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Expression of Recombinant Baculovirus Vetor Double-expressing HA1 Gene from H5 Subtype Avian Influenza Virus and Mature Chicken Interleukin-18 Gene

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Abstract: The Hemagglutinin (*HA1*) gene from H5 subtype Avian Influenza Virus (AIV) and mature Chicken Interleukin-18 (*mChIL-18*) gene were amplified by designing two pairs of primers according to the sequence published on GenBank. The *HA1* gene and *mChIL-18* gene were inserted into the downstreams of PH promoter and P10 promoter of baculovirus expression plasmid pFastBac™Dual, respectively. The recombinant pFastBac™dual-mChIL-18-HA1 (rIL18-HA1) was ransformed into DH10Bac competent cells and expressed in sf9 cells by IFA diction. The results showed that the recombinant protein IL18-HA1 (pIL18-HA1) was simultaneouly expressed in the same sf9 cells with IL18 protein and HA1 protein, respectively. It is the foundation for developing a neotype vaccine against AIV.

Key words: Avian influenza virus, *HA1* gene, mature *ChIL-18* gene, eukaryotic expression, IL18 protein

INTRODUCTION

Highly Pathogenic Avian Influenza (HPAI) is caused by particular Avian Influenza Virus (AIV) subtypes such as H5 and is classified as a disease by the Office Internationale des Epizooties (Alexander, 2000) which has been reported frequently in recent years, leading to substantial economic loss. In 1997, a highly pathogenic H5N1 AIV strain was transmitted directly from birds to humans in Hong Kong (Subbarao *et al*., 1998) and a new outbreak of lethal H5N1 influenza emerged in the Hong Kong poultry markets in May 2001. The genome of AIV is comprised with eight RNA fragments including Hemagglutinin (HA) fragment and Neuraminidase (NA) fragment. AIV representing each of the 15 HA subtypes and 9 NA subtypes have been isolated from aquatic birds in America (Hinshaw *et al*., 1980). HA is the major protective antigen of AIV and HA antibodies against it constitute the primary defense against virus infection. Thus, it is an appropriate gene to produce genetically engineered vaccine. HA that is then split into HA1 subunit and HA2 subunit. In the spatial conformation that the mature HA takes, only the HA1 subunit becomes exposed and it is therefore, where most of its antigenic determinants are found (Wiley and Skehel, 1987). Some of the cytokines have been proven to be effective immunomodulator in animal model or clinical test in many reports. It has been indicated that the recombinated IL18 of chicken has the biologic activity of inducing IFN-γ production (Yu *et al*., 2008). In 2003, Thomas reported that the IL18 of chicken has a great many biologic activities as the Th cell effective activator that it can induce CD4+ T cell to secrete IFN-γ and the proliferation of T cell, so the cytokine adjuvant is widely used lately. The baculovirus expression system has been commonly used in research communities and scientific industries for the production of high levels of recombinant proteins which require posttranslational modifications for functional activity (Gao *et al*., 2007; Azhar and Somashekhar, 2015).

In this study, we have constructed three recombinant baculovirus that double-expressing the *HA1* genes of H5 subtype AIV and the *mChIL-18* gene or single-expressing the *HA1* genes and single-expressing the *mChIL-18* gene. This study reports a significant step in the further development of new AIV vaccines.

MATERIALS AND METHODS

Construction of recombinant pFastBac™dual-mChIL-18 (rIL18): The *mChIL-18* gene was amplified by PCR with the forward primer mChIL-18-BamHI, 5'-CGCGGATCCA TGGCCTTTTGTAAG-3' which contained sequences for a BamHIsite and the reverse primer mChIL-18-Hind III, 5'-CGGAAGCTTTAGT CATAGGTTGTGCCT-3' which included a Hind III site. The amplified product was subcloned into the corresponding sites in pFastBac™Dual plasmid under the control of the baculovirus P10 promoter.

