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# siRNAs targeting PB2 and NP genes potentially inhibit replication of *Highly Pathogenic H5N1 Avian Influenza Virus*

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*Highly Pathogenic Avian Influenza* (HPAI) H5N1 virus is a threat to animal and public health worldwide. Till date, the H5N1 virus has claimed 402 human lives, with a mortality rate of 58% and has caused the death or culling of millions of poultry since 2003. In this study, we have designed three siRNAs (PB2-2235, PB2-479 and NP-865) targeting PB2 and NP genes of avian influenza virus and evaluated their potential, measured by hemagglutination (HA), plaque reduction and Real time RT-PCR assay, in inhibiting H5N1 virus (A/chicken/Navapur/7972/2006) replication in MDCK cells. The siRNAs caused 8- to 16-fold reduction in virus HA titers at 24 h after challenged with 100TCID<sub>50</sub> of virus. Among these siRNAs, PB2-2235 offered the highest inhibition of virus replication with 16-fold reduction in virus HA titer, 80% reduction in viral plaque counts and 94% inhibition in expression of specific RNA at 24 h. The other two siRNAs had 68–73% and 87–88% reduction in viral plaque counts and RNA copy number, respectively. The effect of siRNA on H5N1 virus replication continued till 48h (maximum observation period). These findings suggest that PB2-2235 could efficiently inhibit HPAI H5N1 virus replication.

[Behera P, Nagarajan S, Murugkar HV, Kalaiyarasu S, Prakash A, Gothawal R, Dubey SC, Kulkarni DD and Tosh C 2015 siRNAs targeting PB2 and NP genes potentially inhibit replication of *Highly Pathogenic H5N1 Avian Influenza Virus*. *J. Biosci.* **40** 233–240] DOI 10.1007/s12038-015-9524-6

## 1. Introduction

Avian influenza (AI) is a highly infectious respiratory disease of domestic poultry caused by *Influenza A virus* (IAV) of the family *Orthomyxoviridae*. IAVs are enveloped viruses containing eight segments of single-stranded, negative-sense RNA as viral genome that encodes for at least 13 proteins (Muramoto *et al.* 2013; Dubois *et al.* 2014). The viruses are categorized into distinct subtypes based on the genetic and antigenic make up of two surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). Till date, 18 HA (HA1-18) and 11 NA (NA1-11) subtypes have been identified (Fouchier *et al.* 2005; Tong *et al.* 2012, 2013).

IAVs infecting poultry can further be divided into two major pathotypes on the basis of their ability to cause disease; the very virulent viruses are termed as highly pathogenic avian influenza (HPAI), in which the flock mortality is as high as 100%. These viruses have been restricted to subtypes H5 and H7, although not all viruses of these subtypes cause HPAI. All other viruses cause a much milder disease consisting primarily of mild respiratory disease, depression and problem in egg production and termed as *Low Pathogenic Avian Influenza* (LPAI) (Capua and Alexander 2009).

Outbreaks of HPAI H5N1 virus infection in poultry that started in late 2003 in Southeast Asia are still continuing by infecting domestic poultry, wild birds and sporadic zoonotic

**Keywords.** Avian influenza virus; NP; PB2; siRNA; subtype H5N1

transmission to humans thereby revealing its potential to cause the next pandemic. As on 6th January 2015, H5N1 viruses have killed 402 out of 694 laboratory-confirmed human infections in 16 countries (WHO 2015), with a mortality rate of around 58%. At present, the H5N1 viruses are endemically circulating in poultry in few countries in Asia and Africa (FAO 2011). For control of HPAI in birds, majority of the countries have followed stamping-out method (Swayne et al. 2011). However, few countries have adapted vaccination for control of HPAI in poultry. The use of anti-influenza vaccines and drugs has limitations due to emergence of variants to vaccines (Savill et al. 2006; Domenech et al. 2009; Cattoli et al. 2011; Wang et al. 2012) and anti-influenza drugs (Cheung et al. 2006; Hurt et al. 2007; He et al. 2008; Boltz et al. 2010; Tosh et al. 2011; Govorkova et al. 2013). Besides, the vaccine strain selection is so stringent that vaccination against one lineage/clade of virus does not afford protection to the challenge virus from other clades in poultry (Cha et al. 2013). Because of the global human and animal health importance of the H5N1 HPAI, developing an alternative antiviral agent against H5N1 is urgently needed.

