In Vitro Expression of Native H5 and N1 Genes of Avian Influenza Virus by Using Green Fluorescent Protein as Reporter

RISZA HARTAWAN[1,3], K. ROBINSON[2,3], T. MAHONY[2] and J. MEERS[3]

[1]Indonesian Research Centre for Veterinary Science, Bogor, Indonesia
[2]Department of Primary Industry and Fisheries Queensland, Brisbane, Australia
[3]School of Veterinary Science, The University of Queensland, Brisbane, Australia

ABSTRAK

HARTAWAN, R., K. ROBINSON, T. MAHONY dan J. MEERS. 2011. Ekspresi gen H5 dan N1 dari virus avian influenza secara *in vitro* menggunakan Green Fluorescent Protein sebagai reporter. *JITV* 16(3): 234-241.

Hemaglutinin dan neuraminidase merupakan protein yang bersifat immunogenik dari virus avian influenza yang banyak digunakan dalam penelitian rekombinan. Namun, kedua gen tersebut bermutasi dengan cepat sehingga terbentuk variasi genotipe yang sangat beragam. Hal ini sangat mempengaruhi tingkat ekspresi dalam sistem rekombinan. Tujuan penelitian ini adalah untuk mengetahui tingkat ekspresi gen H5 dan N1 yang diisolasi dari isolat lapang virus avian influenza dengan cara mengkloning kedua gen tersebut pada vektor ekspresi pEGFP-C1. Dua klon pEGFP-C1 yang mengandung gen H5 maupun N1 ditransfeksikan pada sel *chicken embryo fibroblasts* (CEF), *rabbit kidney* (RK13) dan *African green monkey kidney* (VERO) menggunakan reagen Lipofectamine 'Plus'. Hasil penelitian menunjukkan tingkat ekspresi gen pada sel VERO lebih tinggi dibandingkan dengan sel RK13 maupun CEF. Pengamatan dengan mikroskop fluorescent dan Western blotting menunjukkan bahwa gen N1 diekspresikan lebih baik di semua sel dibandingkan dengan gen H5.

Kata Kunci: H5N1 Virus, Hemagglutinin, Neuraminidase, Ekspresi Gen, Green Fluorescent Protein

ABSTRACT

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The hemagglutinin and neuraminidase are important immunogen of avian influenza virus that are suitable for recombinant experimentation. However, both genes have been experienced rapid mutation resulting in diverse variety of genotypes. Hence, gene expression in recombinant systems will be difficult to predict. The objective of the study was to examine expression level of H5 and N1 genes from a field isolate by cloning the genes into expression vector pEGFP-C1. Two clones respresenting full-length of H5 and N1 gene in plasmid pEGFP-C1 were transfected into chicken embryo fibroblasts (CEF), rabbit kidney (RK13) and African green monkey kidney (VERO) cells using Lipofectamine 'Plus' reagent. The experiment showed level of gene expression in the VERO cell was higher than in the RK13 and CEF cells. Observations using fluorescent microscopy and Western blotting revealed that the N1 gene was expressed better in all cells compared to the H5 gene.

Key Words: H5N1 Virus, Hemagglutinin, Neuraminidase, Gene Expression, Green Fluorescent Protein

INTRODUCTION

The genetic features of HPAI virus strain H5N1 have been extensively investigated in an endeavor to gain better understanding of virus nature in order to develop appropriate approaches against the disease. In area of bird vaccination, a recombinant system may provide a suitable alternative since inactivated vaccine appears to be ineffective for preventing outbreak recurrence within vaccinated flocks (CHEN *et al.*, 2008; LIPATOV *et al.*, 2004). Therefore, numerous recombinant investigations have been performed in attempt to improve vaccine performance (CRAWFORD *et al.*, 1999; PAVLOVA *et al.*, 2009; TORO *et al.*, 2008; VEITS *et al.*, 2006; YANG *et al.*, 2007). Since virus possesses several determinants that are capable of inducing immune responses (PERDUE, 2008), many possibilities for recombinant study are available. Nevertheless, hemagglutinin and neuraminidase are the most suitable antigen for recombinant experimentation because both are surface exposed glycoprotein and strongly immunogenic.

