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Original Article

Biological Property of Recombinant Hemagglutinin-Neuraminidase Protein of Avian Paramyxovirus Type 6 Expressed by Recombinant Baculovirus

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Hemagglutination inhibition (HI) test employing whole virus antigen is a prescribed serological test for serotyping, diagnosis and surveillance for avian paramyxoviruses (APMVs). For use as alternative to the virus antigen, hemagglutinin-neuraminidase (HN) protein gene of the wild duck isolate APMV-6/WB12-163FS of APMV serotype 6 (APMV-6) was amplified, cloned and expressed in *Spodoptera frugiperda* insect cells. The HN gene of 1,842 bps in length showed nucleotide and amino acid homology of 93.4% and 97.1%, respectively with that of APMV-6 prototype strain. Putative sialic acid binding motif and potential N-linked glycosylation sites were conserved. In Western blot analysis, the expressed protein had a molecular mass of 66 kDa and reacted specifically with antiserum to APMV-6. In addition, the recombinant HN protein showed biological properties such as hemagglutination (HA) and elution. The recombinant HN protein produced from infected cells showed high HA titers (approximately 2^{13} HA unit/ml). The HA activity of the recombinant HN protein was inhibited by antisera to APMV-6. In cross HA inhibition test, the recombinant HN protein had the highest titers with antisera to homologous APMV serotype, although there was weak cross reaction with some of antisera to other APMV serotypes. Our results indicated that recombinant APMV-6 HN protein would have the potential as alternative to the APMV-6 antigen in HI assays.

Key Words: Avian paramyxovirus-6, Hemagglutinin-neuraminidase protein, Baculovirus expression system, Hemagglutination inhibition test

INTRODUCTION

Avian paramyxoviruses (APMVs) of bird have been isolated from almost all of bird species, including domestic and wild birds (1). The APMVs are classified into the genus Avulavirus in the family *Paramyxoviridae*. Until now, at least 12 serotypes of APMVs have been recognized in a variety of bird species worldwide (2~4). Among them, APMV-1, known as Newcastle disease virus (NDV) is the most important APMV in poultry. Some of APMV serotypes including APMV-2, -3, -6 and -7 also can be associated with

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clinical symptom such as mild respiratory and reproductive disorders in poultry under field or experimental conditions (1, 5~7). Of them, APMV-6 from wild bird might cause a mild respiratory disease and problems in egg production in turkeys (1). In specific pathogen free (SPF) chicken, APMV-6 showed the evidence of viral infection in the trachea, lungs, gut, and pancreas under experimental condition although APMV-6 caused no clinical symptoms (5). The APMV-6 have RNA genomes of 16,230 to 16,236 nts in length, encoding eight proteins, nucleocapsid protein (NP), phosphoprotein (P), V protein, matrix protein (M), fusion protein (F), small hydrophobic (SH) protein, haemagglutinin-neuraminidase (HN) protein and large (L) protein (8, 9). Among the viral proteins of APMV, HN glycoprotein on the surface of the virion mediates attachment to cellular receptors and release from infected cells via its neuraminidase (NA) activity (10). The HN has also activity of hemagglutination (HA) with chicken red blood cells, allowing to HA and HA inhibition (HI) tests.

The HI test is commonly used as a prescribed method for serotyping of unidentified APMV isolates as well as diagnostic purpose and serological surveillance for APMVs in poultry and wild birds (7). The antigen for HI test for APMVs employs whole virus which is grown in embryonated chicken eggs (ECEs) and then inactivated by chemicals. Currently, recombinant HN proteins produced in insect cells by recombinant baculovirus have been successfully applied as alternative to whole virus antigen for HI test in several paramyxoviruses (11~13). APMV-6 is one of APMV serotypes which frequently isolated from migratory birds in Korea. In this study, we expressed recombinant HN protein from APMV-6 using baculovirus expression systems. The expressed HN protein was examined for biological and antigenic properties for assessment of the potential as alternative to diagnostic antigen for HI test.

