J Vet Sci. 2022 Mar;23(2):e24
https://doi.org/10.4142/jvs.21174
pISSN 1229-845X·eISSN 1976-555X

Journal of Veterinary Science

Original Article
Virology

Check for updates

Improving siRNA design targeting nucleoprotein gene as antiviral against the Indonesian H5N1 virus

Risza Hartawan [1,2,*], **Dwi Ari Pujianto** [3], **Ni Luh Putu Indi Dharmayanti** [2], **Amin Soebandrio** [4]

[1]Doctoral Program in Biomedical Science, Faculty of Medicine, Universitas Indonesia, Jakarta 10430, Indonesia
[2]Indonesian Research Center for Veterinary Science, Ministry of Agriculture, Bogor 16114, Indonesia
[3]Department of Medical Biology Pre Clinic, Faculty of Medicine, Universitas Indonesia, Jakarta 10430, Indonesia
[4]Eijkman Institute, Ministry of Research, Technology and Higher Education, Jakarta 10430, Indonesia

OPEN ACCESS

Received: Jun 16, 2021
Revised: Nov 8, 2021
Accepted: Dec 7, 2021
Published online: Jan 10, 2022

***Corresponding author:**
Risza Hartawan
Indonesian Research Center for Veterinary Science, Ministry of Agriculture, Jl RE Martadinata 30 Bogor 16114, Indonesia.
Email: rjoss.dvm@gmail.com
https://orcid.org/0000-0001-9326-6956

ABSTRACT

Background: Small interfering RNA technology has been considered a prospective alternative antiviral treatment using gene silencing against influenza viruses with high mutations rates. On the other hand, there are no reports on its effectiveness against the highly pathogenic avian influenza H5N1 virus isolated from Indonesia.
Objectives: The main objective of this study was to improve the siRNA design based on the nucleoprotein gene (siRNA-NP) for the Indonesian H5N1 virus.
Methods: The effectiveness of these siRNA-NPs (NP672, NP1433, and NP1469) was analyzed *in vitro* in Marbin-Darby canine kidney cells.
Results: The siRNA-NP672 caused the largest decrease in viral production and gene expression at 24, 48, and 72 h post-infection compared to the other siRNA-NPs. Moreover, three serial passages of the H5N1 virus in the presence of siRNA-NP672 did not induce any mutations within the nucleoprotein gene.
Conclusions: These findings suggest that siRNA-NP672 can provide better protection against the Indonesian strain of the H5N1 virus.

Keywords: influenza in birds; H5N1 subtype; Indonesia; RNA interference; antiviral agents

INTRODUCTION

The battles against bird flu from the highly pathogenic avian influenza virus (HPAI) H5N1 are still very far from over because the threats posed by the spontaneous reemergence of these viruses remain. Outbreaks of H5N1-associated disease have been reported across many nations of the Afro-Eurasia continents [1]. The virus can infect various host species, ranging from birds to mammals, including humans. In Indonesia, the initial outbreak occurred in 2003 in poultry farms in East Java Province and then spread widely to most regions [2]. As a member of the influenza A (Orthomyxoviridae family), the H5N1 virus has undergone rapid mutation due to the negative sense, segmented RNA genome of the virus [3,4].

ORCID iDs

Risza Hartawan
https://orcid.org/0000-0001-9326-6956
Dwi Ari Pujianto
https://orcid.org/0000-0002-7274-4151
Ni Luh Putu Indi Dharmayanti
https://orcid.org/0000-0002-9399-8193
Amin Soebandrio
https://orcid.org/0000-0002-5856-4808

Author Contributions

Conceptualization: Hartawan R, Pujianto DA, Dharmayanti NLPI, Soebandrio A; Data curation: Hartawan R, Pujianto DA, Dharmayanti NLPI, Soebandrio A; Formal analysis: Hartawan R, Pujianto DA, Dharmayanti NLPI, Soebandrio A; Funding acquisition: Dharmayanti NLPI; Methodology: Hartawan R; Project administration: Dharmayanti NLPI; Resources: Hartawan R, Dharmayanti NLPI; Supervision: Pujianto DA, Dharmayanti NLPI, Soebandrio A; Validation: Hartawan R, Pujianto DA, Dharmayanti NLPI, Soebandrio A; Writing - original draft: Hartawan R; Writing - review & editing: Hartawan R, Pujianto DA, Dharmayanti NLPI, Soebandrio A.

Conflict of Interest

The authors declare no conflicts of interest.

Funding

This research was supported by the Indonesian Research Center for Veterinary Science Research Projects (18016.020.052.G (2018) and 1806.020.052.E (2019)).

From a public health perspective, the WHO reported that as of 2018, there had been approximately 840 human H5N1 infections worldwide. In addition, Indonesia has reported the second-highest number of cases, with 200 people infected and 168 deaths. Despite the number of human cases decreasing significantly, the threat posed by this virus should not be ignored. The virus still circulates in the field with many exposure routes to humans, such as poultry in human settlements, live bird markets, poultry farms, wild birds, pet birds, poultry products, by-products, and waste [2]. Therefore, specific and effective antiviral against HPAI H5N1 infections is needed to address anticipated disease outbreaks. Thus far, the treatment for influenza infections depends on antiviral based on neuraminidase inhibitors, such as Oseltamivir and Zanamivir [5,6]. Unfortunately, several strains of influenza viruses have become resistant to neuraminidase inhibitors [7-9]. Therefore, the development of new antivirals is essential to anticipate virus mutants resistant to existing antivirals.