The hemagglutinin and neuraminidase are exterior glycoproteins that play important roles in the replication of avian influeza virus (AIV) (COLMAN, 1998; STEINHAUER and WHARTON, 1998). Whereas hemagglutinin is a trimer protein involving in cellular attachment in initial stage (STEINHAUER and WHARTON, 1998; WEIS *et al.*, 1988), neuraminidase is a tetramer protein contributing to release virus in final stage of the

replication (COLMAN, 1998; COLMAN *et al.*, 1983). The hemagglutinin and neuraminidase are encoded by genes located on different segment, which are segment 4 and 6 respectively (HAY, 1998). These genes undergo rapid mutation resulting in massive genetic variance (DUVVURI *et al.*, 2009). As a consequence, expression of these genes can be difficult due to predicted differences in codon usage pattern between the genes and genetic system of the target cell (GREENE, 2004).

The aim of the study was to investigate *in vitro* expression level of the H5 and N1 genes from an Indonesian AIV isolate A/duck/Tangerang/Bbalitvet-ACIAR-TE11/2007. The gene expression was prefered in euracaryotic cells to in procaryotic cells because the outcome of the study was addressed for recombinant purpose with avian herpesvirus vector that is also primarily undertaken in the eucaryotic model. The expression study was undertaken by inserting the native genes into an expression vector pEGFP-C1 that is straightforward to examine gene expression in eucaryotic cells. Therefore, the gene fusion product was utilised to determine the expression level of the H5 and N1 genes from the isolate in order to assess the possibility of utilizing these native genes in a recombinant study.

MATERIALS AND METHODS

Site and time of study

The expression study of the H5 and N1 genes of the field isolate was mainly undertaken in DPIF-QLD Laboratorium (Brisbane, Australia) from August 2009 to February 2010. However, the preliminary studies such as virus isolation, isolation and cloning of genes were carried out in Virology Laboratorium, IRCVS (Bogor, Indonesia). Molecular characterisation of the genes was accomplished in Australia (HARTAWAN *et al.*, 2010).

Source of Hemagglutinin and Neuraminidase gene

The native genes (H5 and N1) that were isolated from the field isolate using an RT-PCR protocol were subsequently cloned into pGEM®-T Easy vector system (Promega Corporation) as previously described (HARTAWAN *et al.*, 2010). The clones derived from the study were utilised in the analysis of gene expression as *in vitro* study.

The full-length of H5 gene (1,707 bp) was assembled from existing overlapped clones {H5.1 (817 bp) & H5.2 (949 bp)} (HARTAWAN *et al.*, 2010). Furthermore, these fragments were joined using a PCR

protocol and subsequently were cloned into pGEM®-T Easy vector system (Promega Corporation). This technique successfully generated new clones carrying full-length H5 gene. The one clone of pGEM-TE/H5 (#26) was selected for the expression trial since it retains identical sequence to its origin. Meanwhile, the expression of N1 gene was performed from clone pGEM-TE/N1#15 containing full-length of the N1 gene.

Construction of Expression Plasmids pEGFP-C1 containing H5 or N1 Gene

Expression Plasmid pEGFP-C1

The commercial expression plasmid pEGFP-C1 (Clontech) were utilised to express the native H5 and N1 genes of the Indonesian isolate. This vector has been already utilised in gene expression study as in cell culture model (XU *et al.*, 2006).

Introduction of a Restriction Site into H5 and N1 Genes

Primer sequences for cloning the H5 and N1 genes are presented in Table 1. The forward primers were added with a *Xho*I restriction site and optimized with Kozak's analysis sequence (KOZAK, 1987; 1990). Meanwhile, the reverse primers encoded for a six histidine peptide tag and stop codon followed by an *Apa*I site at the end of sequence.

The PCR protocol was carried out in a 20 μl mixture containing 2 μl of 10X PCR buffer, 0.75 μl of 10 mM dNTP's, 0.5 μl of 50 mM $MgCl_2$, 0.5 μl of each respective forward and reverse primer (20 μM), 14.25 μl of RNase free water, 0.5 μl of Platinum® *Taq* DNA Polymerase (INVITROGEN™) enzyme mix, and 1 μl of respective gene in the plasmid as template.

