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Cloning and Expression of HA Gene from a Highly Pathogenic Avian Influenza Isolate (H5N1) in Thailand

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ABSTRACT

The avian influenza A virus hemagglutinin (HA) protein is encoded by viral gene segment IV and mediates early steps of the viral replication cycle, receptor binding and membrane fusion. This research was aimed to clone and express HA gene of avian influenza virus (AIV) in insect cell cultures using a baculovirus expression vector system. The viral RNA was extracted from the allantoic fluid of 9 to 11 days old chicken embryonic eggs. The extracted viral RNA was used as template for HA gene synthesis using RT-PCR ~ 1,700 long. The amplicons were cloned into plasmid pFastBac HT and were transformed into *E. coli* strain DH10 Bac to produce the recombinant H5 baculovirus bacmids. The recombinant baculovirus DNA was further used to transfect and express in insect cell cultures. By SDS-PAGE, the recombinant H5 protein was found to be 65 kDa in size. This protein was analyzed by dot blot and Western blotting using goat anti-H5 AIV polyclonal antibody and mouse anti-histidine monoclonal antibody. The results indicated that the HA gene was successfully cloned and the H5 protein could be expressed in insect cell cultures using a baculovirus expression vector system. Therefore, H5 protein could be further developed and applied as a candidate H5 AIV subunit vaccine.

Key words: cloning, expression, HA gene, AIV, baculovirus vector

INTRODUCTION

Avian influenza viruses (AIV) type A belong to the family *Orthomyxoviridae*, and contain eight segments of negative sense RNA encoding 10 different proteins (Lamb and Krug, 1996). The HA gene is the AIV segment IV (Lamb, 1990) and encodes for the synthesis of surface glycoprotein, hemagglutinin (Webster *et al*., 1992), which induces host immune system, responded by producing a specific antibody to inhibit infection (Tamura *et al*., 1990; Johansson *et al*., 1993). This specific antibody is basically used for classifying influenza into 16 distinct antigenic subtypes (Ron *et al*., 2004). Moreover, HA protein is responsible for the attachment and the fusion between viral and cellular membranes as the initiator of infection (Carroll and Paulson,1985; Daniel *et al*., 1987).

The AIV subtypes 5 and 7 (H5 and H7) are unique in that they have undergone rapid phenotypic shifts to highly pathogenic variants both in the field and laboratory conditions

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(Kawaoka and Webster, 1988; Brugh and Perdue, 1991). The AI remains an economic threat to commercial poultry worldwide and its outbreak always results in significant economic losses either in quarantine or import restriction on the country or region of origin (Salem and Odor, 1995).

In recent years, the cloned HA gene has been expressed in eukaryotic cells through different vectors. The insect baculovirus vector with the efficient polyhedron promoter of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) has been successfully developed for HA protein of human influenza virus (Kuroda *et al*., 1986). The expressed HA protein has been found to undergo direct transport to the cell surface and performed many of the posttranslational modifications required for biological activities of many complex protein such as glycosylation, disulfide bond formation, and phosphorylation similar to authentic HA (Kuroda *et al*., 1986; Possee, 1986).

In this paper, the HA gene of H5N1 AIV was cloned and expressed using baculovirus expression vector system. The synthetic recombinant H5 protein was determined by dot blot, SDS-PAGE and Western blot.

MATERIALS AND METHODS

Virus isolation

The H5N1 avian influenza virus, A/Chicken/Nakornpathom/166/04, was isolated from naturally AIV infected chicken by using tracheal swab. These samples were preserved in viral transport media and then inoculated approximately 0.2 ml of $10^{8.7}$ EID_{50}/ml in 9 to 11 days old chicken embryonic eggs via the allantoic cavity. Thereafter, these eggs were incubated at 37°C for 24-72 h. The allantoic fluids of incubated embryonic chicken eggs were, then, harvested and kept at -80°C until used in RT-PCR.

