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# Cloning and *in vitro* Expression of N1 Neuraminidase Gene of Avian Influenza Virus A/Duck/Thailand/KU-KPS/2004(H5N1)

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## ABSTRACT

Avian influenza virus (AIV) can spread rapidly and causes serious disease to animals and humans so it is crucial to have effective diagnostic tools for determining its existence. The purposes of this study were first, to clone N1 neuraminidase (NA) gene of AIV A/Duck/Thailand/KU-KPS/2004 (H5N1), and second, to express recombinant N1 using baculovirus expression system. Viral RNA extracted from positive AIV allantoic fluid was reverse-transcribed to cDNA and then was amplified by PCR using N1 specific primers. The PCR products approximately 1,300 bp that encode the N1 was introduced into baculovirus vector in order to allow N1 expression in insect cell lines. Immuno-dot blot analysis of crude extract of baculovirus infected insect cells using goat-anti H5N1 AIV hyperimmune serum gave a clear immunoreactive spot. SDS-PAGE analysis showed banding patterns from the crude extract contained major protein of 54 kDa. Western blot analysis using goat-anti H5N1 hyperimmune serum and mouse-antihistidine monoclonal antibody also gave a specific band approximately the same size as that of SDS-PAGE. Expression analysis of this gene indicated that recombinant neuraminidase may have correct post-translation modification and folding. The recombinant neuraminidase could be very useful for the development of a test kit that can differentiate infected from vaccinated animals (DIVA).

**Key words:** cloning, N1 gene expression, neuraminidase, AIV, baculovirus system

## INTRODUCTION

Avian influenza (AI) or bird flu or fowl plaque is a highly acute contagious disease of avian species, more specifically to the member species of order *Anseriformes* (chickens, ducks, geese and swans) as well as order *Charadriiformes* (gulls, tern, puffins and guillemots) (De Marco *et al*., 2004). The influenza viruses are divided into three types A, B, and C, based on antigenic differences in nucleoprotein and matrix (M) proteins. Influenza A and B viruses are the major cause of morbidity and mortality world wide. Influenza A virus is further classified into subtypes based on the antigenic differences of the surface proteins hemagglutinin (HA) and neuraminidase (NA). Currently, there are 15 distinct HA (H1 to H15) and 9 NA (N1 to N9) subtypes found (Nicholson *et al*., 2003). Avian influenza is on the list A of the Office International des Epizootics (OIE, 2001).

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The list A disease of OIE contains transmissible diseases that have the potential for very serious and rapid spread, irrespective of national borders. They cause serious socio-economic or public health consequence that are of major importance in the international trade of animals and animal products (Alexander, 1993).

The diagnosis of AIV is important because these viruses can spread rapidly and they are also zoonoses. There are several methods for AIV detection such as hemagglutination-inhibition (HI) (Beby-Defaux *et al*., 2003), agar gel immuno-diffusion (Beard, 1970), immunofluorescence, viral culture in embryonated egg or Madin-Darby canine kidney cells, reverse transcriptase-polymerase chain reaction (RT-PCR) (Lee *et al*., 2001), Taq-man-PCR (Schweiger *et al*., 2000), nucleic acid sequence-based amplification (NASBA) (Collins *et al*., 2003; Lau *et al*., 2004), direct / indirect immunofluorescent antibody test (IFAT) (Capua *et al*., 2002) and enzyme linked immunosorbent assay (ELISA) (Rowe *et al*., 1999). The ELISA technique has advantages over other methods because of its rapid and high sensitivity, being able to handle several samples at the same time and can differentiate infected from vaccinated animal (Capua *et al*., 2002). There are 2 main strategies to control AIV which are stamping out and vaccination. For vaccination strategy, the ability to differentiate infected from vaccinated animal (DIVA) is crucial. In this study, the N1 Neuraminidase genes of H5N1 AVI was cloned and expressed using baculovirus expression system. The recombinant N1 was characterized by immuno-dot blot and SDS-PAGE.

