

## **MODIFYING THE HEMAGGLUTININ GENE WITH THE PACKAGING SIGNALS TO IMPROVE THE ABILITY OF REPLICATION IN EGGS OF IBT-RG02 VACCINE VIRUS STRAIN**

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### **SUMMARY**

The hemagglutinin (HA) and neuraminidase (NA) genes were derived from the popular H5N1 virus, and the remaining six internal segments were derived from the A/Puerto Rico/8/34 strain (H1N1, PR8). However, some of these candidate strains have been reported to produce relatively low yields in vaccine manufacture and do not replicate well in chicken eggs, posing an obstacle to egg-based vaccine production. To address this issue, we explored the possibility that PR8's hemagglutinin (HA) packaging signals mediate the improvement of candidate vaccine virus yield in eggs. We constructed chimeric HA genes with the coding region of IBT-RG02 HA flanked by the 50 packaging signals of PR8's HA and generated variant vaccine viruses by reverse genetics. The growth of candidate vaccine viruses (CVVs) containing the chimeric HA (strains RG2, RG3, and RG4) was tested using the hemagglutination assay. The growth ability was found to be subsequently increased in embryonated chicken eggs, with a nearly 1.5- to 2-fold higher titer than that of the control (RG1). It is concluded that the use of chimeric HA fragments can subsequently improve the replication of reverse genetically derived virus strains in eggs.

**Keywords:** HA chimeric, HA titer, virus replication, avian influenza virus, IBTRG-02

### **INTRODUCTION**

H5N1 from influenza A virus first appeared in Vietnam in 2003, with the third highest number of infected (127) and fatal cases (64) in humans in the world (Chen *et al.*, 2014; Gao *et al.*, 2014; Le, Nguyen, 2014). From 2003 to 2019, the H5N1 pandemic was reported to have affected 50 million poultry from more than 3,000

outbreaks, which caused a 0.5–1.8% annual loss of gross domestic product in the country (Cobbin *et al.*, 2013; Fulvini *et al.*, 2011; Essere *et al.*, 2013). There is a very high risk of viral transmission due to small-scale, conventional methods of producing poultry, unrestricted trade between nearby nations, and close contact with chickens at live bird markets. Therefore, using influenza control techniques to minimize adverse effects on

humans and poultry is essential.

Clade 2.3.2.1c is a great challenge to HPAI A/H5N1 control in Vietnam since the current in-use vaccines show a rapid decline in protection against these viruses, and of course, they present a high risk to public health (Le, Nguyen, 2014; Gerber *et al.*, 2014; Harvey *et al.*, 2010; Watanabe *et al.*, 2003).

The inactivated trivalent influenza A vaccine parts are reassortant viruses, which have the derived gene segments from the master donor strain (A/Puerto Rico/8/1934 (PR8, H1N1)) as well as gene segments hemagglutinin (HA) and neuraminidase (NA) from circulating viruses (Watanabe *et al.*, 2003; Dos Santos Afonso *et al.*, 2005; Fujii *et al.*, 2005; Liang *et al.*, 2005). Even though these reassortants are made up of only the HA and NA from the circulating viruses, in practice, several high-yielding vaccine viruses made by classical reassortment have kept other seasonal virus gene segments, most notably the PB1 segment, showing that this is good for virus production (Wit *et al.*, 2006; Muramoto *et al.*, 2006; Gog *et al.*, 2007; Marsh *et al.*, 2007). Since the reassortant virus contains six gene segments from the donor virus and HA and NA from circulating viruses of different subtypes and lineages, the compatibility of packaging signals in CVVs may impede optimal egg maturation (Liang *et al.*, 2008; Hutchinson *et al.*, 2008; 2009; Harris *et al.*, 2006).

Several studies showed that the 3' and 5' terminal regions, which include the non-coding regions (NCRs) and some open reading frame (ORF) ends, have segment-specific packaging signals (Noda *et al.*, 2006; Noda, Kawaoka, 2010; Fournier *et al.*, 2012a, b). In recent years, the terminal

sequences of the PR8 donor virus's HA or NA have been used to increase the yield of PR8-based CVV in eggs and the growth of PR8-based A/Vietnam/1194/2004 (H5N1) with PR8 HA or NA packaging signals (Bergeron *et al.*, 2010; Harvey *et al.*, 2011; Pan *et al.*, 2012; Wit *et al.*, 2007). This study contributes a strategy regarding the role of PR8 HA packaging signals in the propagation of PR8-based CVVs.

## MATERIALS AND METHODS

### Viruses and reverse genetics vectors

In a previous study, a recombinant H5N1 vaccine strain (designated as IBT-RG02 was successfully generated by a powerful reverse genetics technique that containing two surface proteins (haemagglutinin (HA) and neuraminidase (NA)) from the HPAI H5N1 (A/duck/Vietnam/HT2/2014(H5N1)) of the dominant clade 2.3.2.1c in Vietnam during 2012–2014 (Le, Nguyen, 2014; Hoang *et al.*, 2020). This reverse genetics-based virus was utilized for use in this study.