MATERIALS AND METHODS

Virus and sera

APMV-6 isolate APMV-6/WB12-163FS (abbreviated 163FS) was used in this study. The 163FS virus was isolated from wild duck (species unidentified) during national avian influenza surveillance in 2005 in Korea and confirmed as APMV-6 by cross HI test. The 163FS virus was propagated in SPF ECEs as previously described (1) and stored at -70℃ until use.

Reference APMV antigens and chicken antiserum (to APMV-1,-2,-3,-4,-6,-7,-8, and -9) were purchased from National Veterinary Service Laboratories (NVSL, Ames, IA, USA). Serum from APMV antibody negative SPF chicken was used as negative control.

Cloning of HN protein gene

Viral genomic RNAs were extracted from APMV-6 isolate 163FS using the Viral Gene-spin kit (iNtRON Biotech, Korea) according to the manufacturer's instructions. Reverse transcription of the genomic RNA and polymerase chain reaction (RT-PCR) of the complementary DNA were performed for amplification of full length HN gene of the APMV-6 using the SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) and the Emerald-Amp PCR Master mix kit (TaKaRa Bio, Shiga, Japan), respectively according to the manufacturer's instructions. RT-PCR primer set with incorporated restriction sites *EcoR*I and *Hind*III (underlined) for directional cloning was used to amplify genomic region (1,976 bps) ranging between position 7104 and position 9079 flanking the entire HN open reading frame (ORF): forward; 5'-*GAATTC*ACTCCCCA-GCTGGTGGCAAT-3' and reverse; 5'-*AAGCTT*GCGGG-CTATATGTCACCATCTA-3', respectively. The amplified PCR product was cloned using pCR®-2.1-TOPO® plasmid (Invitrogen) and digested by restriction enzymes *EcoR*I and *Hind*III. Resulting *EcoR*I-*Hind*III digested fragment was inserted into the pFastBac™1 plasmid to generate recombinant plasmid pFB/APMV6HN.

Sequencing analysis of cloned HN gene

PCR assay was performed for sequencing of the HN gene contained in the pFB/APMV6HN by use of four primers; pFastBac vectors polyhedrin leader forward primer (Invitrogen), SV40 polyadenylation reverse primer (Invitrogen) and two APMV-6 HN specific forward primers 7729F

(5'-GGTTGTCACGATGCAGGAGA-3') and 8509F (5'-ACCTGGACAAAGGGCGTATG-3'). Resulting PCR products were subjected to direct sequencing (Macrogen, Korea) with an ABI 3730XL Analyzer (Applied Biosystems, Foster City, CA, USA). Nucleotide sequence editing, amino acid predictions and sequence alignments of the HN ORF were conducted using the MegAlign program of Lasergene 7.0 (DNASTAR Inc, Madison, WI, USA). The sequence obtained was compared with that of APMV-6/duck/Hong Kong/199/77 (Genbank accession no. EU622637).

Generation of recombinant baculovirus

Recombinant baculovirus expressing APMV-6 HN protein was constructed according to the manual of Bac-to-Bac baculovirus expression kit (Invitrogen). Briefly, the pFB/APMV6HN was transformed into competent DH10BAC *E. coli* cells (Invitrogen) which contains the Bacmid DNA. Finally, recombinant Bacmid was transfected into *Spodoptera frugiperda* 9 (Sf9) cells and plaque-purified to generate recombinant baculovirus expressing the HN protein of APMV-6 (rBac/APMV6HN). The expression of the APMV-6 HN protein by the rBac/APMV6HN was confirmed by HI test and Western immunoblot analysis using anti APMV-6 chicken serum.

Expression of APMV-6 HN protein

The recombinant HN protein of APMV-6 (rAPMV-6 HN) was prepared from Sf9 cells infected with rBac/APMV6HN virus as described previously (13). Briefly, Sf9 cells grown in a 175-cm^2 tissue culture flask were infected with rBac/APMV6HN at a multiplicity of infection of 0.1 for 2 hr at 27℃. Following 5 day-incubation at 27℃, infected cells collected were precipitated by centrifugation at 1,500 rpm for 10 min at 4℃. The cells were re-suspended in 1/20 volume of 0.01 M PBS containing a protease inhibitor cocktail (Roche Molecular Biochemicals, Germany) and then lysed by brief sonication. After centrifugation at 11,650× *g* for 20 min, cell supernatants containing APMV6-HN protein were aliquot and kept at -70℃, prior to its use in the study.