RNA isolation and RT-PCR

Viral RNA was extracted from allantoic fluid using phenol-chloroform extraction method (Sambrook and Russell, 2001). Briefly, the exact amount of 100 µl allantoic fluids was mixed with 500 µl denatured solution and 50 µl of 2M sodium acetate and shaken for 5-10 min. The full length cDNA of HA gene was synthesized by using Uni 12 primer and AMV reverse transcriptase (FINNZYMES®) under an extension condition of 42°C for 50 min. Subsequently, the HA DNA was amplified by using a forward primer 5′-GCG CGG ATC CAC CAT GGA GAA AAT AGT GCT TCT TCT TGC-3′ containing *BamH*I cleavage site, and a reverse primer 5′-GCG CAA GCT TTT TAA ATG CAA ATT CTG CAT TGT AAC G-3′ containing *Hind*III cleavage site. The PCR mixture comprising of 1X PCR buffer, 3 mM dNTPs, 2.5 mM $MgCl_2$, 0.5 pmol of each forward and reverse primer, 2.0 U Taq DNA polymerase (Invitrogen®), and DNA template, was amplified in a Primus 96 ^{plus} (Hybaid) thermocycler. The 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. The PCR products were subjected to electrophorese through 1.5% agarose gel at100 volt with a constant current for 25 min and the DNA were visualized under UV illumination (Spectroline).

Construction of expression vector

The amplified HA gene was purified using QIA quick gel extraction kit (QIAGEN®). Thereafter, 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 by PCR and restriction endonuclease assay. These clones were scaled up and used for DNA sequencing. The sequencing result was analyzed using Expasy and ClustalW

programs in DNASIS solfware (Altschul *et al.,* 1997). The inserted transfer vector was used to transform *E. coli* strain DH10-Bac™ (Invitrogen®) competent cells. The transformants were inoculated onto a LB agar containing 50 µg /ml kanamycin, 7 µg/ml gentamicin, and 10 µg/ml tetracycline as well as 50 µg β-Gal and 40 mg IPTG. The white colonies were selected and tested for the presence of HA gene by PCR.

Insect cells transfection and expression of HA gene

Briefly, the Sf21 cell lines *(Spodoptera frugiperda*) were cultured in SF900II medium (Invitgrogen®) supplemented with 4% FBS and 10% penicillin at 27°C. The recombinant expression plasmid was used to transfect Sf21 cells by using Cellfectin® (Invitrogen®). Then, the recombinant baculovirus particles were collected from cell culture at 48 h post transfection (h.p.t.) 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 0.1 in Sf900 II SFM® medium (Invitrogen®), containing 4% fetal bovine serum and 4% penicillin as well as 4% streptomycin (GIBCO®). High Five™ cell lines *(Trichoplusiani*) grown in Express Five serum-free medium (Invitrogen®) supplemented with 9% L-glutamine and 10% penicillin were used to produce hemagglutinin. After 72 hour post inoculation (h.p.i.) and MOI of 2, the 1×10^6 cells/ml of infected High Five™ cell lines were lysed using 10% sodium dodecyl sulfate, and the crude extracted protein was determined for the presentation of HA by dot blot, SDS-PAGE, and Western blot.

Dot blotting

The crude extracted proteins (6 mg/ml) of either the recombinant H5-AcMNPV protein or the recombinant wild type-AcMNPV were dotted on nitrocellulose membrane and incubated with either the goat anti-H5 AIV polyclonal antibody (1:50; V:V) or the mouse anti-histidine IgG monoclonal antibody (1:2,000; V:V) for 2 h. Subsequently, the membrane was incubated with either rabbit anti-goat IgG (1:400; V:V) or mouse anti-goat IgG conjugated with peroxidase (1:250) 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 lysates from the recombinant HA-AcMNPV and wild type-AcMNPV were subjected to 10 % SDS-PAGE and stained with Coomassie Brilliant blue. The proteins on gel were then transferred to a nitrocellulose membrane at the constant current of 400 mAmp at 4°C, and incubated with either goat anti-H5 AIV polyclonal antibody (1:50; V:V) or mouse anti-histidine IgG monoclonal antibody (1:2,000; V:V) for 2 h, followed by either rabbit anti-goat IgG (1:400; V:V) or mouse anti-goat IgG conjugated with peroxidase (1:250) for 1 h. The membrane was finally incubated with diaminobenzidine solution (Sigma®) containing 1% H_2O_2 for 5-10 min.

RESULTS AND DISCUSSION

Cloning and sequencing of HA gene

The PCR product of HA gene showed an amplified band of approximately 1,700 bp (Figure 1). The amplified HA gene was sequenced and aligned (both nucleotide sequence and amino acid sequence) with the HA gene sequences of cat (A/cat/Thailand/KU02/04/H5N1, accession DQ236077), chicken (A/CK/Thailand/9.1/2004/H5N1, accession AY651328) and duck (A/duck/Saraburi/Thailand/CU-74/04/H5N1, accession DQ083581). We found that the cloned HA gene had the 98% similarity when compared with tiger (A/tiger/Thailand/CU-T6/04/H5N1, accession