Construction of recombinant pFastBac™dual-HA1 (rHA1): The *HA1* gene from AIV was amplified by PCR with the forward primer HA1-XhoI, 5'-CGGCTCGAGATGG ATCAGATTTG-3', contained

sequences for a XhoI site and the reverse primer HA1-KpnI, 5'-CGGGCATGCTAGTCTCTTTTTCTTC-3' included a KpnI site. The amplified product was subcloned into the corresponding sites in pFastBac™Dual plasmid under the control of the baculovirus PH promoter.

Construction of recombinant pFastBac™dual-mChIL-18-HA1 (rIL18-HA1): The *HA1* gene was inserted into the downstreams of PH promoter of rIL18 with a pair of primers (HA1-XhoI and HA1-KpnI).

Construction of recombinant bacmid: The purified recombinant plasmid DNA was transformed into DH10Bac *E. coli* (Invitrogen) which contained a baculovirus shuttle vector to obtain the recombinant bacmids DNA: Bacmid-HA1 (rBac-HA1), Bacmid-mChIL18 (rBac-IL18) and Bacmid-HA1-mChIL18 (rBac-HA1-IL18). The identification of recombinant bacmids DNA were carried out by PCR with a pair of primers. The primer sequences were as follows: M13 forward primer, 5'-GTTTTCC CAGTCACGAC-3' and M13 reverse primer, 5'-CAGGA AACAGCTATGAC-3'.

Expression of recombinant protein: Sf9 cells (6×10^5) were infected with the recombinant baculovirus (rBac virus) at 0.1 M.O.I. and recombinant VP2 protein (pHA1), mChIL18 protein (pIL18) and HA1-mChIL18 protein (pHA1-IL) were harvested at 72 h post-infection.

Immuno Fluorescence Test (IFAT) of pHA1, pIL18 and pHA1-IL: The transfected cells containing recombinant proteins were washed with PBS (pH 7.4, 137mM NaCl, 2 mM KH2PO4, 10 mM NaHPO4 and 2.7mM KCl) 3 times and fixed with ice-cold acetone/alcohol (ratio 3:2) for 8 min. After washing with PBS for 3 times, the wells were then overlaid with rabbit anti-mChIL18 polyclonal antibody and positive serum of AIV and then incubated at 37°C for 1 h. The wells were then washed 3 times with PBS and incubated with TRITC anti-rabbit IgG (Jackson) and FITC anti-chicken IgG (Santa Cruz, 1:200) at 37°C for 1 h. The washing steps were repeated, the wells were covered by 50% (v/v) of glycerine and analyzed by an inverted phase contrast microscope with different fluorescence lights.

RESULTS AND DISCUSSION

Identification of recombinant plasmids: The constructed recombinant plasmids were confirmed by PCR amplifications using appropriate primer combinations and enzyme digestions using (Fig. 1). Sequence analysis of rIL18 (Fig. 1a), rHA1 (Fig. 1b) and rIL18-HA1 (Fig. 1c) recombinants also confirmed the sequence identity and the correct reading frames.

Identification of recombinant bacmids: Recombinant bacmid DNA is indentificated by PCR analysis on the basis of Bac-to-Bac Bac Expression System User Manual.