For reverse genetics techniques, the pHW2000 vector containing the dual promoter/terminator system was generously provided by the Department of Infectious Diseases, St. Jude Children's Research Hospital (Memphis, TN, USA).

### Cloning and construction of chimeric NA genes

The 3' forward primer 5'-CCTTGTTCTACTAGC-3' (Wit *et al.*, 2006) with a segment-specific reverse primer was used in an RT-PCR test to deduce the 5' terminal sequence. Similarly, the 3' terminal sequence was determined by sequencing PCR products generated from an RT-PCR

reaction using a segment-specific forward primer and the 5' reverse primer, 5'-CCTGCTTTTGCTAGT-3' (Hoffmann *et al.*, 2000). The chimeric HA genes were created by exchanging the HA and PR8 HA genes' terminal sequences by mega-primer mutagenesis (Barman *et al.*, 2017).

### **Generation of variant vaccine viruses by reverse genetics**

The process of generating the reassortment of 6+2 vaccine viruses involved the co-transfection of 293T cells with eight cDNA plasmids. These included two plasmids that encoded either the wild-type (WT) or chimeric HA and NA of the IBTRG-02, and six contained gene segments of PR8. The cell culture supernatant from transfected 293T cells was then inoculated into 10 to 11-day embryonated chicken eggs, where the viruses multiplied at 37°C for 48 hours before being tittered by the hemagglutination assay, according to Killian (2008).

## **RESULTS AND DISCUSSION**

### **Construction of the chimeric HA gene**

The HA gene segments from the IBTRG02 virus were successfully inserted into the virus rescue plasmid pHW2000. Prior to the advent of cloning, terminal sequences were also ascertained. To investigate how the PR8 HA packing signals impacted on the IBTRG02 CVV development, the chimeric HA gene segments were generated using the methodology outlined by Pan *et al.* (2012). The chimeric IBTRG02 HA was constructed by incorporating packaging signals from PR8 HA. Specifically, based on the packaging signals reported for HA (Fujii *et al.*, 2003), IBTRG02 HA ORF flanked by a 41-nt packaging signal from PR8 HA (32-nt NCR plus 9 nt from ORF, Figure 1) at the

5' end and a 126-nt packaging signal from PR8 HA (81 nt at the 3' end ORF plus 45-nt NCR, Figure 1) at the 3' end was constructed (P-HA-P, Fig. 1). This resulted in a P-HA-P configuration, as depicted in Figure 1. These packaging signals were identified based on the packaging signals reported for HA in a previous study (Fujii *et al.*, 2003). The chimeric constructions utilized the whole open reading frames (ORFs) of the HA gene from the IBTRG02 virus. To prevent amino acid alterations and translation initiation from PR8's ORF, the ATG codon of PR8 HA sequences in chimeric constructions was altered to GCG (Figure 1).

The non-coding region of hemagglutinin in IBTRG02 in this study was functionally similar to the hemagglutinin packaging signals, that were described in Barman *et al.* (2017), which improved the growth of the A/Anhui/1/2013 (H7N9) influenza vaccine virus.

### **Evaluation of the growth of IBTRG02 CVVs in eggs**

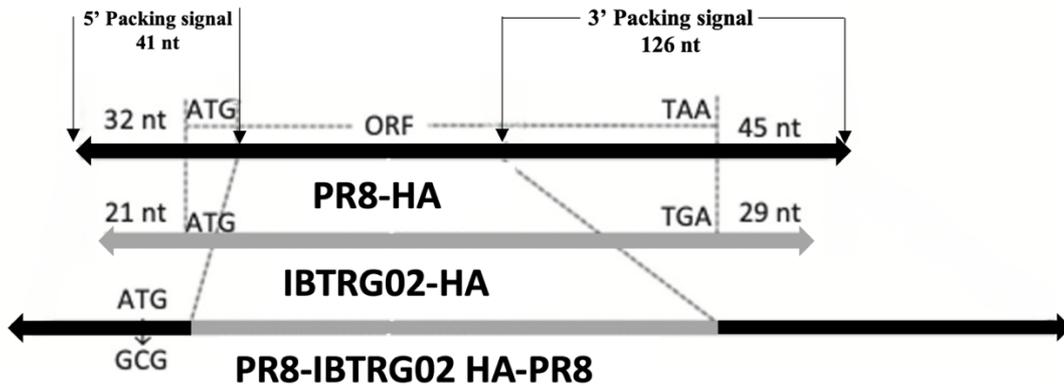
To evaluate the impact of PR8 HA packaging signals on IBTRG02 CVVs, a set of CVVs based on PR8 was created in 293T cells. These CVVs contained either wild-type or chimeric IBTRG02 HA and NA WT, along with the six remaining genes from PR8. The rescued viruses were subjected to a single passage (E1) in eggs, and their titer was determined using the hemagglutination test. The growth level of different variants of vaccine viruses that was evaluated by the virus titration by the hemagglutination test is presented in Table 1. All the RG1, RG2, RG3, and RG4 viruses had the same wild-type neuraminidase (NA(WT)) but different HA gene insertions. Table 1 showed that the RG1, which possessed the HA (WT), has a virus titer of 5.02 (log<sub>2</sub>), and this value is

