infected cells, as indicated: (A), 1 positive and 2 negative control; (B) siRNA/GFP; (C) siRNA/N; (D) siRNA/F; M, leader 1 kb plus; numbers 1–4, 25, 50, 75, 100 nM, respectively.

probe, in a total reaction volume of 25 μL, containing 1 μL of cDNA. Primers for N mRNA were: AMPVN+494 (5′-CAAAAGCCGTCTGCCTTGGAT-3′), AMPVN-567 (5′-GAGGCCAACTTGGTGAAAATG-3′), and the Taqman probe AMPVN+516FAMTAMRA (5′-CTCCCGTTATTCTATTATG-CATTGGTGCCC-3′). Primers for F mRNA were: AMPVF+3643 (5′-TGCCAACTTCATCAGGACAGA-3′), AMPVF-3721 (5′-TCAATATACCAAACCCCTTCCTTCT-3′), and the Taqman probe AMPVF+3667FAMTAMRA (5′-AGTTT-GATGTTGAACAATCGTGCCATGGT-3′). All samples were run in duplicate and carried out on an ABI PRISM 7500 real time PCR cycler (Applied Biosystems, Foster City, CA). For absolute quantification, a standard curve was created based on spectrophotometric determination of PCR products of each gene (Bustin, 2000; Whelan et al., 2003). Copy number was calculated using the formula: molecules/μL = [g/μL DNA/(PCR product length in base pairs × 660) × 6.022×10^{23}]. siRNA/N was compared with the control using Student's *t*-test with a level of significance of $p < 0.05$.

Real time RT-PCR results presented reduction of AMPV N mRNA by 97.8, 99.6, 99.7 and, 99.7%, when transfected with siRNA/N at 25, 50, 75, and 100 nM concentrations, respectively, as compared to siRNA/F and siRNA/GFP treated samples (Fig. 3a). Similar results were obtained with RT-PCR targeting the F protein on samples treated with siRNA/N (Fig. 3b). The absence of PCR inhibitors was confirmed by detection of the internal control (beta-actin gene) in all samples (data not shown).

To confirm that siRNAs can inhibit virus replication, 1 μL of cDNA was applied for RT-PCR by using Taq Polymerase Recombinant (Invitrogen, Carlsbad, CA) with AMPV G gene-specific primers (Bäyon-Auboyer et al., 2000). The RT-PCR conditions were: 94 °C, 3 min; 25 cycles of (94 °C, 30 s, 54 °C, 30 s, 72 °C, 1 min); 72 °C, 10 min. A marked reduction in G mRNA copies was observed only in the cells transfected with siRNA/N (Fig. 4).

The present study reports an siRNA sequence targeting the N gene which was able to inhibit the AMPV production in vitro. We observed a decrease of infectious particles production as well as a reduction of mRNA production by targeting N mRNA with siRNA. The F mRNA and G mRNA were also inhibited by siRNA/N, confirming the essential role of N protein in AMPV replication. Munir et al. (2006) successfully described AMPV subtype C inhibition targeting other component of RNA polymerase complex, the phosphoprotein. In future studies, a combination of siRNAs targeting the RNA polymerase complex should be further explored as a tool to study AMPV infections or as an antiviral therapy.

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