

Continuing Evolution of H3, H4, H6 and H9 Influenza A Viruses in Live Bird Markets in Korea with Potential for Expanding Host Range

Hyun-Jeong Lee¹, Yu-Na Lee¹, Dong-Hun Lee¹, Ha-Na Youn¹, Ji-Sun Kwon², Youn-Jeong Lee², Min-Chul Kim², Ok-Mi Jeong², Hyun-Mi Kang², Jun-Hun Kwon², Joong-Bok Lee², Seung-Young Park¹, In-Soo Choi¹ and Chang-Seon Song^{1*}

¹College of Veterinary Medicine, Konkuk University, Seoul, Korea

²Avian Disease Division, National Veterinary Research & Quarantine Service, Anyang, Korea

INTRODUCTION

- In Korea, there have been extensive gene pools for influenza viruses to generate multiple reassortants.
- Live bird markets (LBMs) are highly productive sources of avian influenza viruses (AIVs) because they provide an ideal environment for viral reassortment and interspecies transmission.
- However, only limited reports provided information about ecology of AIV circulating in Korean LBM.
- In this study, we performed nationwide surveillance on AIV from LBM in Korea from 2006 to 2008
 - To understand the epidemiology of the AIVs in Korean LBM
 - To understand the role of LBMs as sources of AIV evolution
 - To determine the animal species that possess the potentials of expanding host range of AIVs

MATERIALS & METHODS

Sampling and virus isolation

- From September 2006 through March 2008, a total of 644 tissue specimens were collected from different species in 13 different LBMs of South Korea and used for virus isolation.

Phylogenetic and molecular analysis

- Eight gene segments of representative isolates were compared with those of influenza viruses isolated from domestic chickens, wild birds, human, swine and equine.

Animal experiment

- Twelve representative viral isolates were inoculated with $10^{5.0-6.0}$ EID₅₀ of each virus.
- Re-isolate inoculated virus from each animals.

Chicken & Quail (2-4wks old)

In trachea & CT on 3 dpi.

Mouse (5-6wks old)

In lung on 5 dpi.

Dog (10wks old)

In nose daily for 12 days p.i.

Table 1. Summary of avian influenza viruses isolated from LBMs by host species

Order	Species	No. of samples	Viral subtype						Total
			H3N2	H3N8	H4N2	H4N6	H5N2	Unidentified ^a (%)	
Anseriformes	Pekin duck	153	5	2	1	1	6	2	16
	Mallard duck	116	3	1	1	1	6	3	16
	Geese	8							
	Muscovy duck	8							
Galliformes	Korean native chicken	133					17	17	
	Silky fowl	78					11	11	
	Phoenix	34					1	1	
	Turkey	54							
	Quail	25							
	Layer	12					2	2	
Columbiformes	Guinea fowl	4							
	Pigeon	6					1	1	
Carnivora	Dog	7	1						1
	Cat	1							
Total		644	9	3	1	1	24	5	65

^aHA and NA subtype of four isolates were partially identified; one of H3, one of H4, one of H6, and one of N2. One AIV was not identified subtype.

Table 2. Replication of representative influenza viruses in chickens, quails, and mice

Subtype	Isolate	Quails ^a			Chickens ^a			Mice ^b	
		Tra	CT	Tra	CT	Lung			
H3N2	DK/LBM1354/06	2/4	0/4	0/4	0/4	0/4	4/4		
H3N8	DK/LBM347/07	1/4	0/4	0/4	0/4	0/4	0/4		
H3N8	DK/LBM182/07	0/4	0/4	0/4	0/4	0/4	0/4		
H4N6	DK/LBM187/08	1/4	0/4	0/4	0/4	0/4	2/4		
H4N2	MAL/LBM188/08	3/4	0/4	3/4	0/4	0/4	4/4		
H5N2	DK/LBM339/07	1/4	1/4	0/4	0/4	0/4	4/4		
H5N2	DK/LBM1674/07	1/4	0/4	2/4	0/4	0/4	4/4		
H9N2	CK/LBM341/07	0/4	0/4	2/4	0/4	0/4	0/4		
H9N2	DK/LBM186/07	0/4	0/4	3/4	1/4	0/4	0/4		
H9N2	CK/LBM76/07	3/4	0/4	3/4	0/4	0/4	0/4		
H9N2	DK/LBM446/07	4/4	0/4	4/4	1/4	0/4	0/4		
H9N2	CK/Kor/006/96	4/4	3/4	4/4	0/4	0/4	0/4		

^aNumber of virus positive birds/number of inoculated birds. The infectious dose was 10^{5-10} EID₅₀. At 3 days post-infection, Tra and CTs were tested to detect inoculated virus. Tra, trachea; CT, caecal tonsil.