## MATERIALS AND METHODS

### Isolation of virus

The highly pathogenic native AIV strain A/Duck/Thailand/KU-KPS/2004 (H5N1) was isolated from naturally infected duck tissues (lung, spleen, kidney, pancreas, liver and brain) and cloacal swab at the Diagnostic Laboratory, Faculty of Veterinary Medicine, Kasetsart University, Kamphaengsaen Campus, Nakhon Pathom province. Embryonated-chicken eggs, 9-to-12-day-old, were inoculated via the allantoic cavity by injecting with approximately 0.2 ml of extracted viral solution and incubated at 37°C for 24-72 h (Swayne *et al.*, 1998). Embryonic death was daily observed. The allantoic fluid of dead embryonated-chicken eggs was collected and kept at -80°C until used for RT-PCR.

### RNA preparation and reverse-transcription polymerase chain reaction for N1

Viral RNA was extracted from allantoic fluid using acid guanidinium thiocyanate-phenol-chloroform extraction method (Sambrook and Russell, 1998). The exact amount of 100 µl allantoic fluids was mixed with 500 µl of denatured solution and 50 µl of 2M sodium acetate and shaken for 5-10 min. cDNA of whole N1 gene was synthesized using Uni 12 primer (5′-AGC-GAA-AGC-AGG-3′) (WHO, 2002) and AMV reverse transcriptase (FINNZYMES®) under extension condition of 42°C for 50 min. A pair of the N1 specific primers were designed and synthesized according to N1 cDNA sequences published on GenBank (accession AF216714, AF216722, AF216730, AF216738, AF468838, AY075028, AY075031, AY075034). Subsequently, the whole N1 gene except the stop codon at the 3′ end of the gene was amplified using forward primer of 5′-GCG-CTC-TAG-AGC-CAC-CAT-GAA-TCC-AAA-TCA-GAA-GAT-AAC-3′ (Matching the nucleotide sites from 5 to 28 and containing *XbaI* cleavage site), and reverse primer of 5′-GCG-CAA-GCT-TCT-TGT-CAA-TGG-TGA-ATG-GCA-AC-3′ (Matching the nucleotide sites from 1,427 to 1,405 and containing *HindIII* cleavage site). PCR mixture composed of 1X PCR buffer, 3 mM dNTPs, 2.5 mM $MgCl_2$, 0.5 pmol of each forward and reverse primer, 2.0 U Platinum® *Taq* DNA polymerase (Invitrogen®), and DNA

template, was amplified using Primus96 plus (Hybaid) thermocycler. PCR condition was pre-denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 45 sec, annealing at 55° C for 1 min, an extension at 72°C for 3 min, and additional final extension at 72°C for 15 min. PCR products were subjected to 1.5% agarose gel, 100 volt for 45 min and visualized under UV illumination (Spectroline).

**Construction of expression vector**

The amplified N1 gene was purified using QIA quick gel extraction kit (QIAGEN®), and then it was digested and ligated to pFastBac' plasmids (Invitrogen®). The ligated plasmids were used to transform *E. coli* strain DH5∝ (Gibco®) competent cells. The positive clones were checked using PCR and restriction endonuclease assay. These clones were scaled up and used for DNA sequencing using dideoxynucleotide sequencing method at the DNA Technology Laboratory, BIOTEC, Kasetsart University, Kamphaengsaen Campus. The sequencing result was analyzed using DNASIS, Expasy and ClustalW programs. The inserted transfer vector was used to transform *E. coli* strain DH10-Bac™ (Invitrogen®) competent cells. The positive clone was selected using white-blue colony screening in LB agar plates containing kanamycin (50 μg /ml), gentamicin (7μg/ml), and tetracycline (10μg/ml). The presence of N1 gene encoding for neuraminidase was confirmed by PCR.

**Insect cells transfection and expression of N1 gene**

Sf21 cell lines *(Spodoptera frugiperda*) were grown in SF900II medium (Invitgrogen®) supplemented with 4% FBS and 10% antibiotics at 27°C. The recombinant expression plasmid was used to transfect Sf21 cells using cellFECTIN® (Invitrogen®). Then, the recombinant baculovirus particles were collected from cell culture at 72 h post transfection and virus titer was determined by plaque assay. Subsequently, the high-titer seed virus stock of recombinant baculovirus was produced by Sf21 insect cells at a multiplicity of infection (MOI) of 0.01 to 0.1 in Sf900 II SFM, medium (Invitrogen,) containing 4% fetal bovine serum and antibiotic (GIBCO®). High Five™ cell lines *(Trichoplusia ni*) grown in Express Five serum-free medium (Invitrogen®) supplemented with 9% L-glutamine and 10% antibiotics were used to produce neuraminidase. After 72 h post-inoculation (h.p.i.), the infected insect cells were lysed using 10% sodium dodecyl sulfate, and the crude extracted protein was subjected to immuno-dot blot, SDS-PAGE, and Western blot analysis.

