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Impact of the Embryonated Chicken Egg Isolation Process Used For *Avian influenza* Viruses on Subsequent Characterization of Viral Hemagglutinin Gene

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Abstract

Virus isolation is critical for the detailed characterization of *Avian influenza* Viruses (AIVs). In general, AIVs are isolated by inoculating embryonated chicken eggs with the specimens suspected of containing the virus. The characteristics of the AIVs in the original specimens, however, may not be maintained during the virus isolation process. To elucidate the impact of the embryonated chicken egg virus isolation process on the characterization of AIVs, we determined the nucleotide sequences of the open reading frames of the Hemagglutinin (HA) gene in the original specimens from which AIVs of the H5N6 subtype has been isolated and compared these with the sequences from the corresponding embryonated chicken egg isolates. We found that there were differences in nucleotide sequences at a total 24 positions in seven pairs of the viruses (i.e. the original specimen and the corresponding isolate), including nine non-synonymous substitutions in four pairs. In particular, six of the nine non-synonymous substitutions were located in the globular head domain of HA, including four substitutions close to the receptor binding domain. These results suggest that the characteristics of AIVs, such as HA antigenicity and receptor binding specificity, may differ between viruses in the original specimens and in the corresponding isolates. Our findings revealed that virus isolation in embryonated chicken eggs has a critical impact on the characterization of AIVs.

Keywords: *Avian influenza* virus; Embryonated chicken egg; VIrus isolation; Mutation; Hemagglutinin gene

Abbreviations

AIV: *Avian Influenza* Virus; HA: Hemagglutinin; HPAIV: Highly Pathogenic *Avian Influenza* Virus; RT-PCR: Reverse Transcription Polymerase Chain Reaction; ORF: Open Reading Frame; RBD: Receptor Binding Domain

Introduction

Avian Influenza Virus (AIV) is an inclusive term used for influenza A viruses isolated from avian species. AIVs are classified into different subtypes based on the antigenicity of Hemagglutinin (HA) and Neuraminidase (NA), which are viral glycoproteins expressed on the virion surface and are the primary targets of protective immunity in their hosts. Although a total of 18 HA (H1-H18) and 11 NA (N1-N11) subtypes are recognized, viruses of only 16 HA (H1-H16) and 9 NA (N1-N9) subtypes have been isolated as AIVs to date [1-3]. Wild waterfowl, especially wild ducks, from which AIVs of all 16 HA and 9 NA subtypes have been isolated, are believed to be a natural reservoir of AIVs. In contrast, poultry are accidentally infected with AIVs, and do not serve as a natural reservoir. In particular, AIVs of either the H5 or H7 HA subtypes have the potential to cause systemic infections in chickens [4], and thus such Highly Pathogenic AIVs (HPAIVs) pose a threat to poultry and to wild birds that share the nesting and/or overwintering sites with the natural reservoirs. In addition, H5 and H7 AIVs have recently been shown to cause lethal zoonotic infections in humans [5,6]. AIV surveillance in wild waterfowl, therefore, is of the utmost important for the poultry industry, bird species conservation, and public health.

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The Izumi plain in Kagoshima prefecture is one of the largest overwintering sites for wild waterfowls, including endangered cranes, in Japan. In addition, millions of chickens are farmed around the overwintering site. To minimize the potential risks of HPAIV infections in the endangered cranes, chickens, and humans on this plain, we have conducted AIV surveillance and characterized the AIV isolates over the past several years [7-11].

Virus isolation is critical for a detailed characterization of AIVs. In general, AIVs are isolated by inoculating suspected specimens in embryonated chicken eggs. The rationale for this is that AIVs are derived from bird species, and thus fundamental characteristics, such as the receptor binding specificities of AIVs in the specimens, are believed to be maintained during the virus isolation process. In fact, most of the characteristics of AIVs reported to date are based on the isolates from embryonated chicken eggs. Despite this, there is a possibility that AIVs in the original specimens, and those isolated from embryonated chicken eggs, exhibit different characteristics due to either the introduction of mutation(s) in the viral genome during virus replication in the eggs or the selective propagation of viruses that represent only minority of the virus populations present in the original specimens. In fact, a previous study demonstrated that AIVs isolated from chickens and turkeys, but not the AIVs in original specimens, undergo amino acid mutations in various viral gene segments after multiple passages in embryonated chicken eggs [12].