The PCR conditions were designed in two continuous steps with similar conditions for both genes. Step I was 94°C for 2 min (initial DNA denaturation), 10 cycles of 94°C for 15 s (denaturation), 55°C for 30 s (annealing), and 72°C for 1.5 min (extension) with final extension at 72°C for 3 min. The process was directly continued by Step II, which was 25 cycles of 94°C for 15 s (denaturation), and 72°C for 1.5 min (annealing and extension) with final extension at 72°C for 3 min, and hold temperature at 4°C. The DNA products were visualized by electrophoresis in 1% agarose gel. The amplified DNA fragments of H5 and N1 gene were purified by using a QIAquick PCR purification kit (QIAGEN®) and quantified using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc.).

Table 1. Oligonucleotide primers to introduce *Xho*I and *Apa*I restriction sites into the sequence termini of the H5 and N1 genes

Gene	Primer	Primer sequence (5'-3')
Hemagglutinin	H5f-*Xho*I	GGGA**CTCGAG**<u>CCATGGA</u>GAAAATAGTGCTTCTTCTTGC
	H5r-6His-*Apa*I	TCAG**GGGCCC**TTAatgatgatgatgatgatgAATGCAAATTCTGCATTGTAACGATCC
Neuraminidase	N1f-*Xho*I	GGGA**CTCGAG**<u>CCATGAA</u>TCCAAATCAGAAGATAATAACC
	N1r-6His-*Apa*I	GCAG**GGGCCC**TTAatgatgatgatgatgatgCTTGTCAATGGTGAATGGCAACTC

Restriction enzyme sites are shown in bold letter. The consensus Kozak's sequence in the forward primers is underlined. The six-histidine peptide tag added to the reverse primers is shown in lowercase text

Cloning of H5 and N1 genes into plasmid pEGFP-C1

Preparation of gene insertion into plasmid pEGFP-C1 was performed by sequential digestion of all genetic material using *Apa*I and *Xho*I restriction enzymes (NEW ENGLAND Biolabs®Inc.), including plasmid pEGFP-C1 and purified PCR product of the H5 and N1 genes. Briefly, 5 µg respective DNA and 2U of *Apa*I were incubated at 25°C (room temperature) for 1.5 h with supplementation 5 µl of 1x NEB Buffer 3 and 100 µg/ml BSA. Subsequently, 2U of *Xho*I was added into mixture and digestion was continued by incubation at 37°C for 1.5 h. The digestion was visualized by electrophoresis in 1% Agarose gel. The fragments of plasmid and genes were purified from agarose gel using QIAQuick gel extraction kit (QIAGEN®) as per manufacturer's instruction and quantified using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc.).

The cloning of digested H5 or N1 gene into plasmid pEGFP-C1 (Clontech) was performed using the T4 DNA ligation and buffer solution of pGEM®-T Easy vector system (Promega Corporation) as per manufacturer's instruction. Subsequently, the plasmids with gene insertion were transformed into electrocompetent *Escherichia coli* strain DH10B (INVITROGEN™) by electroporation (1.8 KV, 4.10 mS). The transformed cells were inoculated on LB agar plate in presence of 30 µg/ml Kanamycin. Screening test by PCR was performed on colonies in order to ensure correct insertion (HARTAWAN *et al.*, 2010). Several positive clones were propagated in 10 ml of LB broth with addition of 100 µg/ml Kanamycin at 37°C for overnight with gentle agitation. Half of the culture was stored as a glycerol stock, while the recombinant plasmid was purified from the remaining culture using the QIAGEN® Plasmid Mini Kit (QIAGEN®) as per manufacturer's instruction. Subsequently, the purified plasmids were quantified using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc.).

Subsequently, the plasmids with gene inserts were delivered to AGRF Brisbane for sequencing in order to select suitable clones for the expression trial. Several primers were utilised in sequencing, included H5 and N1 internal primers (HARTAWAN *et al.*, 2010) as well as the green fluorescence protein primer (gfp seg C primer: 5' – CATGGTCCTGCTGGAGTTCGTG – 3').