RNA interference (RNAi) is a process by which double-stranded RNA duplexes (21–26 nt long) inhibit the gene expression by inducing sequence-specific degradation of homologous mRNA. The sequence-specific knockdown of viral genes in infected cells without affecting on host gene expression has generated great interest in development of small interfering RNAs (siRNAs)-based antiviral therapeutics (Elbashir et al. 2001; Morris and Rossi 2006). Previous reports have shown that the siRNAs could effectively inhibit IAV replication both *in vivo* and *in vitro* (Ge et al. 2003; Li et al. 2005; Zhou et al. 2007; Sui et al. 2009; Zhang et al. 2009; Li et al. 2011).

Polymerase Basic-2 (PB2) and nucleoprotein (NP) are integral components of ribonucleoprotein (RNP) complex playing crucial roles in influenza virus life cycle in association with other polymerase proteins (PB1 and PA). At the onset of replication, PB2 initiates the cap-snatching process by recognizing and binding to 7mGpppGpNm cap near the 5' termini of host mRNA (Kowalinski et al. 2012; Sikora et al. 2014). The PB2 also play an essential role during genome packaging along with PA and matrix (Gao et al. 2012). NP functions as an adapter between the virus and host cell processes to maintain the viral genome integrity. After release of RNP into the cell cytoplasm, NP interact with many macromolecules of cellular origin (importin  $\alpha$ , actin filament, nuclear export receptors and cellular splicing factors) to facilitate transcription, replication and translation of viral genome (Portela and Digard 2002; Zheng and Tao 2013).

In this study, we designed specific siRNAs in the conserved regions of the PB2 and NP genes, and evaluated their

abilities to inhibit replication of the H5N1 HPAI virus (A/chicken/Navapur/7972/2006) *in vitro*.

## 2. Materials and methods

### 2.1 Cell culture and virus titration

Madin-Darby Canine Kidney (MDCK) cells were maintained in Glasgow Minimum Essential Media (GMEM; Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, USA) and antibiotics (100 U/ml penicillin and 100  $\mu$ g/mL streptomycin) in 5% CO<sub>2</sub> environment at 37°C. Avian influenza H5N1 virus (A/chicken/Navapur/7972/2006) isolated during HPAI outbreak in chickens in Maharashtra, India was used in the study. The

virus was grown initially in the allantoic cavity of 10-day-old embryonated chicken eggs at 37°C and subsequently the virus was propagated in MDCK cells in GMEM supplemented with antibiotics. The virus infectivity titre (50% Tissue Culture Infectivity Dose; TCID<sub>50</sub>) was determined by standard method (Reed and Muench 1938). The infected cell culture supernatants were clarified by centrifugation, aliquoted, and stored at –80°C for further use. All experiments with the H5N1 virus were performed in the containment laboratory (BSL-3) of ICAR-National Institute of High Security Animal Diseases, Bhopal.

### 2.2 siRNAs

siRNAs (table 1) targeting conserved regions in the PB2 and NP genes were designed according to the web-based criteria ([www.ambion.com](http://www.ambion.com)). The siRNAs duplexes were labelled with fluorescent dye (Alexa Fluor 488) at 3' end and synthesized commercially (Sigma, USA).

### 2.3 siRNA transfection and virus challenge

siRNAs at a concentration of 125 pmol and 250 pmol were diluted in serum-free-media (Opti-MEM I; Invitrogen, USA) and transfected in triplicates to logarithmic-phase MDCK

**Table 1.** siRNAs used in this study

siRNA	Sequence
PB2-479	sense 5'- GAUGUCAUCAUGGAGGUCGdTdT- 3', antisense 3'-dTdTTCUACAGUAGUACCUCCAGC- 5'
PB2-2235	sense 5'-ACGGAAACGGGACUCUAGCdTdT- 3', antisense 3'- dTdTUGCCUUUGCCCUGAGAUCG -5'
NP-865	sense 5'-GUCCUGCUUGCCUGCUUGdTdT- 3', antisense 3'- dTdTTCAGGACGAACGGACGAACA -5'

cells (60–70% cell confluency) at 30th passage level in 24-well plates using X-treme® Gene Transfectant (Roche, USA). Mock transfection (no siRNA) was kept as negative control. The transfection efficiency was assayed by direct visualization of cells under UV fluorescent microscope. About 16 hrs after transfection, the transfection medium was removed and cells were inoculated with influenza virus A/chicken/Navapur/7972/2006 (H5N1) with 100 TCID<sub>50</sub>/well. Following 1 h adsorption, cells were washed; wells replaced with 500 µL GMEM without serum and incubated at 37°C under 5% CO<sub>2</sub> environment. Infected cell supernatants were collected at 24, 36 and 48 h post infection (p.i.), clarified by centrifugation, aliquoted, and stored at –80°C for further use. Virus titer was determined by hemagglutination assay, plaque assay and real time PCR assay.