SDS-PAGE and Western blot analysis

SDS-PAGE and Western blot analysis was performed as previously described (13). Briefly, recombinant protein sample was separated through NuPAGE® Novex Bis-Tris Gels. The gels were then either stained with Coomassie blue or transferred onto nitrocellulose membranes using the Xcell II™ Blot Module (Invitrogen) for Western blot analysis according to the manufacturer's instructions. The nitrocellulose membranes were then incubated with 1:50 dilution of APMV-6 reference chicken antiserum (NVSL). Antigen-antibody complexes were visualized on each membrane by applying anti-chicken IgG (H+L) conjugated with alkaline phosphatase (1:1,000 dilution), followed by BCIP/NBT substrate solution (Kirkegaard-Perry Laboratories Inc., Gaithersburg, MD, USA).

Biological activity

Biological activity of the rAPMV-6 HN including HA-elution, neuraminidase (NA) activity and HA thermo-stability were conducted. Wild type APMV-6 isolate 163FS was used as control virus. A standard HA-elution assay was performed in V-bottomed 96 well microtiter plates by standard end-point titration method using 1% (v/v) chicken red blood cells (RBC) as previously described (14, 15). For thermo-stability, the test sample was heat treated at 56℃ for 30 min and then HA titer of the heat-treated sample was compared with that of untreated sample. NA activity assay was examined by a standard NA assay using fetuin substrate (16) and the absorbance was measured at a wavelength of 550 nm.

Immunization of chickens

Immunization antigens including rAPMV-6 HN and wild type APMV-6 (163FS isolate) at the concentration of 2^{13} HA unit/ml were emulsified with Montanide ISA70 (SEPPIC, Paris, France) at a ratio of 30:70 (v/v). Six-week-old SPF chickens (Namduk, Korea) were immunized with APMV-6 HN or 163FS isolate via intramuscular route (0.5 ml per dose). Blood samples were taken from immunized chickens 3 weeks after immunization. The sera collected from

immunized birds were inactivated at 56℃ for 30 min before use. The birds in the study were raised in air-filtered bio-security isolation units (ThreeShine, Korea) with feed and water *ad libitum* during the experiment. This experiment was carried out in accordance with the Korean Guidelines for Animal Welfare.

Hemagglunination inhibition (HI) test

The HI test was performed on sera in V-bottomed micro-titer plates by use of four HA units of antigen and 1% (v/v) chicken RBC, according to the OIE Manual of Standard Diagnostic Tests (17). All tests were repeated in duplicate. The HI titers were expressed as the reciprocal of the highest dilution of serum that completely inhibited HA activity.

Statistical analysis

Mean absorbance and standard deviations obtained by NA activity test were calculated using Microsoft Excel program (version 2010). Differences in values of NA activity between test sample groups tested were analyzed by Student's *t* test using SPSS for Windows version 12.0. A p value of < 0.05 was considered significant.

RERSULTS

Cloning of the entire HN protein gene of APMV-6

As expected, a DNA fragment flanking the entire HN ORF (1,842 bps) of the APMV-6 (isolate 163FS) was successfully amplified by RT-PCR. Amplified DNA product was cloned into pCR®-2.1-TOPO® plasmid. DNA fragment of approximately 1.8 kb in length was digested from the cloned plasmid (pCR/APMV6HN) by two restriction enzymes *Eco*RI and *Hind*III. The *Eco*RI- *Hind*III DNA fragment was finally inserted into the pFastBac™1 vector to generate recombinant plasmid pFB/APMV6HN (Fig. 1).

Sequence analysis of the APMV-6 HN gene

The HN gene contained in the pFB/APMV6HN was sequenced. The HN gene from 163FS isolate had a HN ORF of 1,842 nts long, which is predicted to encode 614 amino acids same as the APMV-6/duck/Hong Kong/199/77 (EU622637). The nucleotide and amino acid homology between the 163Fs and APMV-6/duck/Hong Kong/199/77 were 93.4% and 97.1%, respectively. Also, putative sialic acid binding motif (^{240}NRKSCS^{245}) and potential *N*-linked glycosylation sites (N125, N251, N284, N352, N383, N396, N448 and N495) were conserved when compared to APMV-6 /duck/Hong Kong/199/77 (Fig. 2).