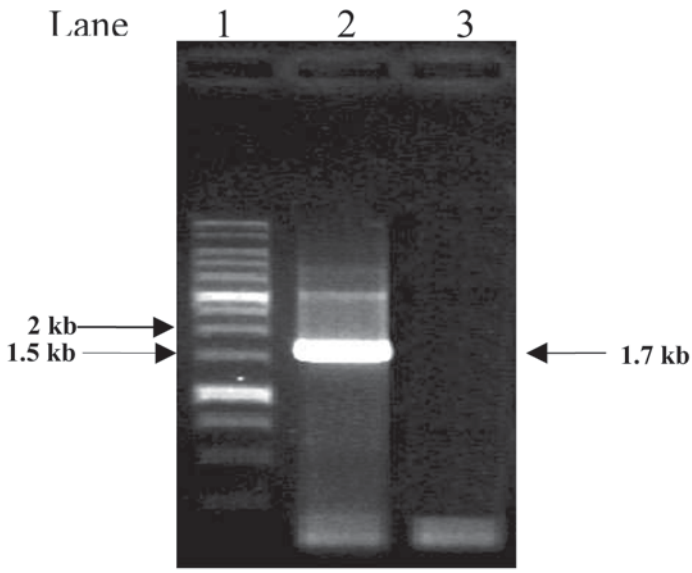

Figure 1 PCR products of HA gene on 1.5% agarose. Lane 1 = DNA marker, Lane 2 = whole HA gene, and Lane 3 = negative control.

AY972541)and human (A/Thailand/2(SP-33)/2004/H5N1, accession AY555153) in both nucleotide alignment (Figure 2) and amino acid alignment (Figure 3). Moreover, the aligned amino acid sequence of cleavage site of the cloned HA gene had 100% similarity when compared with cat (accession DQ236077), chicken (accession AY651328) and duck (accession DQ083581). These results suggested that the H5N1 avian influenza virus which caused the outbreak in animals including cat, chicken, duck, and human in Thailand were the same type of AIV. Besides, the HA gene of Hong Kong and other asian origins had 82-90% similarity with the HA gene of european isolates also are the same type of virus (Puthavathana *et al.,* 2005).

Detection of recombinant protein

Dot blot of the crude protein extracted from the recombinant AcMNPV and infected into High Five™ cells after 72 h.p.i. using the mouse anti-histidine IgG monoclonal antibody and the goat-anti H5N1 AIV polyclonal antibody showed positive results of the recombinant H5-AcMNPV protein (Figure 4). The SDS-PAGE gave a band of approximately 65 kDa which was approximately the size of HA protein reported by several research groups (Gregory *et al.,* 2002; Qiao *et al.,* 2003; Hulse *et al.,* 2004) (Figure 5). Western blot of the crude protein extracted from AcMNPV and infected to High Five™ cells using the mouse anti-histidine IgG monoclonal antibody (Figure 6) and the goat-anti H5 AIV polyclonal antibody (Figure 7) was also positive for the recombinant H5-AcMNPV protein. These results suggested that the recombinant HA protein was similarly glycosylated and folded as authentic HA protein because it could be recognized by goat-anti H5 AIV polyclonal antibody (Kuroda *et al*., 1986).

CONCLUSION

The synthesized whole HA gene products, approximately 1,700 bp showed 98% similarity, especially 100% similarity at cleavage site, comparing to other avian influenza viruses

