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

Kriangkrai Witoonsatian¹, Theera Rukkwamsuk², Thaweesak Songserm³ and Theerapol Sirinarumitr³*

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.
(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 -0°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 BamHI cleavage site, and a reverse primer 5'-GCG CAA GCT TTT TAA ATG CAA ATT CTG CAT TGT AAC G-3' containing HindIII cleavage site. The PCR mixture comprising of 1X PCR buffer, 3 mM dNTPs, 2.5 mM MgCl2, 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 electrophoresis 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 DH5α (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 software (Altschul et al., 1997). The inserted transfer vector was used to transform \textit{E. coli} strain DH10-Bac\textsuperscript{TM} (Invitrogen\textsuperscript{®}) 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.

\textbf{Insect cells transfection and expression of HA gene}

Briefly, the Sf21 cell lines (\textit{Spodoptera frugiperda}) were cultured in SF900II medium (Invitrogen\textsuperscript{®}) supplemented with 4% FBS and 10% penicillin at 27°C. The recombinant expression plasmid was used to transfect Sf21 cells by using Cellfectin\textsuperscript{®} (Invitrogen\textsuperscript{®}). 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 0.01 in SF900 II SFM\textsuperscript{®} medium (Invitrogen\textsuperscript{®}), containing 4% fetal bovine serum and 4% penicillin as well as 4% streptomycin (GIBCO\textsuperscript{®}). High Five\textsuperscript{TM} cell lines (\textit{Trichoplusiani}) grown in Express Five serum-free medium (Invitrogen\textsuperscript{®}) 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\textsuperscript{6} cells/ml of infected High Five\textsuperscript{TM} 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.

\textbf{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 dianimobenzidine solution (Sigma\textsuperscript{®}) containing 1% H\textsubscript{2}O\textsubscript{2} for 5-10 min.

\textbf{RESULTS AND DISCUSSION}

\textbf{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
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.
Cat        ATGGAGAAAATAGTGCTTCTTTTTGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGC   60
Chicken    ATGGAGAAAATAGTGCTTCTTTTTGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGC   60
Duck       ATGGAGAAAATAGTGCTTCTTTTGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGC   60
Cloned     ATGGAGAAAATAGTGCTTCTCTTGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGC   60

Cat        ATTGGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAAAAGAACGTT  120
Chicken    ATTGGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAAAAGAACGTT  120
Duck       ATTGGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAAAAGAACGTT  120
Cloned     ATTGGTTACCATGCAAACAACTCGACAGAGCATGTTGACACAATAATGGAAAAGAACGTT  120

Cat        ACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTA  180
Chicken    ACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTA  180
Duck       ACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTA  180
Cloned     ACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTA  180

Cat        GATGGAGTGAAGCCTCTAATTTTGAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGAAAC  240
Chicken    GATGGAGTGAAGCCTCTAATTTTGAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGAAAC  240
Duck       GATGGAGTGAAGCCTCTAATTTTGAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGAAAC  240
Cloned     GATGGAGTGAAGCCTCTAATTTTGAGAAATTGTAGTGTAGCTGGATGGCTCCTCGGAAAC  240

Cat        CCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAAT  300
Chicken    CCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAAT  300
Duck       CCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAAT  300
Cloned     CCTATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAAT  300

Cat        CCAGTCAATGACCTCTGTTACCCAGGGGATTTCAATGACTATGAAGAATTGAAACACCTA  360
Chicken    CCAGTCAATGACCTCTGTTACCCAGGGGATTTCAATGACTATGAAGAATTGAAACACCTA  360
Duck       CCAGTCAATGACCTCTGTTACCCAGGGGATTTCAATGACTATGAAGAATTGAAACACCTA  360
Cloned     CCAGTCAATGACCTCTGTTACCCAGGGGATTTCAACGACTATGAAGAATTGAAACACCTA  360

Cat        TTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCAGT  420
Chicken    TTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCAGT  420
Duck       TTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCAGT  420
Cloned     TTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTTCAGT  420