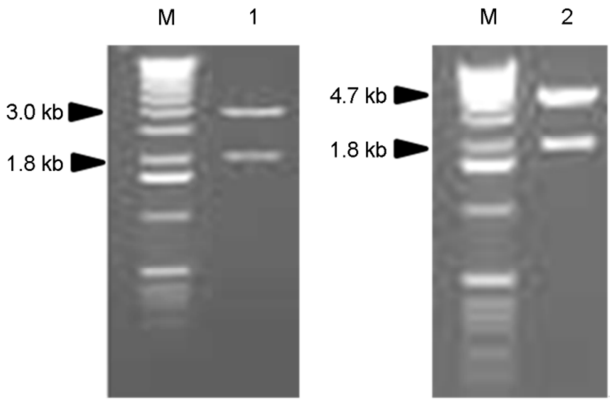

Figure 1. Agarose gel electrophoresis of recombinant plasmids digested with restriction enzymes of *Eco*RI and *Hind*III. M, DNA marker; Lane 1, pCR/APMV6HN; lane 2, pFB/APMV6HN.

Expression of recombinant HN protein of APMV-6

A recombinant baculovirus (rBac/APMV6HN) expressing the APMV-6 HN was generated using Bac-to-Bac® baculo-virus expression systems. The rBac/APMV6HN virus showed distinct cytopathic effects in infected Sf9 cells starting from 3 days post inoculation (dpi). Recombinant protein (rAPMV-6 HN) from the infected cells on 5 dpi showed HA activity for chicken RBCs and the HA activity was inhibited by reference APMV-6 chicken antiserum. When titrated by HA test, the culture supernatant and cell lysates from the infected cells contained amount of rAPMV-6 HN corresponding to HA titers of 128 and 163,840, respectively. However, cell lysates from mock infected Sf9 cells showed no HA activity. In SDS-PAGE and Western blotting, the rAPMV-6 HN preparation contained a distinct protein band of molecular mass of 66 kDa, corresponding to HN protein of wild type APMV-6 antigen. APMV-6 antiserum bound to the protein

Figure 2. Predicted amino acid sequence of HN protein gene of APMV-6/WB12-163FS and comparison with APMV-6 prototype strain (APMV-6/duck/Hong Kong/199/77). Black dot (●) represents putative N-linked glycosylation site. Putative sialic acid binding motif was expressed in a box.

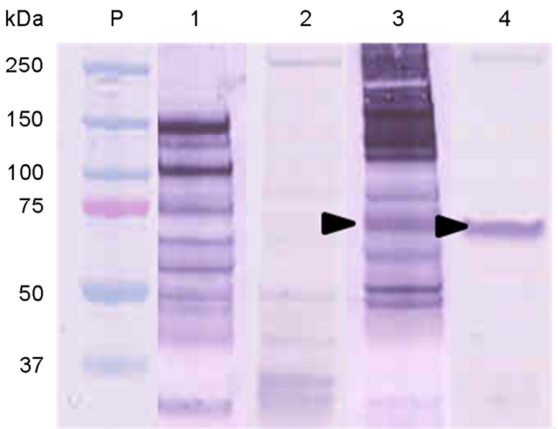

Figure 3. Western blot analysis of recombinant APMV-6 HN protein using APMV-6 reference antiserum. P, protein marker; lane 1, SPF ECE fluid; lane 2, normal Sf9 cell lysate; lane 3, APMV-6/WB12-163Fs; lane 4, rAPMV-6 HN. Arrow represents HN protein of APMV-6.

band on the nitrocellulose membrane (Fig. 3). However, the protein band was not observed in mock infected cell lysates.