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Cat        ATGGAGAAAATAGTGCTTCTTTTTGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGC   60
Chicken    ATGGAGAAAATAGTGCTTCTTTTTGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGC   60
Duck       ATGGAGAAAATAGTGCTTCTTTTTGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGC   60
Cloned     ATGGAGAAAATAGTGCTTCTTCTTGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGC   60
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Cat        ATTGGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAAAAGAACGTT  120
Chicken    ATTGGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAAAAGAACGTT  120
Duck       ATTGGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAAAAGAACGTT  120
Cloned     ATTGGTTACCATGCAAACAACTCGACAGAGCATGTTGACACAATAATGGAAAAGAACGTT  120
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Cat        ACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTA  180
Chicken    ACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTA  180
Duck       ACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTA  180
Cloned     ACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTA  180
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Cat        GATGGAGTGAAGCCTCTAATTTTGAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGAAAC  240
Chicken    GATGGAGTGAAGCCTCTAATTTTGAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGAAAC  240
Duck       GATGGAGTGAAGCCTCTAATTTTGAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGAAAC  240
Cloned     GATGGAGTGAAGCCTCTAATTTTGAGAAATTGTAGTGTAGCTGGATGGCTCCTCGGAAAC  240
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Cat        CCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAAT  300
Chicken    CCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAAT  300
Duck       CCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAAT  300
Cloned     CCTATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAAT  300
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Cat        CCAGTCAATGACCTCTGTTACCCAGGGGATTTCAATGACTATGAAGAATTGAAACACCTA  360
Chicken    CCAGTCAATGACCTCTGTTACCCAGGGGATTTCAATGACTATGAAGAATTGAAACACCTA  360
Duck       CCAGTCAATGACCTCTGTTACCCAGGGGATTTCAATGACTATGAAGAATTGAAACACCTA  360
Cloned     CCAGTCAATGACCTCTGTTACCCAGGGGATTTCAACGACTATGAAGAACTGAAACACCTA  360
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Cat        TTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCAGT  420
Chicken    TTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCAGT  420
Duck       TTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCAGT  420
Cloned     TTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTTCAGT  420
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Cat        CATGAAGCCTCATTAGGGGTGAGCTCAGCATGTCCATACCAGGGAAAGTCCTCCTTTTTC  480
Chicken    CATGAAGCCTCATTAGGGGTGAGCTCAGCATGTCCATACCAGGGAAAGTCCTCCTTTTTC  480
Duck       CATGAAGCCTCATTAGGGGTGAGCTCAGCATGTCCATACCAGGGAAAGTCCTCCTTTTTC  480
Cloned     CATGAAGCCTCATTGGGGGTGAGCTCAGCATGTCCATACCAGGGGAAGTCCTCCTTTTTC  480
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Cat        AGAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAAGAGGAGCTAC  540
Chicken    AGAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAAGAGGAGCTAC  540
Duck       AGAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAAGAGGAGCTAC  540
Cloned     AGAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAAGAGGAGCTAC  540
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Cat        AATAATACCAACCAAGAAGATCTTTTGGTACTGTGGGGGATTCACCATCCTAATGATGCG  600
Chicken    AATAATACCAACCAAGAAGATCTTTTGGTACTGTGGGGGATTCACCATCCTAATGATGCG  600
Duck       AATAATACCAACCAAGAAGATCTTTTGGTACTGTGGGGGATTCACCATCCTAATGATGCG  600
Cloned     AATAATACCAACCAAGAAGATCTTTTGGTACTGTGGGGGATTCACCATCCTAATGATGCG  600
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Cat        GCAGAGCAGACAAAGCTCTATCAAAACCCAACCACCTATATTTCCGTTGGGACATCAACA  660
Chicken    GCAGAGCAGACAAAGCTCTATCAAAACCCAACCACCTATATTTCCGTTGGGACATCAACA  660
Duck       GCAGAGCAGACAAAGCTCTATCAAAACCCAACCACCTATATTTCCGTTGGGACATCAACA  660
Cloned     GCAGAGCAGACAAAGCTCTATCAAAACCCAGCCACCTATATTTCCGTTGGGACATCAACA  660
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Cat        CTAAACCAGAGATTGGTACCAAGAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGA  720
Chicken    CTAAACCAGAGATTGGTACCAAGAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGA  720
Duck       CTAAACCAGAGATTGGTACCAAGAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGA  720
Cloned     CTGAACCAGAGATTGGTACCAGAAATAGCTACTAGACCTAAAGTAAACGGGCAAAGTGGA  720
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Cat        AGGATGGAGTTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAAT  780
Chicken    AGGATGGAGTTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAAT  780
Duck       AGGATGGAGTTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAAT  780
Cloned     AGGATGGAGTTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAAT  780
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Cat        GGAAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATT  840
Chicken    GGAAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATT  840
Duck       GGAAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATT  840
Cloned     GGGAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCGAGAATT  840
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Cat        ATGAAAAGTGAATTGGAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCG  900
Chicken    ATGAAAAGTGAATTGGAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCG  900
Duck       ATGAAAAGTGAATTGGAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCG  900
Cloned     ATGAAAAGTGAATTGGAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCG  900
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Cat        ATAAACTCTAGTATGCCATTCCACAATATACACCCTCTCACCATCGGGGAATGCCCCAAA  960
Chicken    ATAAACTCTAGTATGCCATTCCACAATATACACCCTCTCACCATCGGGGAATGCCCCAAA  960
Duck       ATAAACTCTAGTATGCCATTCCACAATATACACCCTCTCACCATCGGGGAATGCCCCAAA  960
Cloned     ATAAACTCTAGTATGCCATTCCACAATATACACCCTCTCACCATCGGGGAATGCCCCAAA  960
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Cat        TATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAG 1020
Chicken    TATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAG 1020
Duck       TATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAG 1020
Cloned     TATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAG 1020
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Cat        AGAAGAAGAAAAAAGAGAGGATTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGG 1080
Chicken    AGAAGAAGAAAAAAGAGAGGATTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGG 1080
Duck       AGAAGAAGAAAAAAGAGAGGATTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGG 1080
Cloned     AGAAGAAGAAAAAAGAGAGGATTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGG 1080
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Cat        CAGGGAATGGTAGATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTAC 1140
Chicken    CAGGGAATGGTAGATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTAC 1140
Duck       CAGGGAATGGTAGATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTAC 1140
Cloned     CAGGGAATGGTAGATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTAC 1140
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Cat        GCTGCAGACAAAGAATCCACTCAAAAGGCAATAGATGGAGTCACCAATAAGGTCAACTCG 1200
Chicken    GCTGCAGACAAAGAATCCACTCAAAAGGCAATAGATGGAGTCACCAATAAGGTCAACTCG 1200
Duck       GCTGCAGACAAAGAATCCACTCAAAAGGCAATAGATGGAGTCACCAATAAGGTCAACTCG 1200
Cloned     GCTGCAGACAAAGAATCCACTCAAAAGGCAATAGATGGAGTCACCCATAAGGTCAACTCG 1200
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Cat        ATCATTGACAAAATGAACACTCAGTTTGAGGCCGTTGGAAGGGAATTTAATAACTTAGAA 1260
Chicken    ATCATTGACAAAATGAACACTCAGTTTGAGGCCGTTGGAAGGGAATTTAACAACTTAGAA 1260
Duck       ATCATTGACAAAATGAACACTCAGTTTGAGGCCGTTGGAAGGGAATTTAACAACTTAGAA 1260
Cloned     ATCAGTGACAAAATGAACACTCAGTTTGAGGCCGTTGGAAGGGAATTTAACAACTTAGAA 1260
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Cat        AGGAGAATAGAGAATTTAAACAAGAAGATGGAAGACGGGTTCCTAGATGTCTGGACTTAT 1320
Chicken    AGGAGAATAGAGAATTTAAACAAGAAGATGGAAGACGGGTTCCTAGATGTCTGGACTTAT 1320
Duck       AGGAGAATAGAGAATTTAAACAAGAAGATGGAAGACGGGTTCCTAGATGTCTGGACTTAT 1320
Cloned     AGGAGAATAGAGAATTTAAACAAGAAGATGGAAGACGGGTTCCTAGATGTCTGGACTTAT 1320
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Cat        AATGCTGAACTTCTGGTTCTCATGGAAAATGAGAGAACTCTAGACTTTCATGACTCAAAT 1380
Chicken    AATGCTGAACTTCTGGTTCTCATGGAAAATGAGAGAACTCTAGACTTTCATGACTCAAAT 1380
Duck       AATGCTGAACTTCTGGTTCTCATGGAAAATGAGAGAACTCTAGACTTTCATGACTCAAAT 1380
Cloned     AATGCTGGACTTCTGGTTCTCATGGAGAATGAGAGAACTCTAGACTTTCATGACTCAAAT 1380
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Cat        GTCAAGAACCTTTACGACAAGGTCCGACTACAGCTTAGGGATAATGCAAAGGAGCTGGGT 1440
Chicken    GTCAAGAACCTTTACGACAAGGTCCGACTACAGCTTAGGGATAATGCAAAGGAGCTGGGT 1440
Duck       GTCAAGAACCTTTACGACAAGGTCCGACTACAGCTTAGGGATAATGCAAAGGAGCTGGGT 1440
Cloned     GTCAAGAACCTTACGTACAAGGTCCGACTACAGCTTAGGGATAATGCAAAGGAGCTGGGT 1440
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Cat        AACGGTTGTTTCGAGTTCTATCATAAATGTGATAATGAATGTATGGAAAGTGTAAGAAAC 1500
Chicken    AACGGTTGTTTCGAGTTCTATCATAAATGTGATAATGAATGTATGGAAAGTGTAAGAAAC 1500
Duck       AACGGTTGTTTCGAGTTCTATCATAAATGTGATGATGAATGTATGGAAAGTGTAAGAAAC 1500
Cloned     AACGGTTGTTTCGAGTTCTATCATAAATGTGATAATGAATGTATGGAAAGTGTAAGAAAC 1500
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Cat        GGAACGTATGACTACCCGCAGTATTCAGAAGAAGCAAGACTAAAAAGAGAGGAAATAAGT 1560
Chicken    GGAACGTATGACTACCCGCAGTATTCAGAAGAAGCAAGACTAAAAAGAGAGGAAATAAGT 1560
Duck       GGAACGTATGACTACCCGCAGTATTCAGAAGAAGCAAAACTAAAAAGAGAGGAAATAAGT 1560
Cloned     GGAACGTATGACTACCCGCAGTATTCAGAAGAAGCAAGACTAAATAGAGAGGAAATAAGT 1560
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Cat        GGAGTAAAATTGGAATCAATAGGAATTTACCAAATACTGTCAATTTATTCTACAGTGGCG 1620
Chicken    GGAGTAAAATTGGAATCAATAGGAATTTACCAAATACTGTCAATTTATTCTACGGTGGCG 1620
Duck       GGAGTAAAATTGGAATCAATAGGAATTTACCAAATACTGTCAATTTATTCTACAGTGGCG 1620
Cloned     GGAGCAAAATTGGAATCAATAGGAATTTACCAAATACTGTCAATTTATTCTACAGTGGCG 1620
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Cat        AGTTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCCTTATGGATGTGCTCCAATGGG 1680
Chicken    AGTTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCCTTATGGATGTGCTCCAATGGG 1680
Duck       AGTTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCCTTATGGATGTGCTCCAATGGA 1680
Cloned     AGTTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCCTTATGGATGTGCTCCAATGGA 1680
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Cat        TCGTTACAATGCAGAATTTGCATTTAA 1707
Chicken    TCGTTACAATGCAGAAT---------- 1697
Duck       TCGTTACAATGCAGAATTTG------- 1700
Cloned     TCGTTACAATGCAGAATTTGCATTTAA 1707
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Figure 2 Nucleotide alignments of cloned HA gene with Genbank database of cat (A/cat/Thailand/KU02/04/H5N1,accessionDQ236077),chicken(A/CK/Thailand/9.1/2004/H5N1, accession AY651328) and duck (A/duck/Saraburi/Thailand/CU-74/04/H5N1,accession DQ083581), by using ClastalW program.