#### 2.4 Hemagglutination assay

Titration of the virus in the infected cell supernatants were carried out as described previously (OIE 2012). The hemagglutination (HA) assay was carried in V-bottom 96-well micro-titre plate using 1% chicken RBC as indicator system.

#### 2.5 Plaque assay

Plaque assay for virus titrations was performed as described previously (Bright *et al.* 2008) with little modification. Confluent monolayers of MDCK cells in 24-well plates were inoculated with 50 µL of virus serially diluted (10-fold) in GMEM without serum. Virus was allowed to adsorb onto cells for 1 h at 37°C and 5% CO<sub>2</sub> environment with gentle rocking every 15 min. The inoculum was removed and cells were washed and overlay with 1% SeaPlaque Agarose (Lonza, USA) containing 2.5% FBS and antibiotics, and incubated at 37°C under 5% CO<sub>2</sub> environment. Three days after infection, the plaques were visualized by staining with 0.1% crystal violet solution. Plaque-forming units (PFU) was counted from three independent experiments and the drop in plaque counts was expressed in percentage reduction.

#### 2.6 Cloning and *in vitro* transcription of PB2 and NP genes

Viral RNA was isolated from infected cell culture supernatant using QIAamp viral RNA mini kit (QIAGEN, Germany). The viral RNA was reverse-transcribed using AMV Reverse Transcriptase (Promega, USA) and IAV universal primer (Hoffmann *et al.* 2001). Full length PCR amplification of the PB2 and NP genes was carried out with *Taq* DNA polymerase (Promega, USA) using segment-

specific primers (Hoffmann *et al.* 2001). The PCR amplified products were purified with the QIAquick gel extraction kit (Qiagen, Germany) and cloned into pTZ57R/T vector (Fermentas, USA). Recombinant plasmids containing the full length sequences of the PB2 and NP genes of A/chicken/Navapur/7972/2006 in pTZ57R/T vector were linearized using vector specific *Xba*I restriction enzyme (MBI Fermentas, USA). The plasmids containing the genes were *in vitro* transcribed (IVT) using T7 Transcription Kit (Fermentas, USA). The quantification of IVT RNA was carried out using Qubit® Fluorometer and Quant-iT™ RNA Assay Kit (Invitrogen, USA) according to the instructions of the manufacturer. The copy numbers of the RNA transcripts were determined as previously reported (Nagarajan *et al.* 2012).

#### 2.7 SYBR Green real-time RT-PCR

The concentrations of IVT RNAs and their deduced RNA copies were estimated as described previously (Nagarajan *et al.* 2012). With modification of the protocol described earlier (Santhosh *et al.* 2007), standard curves were prepared from ten-fold serially diluted IVT RNA of PB2 and NP genes in presence of gene specific primers using Brilliant II SYBR® Green QRT-PCR Master Mix (Stratagene, USA) in Light Cycler® 480 Real Time PCR System II (Roche, USA). One set of primer for PB2 gene (Forward: 469-AAAGAA GCACAAGATGTCATCATGGA-494 and Reverse: 947-CCCATTGCTGCTTTGCATATA-927 designed using LightCycler Probe Design Software 2.0, Version 1.0 Roche, USA) and previously reported NP gene primer (Nagarajan *et al.* 2012) were used in the study. A single-Step QRT-PCR of 12.5 µL containing 6.25 µL of 2× master mix, 0.4 µL of passive reference dye (1:500 dilution), 0.5 µL each of the forward and reverse primers (5 pmole), 1 µL of RNA, 0.5 µL of Enzyme mixture (RT/RNase Block Enzyme) and 3.35 µL of nuclease free water. No template, no primer and buffer controls were also included in the test. The thermal profile included one cycle of reverse transcription at 50°C for 45 min, one cycle of polymerase activation at 95°C for 10 min followed by 40 PCR cycles of denaturation (95°C for 30 s), annealing (50°C for 30 s) and extension (72°C for 30 s) and one cycle of final extension at 72°C for 10 min. A melting curve analysis was performed, where the one cycle of 95°C for one min and cooling to 50°C temperature was followed by increase in temperature by 0.5°C every 10 s, till 95°C under continuous data acquisition mode. Data analysis for estimated viral copies was performed using the second derivative method of the instrument extrapolating the standard curves.