Transfection of recombinant expression plasmids into cell cultures

Three different eukaryotic cell cultures were utilised, including CEF, RK13, and VERO. Whereas CEF cell was cultured in Media 199 (INVITROGEN™) with 10% fetal calf serum, RK13 and VERO were cultured in MEM (INVITROGEN™) with of 10% fetal calf serum. All cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2.

The recombinant expression plasmids were transfected into cells using the Lipofectamine enhanced with 'Plus' reagent (INVITROGEN™). Since presence of serum can inhibit the transfection process, OptiMEM media (INVITROGEN™) was utilised for transfection. Briefly, the cells was seeded in 6 well/plate flask (NUNC) with concentration of 5×10^5 cells/ml and subsequently incubated for 18-24 h to achieve about 90% confluence. Each of the respective recombinant plasmid (1 µg) was diluted with OptiMEM into a final volume of 100 µl following the addition 16 µl of 'PLUS' reagent. The mixtures were incubated at room temperature for 15 min. At the same time, 8 µl of Lipofectamine transfection reagent was diluted with OptiMEM into volume of 100 µl. Subsequently, the diluted Lipofectamine was added into the mixture of DNA and 'PLUS' reagent, followed by 45 min incubation at room temperature.

Meanwhile, the cultures were washed twice with 1 x RT-PBS and once with RT-OptiMEM. Approximately 800 µl of OptiMEM was added into each transfection well. Then, ~200 µl of DNA/Lipofectamine Plus reagent mixtures were gently added to the respective

washed culture. All cultures were then incubated at $37^{o}C$, in a humidified atmosphere, and 5% CO_2. After 20 h, culture integrity was checked by microscopy. If the cells appeared disrupted, the OptiMEM was replaced with a new culture media (Media 199 for CEF and MEM for VERO & RK13).

The results of the transfection were observed for 72 h with daily observation. Every 24 h, sample cell culture for each plasmid was collected for analysis. For collection, the culture was washed with once with 1XRT-PBS and the monolayer was scraped. The cell suspension in microfuge was pelleted by centrifugation in maximum speed at $4^{o}C$. After PBS was removed, the pellet was stored at $-20^{o}C$.

Detection of gene expression in cell cultures

The level of protein expression of the respective recombinant clone of the pEGFP-C1 vector was analyzed using fluorescent microscope and Western blotting. The level of the GFP fusion protein was examined directly from transfected cell culture in 6 well/plate flask (NUNC) under flurescence microscope using a GFP special filter.

For Western blotting, initially total protein of each sample was extracted from PBS resuspended cell pellets by dissolving in 50 µl SDS reducing buffer (50 mM Tris-HCl pH 6.8, 100 mM DTT, 2% SDS, 20% glycerol & 0.2 mg/ml bromophenol blue). After heating at $96^{o}C$ for 10 min, the mixture was centrifuged at 14,000 RPM in room temperature to separate the protein from the cell debris. The proteins were separated on a 10% SDS polyacrylamide gel by electrophoresis and transferred onto nitrocellulose membranes (Amersham Pharmacia). The transferred membrane was immunoblotted with primary antibodies (goat anti-GFP, Roche) at 1:10000 in blocking buffer (PBST-5% skim milk). Subsequently, the membrane was incubated with secondary antibody (goat anti rabbit IgG) at final dilution 1:10000 in blocking buffer. Immunodetection was performed by the chemiluminescence method using ECL reagents kit (GE Healthcare), and then exposed to light sensitive film.

RESULTS AND DISCUSSION

Fusion of H5 gene to the C-terminus of GFP

The H5 gene of the H5N1 isolate was successfully inserted into the pEGFP-C1 expression vector. The screening test by PCR identified several clones that positively contained full-length of H5 gene. These positive clones were identified as pEGFP-C1/H5 (Figure 1A). In addition, digestion with double restriction endonucleases (*Apa*I & *Xho*I) demonstrated a 1,707 bp insertion of H5 genes along with the expected 4.7 kbp of vector fragment (Figure 1C). The sequencing of these recombinants recognized several single nucleotide polymorphisms (SNP's) between these clones. Therefore, one clone of pEGFP-C1/H5#26.8 was selected for further gene expression study in cell culture since it comprises similar sequence as the parental gene.