**Immuno-dot blot analysis**

The crude extracted protein was dotted on nitrocellulose membrane and incubated with either goat anti-H5N1 AIV hyperimmune sera (1:50) or mouse anti-histidine IgG monoclonal antibody (1:150) for 2 h. Subsequently, the membrane was incubated with either rabbit anti-goat IgG (1:1,000) or goat anti-mouse IgG conjugated with peroxidase (1:500) for 1 h. The membrane was finally incubated with diaminobenzidine solution (Sigma®) containing 1% $H_2O_2$ for 5-10 min.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting**

The crude extracted protein was analyzed using 12 % SDS-PAGE and stained with Coomassie brilliant blue. For Western blot analysis, proteins on SDS-PAGE were transferred onto nitrocellulose membrane under condition 400 mAmp for 5 h, keep at 4°C, then the membrane was incubated with either goat-anti H5N1 AIV hyperimmune sera (1:50) or mouse anti-histidine IgG monoclonal antibody (1:3,000). Subsequently, the nitrocellulose membrane was incubated with either rabbit anti-goat IgG (1:1,000) or goat anti-mouse IgG (1:500) conjugated with peroxidase. The membrane was then incubated with diaminobenzidine solution (Sigma®) containing

1% $H_2O_2$ for 5-10 min.

## RESULTS AND DISCUSSION

### Cloning and sequencing of N1 gene

The PCR of neuraminidase gene showed an amplified band of approximately 1,300 bp (Figure 1). The N1 gene of AIV from different species as reported in several countries around the world was found to be 1,350-1,417 bp (Cauthen *et al.*, 2000; Guan *et al.*, 2002; Tumpey *et al.*, 2002; GenBank, 2005; Lee *et al.*, 2005). It was found that the length of N1 gene of H5N1 AIV from a local duck (GenBank accession DQ103713), chicken (GenBank accession AY649383) and human (GenBank accession AY555152) was similar. Comparing the N1 gene sequences of these 3 isolates, it was shown that the N1 gene sequence of duck had 99% homology with those of chicken and human. There were 5 nucleotide changes on this gene (GenBank, 2005) which resulted in 4 amino acids changes at amino acid number 5, 8, 80 and 418 from the start codon (Figure 2). Furthermore, the nucleotide sequence of N1 gene from all local isolates had more than 97% homology with N1 gene of Hong Kong and other Asian origins but less homology with that of European isolates (82-90%). The sequence of this H5N1 AIV was also found to have 99% homology with this virus in a leopard and a tiger isolates (GenBank accession AY646168 and AY646176). These results suggested that the H5N1 AIV which caused the outbreak in animals and human in Thailand may have the same source of origin. This type of outbreak was also found in other Asian countries (Claas *et al.*, 1998; Matrosovich *et al.*, 1999; Subbarao *et al.*, 1998; Lin *et al.*, 2005; Puthavathana *et al.*, 2005).