Cat        CATGAAGCCTCATTAGGGGTGAGCTCAGCATGTCCATACCAGGGAAAGTCCTCCTTTTTC  480
Chicken    CATGAAGCCTCATTAGGGGTGAGCTCAGCATGTCCATACCAGGGAAAGTCCTCCTTTTTC  480
Duck       CATGAAGCCTCATTAGGGGTGAGCTCAGCATGTCCATACCAGGGAAAGTCCTCCTTTTTC  480
Cloned     CATGAAGCCTCATTGGGGGTGAGCTCAGCATGTCCATACCAGGGAAAGTCCTCCTTTTTC  480

Cat        AATAATACCAACCAAGAAGATCTTTTTGGTACTGTGGGGGATTCACCATCCTAATGATGCG  540
Chicken    AATAATACCAACCAAGAAGATCTTTTTGGTACTGTGGGGGATTCACCATCCTAATGATGCG  540
Duck       AATAATACCAACCAAGAAGATCTTTTTGGTACTGTGGGGGATTCACCATCCTAATGATGCG  540
Cloned     AATAATACCAACCAAGAAGATCTTTTTGGTACTGTGGGGGATTCACCATCCTAATGATGCG  540

Cat        GCAGAGCAGACAAAGCTCTATCATCATAACCAACCAACCAACAATAAAAAGGAGAGCTAC  600
Chicken    GCAGAGCAGACAAAGCTCTATCATCATAACCAACCAACCAACAATAAAAAGGAGAGCTAC  600
Duck       GCAGAGCAGACAAAGCTCTATCATCATAACCAACCAACCAACAATAAAAAGGAGAGCTAC  600
Cloned     GCAGAGCAGACAAAGCTCTATCATCATAACCAACCAACCAACAATAAAAAGGAGAGCTAC  600
<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat</td>
<td>CTAACCGAGAGATTGGTACCAAAGAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGA</td>
<td>720</td>
</tr>
<tr>
<td>Chicken</td>
<td>CTAACCGAGAGATTGGTACCAAAGAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGA</td>
<td>720</td>
</tr>
<tr>
<td>Duck</td>
<td>CTAACCGAGAGATTGGTACCAAAGAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGA</td>
<td>720</td>
</tr>
<tr>
<td>Cloned</td>
<td>CTGAACCGAGAGATTGGTACCAGAATAGCTACTAGCTAAAGTAAACGGGCAAAGTGGA</td>
<td>720</td>
</tr>
<tr>
<td>Cat</td>
<td>AGGATGGAGTTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAAT</td>
<td>780</td>
</tr>
<tr>
<td>Chicken</td>
<td>AGGATGGAGTTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAAT</td>
<td>780</td>
</tr>
<tr>
<td>Duck</td>
<td>AGGATGGAGTTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAAT</td>
<td>780</td>
</tr>
<tr>
<td>Cloned</td>
<td>AGGATGGAGTTCTTCTGGACAATTTTAAAACCGAATGATGCAATCAACTTCGAGAGTAAT</td>
<td>780</td>
</tr>
<tr>
<td>Cat</td>
<td>GGAAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATT</td>
<td>840</td>
</tr>
<tr>
<td>Chicken</td>
<td>GGAAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATT</td>
<td>840</td>
</tr>
<tr>
<td>Duck</td>
<td>GGAAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATT</td>
<td>840</td>
</tr>
<tr>
<td>Cloned</td>
<td>GGGAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAACAATT</td>
<td>840</td>
</tr>
<tr>
<td>Cat</td>
<td>ATGAAAAGTGAATTGGAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCG</td>
<td>900</td>
</tr>
<tr>
<td>Chicken</td>
<td>ATGAAAAGTGAATTGGAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCG</td>
<td>900</td>
</tr>
<tr>
<td>Duck</td>
<td>ATGAAAAGTGAATTGGAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCG</td>
<td>900</td>
</tr>
<tr>
<td>Cloned</td>
<td>ATGAAAAGTGAATTGGAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCG</td>
<td>900</td>
</tr>
<tr>
<td>Cat</td>
<td>ATAAACTCTAGTATGCCATTCCACAATATACACCCTCTCACCATCGGGGAATGCCCCAAA</td>
<td>960</td>
</tr>
<tr>
<td>Chicken</td>
<td>ATAAACTCTAGTATGCCATTCCACAATATACACCCTCTCACCATCGGGGAATGCCCCAAA</td>
<td>960</td>
</tr>
<tr>
<td>Duck</td>