The recent progress in the epigenetic field provides an alternative for antiviral developments based on gene silencing using RNA interference, such as small interfering RNA (siRNA) that is designed efficiently, relatively inexpensive, and flexible for changes with high potency and specificity against the influenza virus infection [10,11]. Several types of research have been dedicated to exploring the effectiveness of siRNA against influenza virus infections by targeting six viral genes, including polymerase base 2 (PB2), polymerase base 1 (PB1), polymerase acidic (PA), nucleoprotein (NP), matrix (M), non-structural (NS) genes [12-15]. These studies revealed worthy prospects against influenza virus infections, particularly siRNAs targeting the NP gene. On the other hand, there are no reports on the siRNA studies of the H5N1 viruses circulating in Indonesia. The circumstances of the Indonesian HPAI H5N1 showed significant alterations, such as clade evolution, genotypic and phenotypic changes [16-18]. Therefore, this study aimed to improve the siRNA design targeting the NP gene (siRNA-NP) against the Indonesian H5N1 virus.

MATERIALS AND METHODS

Study design, time, and location

This paper presents the results of experimental laboratory research to design an antiviral based on the siRNA-NP that inhibits Indonesian H5N1 virus infections as *in vitro* challenge in Madin-Darby canine kidney (MDCK) cell cultures. The study was conducted in the Virology Laboratorium of the Indonesian Research Center for Veterinary Science (IRCVS), Bogor, Indonesia, with the time of research from 2018–2020.

Design and construction of siRNA-NP for the Indonesian H5N1 viruses

The sequences of siRNA-NP were designed by the siDirect version 2.0 software (http://sidirect2.rnai.jp) using the consensus of 210 NP gene sequences of the H5N1 viruses circulating in Indonesia that retrieved from the Genbank NCBI database on February 19, 2018. **Fig. 1** presents the scheme of this siRNA design. Subsequently, two sequences of siRNA-NP from the software were selected for further analyses. Moreover, the siRNA-NP1496 sequence was included for comparison [13]. The siRNA oligonucleotides were synthesized, desalted, and duplexed before shipment using HP Custom siRNA without modification (Qiagen, Germany). **Table 1** lists the sequences of this designed siRNA-NP.