Biological activity of the rAPMV-6 HN protein

For the rAPMV-6 HN protein, biological activity such as HA-elution, NA activity and HA thermostabilty was examined and compared to those of wild type APMV-6 (isolate 163FS). The rAPMV-6 HN used in the study had HA titers of 256 before heat treatment. However, the HA activity was almost lost after 30 min incubation at 56℃. Meanwhile wild type APMV-6 maintained still its HA activity after heat treatment (Table 1). Both rAPMV-6 HN and wild type APMV-6 maintained HA activity after additional 24 h incubation at 4℃ following HA test (Table 1). The rAPMV-6 HN showed NA activity and the NA activity was comparable to wild type APMV-6 (Fig. 4). Two controls (fluid samples from SPF ECEs and mock-infected Sf9 cells) did not have any NA activity.

Antigenic specificity of the rAPMV-6 HN protein

Whether the HA activity of rAPMV-6 HN shows antigenic specificity for APMV-6 antiserum was investigated. Cross

Table 1. Heat stability and hemagglutination elution pattern of the rAPMV-6 HN protein

	HA titer			
	Heat treatment[a]		Incubation at 4℃	
	Before	After	1 h	24h
APMV-6[b]	256	128	256	256
rAPMV6 HN	256	4	256	256

[a] Heat treatment represents treatment of test sample for 30 min at 56℃.
[b] APMV-6 isolate (APMV-6/WB12-163FS) used as genetic template for rAPMV-6 HN.

HI tests employing reference panel of APMV antigens and antisera was performed (Table 2). Two APMV-6 (163Fs, reference APMV-6) antigens showed the highest HI titers (64 to 1024) with antisera to homologous APMV serotype and rAPMV-6 HN antigen. However, weak cross reactivity between APMV-6 antigen and other APMV serotypes was observed. Similar HI pattern was observed in rAPMV-6 HN antigen. The rAPMV-6 HN antigen also showed the highest HI titers of ≥128 for antisera to homologous APMV serotype and rAPMV-6 HN antigen. However, some of cross reaction against other APMV serotypes were not found in the rAPMV-6 HN antigen. Our results indicate that the rAPMV-6 HN antigen might have the specificity of HA activity corresponding to wildtype APMV-6 antigen.

DISCUSSION

With APMV/avian influenza surveillance program world-wide, newly emerging or unidentified APMV serotypes have reported continuously in nature elsewhere (2, 3). Currently at least twelve APMV serotypes have been recognized in a variety of domestic and wild bird species worldwide (4). For APMV, the HN protein on the virus is important target for diagnostic assay such as HA and HI tests target since the HN protein has multiple biological property such HA and NA activity (18).

For APMV surveillance, HI test is the sole and prescribed method for serological test and serotyping of APMV isolate.

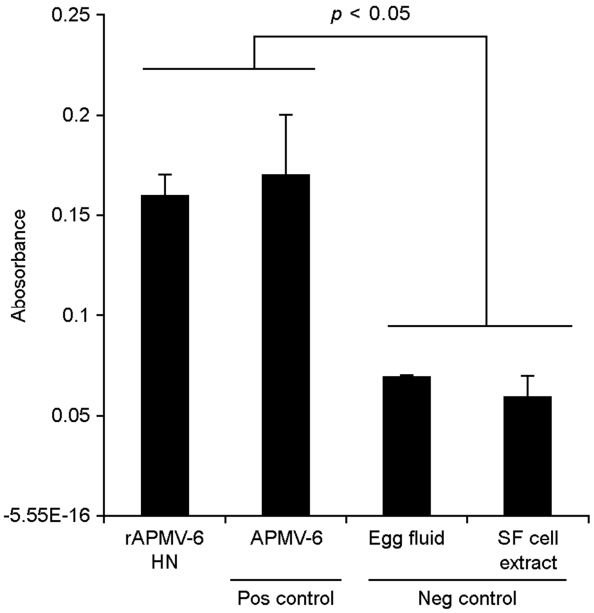

Figure 4. Measurement of NA activity of the rAPMV-6 HN protein expressed in insect cells.