To elucidate the impact of the embryonated chicken egg virus isolation process on the characterization of AIVs, we attempted to determine the nucleotide sequences of AIV genes from the original specimens collected from the Izumi plain and to compare these sequences with those from the corresponding isolates obtained using embryonated chicken eggs. To this end, we focused on the HA gene which is known to be readily-mutated [13,14], partly because the HA protein encoded by this gene is exposed to selective pressure due to its ability to bind to both cellular receptors and neutralizing antibodies via its globular head domain [15,16].

Materials and Methods

Samples

We isolated a total of 33 highly pathogenic avian influenza viruses of the H5N6 subtype from debilitated or dead cranes, dead ducks, duck feces, and environmental water samples collected from the Izumi plain, Japan during the 2016/2017 winter season [9,17]. Among the original specimens subjected to virus isolation, 13 samples collected in November 2016 (Table 1) were used for the genetic analysis in this study.

Amplification of HA genes

HA genes present in the original AIV specimens were amplified by means of Reverse Transcription Polymerase Chain Reaction (RT-PCR). Briefly, RNA was extracted from the original specimens using the High Pure Viral Nucleic Acid Kit (Roche, Basel, Switzerland) and reverse transcribed using the Super Script IV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA) with primers specific for the nucleotide sequence at the 5′ end of viral gene segments [18]. The full-length HA gene Open Reading Frames (ORFs) were amplified by PCR using TksGflex DNA Polymerase (Takara Bio, Shiga, Japan), with the synthesized cDNA as templates. According to the manufacturer's instructions, the mutation frequency of TksGflex DNA Polymerase was estimated 0.0131% (42/320,204 nucleotides) (https://www.takarabio.com/). The PCR conditions, including primer sequences, are available upon request.

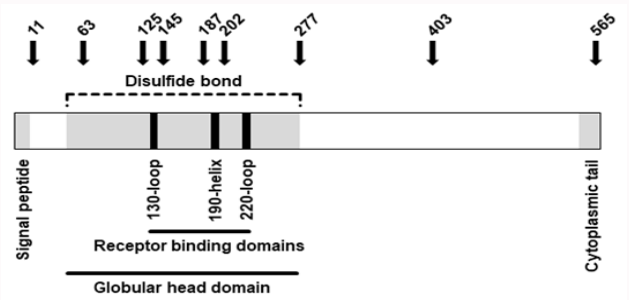

Figure 1: Amino acid positions of the nine non-synonymous substitutions. A schematic diagram of HA with representative protein domains (gray and black bars) is shown. The numbers with black arrows represent the amino acid positions of the nine non-synonymous substitutions between the original specimens and the corresponding isolates.

Molecular cloning and sequence analysis of HA genes

The PCR products for the amplified HA gene were ligated with a plasmid vector pCR-Blunt II-TOPO (Thermo Fisher Scientific) and transformed into competent *E.coli* DH5α cells. The bacterial colonies were screened for plasmids harboring the full-length HA gene ORFs by colony PCR using Go-Taq G2 Hot Start Polymerase (Promega, Madison, WI). The colony PCR conditions, including primer sequences, are available upon request. Four to six PCR-positive colonies from each of the original specimens were randomly selected for plasmid extraction using Wizard Plus SV Minipreps DNA Purification System (Promega). The nucleotide sequences of the full-length HA gene ORFs cloned in the plasmid vectors were determined using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and an Applied Biosystems 3500xL Genetic Analyzer (Thermo Fisher Scientific).

Results

Nucleotide sequences of the HA gene ORFs from the original specimens

To determine the nucleotide sequences of the HA gene ORFs of H5N6 HPAIVs from the original specimens, we attempted to amplify the full-length HA genes from a total of 13 original specimens by RT-PCR. As a result, we obtained amplified DNAs of the correct size from only nine specimens; five crane specimens (specimens No. 1-5) and four duck specimens (specimens No. 7-10) (Table 1). To determine the nucleotide sequences of not only the major, but also minor virus populations, the nine RT-PCR products were ligated to plasmid vectors for molecular cloning. The full-length HA gene ORFs cloned into four to six randomly-selected colonies obtained from each of the original specimens were then subjected to sequence analysis. The nucleotide sequences of the HA gene ORFs from the original specimens were then compared with those from the corresponding isolates (Tables 2-8). Note that these isolates were passaged in embryonated chicken eggs only once and the nucleotide sequences of their HA gene ORFs were determined by direct sequencing of the RT-PCR products.

Differences in nucleotide sequences in the full-length HA gene ORFs

We found that at least one molecular clone each from seven [three crane and four duck specimens (Tables 2-8)] of the nine original specimens tested encoded full-length HA gene ORFs whose

Table 1: Original specimens used in this study.