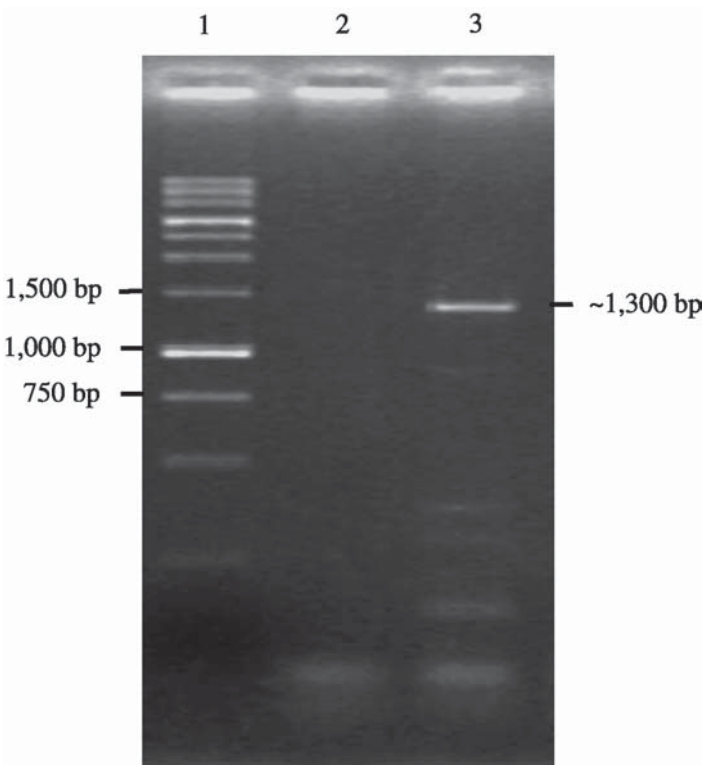


**Figure 1** Analysis of PCR products of N1 gene using 1.5% agarose gel electrophoresis. A 10 μl of PCR mixture was loaded onto each lane of agarose gel. Lane 1 = DNA marker, Lane 2 = negative control and Lane 3 = PCR products of whole N1 gene.

**Detection of recombinant protein**

The N1 cDNA was subcloned in baculovirus transfer vector (pFastBac™, Invitrogen®) for expression in insect cells. The crude protein was recovered from the insect cell lysate and identified in immuno-dot blot by the reaction with goat hyperimmune serum raised against H5N1 AIV or monoclonal antibody against histidine. A clear immunoreactive spot was observed in both assays confirming reactivity with the goat hyperimmune serum and monoclonal antibody to the crude protein (Figure 3). In addition, the crude protein extract was subjected to the SDS-PAGE and Western blot analysis in order to demonstrate the expression pattern of the recombinant neuraminidase. As shown in Figure 4, the band around 54 kDa (approximately the size of NA) was made visible by SDS-PAGE analysis with Coomassie brilliant blue staining. Similarly, Western blotting with antibodies to goat

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Chicken     MNPNKKIITIGSICMVTGMVSLMLQIGNLISIWVSHSIHTGNQHKAEPISNTNFLTEKAV 60
Duck        MNPNQKITTIGSICMVTGMVSLMLQIGNLISIWVSHSIHTGNQHKAEPISNTNFLTEKAV 60
Human       MNPNKKIITIGSICMVTGMVSLMLQIGNLISIWVSHSIHTGNQHKAEPISNTNFLTEKAV 60
            ****▲**▲**********************************************************

Chicken     ASVKLAGNSSLCPINGWAVYSKDNSIRIGSKGDVFVIREPFISCSHLECRTFFLTQGALL 120
Duck        ASVKLAGNSSLCPINGWAVYSKDNSIRIGSKGDVFVIREPFISCSHLECRTFFLTQGALL 120
Human       ASVKLAGNSSLCPINGWAVHSKDNSIRIGSKGDVFVIREPFISCSHLECRTFFLTQGALL 120
            ********************▲**************************************************

Chicken     NDKHSNGTVKDRSPHRTLMSCPVGEAPSPYNSRFESVAWSASACHDGTSWLTIGISGPDN 180
Duck        NDKHSNGTVKDRSPHRTLMSCPVGEAPSPYNSRFESVAWSASACHDGTSWLTIGISGPDN 180
Human       NDKHSNGTVKDRSPHRTLMSCPVGEAPSPYNSRFESVAWSASACHDGTSWLTIGISGPDN 180
            ************************************************************

Chicken     GAVAVLKYNGIITDTIKSWRNNILRTQESECACVNGSCFTVMTDGPSNGQASHKIFKMEK 240
Duck        GAVAVLKYNGIITDTIKSWRNNILRTQESECACVNGSCFTVMTDGPSNGQASHKIFKMEK 240
Human       GAVAVLKYNGIITDTIKSWRNNILRTQESECACVNGSCFTVMTDGPSNGQASHKIFKMEK 240
            ************************************************************

Chicken     GKVVKSVELDAPNYHYEECSCYPDAGEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSG 300
Duck        GKVVKSVELDAPNYHYEECSCYPDAGEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSG 300
Human       GKVVKSVELDAPNYHYEECSCYPDAGEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSG 300
            ************************************************************