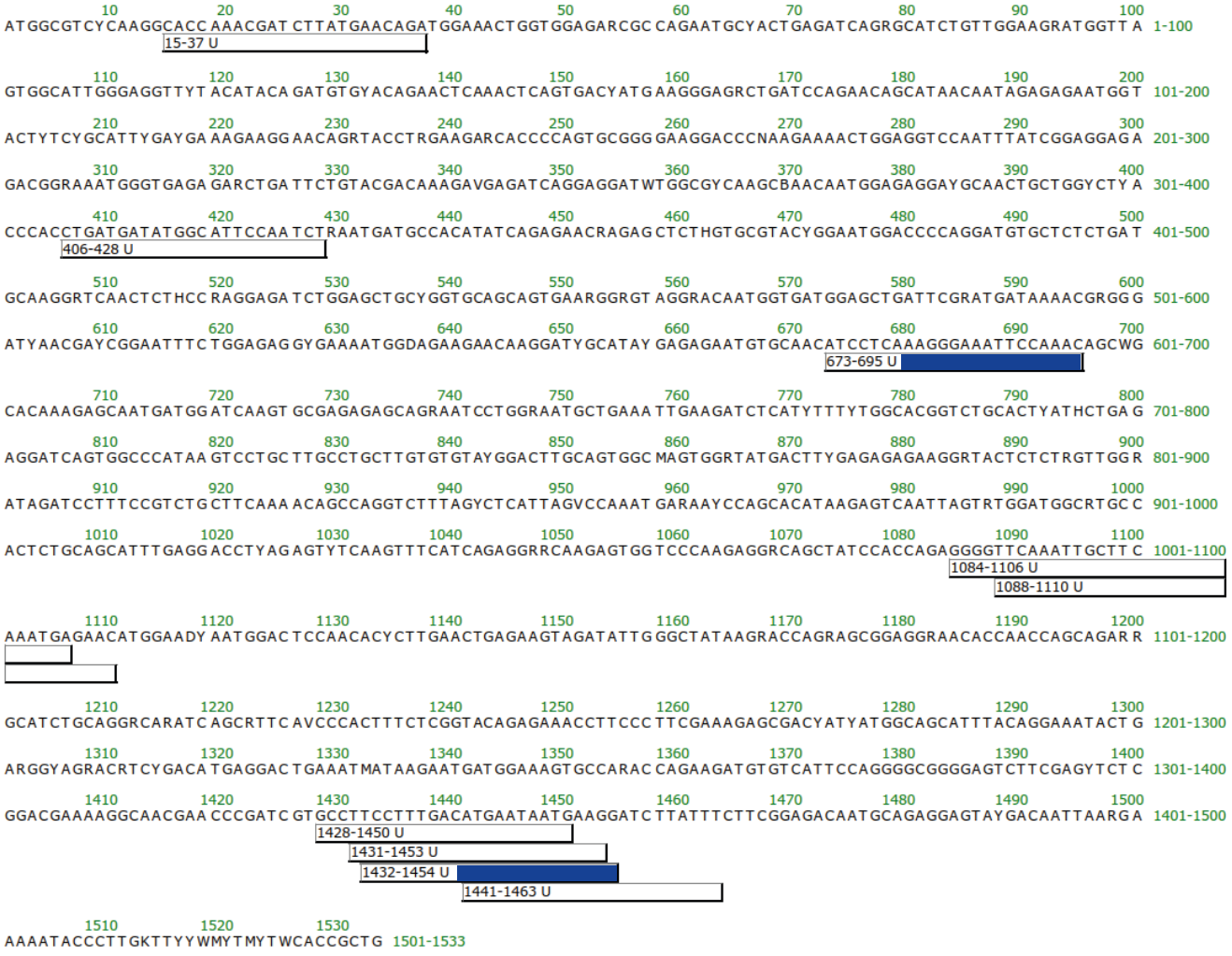

Fig. 1. siRNA design for the nucleoprotein gene of the Indonesian H5N1 viruses using the siDirect Version 2.0 software. Two selected siRNA sequences were marked by blue color.
siRNA, small interfering RNA.

Table 1. Design of the siRNA-NPs used in the study

siRNA-NP	Orientation	Sequence (5′-3′)
siRNA-NP672*	Target	CATCCTCAAAGGGAAATTCCA
	Sense	UCCUCAAAGGGAAAUUCCATT
	Antisense	UGGAAUUUCCCUUUGAGGATG
siRNA-NP1433*	Target	CCTTTGACATGAATAATGAAG
	Sense	UUUGACAUGAAUAAUGAAGTT
	Antisense	CUUCAUUAUUCAUGUCAAAGG
siRNA-NP1496†	Target	GGATCTTATTTCTTCGGAG
	Sense	AUCUUAUUUCUUCGGAGTT
	Antisense	CUCCGAAGAAAUAAGAUCC

siRNA-NP, small interfering RNA design targeting the nucleoprotein gene.
*siRNA-NP from siDirect version 2.0 (http://sidirect2.rnai.jp/); †The control of siRNA-NP.

Selection, propagation, and titration of the Indonesian H5N1 virus

The Indonesian H5N1 virus was selected from the IRCVS collection. The selected virus isolate was A/bird/Bogor/BR7.Lovebird/2015 belonging to clade 2.1.3 was already confirmed positive by reverse transcriptase-polymerase chain reaction (RT-PCR) for the H5N1 virus

and free from the Newcastle disease virus contamination [19-23]. The viral stock was grown in the allantoic cavity of 10-day-old embryonated chicken eggs in 37°C incubators [24]. The allantoic fluid was harvested 48 h after inoculation and stored at −76°C. Two methods of viral titration were used: hemagglutination (HA) and 50% tissue culture infectious dose per mL ($TCID_{50}$/mL) tests. The HA test based on the red blood cell agglutination of hemagglutinin was conducted using the OIE standard method in a 96-well microplate V shape with two serial dilutions. The $TCID_{50}$/mL was conducted in MDCK cells in the 96-well/plate with 10 serial dilutions using Reed and Muench methods. Subsequently, the multiplicity of infection (MOI) was derived empirically from the $TCID_{50}$/mL titer.

Cell culture for *in vitro* studies

The MDCK cells were used because the highly pathogenic influenza virus could grow well, resulting in an observable cytopathic effect without trypsin supplementation. The MDCK cells were grown in Dulbecco's modified eagle medium at 37°C in the presence of 5% CO_2 with supplementation of 10% fetal bovine serum, 50 μg/mL gentamicin, and 250 ng/mL amphotericin B.

In vitro test of siRNA-NP against the Indonesian H5N1 virus

As an antiviral substance, the designed siRNA-NP should effectively inhibit the viral H5N1 replication. For *in vitro* studies, the MDCK cells grown in the 24 wells/plate were transfected with each siRNA-NPs at a concentration of 32 nM in a volume of 500 μL of OptiMEM using Lipofectamine 2000 (Invitrogen, USA). Approximately 24 h after transfection, the MDCK cells were infected with the H5N1 virus at an MOI = 0.1. The effectiveness of the siRNA-NPs against the H5N1 infection was analyzed at 24, 48, and 72 h post-infection for both the control and treatment groups. By contrast, the supernatant was analyzed for virus titer using the HA and $TCID_{50}$/mL test; the cells were analyzed for the viral gene expression level.

Analyses of viral gene expression level

The two-step quantitative RT-PCR based on SYBR Green was conducted to measure the expression level of the NP gene as the primary target of the siRNA-NPs. The expression level of other genes, such as PA, H5, N1, M, and NS was also analyzed as an indirect effect of the NP gene silencing. Briefly, the total RNA was extracted from MDCK cells using a total RNA Minikit (Blood/Cultured Cell) (Geneaid, RB100). The complementary DNA (cDNA) was synthesized using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, FSQ30-301). qPCR was conducted from the cDNA using PowerUp SYBR Green Master Mix (Applied Biosystem, 4391178) in Applied Biosystem 7300 Real-Time PCR systems. **Table 2** lists the set of primers for the H5N1 viral genes. The gene expression level was calculated as a relative expression using the Livak method ($2^{-\Delta\Delta ct}$) with the housekeeping gene (γ-Actin) as a normalizer.

Effect of siRNA-NP repeat exposure for virus mutation

As an antiviral substance, the designed siRNA-NPs should effectively inhibit the H5N1 virus infection without a significant virus mutation outcome. The effect of the siRNA-NPs on the NP gene was analyzed by inhibiting the virus infection in the MDCK cells repeatedly using respective siRNA-NPs for three passages. Briefly, the MDCK cells grown in the 24 wells/plate were transfected with each siRNA-NPs with a concentration of 32 nM in volume 500 μL of OptiMEM using Lipofectamine 2000 (Invitrogen). Approximately 24 h after transfection, the cells were infected with the H5N1 viruses. Forty-eight hours post-infection, the cell media were harvested for the next passage of infection. After the three serial passages of infection, the virus RNA was extracted from the cell media using QIAamp Viral Mini Kit (Qiagen).