Specimen No.	Host	Specimen source	Collection date	Isolate
1	Dead crane	Tracheal swab	November 18, 2016	A/crane/Kagoshima/KU-4/2016 (H5N6)
2	Dead crane	Tracheal swab	November 19, 2016	A/crane/Kagoshima/KU-6/2016 (H5N6)
3	Sick crane	Conjunctival swab	November 20, 2016	A/crane/Kagoshima/KU-8/2016 (H5N6)
4	Sick crane	Tracheal swab	November 20, 2016	A/crane/Kagoshima/KU-9/2016 (H5N6)
5	Dead crane	Tracheal swab	November 21, 2016	A/crane/Kagoshima/KU-10/2016 (H5N6)
6	Dead duck	Mixture of conjunctival, tracheal, and cloacal swabs	November 23, 2016	A/eurasian wigeon/Kagoshima/KU-20/2016 (H5N6)
7	Dead duck	Mixture of conjunctival, tracheal, and cloacal swabs	November 23, 2016	A/eurasian wigeon/Kagoshima/KU-21/2016 (H5N6)
8	Dead duck	Mixture of conjunctival, tracheal, and cloacal swabs	November 22, 2016	A/northern pintail/Kagoshima/KU-23/2016 (H5N6)
9	Dead duck	Cloacal swab	November 24, 2016	A/eurasian wigeon/Kagoshima/KU-32/2016 (H5N6)
10	Duck	Feces	November 20, 2016	A/duck/Kagoshima/KU-d35/2016 (H5N6)
11	Duck	Feces	November 20, 2016	A/duck/Kagoshima/KU-d42/2016 (H5N6)
12	Duck	Feces	November 20, 2016	A/duck/Kagoshima/KU-d66/2016 (H5N6)
13	Duck	Feces	November 20, 2016	A/duck/Kagoshima/KU-d79/2016 (H5N6)

Table 2: Differences in nucleotide and amino acid sequences in HA gene ORFs in the original specimen and corresponding isolate for specimen No. 2.

Source of nucleotide sequence		Amino acid (nucleotide) at position[b]:		
		81 (264)	145 (466)	277 (865)
A/crane/Kagoshima/KU-6/2016 (H5N6)[a]		R (A)	P (C)	C (T)
Molecular clone from the original specimen No. 2	Clone 1	-[c](G)	- (-)	R (C)
	Clone 2	- (-)	S (T)	- (-)
	Clone 3	- (-)	- (-)	- (-)
	Clone 4	- (-)	- (-)	- (-)
	Clone 5	- (-)	- (-)	- (-)

[a]Isolate from original specimen No. 2.
[b]Amino acid positions are shown by H3 numbering.
[c]Identical amino acid or nucleotide to those of the corresponding isolate.

Table 3: Differences in nucleotide and amino acid sequences in HA gene ORFs in the original specimen and corresponding isolate for specimen No. 4.

Source of nucleotide sequence		Amino acid (nucleotide) at position[b]:
		137 (434)
A/crane/Kagoshima/KU-9/2016 (H5N6)[a]		P (G)
Molecular clone from the original specimen No. 4	Clone 1	-[c](A)
	Clone 2	- (-)
	Clone 3	- (-)
	Clone 4	- (-)

[a]Isolate from original specimen No. 4.
[b]Amino acid positions are shown by H3 numbering.
[c]Identical nucleotide or amino acid to those of the corresponding isolate.

nucleotide sequences were different from those of the corresponding isolates. This indicates that the virus isolation process in embryonated chicken eggs does indeed affect the genetic characteristic of AIVs.

In contrast, the nucleotide sequences of the full-length HA gene ORFs in all the molecular clones from the remaining two original specimens were identical to those of the corresponding isolates. This suggests that the differences in nucleotide sequences found in the seven specimens described above were not introduced during the genetic analysis, such as a PCR error, but rather occurred during the virus isolation process.

Differences in HA amino acid sequences

We found that nucleotides at a total of 24 positions in the full-length HA gene ORFs from 20 molecular clones derived from the seven specimens were different from those of the corresponding isolates (Tables 2-8). Among these 24 different nucleotides, 15 nucleotides were synonymous substitutions, suggesting that their impact on the characteristics of the virus is limited. In contrast, the remaining nine nucleotides were non-synonymous substitutions (Tables 2,5,6 and 8). These nine non-synonymous substitutions were found in the four original specimens; one crane specimen (specimen No. 2) and three duck specimens (specimens No. 7, 8 and 10).