Chicken     VFGDNPRPNDGTGSCGPVSSNGAYGVKGFSFKYGNGVWIGRTKSTNSRSGFEMIWDPNGW 360
Duck        VFGDNPRPNDGTGSCGPVSSNGAYGVKGFSFKYGNGVWIGRTKSTNSRSGFEMIWDPNGW 360
Human       VFGDNPRPNDGTGSCGPVSSNGAYGVKGFSFKYGNGVWIGRTKSTNSRSGFEMIWDPNGW 360
            ************************************************************

Chicken     TETDSSFSVKQDIVAITDWSGYSGSFVQHPELTGLDCIRPCFWVELIRGRPKESTIWTSG 420
Duck        TETDSSFSVKQDIVAITDWSGYSGSFVQHPELTGLDCIRPCFWVELIRGRPKESTIWTSG 420
Human       TETDSSFSVKQDIVAITDWSGYSGSFVQHPELTGLDCIRPCFWVELIRGRPKESTIWASG 420
            ************************************************************▲**

Chicken     SSISFCGVNSDTVGWSWPDGAELPFTIDK 449
Duck        SSISFCGVNSDTVGWSWPDGAELPFTIDK 449
Human       SSISFCGVNSDTVGWSWPDGAELPFTIDK 449
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**Figure 2** Amino acid sequence alignment of the expected neuraminidase of AIV isolated from a chicken case (upperline) compared to those of a naturally infected native duck and a human cases using Expasy and ClustalW program. ▲ = a different amino acid of neuraminidase of a chicken, a duck and a human isolates of AI virus.

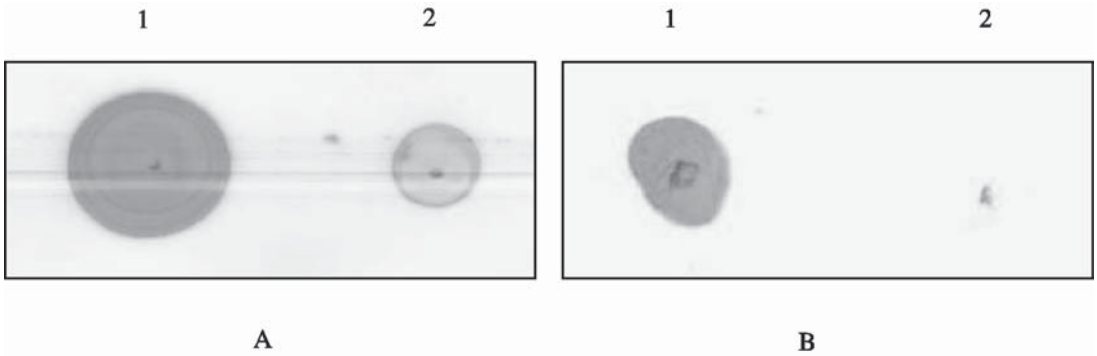


**Figure 3** Immuno-dot blot analysis of crude protein extracted from recombinant baculovirus infected cells (1) and wild type baculovirus infected High Five™ cells (2) using goat hyperimmune serum against H5N1 AIV (A) and monoclonal antibody against histidine (B).

hyperimmune serum raised against H5N1 AIV or monoclonal antibody against histidine revealed immunoreactive bands with apparent molecular masses at the same position (Figure 5). These results indicated that the insect cell lysate yielded a protein (presuming the recombinant neuraminidase) that possessed conformational epitopes of H5N1 AIV.

## CONCLUSION

In the present study, recombinant N1 gene was expressed using baculovirus in order to obtain the bioactive neuraminidase. So far, this has been the first report on N1 gene sequence of H5N1 AIV isolated from local ducks in Thailand and its recombinant protein was produced in a baculovirus expression system. This N1 gene was similar to those of chicken and human in size. The nucleotide sequences synthesized from this gene showed high similarity comparing to other AIV isolated from chicken and human which suggested that the outbreak H5N1 in Thailand may come from the same virus origin. However, the nucleotide sequences of N1 product isolated from duck had 4 amino acids changes at position 5, 8, 80 and 418 compared to that of the chicken and human, but more similar to Asian isolates than European origin. The insect cell-derived proteins were