Table 2. Set of primers for RT-PCR and qPCR based on SYBR green

Gene	Primer name	Sequence (5′-3′)
NP	NP-F	GATCAGTGGCCCATAAGTC
	NP-R	AATTTGAACCCCTCTGGTG
	NPseqF*	GGATGATAAAACGAGGGATCA
	NPseqR*	TTGTCATACTCCTCTGCATTG
PA	PA-F	GCTTCTTATCGTTCAGGCTCTTAGG
	PA-R	CCGAGAAGCATTAAGCAAAACCCAG
H5	H5-F	CGAATTCACCAATGTGCCAG
	H5-R	GAGTCTGACACCTGGTGTTG
N1	N1-F	TTGCTTGGTCAGCAAGTGCA
	N1-R	TCTGTCCATCCATTAGGATCC
M	M-F	CTTCTAACCGAGGTCGAAACG
	M-R	AGGGCATTTTGGACAAAKCGTCTA
NS	NS-F	CAGGACATACTGATGAGGATG
	NS-F	GTTTCAGAGACTCGAACTGTG
γ-Actin	γ-Actin-F	CATCCGGAAGGACCTTTAC
	γ-Actin-R	ATCTTGATCTTCATCGTGCT

NP, nucleoprotein; PA, polymerase acidic; H5, hemagglutinin; N1, neuraminidase; M, matrix; NS, non-structural.
*Set primer was used for the NP gene sequencing.

The NP gene fragment was amplified from position 629 to 1538 by the RT-PCR using the SuperScript III one-step RT-PCR system with Platinum Taq DNA polymerase (Invitrogen). **Table 2** lists the primer set used for the NP gene sequencing. Subsequently, the RT-PCR product was purified using a QIAquick PCR purification kit (Qiagen) and delivered to Macrogen for DNA sequencing.

Statistical analysis

Parametric or non-parametric statistical analyses were used to compare the control and treatment groups using IBM SPSS statistic 21 software. The *p* values less than 0.05 were considered significant.

RESULTS

Three siRNA designs targeting the conserved regions of the NP gene (siRNA-NP672, siRNA-NP1433, and siRNA-NP1496) were evaluated to study the inhibition effect for the Indonesian avian influenza H5N1 virus clade 2.1.3 (A/bird/Bogor/BR7.Lovebird/2015) replication *in vitro* in MDCK cells. As shown in **Table 3**, virus production (titer in HA units) in infected cell culture supernatants was significantly lower in the specific siRNAs-treated cells than in the control group with no siRNA-NP (mock) at 72 h post-infection ($p < 0.05$), except for siRNA-NP1433. Among these siRNAs, the siRNA-NP672 showed the highest inhibition activity at 72 h post-infection. The virus production inhibited with this siRNA was approximately 41 times lower than virus infection without intervention (mock). The siRNA-NP1433 and siRNA-NP1496 showed similar results in inhibiting the H5N1 viral production by approximately four times lower than the virus control (mock) at 72 h post-infection. The viral infectivity analysis reflected by the $TCID_{50}$/mL titer also showed similar results (**Table 3**) as the previous stage of the study, where the siRNA-NPs caused a significant decrease in the infectious viral particles ($p < 0.05$). Furthermore, the siRNA-NP672 also provides the highest reduction of the infectious viral titer among all treatment groups in the 24, 48, and 72 h post-infection in approximately 3,160 times that of the virus control (mock) at 72 h post-infection. Moreover, the siRNA-NP1433 and siRNA-NP1496 had a comparable degree of infectious virion titer reduction of approximately 32–50 times lower than the virus control (mock).

Table 3. Effect of siRNA-NPs on virus production in infected Marbin-Darby canine kidney cell supernatants

Group	No.	Viral production									
		Median titre in HA units*					Median titer in log_{10} $TCID_{50}$/mL†				
		24 hpi	48 hpi	72 hpi	*p* value‡	FR§	24 hpi	48 hpi	72 hpi	*p* value‡	FR§
siRNA-NP672	3	1.0 (1.0–1.0)	1.0 (1.0–2.0)	1.0 (1.0–2.0)	0.043	41	1.0 (1.0–1.0)	1.5 (1.0–1.5)	2.3 (1.5–2.5)	0.046	3,160
siRNA-NP1433	3	2.0 (2.0–4.0)	4.0 (4.0–5.7)	5.7 (4.0–32.0)	0.072	4	1.7 (1.5–1.7)	2.5 (2.5–2.5)	4.3 (3.5–4.5)	0.046	32
siRNA-NP1496	3	4.0 (2.8–4.0)	4.0 (4.0–8.0)	16.0 (8.0–16.0)	0.043	4	1.5 (1.3–1.7)	3.3 (1.5–3.3)	3.7 (3.5–4.5)	0.046	50
mock	3	16.0 (8.0–16.0)	45.2 (16.0–64.0)	64.0 (32.0–64.0)	NA	NA	3.7 (3.5–3.7)	4.5 (4.3–4.5)	5.5 (5.5–5.7)	NA	NA

siRNA-NP, small interfering RNA design targeting the nucleoprotein gene; HA, hemagglutination; $TCID_{50}$/mL, 50% tissue culture infectious dose per mL; NA, not applicable; hpi, h post-infection; FR, fold reduction.
*The virus titer expressed as HA unit was the median value (minimum-maximum); †the virus titer expressed as log_{10} $TCID_{50}$/mL was the median value (minimum-maximum); ‡*p* value, the difference between the treatment groups and the mock at 72 hpi was analyzed with Mann Whitney *U* test; §FR is calculated by comparing the mean value of the treatment groups and the mock (data not shown) at 72 hpi.