In vitro expression level of hemagglutinin gene

The expression of the H5 gene fused to GFP demonstrated low expression in three different cells. The native H5 gene in the GFP fusion system was most poorly expressed in CEF and RK13. The expression level of GFP fusion protein slightly increased in the VERO culture. The level of *in vitro* expression of GFP-H5 fusion protein in cell cultures is shown in Figure 2B, 2E and 2H. Although, expression was demonstrated in all cultures by fluorescence, Western blotting revealed production of GFP-H5 fusion protein only in VERO (Figures 3C).

Fusion of N1 gene to the C-terminus of GFP

The cloning the N1 gene into plasmid pEGFP-C1 expression vector was successfully carried out from clone pGEM-TE/N1#15. The PCR test was utilised to identify clones of pEGFP-C1 expression vector carrying N1 full-gene. The positive clones of plasmid pEGFP-C1 were identified as pEGFP-C1/N1#15 (Figure 1B). The restriction pattern of this recombinant clone using *Apa*I & *Xho*I double digestion demonstrated the insertion of 1,350 bp of N1 gene into 4.7 kbp of vector fragment (Figure 1D). Sequencing of these clones confirmed that all clones were consistent to the parental gene.

### *In vitro* expression level of neuraminidase gene

The expression of the N1 gene products fused to GFP was observed in all transfected cell lines. The observation using fluorescence microscopy on GFP fusion protein confirmed for maximum expression at 48 h after transfection. In addition, the expression level of GFP fusion protein was higher using the cell line (RK13 & VERO) compared the CEF primary cell culture (Figures 2C, 2F & 2I). Moreover, the Western blotting analysis in this N1 clone was detected GFP-N1 fusion protein in all three types of cell culture (Figures 3A-C).

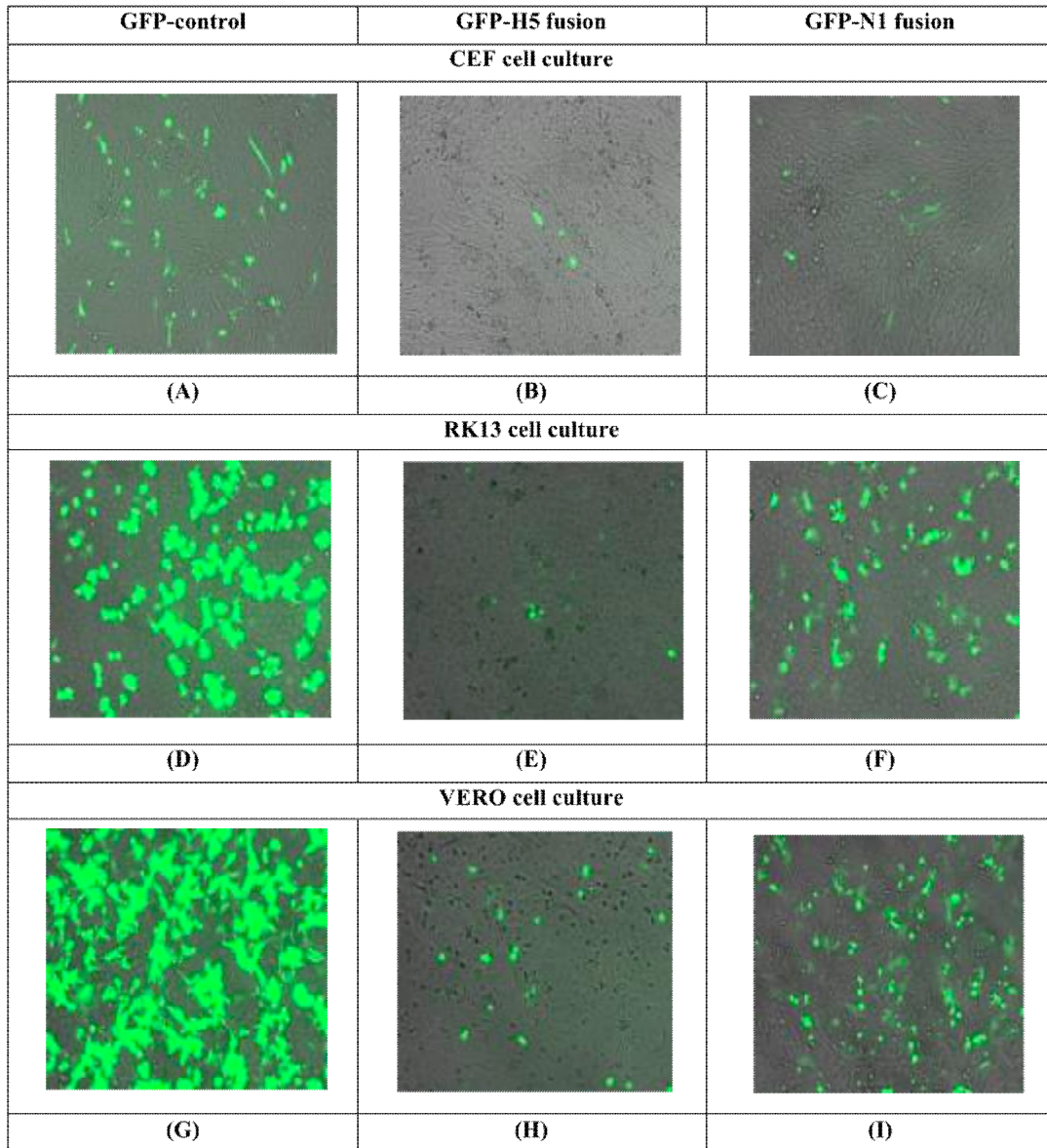### Comparison of expression level between H5 and N1 genes