## 2.8 Preparation of standard curve

Ten-fold serial dilutions (from  $\log 10^{-1}$  to  $\log 10^{-10}$ ) of the quantified RNA standards of the PB2 and NP genes were tested in triplicate using SYBR green Real Time RT-PCR protocol described under section 2.7. The  $C_p$  values obtained against the known concentration of the RNA standards were used for construction of standard curve.

## 2.9 Statistical analysis

One-way analysis of variance (ANOVA) was used to compare multiple groups using SPSS 16.0 software. Pair wise comparison between different groups was done by Tukey's *post hoc* analysis.  $p < 0.05$  was considered as statistically significant level.

## 3. Results

siRNAs targeting three conserved regions (PB2-479, PB2-2235 and NP-865) were designed to study the inhibition of avian influenza H5N1 virus (A/chicken/Navapur/7972/2006) replication *in vitro*. In the blast search at National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), none of the siRNAs were found sequence homology with human genome or any other host genome. All the siRNAs were found to have sequence homology with human, avian and swine strains of IAVs. To test whether these siRNAs inhibited influenza virus replication, they were transfected into a monolayer of MDCK cells. As the siRNAs were labeled with Alexa Fluor 488 at 3' position, the efficiency of transfection was visualized under UV microscope after 16 h post transfection and the fluorescence intensity was almost double in wells with 250 pmol of siRNA compared to that of 125 pmol (data not shown).

As shown in table 2, virus production (titre in HA units) in the infected cell culture supernatants was significantly lower in the three specific siRNAs-treated cells than in the control (no siRNA). Moreover, siRNAs caused a reduction of 8- to 16-fold in virus HA titers compared with the control at 24 h. Among these siRNAs, PB2-2235 showed the highest inhibition activity (16-fold reduction) even at 48 h of virus challenge (maximum observation period). With PB2-2235, the inhibition was so pronounced that culture supernatants lacked detectable HA activity at 24 h with 125 pmol of siRNA (Table 2). From the result it is evident that the extent of inhibition of virus production was siRNA dose-dependent manner. For example, treatment of 125 pmole of PB2-479 and NP-865 reduced virus production to 2 HA titre, whereas there was no detectable HA titre with treatment of 250 pmol of the same siRNA. The effect of siRNA on influenza virus

**Table 2.** Effects of siRNAs on H5N1 virus production in MDCK cells

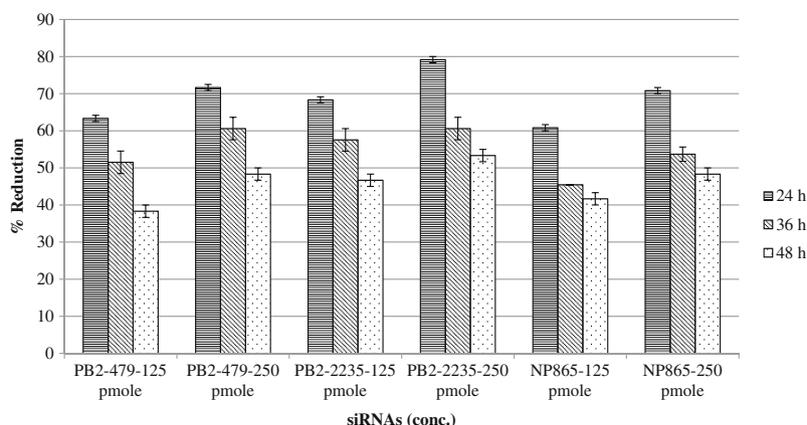
siRNA	siRNA concentration (pmol)	Virus production (titre in HA units) at:		
		24 h	36 h	48 h
PB2-2235	125	1	8	32
	250	1	4	16
PB2-479	125	2	8	64
	250	1	4	32
NP-865	125	2	16	64
	250	1	8	32
mock	-	16	32	256

production was continued till 48 h, the maximum observation period.

To further evaluate the antiviral effect of the siRNAs, plaque assay and real time RT-PCR assay were conducted in infected cell culture supernatants. In the plaque assay, there was reduction of 50–80% of viral plaque counts with treatment of 250 pmol of siRNAs compared to the control (figure 1). However, with treatment of 125 pmol of siRNAs, the reduction of plaque count was 40–68%. The PB2-2235 offered the highest inhibition of virus production compared to the PB2-479 and NP-865 (figure 1). With treatment of 250 pmol of siRNA, highest reduction of 80% in viral plaque counts was observed at 24 h with PB2-2235 compared to 68–73% with PB2-479 and NP-865. The effect of siRNAs on influenza virus replication was observed till end of the experiment (48 h) with reduction of virus plaques of 50–55% and 40–45% with 250 and 125 pmol of siRNAs, respectively. Plaque reduction was highly significant ( $p < 0.0001$ ) between groups at all three time intervals. Tukey's *post hoc* analysis revealed that pair wise comparison also significantly different between groups. Mean plot analysis revealed that treatment of 250 pmole of siRNA PB2-2235 was significantly better than other groups at all three time intervals.