The effects of these siRNA-NPs on the H5N1 gene expression level were analyzed by measuring viral mRNA transcript as a relative expression with the housekeeping gene (γ-actin). The siRNA-NP intervention in the viral infection in the MDCK cell caused a significant decrease in the viral gene expression, including NP, PA, H5, N1, M, and NS genes (**Fig. 2**). In particular, siRNA-NP672 caused the highest reduction consistently. As the main target of the siRNA-NPs, the level of viral NP gene expression was reduced significantly ($p < 0.05$) compared to mock in 24, 48, and 72 h post-infection. Despite no significant difference between these siRNA-NPs, the siRNA-NP672 caused the highest reduction in the NP gene expression level compared to the other two siRNA-NPs. Subsequently, the indirect effects of the siRNA-NPs also caused a decrease in the expression level of the other genes, such as PA, H5, N1, M, and NS.

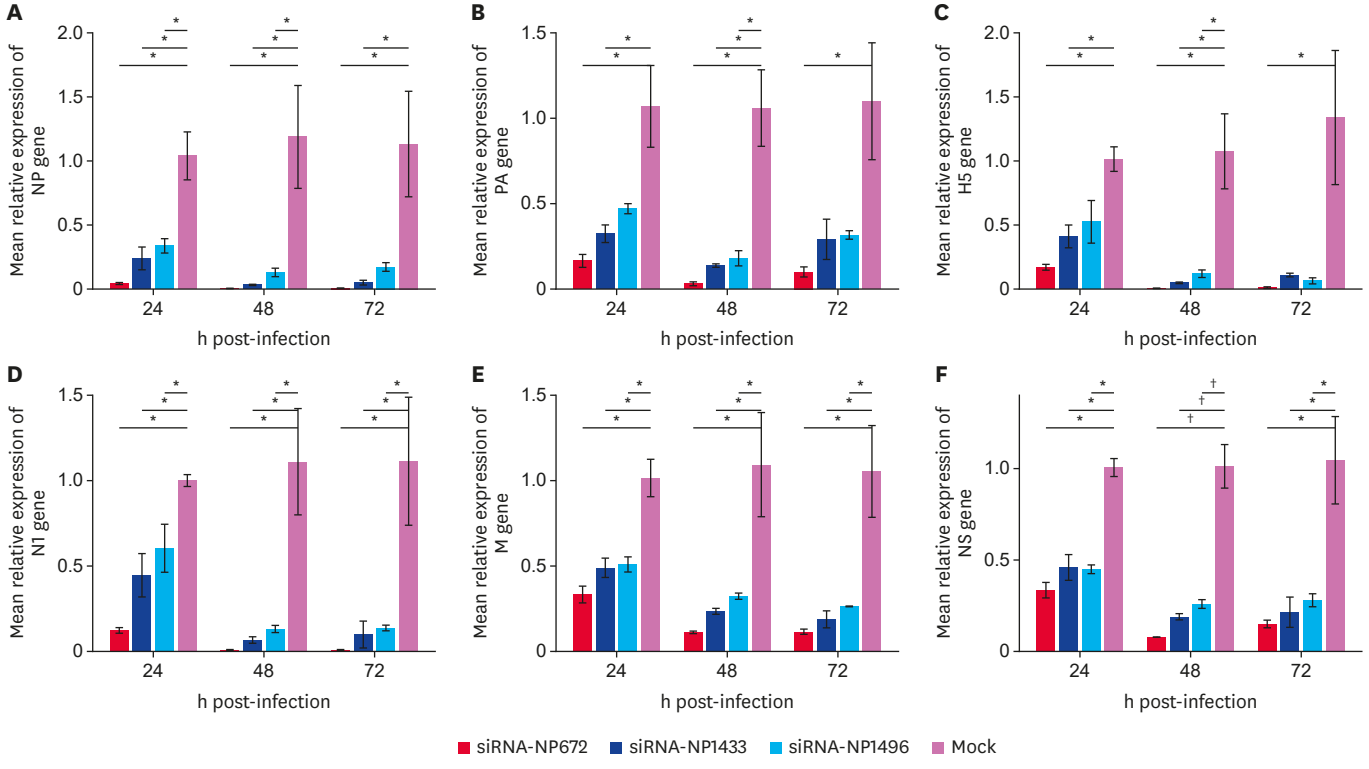

Fig. 2. Relative expression of the H5N1 genes in the Marbin-Darby canine kidney cell as effects of the siRNA-NPs inhibition, including NP (A), PA (B), H5 (C), N1 (D), M (E), and NS (F). The differences between groups were analyzed with one-way ANOVA post hoc Bonferroni.
siRNA-NP, small interfering RNA design targeting the nucleoprotein gene; NP, nucleoprotein; PA, polymerase acidic; H5, hemagglutinin; N1, neuraminidase; M, matrix; NS, non-structural.
*$p < 0.05$, †$p < 0.01$.