The experimental results demonstrated clear differences of *in vitro* expression levels of the H5 and

N1 genes in CEF, RK13, and VERO cell culture systems. It appears that the recombinant proteins were better expressed in the VERO mammalian cell, suggesting this cell is more suitable for expression of foreign gene. Although the H5 and N1 genes were originally derived from an avian virus, these genes were poorly expressed on primary CEF cell culture. Apparently, the primary CEF cells were not suitable for gene expression studies, which might be due to low transfection efficiency of this cell. As the control GFP worked well, the expression of H5 gene was generally observed to be low in these cultures with only a small quantity of the protein detected by Western blotting analysis. In contrast, the N1 gene was expressed at a higher level compared to H5. This evidence supports the premise that the highly variance of codon usage pattern in the H5 gene sequence may negatively affect the expression level of the gene.

It is also suspected that the regulation of H5 gene expression in the naive infection involves viral helper genes. If the production of H5 protein requires processing of some sort by other viral proteins, the individual expression of H5 gene will be negatively affected by the absence of these proteins. In the studies by FODOR *et al.* (1999) and HOWARD *et al.* (2007), the presence of four additional protein expression plasmids containing PB1, PB2, PA, and NP gene increased the efficiency of the rescue system of all eight genomic segments of influenza virus in the reverse genetics studies. However, the significance of these four genes still requires clarification for influencing different levels of expression the H5 and N1 genes demonstrated in this study. These reverse genetics studies are more reflective of native virus replication where co-expression of all eight genes occurs at the same time rather than individual gene expression as conducted in this study.
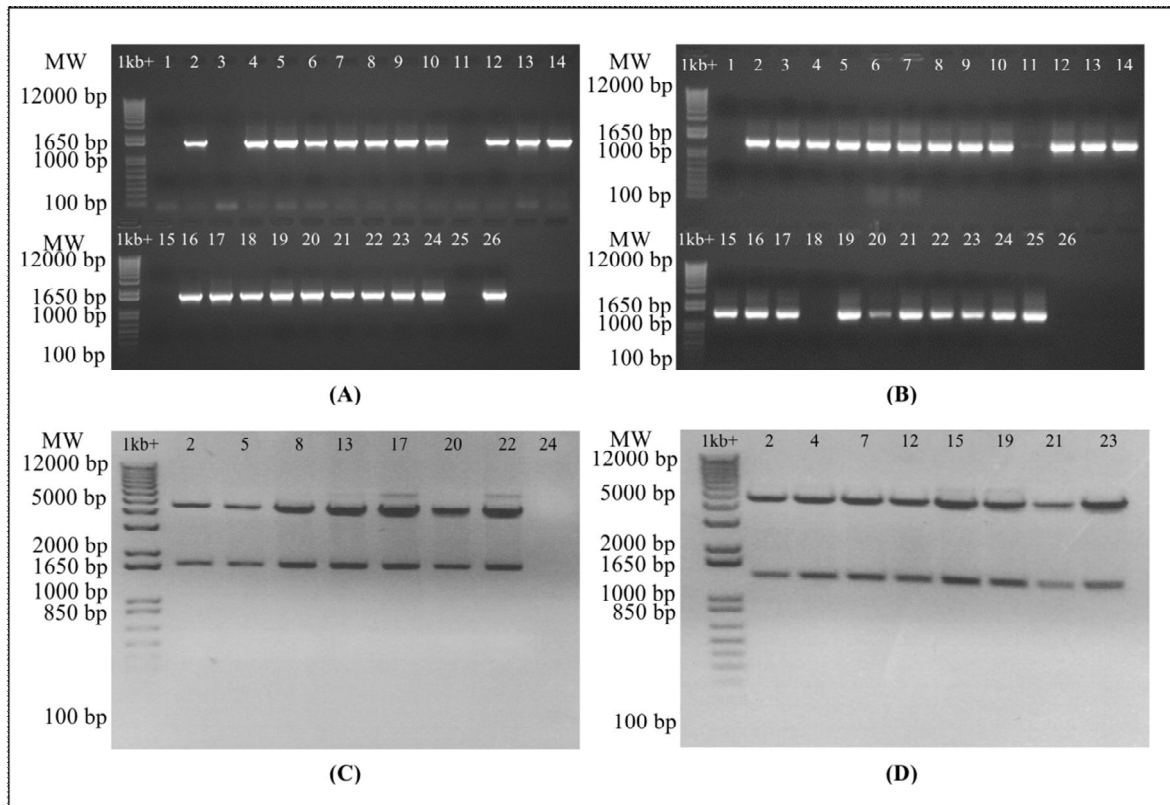

Figure 1. Construction of expression plasmid pEGFP-C1 carrying full-length of H5 and N1 genes: **(A)** PCR screening of 26 clones for identification H5 full gene of the recombinant pEGFP-C1/H5#26; **(B)** PCR screening of 26 clones for identification N1 full gene of the recombinant pEGFP-C1/N1#15; **(C)** Double digestion with *Xho*I & *Apa*I for the recombinant pEGFP-C1/H5#26 (clone 2, 5, 8, 13, 17, 20, 22, 24); **(D)** Double digestion with *Xho*I & *Apa*I for the recombinant pEGFP-C1/N1#15 (clone 2, 4, 7, 12, 15, 19, 21, 23)

GFP-control	GFP-H5 fusion	GFP-N1 fusion
CEF cell culture		
(A)	(B)	(C)
RK13 cell culture		
(D)	(E)	(F)
VERO cell culture		
(G)	(H)	(I)

Figure 2. Expression level of the pEGFP-C1 carrying H5 and N1 gene in cell cultures after 48 h incubation: **(A)** pEGFP-C1 (control) in CEF cells; **(B)** pEGFP-C1/H5 in CEF cells; **(C)** pEGFP-C1/N1 in CEF cells; **(D)** pEGFP-C1 (control) in RK13 cells; **(E)** pEGFP-C1/H5 in RK13 cells; **(F)** pEGFP-C1/N1 in RK13 cells; **(G)** pEGFP-C1 (control) in VERO cells**; (H)** pEGFP-C1/H5 in VERO cells; **(I)** pEGFP-C1/N1 in VERO cells