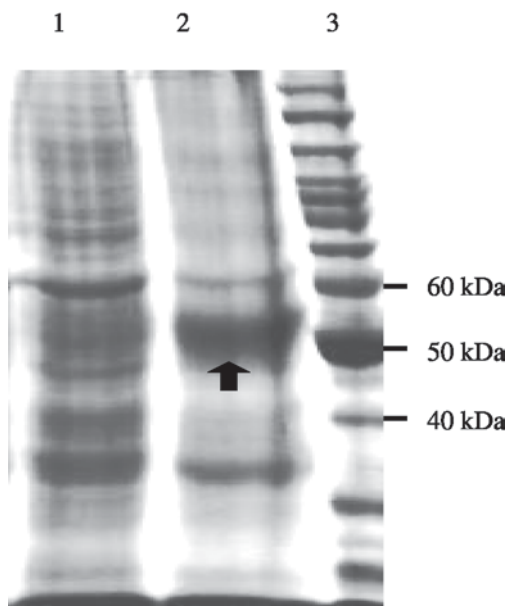


**Figure 4** 12% SDS-PAGE analysis of crude protein from wild type infected (Lane 1), recombinant baculovirus infected High Five™ cells (Lane 2) and protein standard (Lane 3). The arrow indicated the major band of 54 kDa protein extracted from the recombinant inoculated High Five™ cells.

reactive in Western blots with goat hyperimmune serum raised against H5N1 AIV as well as monoclonal antibody against histidine. The 54 kDa

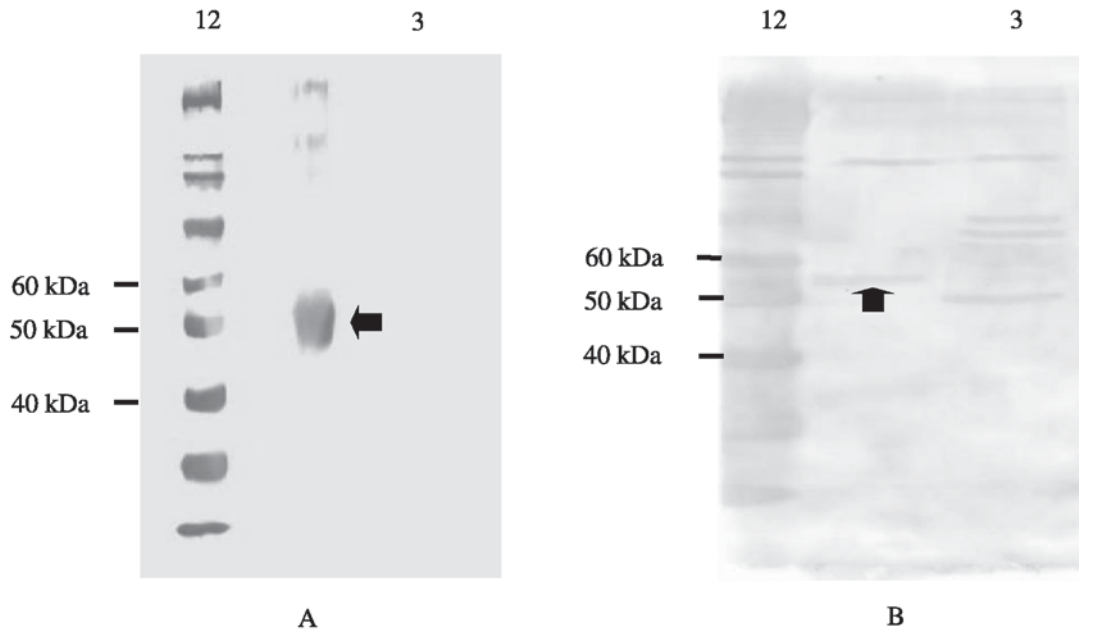


**Figure 5** Western blot analysis of crude protein from recombinant baculovirus infected (Lane 2) and wild type infected High Five™ cells (Lane 3) using monoclonal antibody against histidine (A) and goat hyperimmune serum against H5N1 AIV(B). Lane 1 was the protein standard marker. The arrow indicated 54 kDa protein band reacted with monoclonal antibody against histidine and goat hyperimmune serum.

band was detected and presumed to be the N1 protein. These observations provide evidence that the N1 protein containing conformational epitopes sufficient for further development of an antibody-based antigen capture kit as a tool for an early diagnosis of AIV infection.

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