considered the control. As a result, the RG2 with the HA-PR8 had a virus titer of 9.62 ( $\log_2$ ), which is 191% higher than that of the RG1, and the RG3 with the PR8-HA had a virus titer of 7.16 ( $\log_2$ ), which is 142% higher. The RG4 with the PR8-HA-PR8 had a virus titer of 9.91 ( $\log_2$ ), which is the highest value and is 197% higher than the RG1 control.

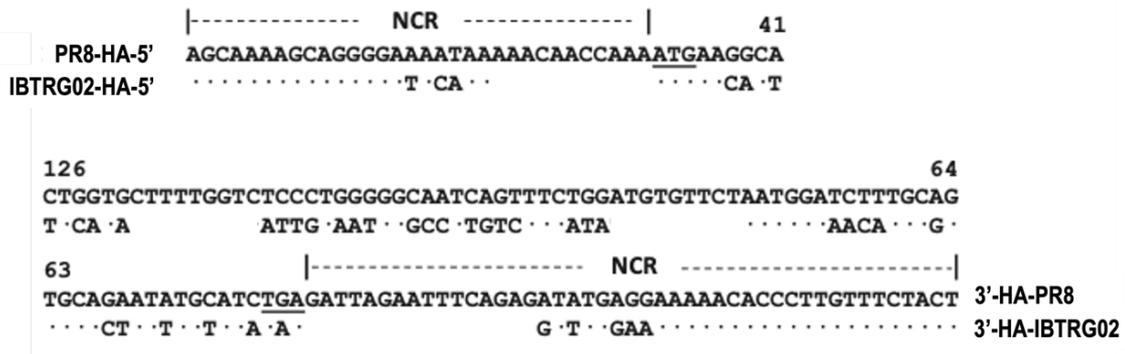
All of these findings indicated that the PR8 HA 5' and 3' packaging signals, which were surrounded the HA ORF of the

IBTRG02 viruses, had a substantial impact on the growth of CVVs (i.e., RG2, RG3, and RG4) when compared to the growth of the wild-type CVV RG1. Specifically, the titers increased value to 2-fold that of the RG1 resulted from the RG4, which indicated the utilization of the PR8 HA 5' and 3' packaging signals with the HA gene in the reassortant vaccine viruses.

The findings align with the results reported for the A/Anhui/1/2013 (H7N9) influenza vaccine virus (Barman *et al.*, 2017).



**Figure 1.** Schematic diagram of IBTRG02 chimeric HA. The IBT-RG02's HA open reading frame (ORF) was flanked by PR8's HA. In chimeric constructs, the ATG translation initiation codon of PR8's HA sequences was mutated to GCG. IBT-RG02 HA ORF is flanked by a 41-nt packaging signal from PR8 HA (32-nt NCR plus 9 nt from ORF) at the 5' end and a 126-nt packaging signal from PR8 HA (81 nt at the 3' end ORF plus 45-nt NCR) at the 3' end.



**Figure 2.** Packaging signal sequences of PR8's and IBTRG02. The numbers shown above the sequences are nucleotide numbers counted from their respective ends. NCR, non-coding region. IBTRG02 HA ORF is flanked by a 41-nt packaging signal from PR8 HA at the 5' end and a 126-nt packaging signal from PR8 HA at the 3' end.

**Table 1.** Growth of IBTRG02 candidate vaccine viruses containing chimeric HA and NA-wildtype.

Candidate vaccines (designated: RG)	NA	HA	VIRUS TITER	RELATIVE TO RG1 (%)
RG1	NA (WT)	HA (WT)	5.02 (log <sub>2</sub> )	100
RG2	NA (WT)	HA-PR8	9.62 (log <sub>2</sub> )	191
RG3	NA (WT)	PR8-HA	7.16 (log <sub>2</sub> )	142
RG4	NA (WT)	PR8-HA-PR8	9.91 (log <sub>2</sub> )	197

## CONCLUSION

The ability of replication in eggs of the HA-modified IBT-RG02 virus vaccine strain was significantly improved compared to the unmodified strain, reaching 9.91 log<sub>2</sub> HA titer. The change in hemagglutinin packaging signals of the A/H5N1 virus vaccine strain, which led to the HA titer increasing in eggs, showed the influential function of the HA fragment in virus growth in eggs. Modifying the functional gene by changing the packaging signals as a method of improving the HA titer by using the chimeric HA gene in this study can be applied to enhancing replication for other virus vaccine strains.

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