<td>ATAAACTCTAGTATGCCATTCCACAATATACACCCTCTCACCATCGGGGAATGCCCCAAA</td>
<td>960</td>
</tr>
<tr>
<td>Cloned</td>
<td>ATAAACTCTAGTATGCCATTCCACAATATACACCCTCTCACCATCGGGGAATGCCCCAAA</td>
<td>960</td>
</tr>
<tr>
<td>Cat</td>
<td>TATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAG</td>
<td>1020</td>
</tr>
<tr>
<td>Chicken</td>
<td>TATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAG</td>
<td>1020</td>
</tr>
<tr>
<td>Duck</td>
<td>TATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAG</td>
<td>1020</td>
</tr>
<tr>
<td>Cloned</td>
<td>TATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAAGAGAG</td>
<td>1020</td>
</tr>
<tr>
<td>Cat</td>
<td>CAGGGAATGGTAGATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTAC</td>
<td>1140</td>
</tr>
<tr>
<td>Chicken</td>
<td>CAGGGAATGGTAGATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTAC</td>
<td>1140</td>
</tr>
<tr>
<td>Duck</td>
<td>CAGGGAATGGTAGATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTAC</td>
<td>1140</td>
</tr>
<tr>
<td>Cloned</td>
<td>CAGGGAATGGTAGATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTAC</td>
<td>1140</td>
</tr>
<tr>
<td>Cat</td>
<td>GCTGCAGACAAAGAATCCACTCAAAACGGGCAATAGATGGGACTCAGAACATAGCTCAAAAGAGAG</td>
<td>1200</td>
</tr>
<tr>
<td>Chicken</td>
<td>GCTGCAGACAAAGAATCCACTCAAAACGGGCAATAGATGGGACTCAGAACATAGCTCAAAAGAGAG</td>
<td>1200</td>
</tr>
<tr>
<td>Duck</td>
<td>GCTGCAGACAAAGAATCCACTCAAAACGGGCAATAGATGGGACTCAGAACATAGCTCAAAAGAGAG</td>
<td>1200</td>
</tr>
<tr>
<td>Cloned</td>
<td>GCTGCAGACAAAGAATCCACTCAAAACGGGCAATAGATGGGACTCAGAACATAGCTCAAAAGAGAG</td>
<td>1200</td>
</tr>
<tr>
<td>Cat</td>
<td>ATCATTGCCAAAATGGAACATCTCAATTGATGGTTGGTGGTAC</td>
<td>1260</td>
</tr>
<tr>
<td>Chicken</td>
<td>ATCATTGCCAAAATGGAACATCTCAATTGATGGTTGGTGGTAC</td>
<td>1260</td>
</tr>
<tr>
<td>Duck</td>
<td>ATCATTGCCAAAATGGAACATCTCAATTGATGGTTGGTGGTAC</td>
<td>1260</td>
</tr>
<tr>
<td>Cloned</td>
<td>ATCATTGCCAAAATGGAACATCTCAATTGATGGTTGGTGGTAC</td>
<td>1260</td>
</tr>
<tr>
<td>Cat</td>
<td>AGGAGAATAGGAATTTAAAAATGGAACATCTCAATTGATGGTTGGTGGTAC</td>
<td>1320</td>
</tr>
<tr>
<td>Chicken</td>
<td>AGGAGAATAGGAATTTAAAAATGGAACATCTCAATTGATGGTTGGTGGTAC</td>
<td>1320</td>
</tr>
<tr>
<td>Duck</td>
<td>AGGAGAATAGGAATTTAAAAATGGAACATCTCAATTGATGGTTGGTGGTAC</td>
<td>1320</td>
</tr>
<tr>
<td>Cloned</td>
<td>AGGAGAATAGGAATTTAAAAATGGAACATCTCAATTGATGGTTGGTGGTAC</td>
<td>1320</td>
</tr>
</tbody>
</table>
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.
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) giving 100% 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).

ACKNOWLEDGEMENT

The authors gratefully acknowledge Kasetsart University Research and Development Institute (KURDI) and the Center for Agricultural Biotechnology (CAB), Kasetsart University, Thailand, for funding this research project. We are also very thankful to the Veterinary Diagnostic Laboratory, Faculty of Veterinary Medicine, Kasetsart University, for providing the avian influenza virus isolate and facilities used in this project.
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).
LITERATURE CITED


Delaware and Maryland.