https://doi.org/10.4142/jvs.21174

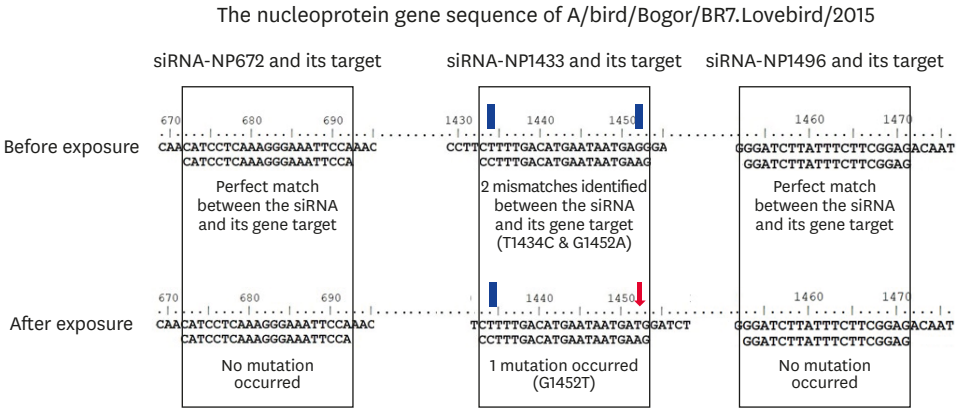

Fig. 3. Mutation analysis of the nucleoprotein gene as the effect of three serial exposure of siRNA-NPs. The difference between the siRNA-NP and its target sequence was shown by bar marking. The mutations that occurred after siRNA-NP exposures are indicated by the arrows.
siRNA-NP, small interfering RNA design targeting the nucleoprotein gene.

Sequence analysis of the original NP gene of A/bird/Bogor/BR7.Lovebird/2015 showed 100% similarity between siRNA-NP672 and siRNA-NP1496 with its target sequence. By contrast, there is two nucleotides difference between siRNA-NP1433 and its target sequence. **Fig. 3** presents the outcomes of the three serial siRNA-NP exposure. For siRNA-NP672 and siRNA-NP1496, there was no mutation on the target sequence after three serial siRNA-NP exposures. On the other hand, one nucleotide mutation occurred in the NP gene on position G1452T after three exposures to siRNA-NP1433.

DISCUSSION

The influenza virus utilizes its material genetics to hijack the host cell manufacturers to produce various properties for generating new virions [4,25]. The application of gene silencing based on siRNA technology is a powerful strategy for limiting viral infections by targeted degradation of viral messenger RNA, providing its sequence matched with the target [11]. The HPAI H5N1 viruses have been highly mutated over time. Thus, the siRNA for these viruses should be targeted to conserved genes. The NP gene is a promising target for gene silencing because it has several conserved regions present in several strains of influenza viruses [13,14]. The other rationale for targeting the NP gene for silencing is its important function in virus replication as templates for maintaining the viral genome integrity [12].

Three siRNA designs targeting the NP gene (siRNA-NP672, siRNA-NP1433, and siRNA-NP1496) were evaluated against the Indonesian H5N1 virus clade 2.1.3. Overall, the siRNA-NP672 provides the best result in reducing the viral expression gene level, as well as virus production and infectivity. The siRNA-NP1433 and siRNA-NP1496 provide a similar outcome in reducing the viral infection parameters. These results may be related to the position of the gene targets of these siRNA-NPs. Although the target gene of siRNA-NP672 is located in the middle part of the mRNA, both gene targets of siRNA-NP1433 and siRNA-NP1496 are located in the distal part, where high genetic variations occur. The siRNA-NP1496 was originally designed for subtype H1N1. Hence, it was also evaluated against subtypes H5N1, H6N2, H7N7, H8N4, and H9N2 with various reductions of viral infection [13,26-28]. This study confirmed that the siRNA-NP1496 is still effective in decreasing the Indonesian H5N1 viral infection, but the siRNA-NP672 caused a better reduction against the respective virus.

https://doi.org/10.4142/jvs.21174

The effects of the siRNA-NPs should be disrupted directly only for the NP gene expression level, where the expression of the other genes should not be interrupted. Nevertheless, this study showed that the interruption of viral gene expression occurred in the NP gene and other genes, including PA, H5, N1, M, and NS genes. Reducing the expression level of other genes may be related to its role in virus replication. This finding confirmed that the NP gene is one of the best targets for gene silencing [13,14,27].

The following matter related to siRNA efficacy to disturb the expression of gene target is the perfect match between their nucleotide sequences. A previous study showed that even a single nucleotide mismatch between siRNA and its target caused interference efficiency that reduced its effect significantly [29]. On the other hand, this study confirmed that siRNA-NP1433 with two nucleotide mismatches exhibited comparable viral infection inhibition with the siRNA-NP1496 already used in several studies against the Indonesian H5N1 virus. siRNA NP1433 binds its target effectively and affects virus replication despite two nucleotide mismatches, which is a significant result because the field strains of the influenza virus can have considerable variations.

The last concern regarding the design of antivirals for influenza viruses is related to their natural behavior and properties, which are easy to mutate for surviving in the environment [1,4]. There is considerable evidence of influenza viruses surviving biological pressures, such as innate immunity, vaccination, and antiviral therapies, through escape mutations [7,30-32]. This concern should also be addressed for the siRNA design for antiviral agents against the influenza virus. In this study, a mutation of the NP gene occurred for the Indonesian H5N1 virus after three serial exposures with the siRNA-NP1433. The two other siRNA-NPs did not show any mutation after three serial exposures in the viral infection in the MDCK cells.