In SYBR Green Real time RT PCR, the minimum detection limit of PB2 gene was found to be  $2.34 \times 10^4$  ( $SD \pm 0.044$ ) and the 478 bp product displayed melting peak at  $82(\pm 0.5)^\circ\text{C}$ . Similarly for NP gene, we found the detection limit to be  $2.11 \times 10^3$  ( $SD \pm 0.106$ ) and the 445bp amplicon shows a melting temperature of  $83(\pm 0.5)^\circ\text{C}$ .

Evaluating the inhibition in real time RT-PCR assay, siRNAs caused 66–94% reduction in RNA copy number at 24 h with treatment of 250 pmole siRNA, whereas the reduction was 47–91% with 125 pmol of siRNAs (figure 2). Out of the three siRNAs, PB2-2235 offered the highest inhibition with 91% and 94% reduction in RNA copy number compared to control with 125 and 250 pmol of siRNAs, respectively. The other two siRNAs had similar anti-influenza activity with maximum (87–88%) inhibition in



**Figure 1.** Reduction (%) in viral plaque counts in the infected supernatants.

RNA copy number at 24 h with 250 pmole of siRNAs. Specific anti-influenza activity persisted for 48 h (maximum observation period) as seen from the inhibition of RNA copy number compared to the control.

Copy number was highly significant ( $p < 0.0001$ ) between groups at all three time intervals. Tukey's post hoc analysis revealed that pair wise comparison is also significant ( $p < 0.001$ ) among the groups except between 125 pmol of siRNA PB2-479 and 250 pmole of siRNA NP-865. Mean plot analysis revealed that treatment of 250 pmol of siRNA PB2-2235 was significantly better than others at all three time intervals.

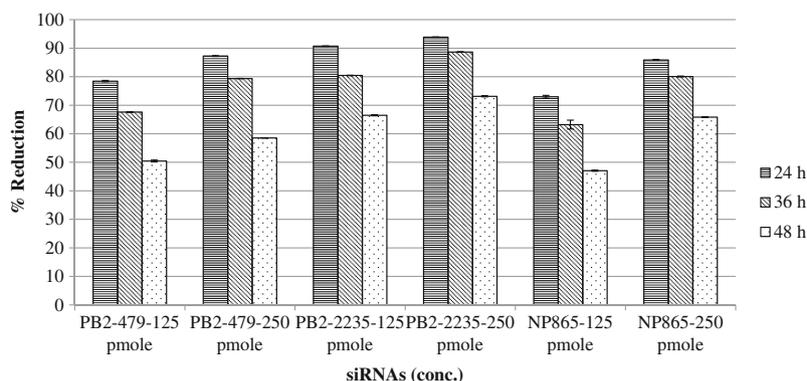
#### 4. Discussion

HPAI H5N1 virus continues to be a significant threat to animal and public health worldwide. The anti-influenza vaccines and drugs have their limitations due to emergence of variants to vaccines and anti-influenza drugs. Development of novel antivirals to combat the economic and public health

impact of HPAI H5N1 virus remains a priority. RNA interference had been used as an effective strategy for its specific silencing of viral gene expression in mammalian cells.

For an effective RNAi study, the siRNAs should be designed targeting the conserved regions of viral mRNAs. Due to high sequence variability, studies related to HA and NA genes have not been reported so far. Previous studies with siRNAs directed against NP, PA, NS1 and M2 genes have established that conserved genes offer higher protection rate during H5N1 infection *in vivo* and NP, PA, M, PB1 and PB2 genes *in vitro* (Ge *et al.* 2003; Zhou *et al.* 2007; Zhang *et al.* 2009; Stewart *et al.* 2011). Since recent studies have indicated that PB2 and NP genes play a crucial role in the virus replication and packaging, siRNAs against these two genes should inhibit most influenza virus replication and hence used in this study.