isolated from duck, cat, tiger and human, suggesting that H5N1 in Thailand might be the same type of virus. The synthesized whole HA gene products constructed into pFastBac HT plasmids were transformed into *E. coli* strain DH10 Bac to produce the recombinant H5 baculovirus bacmids. These bacmids were transfected and expressed in insect cell cultures. The expressed H5 protein was primarily determined by dot blotting using goat anti-H5N1 AIV polyclonal antibody. Subsequently, the deduced protein showed to be approximately 65 kDa fraction by using 10% SDS-PAGE and Western blotting with goat anti-H5N1 AIV polyclonal antibody and the mouse anti-histidine monoclonal antibody.

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Chicken     MEKIVLLFAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDL  60
Duck        MEKIVLLFAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDL  60
Cat         MEKIVLLFAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDL  60
Cloned      MEKIVLLLAIVSLVKSDQICIGYHANNSTEHVDTIMEKNVTVTHAQDILEKTHNGKLCDL  60
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Chicken     DGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKANPVNDLCYPGDFNDYEELKHL 120
Duck        DGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKANPVNDLCYPGDFNDYEELKHL 120
Cat         DGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKANPVNDLCYPGDFNDYEELKHL 120
Cloned      DGVKPLILRNCSVAGWLLGNPMCDEFINVPEWSYIVEKANPVNDLCYPGDFNDYEELKHL 120
            *********:**************************************************

Chicken     LSRINHFEKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRNVVWLIKKNSTYPTIKRSY 180
Duck        LSRINHFEKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRNVVWLIKKNSTYPTIKRSY 180
Cat         LSRINHFEKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRNVVWLIKKNSTYPTIKRSY 180
Cloned      LSRINHFEKIQIIPKSSWFSHEASLGVSSACPYQGKSSFFRNVVWLIKKNSTYPTIKRSY 180
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Chicken     NNTNQEDLLVLWGIHHPNDAAEQTKLYQNPTTYISVGTSTLNQRLVPRIATRSKVNGQSG 240
Duck        NNTNQEDLLVLWGIHHPNDAAEQTKLYQNPTTYISVGTSTLNQRLVPRIATRSKVNGQSG 240
Cat         NNTNQEDLLVLWGIHHPNDAAEQTKLYQNPTTYISVGTSTLNQRLVPRIATRSKVNGQSG 240
Cloned      NNTNQEDLLVLWGIHHPNDAAEQTKLYQNPATYISVGTSTLNQRLVPEIATRPKVNGQSG 240
            *******************************:****************.****.*******