The expression study of native H5 and N1 genes in RK13 cell using pCDNA3 by PAVLOVA *et al.* (2009) generated poor expression for both genes. This result concurred with the expression of H5 gene of the current study but it displayed different outcome for the expression of N1 gene. However, these reseachers were able to enhance the individual expression of H5 and N1 genes in RK13 cell by inserting a synthetic intron sequence in the upstream region. Therefore, this outcome sugest that testing wider range of expression vector genetic elements may improve the expression level of HA and NA genes.
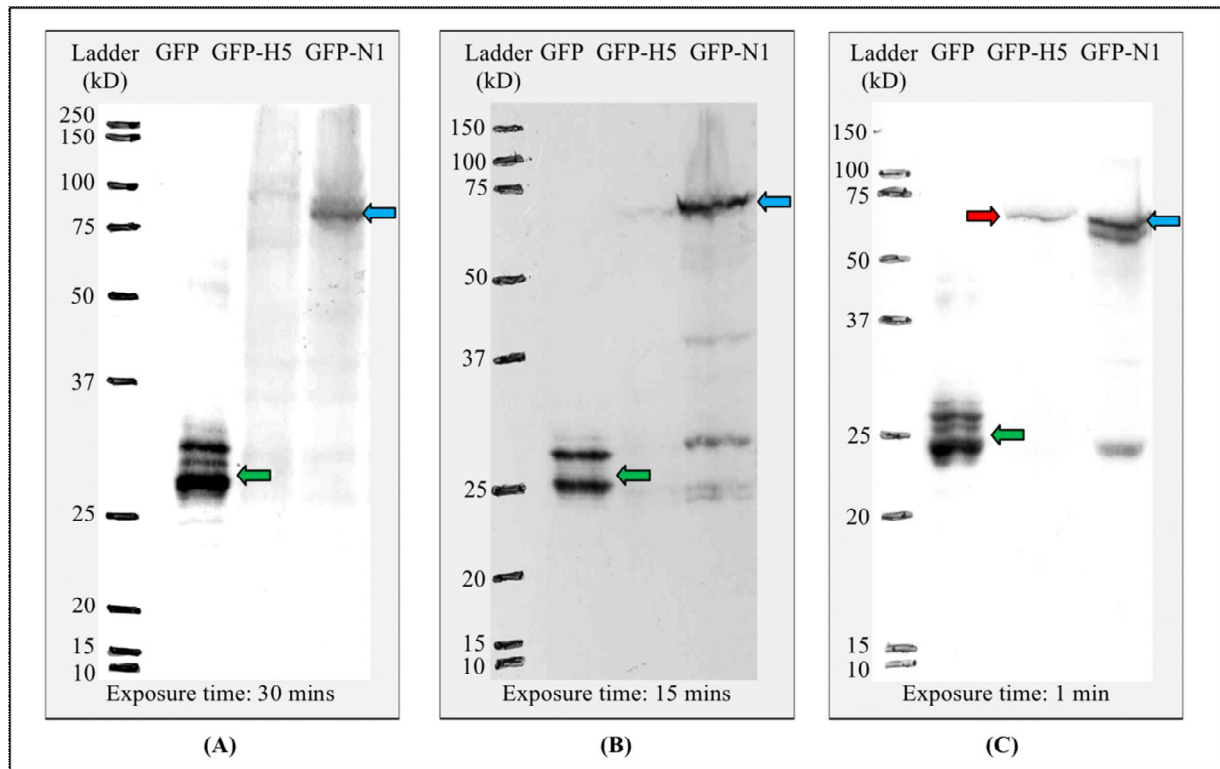
Figure 3. Western blotting analysis of GFP-H5/N1 fusion protein in cell culture after 48 h incubation: **(A)** gene expression in CEF culture; **(B)** gene expression in RK13 culture; **(C)** gene expression in VERO culture. Green arrow indicates presence of GFP protein; red arrow indicates GFP-H5 fusion protein, whilst blue arrow indicates GFP-N1 fusion protein

CONCLUSIONS

The native N1 gene was expressed higher by comparison with the native H5 gene in GFP recombinant system. In point of fact, the level expression of H5 gene as GFP fused protein was very low. These findings have implications for the utilisation of native H5 and N1 genes in recombinant systems, whereas the native N1 gene could be utilised in recombinant system, the native H5 gene would need to be optimised before it could be used for recombinant purposes.

ACKNOWLEDGEMENTS

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