As a limitation, this study only conducted three serial passages, so future studies should prolong the serial passage because the mutation effect of siRNA could occur by positive selection, particularly for the NP gene with a lower mutation rate compared to the H5 and N1 genes. Moreover, the suitable siRNAs for the influenza virus should be selected carefully based on recent circulating viruses in the field to avoid any drawbacks in the future. Another strategy to address the potential variation in circulating field strains of H5N1 would be to deliver multiple siRNAs simultaneously. For example, the cocktail siRNA application targeting NP and other influenza viral genes provides a better knockdown effect for influenza virus infections [14,27]. Therefore, using multiple siRNAs may account for natural variations and reduce the likelihood of siRNA escape mutations emerging.

In summary, this study obtained promising results in reducing HPAI H5N1 virus infections with the siRNA-NPs, particularly the siRNA-NP672, which exhibited the highest inhibition of virus replication. Future studies should focus on the most suitable systems to deliver siRNAs to the target cells against acute viral infection, such as the H5N1 virus, particularly the challenge test in animal models.

ACKNOWLEDGEMENTS

The authors acknowledge all the staff and technicians of the Virology Department for the laboratory and technical support.

REFERENCES

1. Sims LD, Brown IH. Multi-continental panzootic of H5 highly pathogenic avian influenza (1996–2015). In: Swayne DE, editor. *Animal Influenza.* 2nd ed. Iowa: John Wiley & Sons, Inc.; 2016, 204-247.
2. Dharmayanti NLPI, Hewajuli DA, Ratnawati A, Hartawan R. Genetic diversity of the H5N1 viruses in live bird markets, Indonesia. J Vet Sci. 2020;21(4):e56.
PUBMED | CROSSREF
3. Shaw ML, Palese P. Orthomyxoviridae. In: Knipe DM, Howley PM, editors. *Fields Virology*. 6th ed. Baltimore: Wolter Luwer; 2013, 1151-1185.
4. Suarez DL. Influenza A virus, In: Swayne DE, editor. *Animal Influenza.* 2nd ed. Iowa: John Wiley & Sons, Inc.; 2016, 3-30.
5. Gasparini R, Amicizia D, Lai PL, Bragazzi NL, Panatto D. Compounds with anti-influenza activity: present and future of strategies for the optimal treatment and management of influenza. Part II: future compounds against influenza virus. J Prev Med Hyg. 2014;55(4):109-129.
PUBMED
6. Saladino R, Barontini M, Crucianelli M, Nencioni L, Sgarbanti R, Palamara AT. Current advances in anti-influenza therapy. Curr Med Chem. 2010;17(20):2101-2140.
PUBMED | CROSSREF
7. de Jong MD, Tran TT, Truong HK, Vo MH, Smith GJ, Nguyen VC, Bach VC, Phan TQ, Do QH, Guan Y, Peiris JS, Tran TH, Farrar J. Oseltamivir resistance during treatment of influenza A (H5N1) infection. N Engl J Med. 2005;353(25):2667-2672.
PUBMED | CROSSREF
8. Earhart KC, Elsayed NM, Saad MD, Gubareva LV, Nayel A, Deyde VM, Abdelsattar A, Abdelghani AS, Boynton BR, Mansour MM, Essmat HM, Klimov A, Shuck-Lee D, Monteville MR, Tjaden JA. Oseltamivir resistance mutation N294S in human influenza A(H5N1) virus in Egypt. J Infect Public Health. 2009;2(2):74-80.
PUBMED | CROSSREF
9. Okomo-Adhiambo M, Fry AM, Su S, Nguyen HT, Elal AA, Negron E, Hand J, Garten RJ, Barnes J, Xiyan X, Villanueva JM, Gubareva LV2013–14 US Influenza Antiviral Working Group. Oseltamivir-resistant influenza A(H1N1)pdm09 viruses, United States, 2013–14. Emerg Infect Dis. 2015;21(1):136-141.
PUBMED | CROSSREF
10. Gavrilov K, Saltzman WM. Therapeutic siRNA: principles, challenges, and strategies. Yale J Biol Med. 2012;85(2):187-200.
PUBMED
11. Pawitan JA. Molecular pathogenesis of avian influenza and prospect of theraphy using small interfering RNA. In: Haugan S, Bjornson W, editors. *Avian Influenza: Etiology, Pathogenesis and Interventions*. New York: Nova Science Publishing; 2010, 69-82.
12. Behera P, Nagarajan S, Murugkar HV, Kalaiyarasu S, Prakash A, Gothalwal R, Dubey SC, Kulkarni DD, Tosh C. siRNAs targeting PB2 and NP genes potentially inhibit replication of highly pathogenic H5N1 avian influenza virus. J Biosci. 2015;40(2):233-240.
PUBMED | CROSSREF
13. Ge Q, McManus MT, Nguyen T, Shen CH, Sharp PA, Eisen HN, Chen J. RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. Proc Natl Acad Sci U S A. 2003;100(5):2718-2723.
PUBMED | CROSSREF
14. Zhou H, Jin M, Yu Z, Xu X, Peng Y, Wu H, Liu J, Liu H, Cao S, Chen H. Effective small interfering RNAs targeting matrix and nucleocapsid protein gene inhibit influenza A virus replication in cells and mice. Antiviral Res. 2007;76(2):186-193.
PUBMED | CROSSREF
15. Zhou K, He H, Wu Y, Duan M. RNA interference of avian influenza virus H5N1 by inhibiting viral mRNA with siRNA expression plasmids. J Biotechnol. 2008;135(2):140-144.
PUBMED | CROSSREF
16. Dharmayanti NLPI, Hartawan R, PudjiatmokoWibawa H, HardimanBalish A, Donis R, Davis CT, Samaan G. Genetic characterization of clade 2.3.2.1 avian influenza A(H5N1) viruses, Indonesia, 2012. Emerg Infect Dis. 2014;20(4):671-674.
PUBMED | CROSSREF
17. Dharmayanti NLPI, Ibrahim F, Soebandrio A. Amantadine resistant of Indonesian H5N1 subtype influenza viruses during 2003-2008. Microbiol Indones. 2010;4:1-6.