Table 4: Differences in nucleotide and amino acid sequences in HA gene ORFs in the original specimen and corresponding isolate for specimen No. 5.

Source of nucleotide sequence		Amino acid (nucleotide) at position[b]:
		215 (678)
A/crane/Kagoshima/KU-10/2016 (H5N6)[a]		P (A)
Molecular clone from the original specimen No. 5	Clone 1	-[c](C)
	Clone 2	- (-)
	Clone 3	- (-)
	Clone 4	- (-)
	Clone 5	- (-)

[a]Isolate from original specimen No. 5.
[b]Amino acid positions are shown by H3 numbering.
[c]Identical nucleotide or amino acid to those of the corresponding isolate.

Two non-synonymous substitutions were found in the full-length HA gene ORFs from specimen No. 2 (Table 2). One of the five molecular clones (Clone 2) from this specimen encoded a serine at position 145 in HA (H3 numbering), while the corresponding isolate, A/crane/Kagoshima/KU-6/2016 (H5N6), possessed a proline at this position. The amino acid at position 145 in HA is located close to the so-called "130-loop", which is one of three secondary structure elements in the Receptor Binding Domain (RBD) [19], and forms a conformational epitope [20] together with the amino acids at positions 140, 171, and 172, suggesting that HA antigenicity and/or receptor specificity may differ between the viruses in the original specimen and the corresponding isolate. Another molecular clone (Clone 1) from the same specimen encoded an arginine at position 277 in HA, while the corresponding isolate possessed a cysteine at this position. The cysteine at position 277 in HA is one of the components of the globular head domain, and is linked with cysteine at position 52 by a disulfide bond [21]. Since these cysteines are highly conserved

Table 5: Differences in nucleotide and amino acid sequences in HA gene ORFs in the original specimen and corresponding isolate for specimen No. 7.

Source of nucleotide sequence		Amino acid (nucleotide) at position[b]:									
		11 (49)	63 (208)	125 (400)	150 (483)	161 (516)	187 (592)	299 (933)	302 (942)	445 (1380)	494 (1527)
A/eurasian wigeon/Kagoshima/KU-21/2016 (H5N6)[a]		D (G)	D (G)	P (C)	N (C)	Y (C)	N (A)	P (C)	I (T)	K (A)	E (A)
Molecular clone from the original specimen No. 7	Clone 1	-[c](-)	N (A)	S (T)	- (T)	- (T)	- (-)	- (T)	- (C)	- (G)	- (G)
	Clone 2	- (-)	N (A)	- (-)	- (T)	- (T)	D (G)	- (T)	- (C)	- (G)	- (G)
	Clone 3	- (-)	N (A)	- (-)	- (T)	- (T)	D (G)	- (T)	- (C)	- (G)	- (G)
	Clone 4	- (-)	N (A)	- (-)	- (T)	- (T)	D (G)	- (T)	- (C)	- (G)	- (G)
	Clone 5	N (A)	N (A)	- (-)	- (T)	- (T)	- (-)	- (T)	- (C)	- (G)	- (G)

[a]Isolate from original specimen No. 7.
[b]Amino acid positions are shown by H3 numbering.
[c]Identical nucleotide or amino acid to those of the corresponding isolate.

Table 6: Differences in nucleotide and amino acid sequences in HA gene ORFs in the original specimen and corresponding isolate for specimen No. 8.

Source of nucleotide sequence		Amino acid (nucleotide) at position[b]:					
		55 (186)	79 (258)	146 (471)	178 (564)	202 (637)	403 (1252)
A/northern pintail/Kagoshima/KU-23/2016 (H5N6)[a]		G (A)	F (C)	S (C)	L (G)	V (G)	E (G)
Molecular clone from the original specimen No. 8	Clone 1	-[c](-)	- (-)	- (-)	- (-)	- (-)	K (A)
	Clone 2	- (-)	- (-)	- (-)	- (-)	- (-)	K (A)
	Clone 3	- (-)	- (T)	- (T)	- (A)	- (-)	- (-)
	Clone 4	- (-)	- (-)	- (-)	- (-)	I (A)	- (-)
	Clone 5	- (G)	- (-)	- (-)	- (-)	- (-)	- (-)

[a]Isolate from original specimen No. 8.
[b]Amino acid positions are shown by H3 numbering.
[c]Identical nucleotide or amino acid to those of the corresponding isolate.

among the isolates, the cysteine-to-arginine substitution at position 277 should have a significant impact on the HA structure. This mutant HA gene, therefore, might be detected as one of the genes from replication-incompetent quasi-species in the original specimen.