Cross HI tests for serotype determination of APMV isolate are required since there are some cross reaction between some APMV serotypes. Currently HI test relies still on the use of APMV grown in ECEs. For the recent, recombinant HN proteins of several APMV serotypes such as APMV-1 and APMV-2 have been successfully produced using a baculovirus expression system and showed APMV serotype-specific HA activity as alternative to APMV antigen in HI test (13, 16). Importantly, some isolates of several APMVs were reported to be a potential to cause mild clinical symptom in poultry (1, 6, 7, 19). APMV-6 isolates are also considered to be capable of causing disease in poultry since there are several reports of causing mild clinical signs such as mild respiratory signs and reduced egg production in turkey (1, 20). APMV-6 is one of APMV serotypes which were frequently isolated from wild migratory birds in Korea and other several countries especially wild ducks and geese worldwide (9, 21, 22). Here we attempted to express the HN protein gene of APMV-6 in insect cells using a baculovirus expression system for the use of alternative to APMV-6 antigen used in HI test. In the study, we used genomic

Table 2. Results of cross HI test using recombinant APMV-6 HN protein and representative APMV serotypes

Antiserum	Antigen									
	rAPMV-6HN	APMV-1	APMV-2	APMV-3	APMV-4	APMV-6	163FS[c]	APMV-7	APMV-8	APMV-9
rAPMV-6 HN	**256**[a]	**2**	8	<2	2	**256**	**256**	2	4	<2
APMV-1	<2	128	NT[b]	NT	NT	<2	<2	NT	NT	NT
APMV-2	<2	NT	512	NT	NT	8	32	NT	NT	NT
APMV-3	<2	NT	NT	512	NT	16	<2	NT	NT	NT
APMV-4	<2	NT	NT	NT	32	<2	<2	NT	NT	NT
APMV-6	**128**	<2	16	<2	<2	**512**	**64**	4	2	<2
163FS	**512**	8	32	16	2	**1024**	**256**	8	16	2
APMV-7	16	NT	NT	NT	NT	<2	16	256	NT	NT
APMV-8	<2	NT	NT	NT	NT	2	4	NT	256	NT
APMV-9	<2	NT	NT	NT	NT	8	8	NT	NT	256
Negative serum	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2

[a] HI titers
[b] NT, not tested
[c] APMV-6 isolate (APMV-6/WB12-163FS) used as genetic template for rAPMV-6 HN.

RNAs from APMV-6 isolate 163FS as genetic template for expression of the recombinant HN protein (rAPMV-6 HN). The HN protein of APMV-6 isolate 163FS showed amino nucleotide and acid similarity of 93.4% and 97.1% with prototype strain of APMV-6 (APMV-6/duck/Hong Kong/199/77), suggesting genetic diversity among APMV-6 isolates in nature. Nevertheless biologically important sites on the HN protein such as putative sialic acid binding motif and potential N-linked glycosylation sites were conserved on both viruses.

The recombinant protein expressed by the rBac/APMV6HN in the study reacted strongly with APMV-6 reference antiserum in Western blotting and the molecular weight corresponds to that of APMV-6 HN protein. This indicates that the rAPMV-6 HN protein resembles antigenic structure of APMV-6 HN protein. In the study, slight antigenic variation between two APMV-6s was observed. This suggests the antigenic variation among APMV-6 isolates, as in several APMV serotypes (1, 21). In addition, the rAPMV-6 HN showed both HA and NA activity, indicating that the recombinant protein might be biologically active as in wild type APMV-6. Importantly rBac/APMV6HN produced high yield of the rAPMV-6 HN protein (approximately 164,000 HA units per 175 cm^2 culture flask). The amount of the expressed HN protein corresponds to that of APMV-6 antigen produced using several tens of expensive ECEs. This indicates that recombinant HN protein could be produced using *in vitro* cell culture system in economic way. Here we should note that HA activity of the rAPMV-6 HN is APMV-serotype specific, indicating the usefulness of alternative to APMV antigen in HI test. More importantly the HN protein antigen showed less cross reaction with other APMV serotypes than two APMV-6 antigens. Unfortunately we could not evaluate the sensitivity and specificity of the rAPMV-6 HN for the efficacy as diagnostic reagent because of the lack of field samples available. Nevertheless our results indicate that the rAPMV-6 HN in the study would have the potential as alternative to APMV-6 antigen for serological and diagnostic tests.

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