18. Dharmayanti NLPI, Thor SW, Zanders N, Hartawan R, Ratnawati A, Jang Y, Rodriguez M, Suarez DL, Samaan G, Pudjiatmoko , Davis CT. Attenuation of highly pathogenic avian influenza A(H5N1) viruses in Indonesia following the reassortment and acquisition of genes from low pathogenicity avian influenza A virus progenitors. Emerg Microbes Infect. 2018;7(1):147.
PUBMED | CROSSREF

19. Dharmayanti NLPI, Hartawan R, Hewajuli DA. Development of primers for reverse transcriptase polymerase chain reaction to detect avian influenza virus in Indonesia. J Vet. 2016;17:183-196.
CROSSREF

20. Fouchier RA, Bestebroer TM, Herfst S, Van Der Kemp L, Rimmelzwaan GF, Osterhaus AD. Detection of influenza A viruses from different species by PCR amplification of conserved sequences in the matrix gene. J Clin Microbiol. 2000;38(11):4096-4101.
PUBMED | CROSSREF

21. Lee MS, Chang PC, Shien JH, Cheng MC, Shieh HK. Identification and subtyping of avian influenza viruses by reverse transcription-PCR. J Virol Methods. 2001;97(1-2):13-22.
PUBMED | CROSSREF

22. Stäuber N, Brechtbühl K, Bruckner L, Hofmann MA. Detection of Newcastle disease virus in poultry vaccines using the polymerase chain reaction and direct sequencing of amplified cDNA. Vaccine. 1995;13(4):360-364.
PUBMED | CROSSREF

23. Wright KE, Wilson GA, Novosad D, Dimock C, Tan D, Weber JM. Typing and subtyping of influenza viruses in clinical samples by PCR. J Clin Microbiol. 1995;33(5):1180-1184.
PUBMED | CROSSREF

24. Spackman E, Killian ML. Avian influenza virus isolation, propagation, and titration in embryonated chicken eggs. In: Spackman E, editor. *Animal Influenza Virus*. 2nd ed. New York: Humana Press; 2014, 125-140.

25. Whittaker GR. Intracellular trafficking of influenza virus: clinical implications for molecular medicine. Expert Rev Mol Med. 2001;2001(5):1-13.
PUBMED | CROSSREF

26. Khantasup K, Kopermsub P, Chaichoun K, Dharakul T. Targeted small interfering RNA-immunoliposomes as a promising therapeutic agent against highly pathogenic avian influenza A (H5N1) virus infection. Antimicrob Agents Chemother. 2014;58(5):2816-2824.
PUBMED | CROSSREF

27. Linke LM, Wilusz J, Pabilonia KL, Fruehauf J, Magnuson R, Olea-Popelka F, Triantis J, Landolt G, Salman M. Inhibiting avian influenza virus shedding using a novel RNAi antiviral vector technology: proof of concept in an avian cell model. AMB Express. 2016;6(1):16.
PUBMED | CROSSREF

28. Tompkins SM, Lo CY, Tumpey TM, Epstein SL. Protection against lethal influenza virus challenge by RNA interference in vivo. Proc Natl Acad Sci U S A. 2004;101(23):8682-8686.
PUBMED | CROSSREF

29. Huang DT, Lu CY, Shao PL, Chang LY, Wang JY, Chang YH, Lai MJ, Chi YH, Huang LM. In vivo inhibition of influenza A virus replication by RNA interference targeting the PB2 subunit via intratracheal delivery. PLoS One. 2017;12(4):e0174523.
PUBMED | CROSSREF

30. Gillman A, Muradrasoli S, Söderström H, Holmberg F, Latorre-Margalef N, Tolf C, Waldenström J, Gunnarsson G, Olsen B, Järhult JD. Oseltamivir-resistant influenza A (H1N1) virus strain with an H274Y mutation in neuraminidase persists without drug pressure in infected mallards. Appl Environ Microbiol. 2015;81(7):2378-2383.
PUBMED | CROSSREF

31. Govorkova EA, Baranovich T, Seiler P, Armstrong J, Burnham A, Guan Y, Peiris M, Webby RJ, Webster RG. Antiviral resistance among highly pathogenic influenza A (H5N1) viruses isolated worldwide in 2002–2012 shows need for continued monitoring. Antiviral Res. 2013;98(2):297-304.
PUBMED | CROSSREF

32. He G, Qiao J, Dong C, He C, Zhao L, Tian Y. Amantadine-resistance among H5N1 avian influenza viruses isolated in Northern China. Antiviral Res. 2008;77(1):72-76.
PUBMED | CROSSREF