Four non-synonymous substitutions were found in the full-length HA gene ORFs from specimen No. 7 (Table 5). One of the five molecular clones (Clone 5) from this specimen encoded an asparagine at position 11 in HA, while the corresponding isolate, A/eurasian wigeon/Kagoshima/KU-21/2016(H5N6), possessed an aspartic acid at this position. The amino acid at position 11 in HA is located immediately downstream of the signal peptide required for protein maturation. Moreover, the aspartic acid at this position is known to be a main cleavage target for H5 HA maturation [22]. To assess the impact of this non-synonymous substitution on H5 HA maturation, we predicted the signal peptide cleavage sites in the HA amino acid sequences containing either asparagine or aspartic acid at position 11 by using the online software SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The results suggested that there was no difference in the HA cleavage site between the viruses in the original specimen and in the corresponding isolate. All five of the molecular clones from the same specimen encoded an asparagine at position 63 in HA, while the corresponding isolate possessed an aspartic acid at this position. The amino acid at position 63 in HA is located in the globular head domain, and is part of the HA antigenic site [23], suggesting that HA antigenicity differs between the viruses in the original specimen and the corresponding isolate. One of the five molecular clones (Clone 1) from the same specimen encoded a serine at position 125 in HA, while the corresponding isolate possessed a proline at this position. The amino acid at position 125 in HA is located close to the 130-loop [19], and mutation at this position has been found in viral escape mutants from anti-HA monoclonal antibodies [24], suggesting that HA antigenicity may differ between

Table 7: Differences in nucleotide and amino acid sequences in HA gene ORFs in the original specimen and corresponding isolate for specimen No. 9.

Source of nucleotide sequence		Amino acid (nucleotide) at position[b]:
		377 (1176)
A/eurasian wigeon/Kagoshima/KU-32/2016 (H5N6)[a]		T (V)
Molecular clone from the original specimen No. 9	Clone 1	-[c](C)
	Clone 2	- (-)
	Clone 3	- (-)
	Clone 4	- (-)

[a]Isolate from original specimen No. 9.
[b]Amino acid positions are shown by H3 numbering.
[c]Identical nucleotide or amino acid to those of the corresponding isolate.

the viruses in the original specimen and the corresponding isolate. Three of the five molecular clones (Clones 2, 3 and 4) from the same specimen encoded an aspartic acid at position 187 in HA, while the corresponding isolate possessed an asparagine at this position. The amino acid at position 187 is located close to the so-called "190 helix", which is also one of the three secondary elements of the RBD [19], suggesting that the receptor binding specificity may differ between the viruses in the original specimen and the corresponding isolate.

Two non-synonymous substitutions were found in the full-length HA gene ORFs from specimen No. 8 (Table 6). One of the five molecular clones (Clone 4) from this specimen encoded an isoleucine at position 202 in HA, while the corresponding isolate, A/northern pintail/Kagoshima/KU-23/2016 (H5N6), possessed a valine at this position. The amino acid at position 202 in HA is located close to the 190 helix [19], and is one of the critical residues for receptor binding specificity [25], suggesting that the receptor binding specificity may differ between the viruses in the original specimen and the corresponding isolate. Two molecular clones (Clones 1 and 2) from the same specimen encoded a lysine at position 403 in HA, while

Table 8: Differences in nucleotide and amino acid sequences in HA gene ORFs in the original specimen and corresponding isolate for specimen No. 10.

Source of nucleotide sequence		Amino acid (nucleotide) at position[b]:										
		63 (208)	125 (400)	150 (483)	161 (516)	187 (592)	213 (672)	299 (933)	302 (942)	445 (1380)	494 (1527)	565 (1694)
A/duck/Kagoshima/KU-d35/2016 (H5N6)[a]		D (G)	P (C)	N (C)	Y (C)	N (A)	L (A)	P (C)	I (T)	K (A)	E (A)	C (G)
Molecular clone from the original specimen No. 10	Clone 1	N (A)	-[c](-)	- (T)	- (T)	- (-)	- (G)	- (T)	- (C)	- (G)	- (G)	Y (A)
	Clone 2	N (A)	- (-)	- (T)	- (T)	- (-)	- (G)	- (T)	- (C)	- (G)	- (G)	- (-)
	Clone 3	N (A)	- (-)	- (T)	- (T)	- (-)	- (G)	- (T)	- (C)	- (G)	- (G)	- (-)
	Clone 4	N (A)	- (-)	- (T)	- (T)	D (G)	- (G)	- (T)	- (C)	- (G)	- (G)	- (-)
	Clone 5	N (A)	S (T)	- (T)	- (T)	- (-)	- (G)	- (T)	- (C)	- (G)	- (G)	- (-)