Chicken     RMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKKGDSTIMKSELEYGNCNTKCQTPMGA 300
Duck        RMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKKGDSTIMKSELEYGNCNTKCQTPMGA 300
Cat         RMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKKGDSTIMKSELEYGNCNTKCQTPMGA 300
Cloned      RMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKKGDSRIMKSELEYGNCNTKCQTPMGA 300
            ************************************ ********************

Chicken     INSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRERRRKKRGLFGAIAGFIEGGW 360
Duck        INSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRERRRKKRGLFGAIAGFIEGGW 360
Cat         INSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRERRRKKRGLFGAIAGFIEGGW 360
Cloned      INSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRERRRKKRGLFGAIAGFIEGGW 360
            ************************************************************

Chicken     QGMVDGWYGYHHSNEQGSGYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLE 420
Duck        QGMVDGWYGYHHSNEQGSGYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLE 420
Cat         QGMVDGWYGYHHSNEQGSGYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLE 420
Cloned      QGMVDGWYGYHHSNEQGSGYAADKESTQKAIDGVTHKVNSISDKMNTQFEAVGREFNNLE 420
            ***********************************:***** ******************

Chicken     RRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQLRDNAKELG 480
Duck        RRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQLRDNAKELG 480
Cat         RRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQLRDNAKELG 480
Cloned      RRIENLNKKMEDGFLDVWTYNAGLLVLMENERTLDFHDSNVKNLTYKVRLQLRDNAKELG 480
            ********************** ********************  **************

Chicken     NGCFEFYHKCDNECMESVRNGTYDYPQYSEEARLKREEISGVKLESIGIYQILSIYSTVA 540
Duck        NGCFEFYHKCDDECMESVRNGTYDYPQYSEEAKLKREEISGVKLESIGIYQILSIYSTVA 540
Cat         NGCFEFYHKCDNECMESVRNGTYDYPQYSEEARLKREEISGVKLESIGIYQILSIYSTVA 540
Cloned      NGCFEFYHKCDNECMESVRNGTYDYPQYSEEARLNREEISGAKLESIGIYQILSIYSTVA 540
            ***********:********************:*:******.******************