All the three siRNAs inhibit the influenza virus production, but with varying degrees. Anti-influenza properties of the siRNAs continued till the end of the experiment (48 h) as revealed from the reduction in HA titre, viral plaque counts and RNA copy number in the virus infected cell culture



**Figure 2.** Reduction (%) in viral RNA copy number in the infected supernatants.

supernatants. Out of the three siRNAs, the PB2-2235 offered the highest anti-influenza effects with no detectable HA titre leading to 68–80% reduction in viral plaque counts at 24 h with both the concentrations (125 and 250 pmol) of siRNA (table 1 and figure 1). The real time PCR assay estimated 91–94% reduction in RNA copy number with same concentrations of PB2-2235 at 24 h. However, despite of a rational design in the conserved regions of PB2 gene, the extent of antiviral activity of another siRNA against PB2 (PB2-479) is less prominent (79–87% reduction in RNA copy number at 24 h with both the concentrations). PB2-2235 inhibited H5N1 virus replication more efficiently than PB2-479 indicating that this difference in efficacy cannot be ascribed solely due to the differences in transfection efficiency and further signifies that PB2-2235 is a potent site for further *in vivo* experiments. Here both the siRNAs shared complete sequence match to H5N1 challenge virus (A/chicken/Navapur/7972/2006), thus possible reason could be the target site accessibility to RISC (RNA-induced silencing complex) for binding of siRNAs, as the GC-content in both cases are close 50% (PB2-2235: 52% and PB2-479: 48%). Previous studies have shown that target site accessibility/mRNA secondary structure is equally important to the GC-content for efficient RNA interference (Chan *et al.* 2009), as RISC involved in mRNA degradation is unable to unfold the structured RNA (Ameres *et al.* 2007). The PB2 RNA secondary structure prediction (data not shown) by mfold server (<http://mfold.rutgers.edu/?q=mfold/download-mfold>) showed that both the siRNAs are targeted to different regions; PB2-2235 – loop region, PB2-479 – hairpin structure. Our study is also in agreement with the result of Luo and Chang (2004), where they have shown that siRNA forming a hairpin structure are usually less effective in gene silencing. In case of NP-865, like PB2-479, had moderate anti-influenza effect with 60–68% reduction in viral plaque counts and 73–88% reduction in RNA copy number at 24 h. Our findings demonstrate that the inhibition of the viral replication by siRNA is inversely correlated to the time after virus challenge but directly correlated to the dose of the siRNA. The inverse correlation of the viral replication by siRNA directed against PB2 to the time after virus challenge has also been reported by Ge *et al.* (2003). Even though the method of virus titration is different (TCID<sub>50</sub> in our study and multiplicity of infection (moi) in the reported study), both the studies have used MDCK cell line. In this case, 1 mL of virus stock would be expected to have about half of the number of plaque forming units (PFUs) as TCID<sub>50</sub> ([http://www.atcc.org/~media/PDFs/webinars/QA\\_Webinar\\_Influenza.ashx](http://www.atcc.org/~media/PDFs/webinars/QA_Webinar_Influenza.ashx)). As Ge *et al.* (2003) used 0.01 moi (50 pfu), the estimated TCID<sub>50</sub> is 100, which is the same virus titre used in our study. However, Ge *et al.* (2003) used 2.5 nmol of PB2-2240 siRNA compared to 250 pmol of PB2-2235 siRNA used in this study, indicating that the PB2-2235 could inhibit H5N1 virus replication at 10-fold lesser dose. Stewart

*et al.* (2011) reported that addition of immunostimulatory motifs to the 5' end of the sense strand of siRNAs enhanced anti-H5N1 HPAI viral targeting by increasing the expression of IFN- $\beta$  in chicken cells. Further studies are needed to ascertain the mechanism by which the siRNA PB2-2235 inhibits the replication of H5N1 HPAIV *in vitro* and whether addition of immunostimulatory motifs to it will enhance inhibition of H5N1 virus replication.

In summary, the study shows promising result in treating HPAI H5N1 virus infection with siRNAs. Among the siRNAs studied PB2-2235 exhibited the highest inhibition of H5N1 virus replication in MDCK cells. However, further *in vivo* experiments are needed to prove its potential in therapeutic application against influenza virus infection.

### Acknowledgements

We thank the Director, Indian Veterinary Research Institute and Indian Council of Agricultural Research, New Delhi, for providing necessary facilities to carry out this work. We are thankful to the Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture, India, for financial support through CDDL for Avian influenza diagnosis and research grant.

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*MS received 24 October 2014; accepted 23 March 2015*

Corresponding editor: INDRANIL DASGUPTA