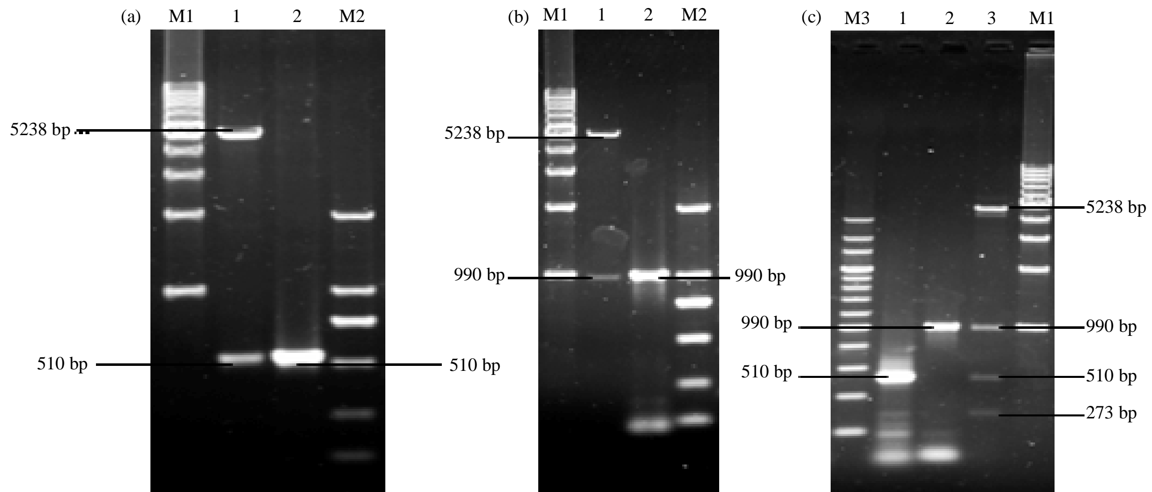

Fig. 1: Agarose gel images of PCR and digested products of recombinant plasmids. M1: 1 kbp DNA Ladder Marker; M2: DL2,000 DNA Marker; M3: 200 bp DNA Ladder Marker: a) Lane 1: BamHI-HindIII digestion of rIL18; Lane 2: PCR products of *IL18* gene; b) Lane 1: Kpn I-Xho I digestion of rHA1; Lane 2: PCR products of *HA1* gene; c) Lane 1: PCR products of *IL18* gene; Lane 2: PCR products of *HA1* gene; Lane 3: BamHI-Hind III and KpnI-XhoI digestion of rIL18-HA1

The size of PCR product was calculated by 2560 bp mini-attTn7 plus size of insert gene. The size of amplified fragments for rBac-IL18, rBac-HA1 and rBac-HA1-IL18 was respectively 3070, 3550 and 4060 bp (Fig. 2).

IFAT of recombinant protein: IFAT was performed to further confirm the expression of the recombinant protein.

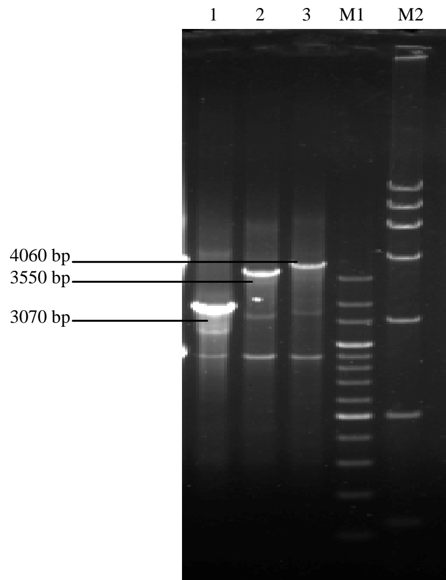

Fig. 2: PCR identification of recombinant bacmids. M1: 200 bp DNA Ladder Marker; M2: DL15,000 DNA Marker; Lane 1: PCR products of rBac-IL18; Lane 2: PCR products of rBac-HA1; Lane: 3 PCR products of rBac-HA1-IL18

The cells transfected with rBac-HA1-IL18 exhibited bright red fluorescence and bright green fluorescence, respectively by rabbit anti-mChIL18 polyclonal antibody linked TRITC anti- rabbit IgG (Fig. 3a) and positive serum of AIV linked FITC anti-chicken IgG (Fig. 3b) in the same eyesight. It demonstrated that IL18 protein and HA1 protein were simultaneously expressed in sf9 cells. No fluorescent staining was detected from nontransfected cells (Fig. 3f) and cells transfected with pFastBac^{TM}Dual only (Fig. 3c). The cells transfected with rBac-HA1 only exhibited bright green fluorescence (Fig. 3d) but not bright red fluorescence while the cells transfected with rBac-IL18 only exhibited bright red fluorescence (Fig. 3e) but not bright green fluorescence by the same treament.