Chicken     SSLALAIMVAGLSLWMCSNGSLQCR--- 565
Duck        SSLALAIMVAGLSLWMCSNGSLQCRI-- 566
Cat         SSLALAIMVAGLSLWMCSNGSLQCRICI 568
Cloned      SSLALAIMVAGLSLWMCSNGSLQCRICI 568
```

Figure 3 Amino acid alignments of HA gene from Genbank database, cat (accession DQ236077),chicken (accession AY651328) and duck (accession DQ083581), and the cloned HA gene, showing 98% similarity, whereas the cleavage site of each HA gene (underlined sequence) giving100 % similarity.

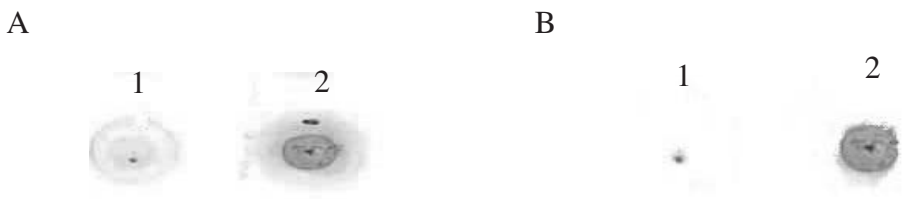

Figure 4 Dot blot of the expressed crude protein from the recombinant wild type- AcMNPV protein (1) and the recombinant H5-AcMNPV protein (2), reacted with goat-anti H5 AIV polyclonal antibody (A) and using mouse antihistidine IgG monoclonal antibody (B).

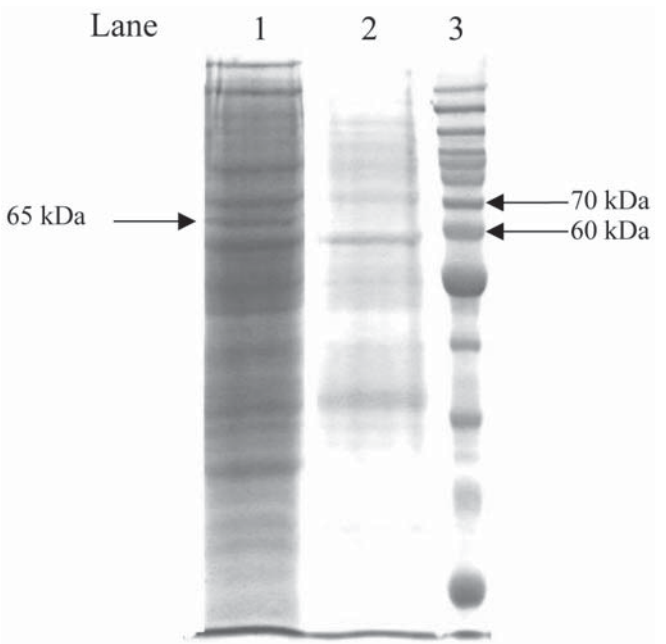

Figure 5 Crude recombinant protein on 10% SDS-PAGE showing 65 kDa of the recombinant H5-AcMNPV protein (Lane1), recombinant wild type-AcMNPV protein as the negative control (Lane 2), and lane 3 is the protein ladder (Lane 3).

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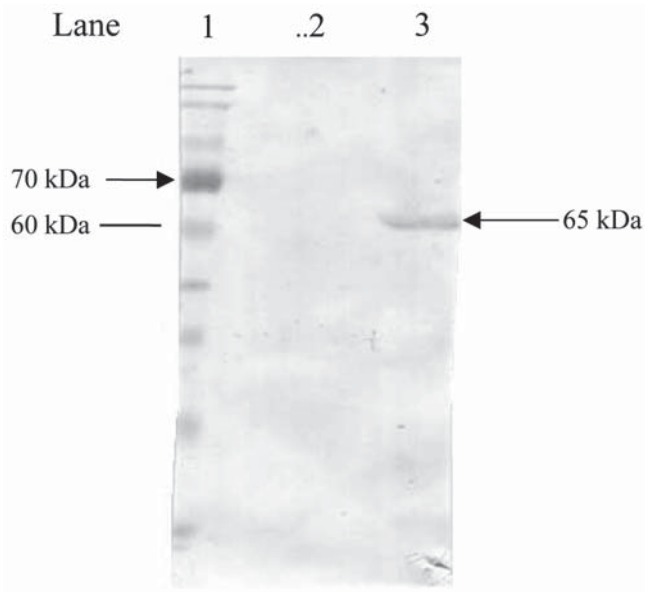

Figure 6 Western blot of the expressed recombinant crude proteins bind with mouse anti-histidine IgG monoclonal antibody: protein ladder marker (Lane 1), wild type-AcMNPV protein (Lane 2) as negative control, and 65 kDa band of the recombinant H5-AcMNPV protein (Lane 3).

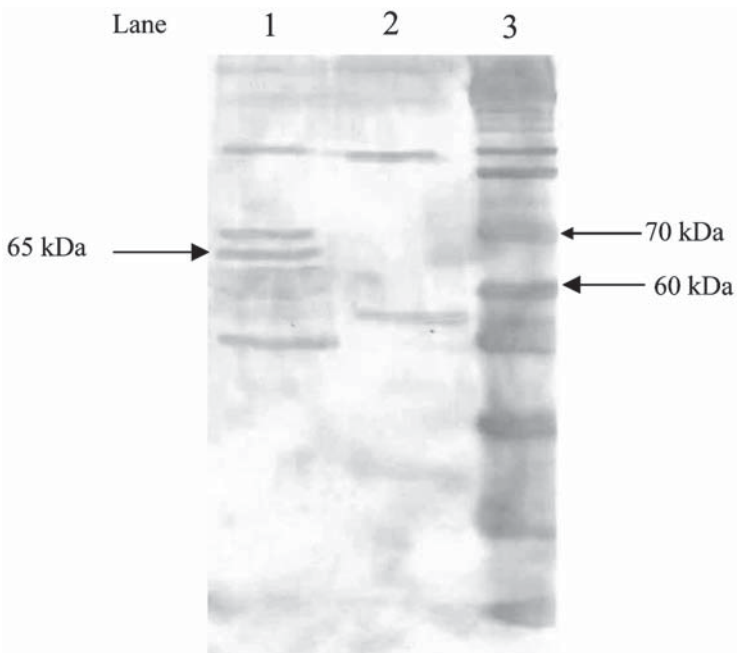

Figure 7 Western blot of the expressed recombinant crude proteins bind with goat anti-H5 AIV polyclonal antibody: 65 kDa band of the recombinant H5-AcMNPV protein (Lane 1) wild type-AcMNPV protein (Lane 2) as negative control, and protein ladder marker (Lane 3).

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