[a]Isolate from original specimen No. 10.
[b]Amino acid positions are shown by H3 numbering.
[c]Identical nucleotide or amino acid to those of the corresponding isolate.

the corresponding isolate possessed a glutamic acid at this position. While the amino acid at position 403 in HA is located in the HA stalk region, its specific function has not been described.

Four non-synonymous substitutions were found in the full-length HA gene ORFs from specimen No. 10 (Table 8). All five of the molecular clones from this specimen encoded an asparagine at position 63 in HA, while the corresponding isolate, A/duck/Kagoshima/KU-d35/2016 (H5N6), possessed an aspartic acid at this position. One of the five molecular clones (Clone 5) from the same original specimen encoded a serine at position 125 in HA, while the corresponding isolate possessed a proline at this position. The other molecular clone (Clone 4) from the same original specimen encoded an aspartic acid at position 187 in HA, while the corresponding isolate possessed an asparagine at this position. It is worth noting these three non-synonymous substitutions were also found between the HA gene ORFs from specimen No. 7 and the corresponding isolate. One of the five molecular clones (Clone 1) from the same specimen encoded a tyrosine at position 565 in HA, while the corresponding isolate possessed a cysteine at this position. The cysteine at position 565 is known to be one of the palmitoylated residues found in the HA cytoplasmic tail and to play a key role in virus replication [26]. Nevertheless, the cysteine-to-tyrosine substitution at this position has been suggested to compensate for the loss of palmitate [26]. Although it is possible that this mutant HA gene was detected as one of the genes from the replication-incompetent quasi-species in the original specimen, the replication ability of the viruses in the original specimen may be equivalent to that of the corresponding isolate.

Discussion

It is well known that the embryonated chicken egg isolation process causes mutations in influenza viral genes from mammalian hosts, including humans and pigs [27]. Here, we demonstrated similar bias effect of the isolation process for HPAIVs.

We compared the nucleotide sequences of the full-length HA gene ORFs of H5N6 HPAIVs between viruses in the original specimens and the corresponding isolates. As a result, we found that there were nucleotide differences at a total of 24 positions in seven pairs of viruses from the original specimens and their corresponding isolates, including nine non-synonymous substitutions in four pairs (Tables 2,5,6 and 8). Among the nine non-synonymous substitutions that we found in these four pairs, six substitutions (at amino acids 63, 125, 145, 187, 202 and 277) are located in the globular head domain (Figure 1). In particular, four amino acids, at positions 125, 145, 187, and 202, are located close to the secondary structure elements of the RBD. The globular head domain of HA plays a critical role in both HA antigenicity and receptor binding specificity [19,23]. Our findings suggest that the embryonated egg isolation process used for AIVs has the potential to significantly affect the characteristics of AIVs, although further study is needed to confirm these possibilities.

We employed a molecular cloning technique to determine the nucleotide sequences of the HA gene ORFs from minor virus populations present in the original specimens. Although we picked only four to six molecular clones from each of the original specimens, we found several variations in the HA gene ORFs in these individual clones. These results highlight the genetic diversity of AIVs in nature.

Initially, we attempted to amplify the full-length HA genes from a total of 13 original specimens by RT-PCR. Amplified DNA samples of the correct size, however, were obtained from only nine specimens. Nevertheless, we did isolate H5N6 HPAIVs from the remaining four specimens by inoculating them into embryonated chicken eggs. Although the high sensitivity of PCR-based viral gene detection is well-known, these results suggest that virus isolation in embryonated chicken eggs is more sensitive than PCR-based viral gene analysis in terms of the AIV detection, even though the genetic characteristics of the isolates may not accurately reflect those of the viruses in the original specimens.

In conclusion, we have revealed the impact of the embryonated chicken eggs virus isolation process on the characterization of AIVs. Although embryonated chicken eggs are globally accepted as the gold standard for AIV isolation, we should understand the potential risks of overlooking AIV genetic variants in nature. More importantly, we should consider using AIV genetic analyses in the original specimens in addition to those of the corresponding isolates.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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