Only type A influenza viruses are known to cause natural infections in birds but viruses of all 15 haemagglutinin and all nine neuraminidase influenza A subtypes in the majority of possible combinations have been isolated from avian species. Influenza A viruses infecting poultry can be divided into two distinct groups on the basis of their ability to cause disease. The very virulent viruses cause HPAI in which mortality may be as high as 100%. These viruses have been restricted to subtypes H5 and H7, although, not all viruses of these subtypes cause HPAI (Alexander, 2000). HA1 subunit which is the major antigen of AIV and contains major epitopes responsible for protection against AIV can stimulate neutralizing antibodies and accordingly induce humoral immuniny *in vivo*.

Vaccine adjuvants are essential to stimulate the host’s immune response to antigens that lack immunogenicity. Traditional adjuvants come in many

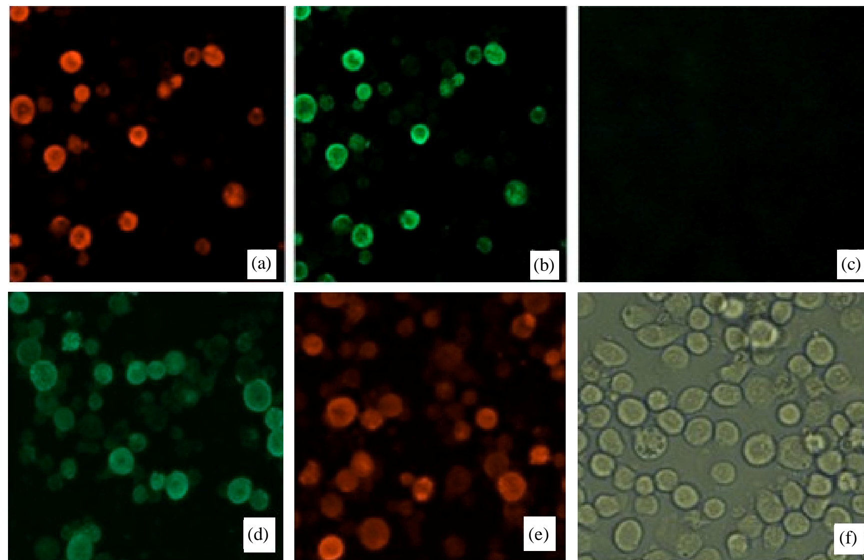

Fig. 3: a-f) IFAT of pHA1, pIL18 and pHA1-IL

flavours but for most substances little is known about their mechanism of activity. Theoretically, adjuvants can be divided into either facilitators of signal 1, enhancing the duration or magnitude of either whole antigen or its peptide fragments presented by MHC molecules on antigen presenting cells in lymphoid organs and/or as inducers of endogenous signal 2 molecules including cytokines, membrane-bound co-stimulatory molecules or other host-derived natural adjuvants (Degen *et al*., 2003). In 2000, the cDNA encoding IL18 of chicken was cloned for the first time (Schneider *et al*., 2000) and expressed in the bacterium coli, it indicated that the recombinated IL18 of chicken has the biologic activity of inducing IFN-γ production. Thomas reported that the IL18 of chicken has a great many biologic activities as the Th cell effective activator that it can induce CD4+ T cell to secrete IFN-γ and the proliferation of T cell, in addition, it has the positive accommodation effect for the composition of MHC ClassIIAg.

It had been shown that recombinant vaccination expressed by many prokaryotic and eukaryotic expression systems could induce virus-neutralizing antibody response and protected chickens against virus infection (Wu *et al*., 2004). But those productions of subunit vaccine were less biology safe than Baculovirus expression system was employed. So, it could be safe for us to produce the protein vaccine using the new expression system.

CONCLUSION

In this study, the cells transfected with rBac-HA1-IL18 exhibited bright red fluorescence and bright green fluorescence, respectively by rabbit anti-mChIL18 polyclonal antibody linked TRITC anti-rabbit IgG (Fig. 3a) and positive serum of AIV linked FITC anti-chicken IgG (Fig. 3b) in the same eyesight. It demonstrated that IL18 protein and HA1 protein were simultaneously expressed in sf9 cells. This study reports a significant step in the further development of new AIV vaccines.

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