^bNumber of virus positive mice/number of inoculated mice. The infectious dose was $10^{6EID_{50}}$. At 5 days post-infection, lungs were tested to detect inoculated virus.

Table 3. Replication of H3 influenza viruses in dogs

Subtype/Isolate	Source	Clinical signs			Virus replication ^b	Seroconversion ^c	
		Fever ^a	Cough	Sneeze			
H3N2	Canine/LBM412/08	3/3	3/3	3/3	3/3	3/3	
H3N2	DK/LBM1354/06	Duck	0/3	3/3	3/3	0/3	3/3
H3N8	DK/LBM347/07	Duck	0/3	3/3	3/3	3/3	3/3

^aFever is defined by rectal temperature over 39.5°C.

^bNumber of virus positive dogs/number of inoculated dogs. The infectious dose was $10^{6EID_{50}}$. For 12 days, nasal swabs were examined for virus shedding by RRT-PCR.

^cNumber of virus positive dogs/number of inoculated dogs.

At 17 days post-infection, ELISA antibody titers were regarded as positive if percent inhibition (PI) was >50.

RESULTS

Virus isolation and distribution of subtypes (Table 1)

- Sixty-five AIVs (H3, H4, H6 and H9) were isolated from 644 tissue samples collected in LBMs.
- Prevalence of subtypes: H9 (44) > H3 (13) > H4 & H6 (3)
- Most H9 subtypes were isolated from *Galliformes* and other subtypes were isolated from *Anseriformes*.
- A single H3N2 virus isolated from nasal swabs of farmed dogs sold in LBMs.

Phylogenetic analysis (Figure 1 & 2)

- In H9 HA tree, all H9 viruses belonged to the Korea lineage (CK/Korea/96-like lineage) that has been prevalent in chicken farms in Korea and genetically far from other Asian H9 lineage (Fig 2-a).
- In H3 HA tree, all H3 viruses formed Korea LBM lineage within Eurasian avian lineage (Fig 2-b).
- N2 genes of the AIVs in LBMs were divided into two clusters, LBM lineage and Korea lineage (Fig 2-c).
- Six internal genes of H9N2 viruses of Korea lineage were widely dispersed in AIV gene pool together with those of AIVs in Eurasian aquatic birds.
- There were genetic diversity of AIV in LBMs leading to generate numerous reassortants including CIV (Figure 1).

Animal experiments (Table 2 & 3)

- Quail (Table 2)**
 - Most isolates replicated in the respiratory tracts.
- Chicken (Table 2)**
 - All the H9N2 viruses from chickens replicated well in trachea of chickens, as well as single H4N2 and H6N2 virus from ducks. However, none of H3 viruses replicated in chickens.
- Mouse (Table 2)**
 - H3, H4, and H6 viruses from ducks replicated well without pre-adaptation.
 - In contrast, none of H9 viruses from chickens were reisolated in the lung of inoculated mice.
- Dog (Table 3)**
 - CIV and two AIV examined replicate in dogs.
 - Clinical sign: fever (>39.5°C), sneezing, nasal discharge, and coughing.
 - CIV induced severe clinical signs, but 2 H3 viruses from ducks induced mild clinical signs without fever.
 - Virus shedding: detected both in dogs inoculated with CIV and one AIV.
 - Seroconversion: observed in all dogs inoculated with CIV or AIV.
 - Gross lesions: multifocal to coalescing reddish consolidations observed in lung of all infected dogs.

Molecular characterization

- Amino acid alteration observed in several AIVs showing ability to replicate in mice
 - M1 protein: V151 (associated with pathogenicity in mice).
- Amino acid alteration observed in CIV
 - HA protein
 - Lys-to-Arg substitution at position -4 from the cleavage site (RQTR*GLF)
 - Q226L and G288S in receptor binding site (preferentially recognize SAα2,6Gal)
 - PB1-F2 protein: N66S (correlated with pathogenicity in mouse)

DISCUSSIONS

Phylogenetic study & molecular analysis

- H9N2 viruses circulating in chickens have continued to maintain its lineage since the first description, and internal genes of them were introduced into H3, H4, and H6 viruses in ducks.
- There were genetic diversity of AIV circulating in duck population.
- Seven genes of CIV was closely related to those of at least one isolate of H3 and H4 viruses from ducks in LBMs (Fig 1).
- Therefore, we suggest that ducks in LBM play a role in mixing vessels for AIVs to generate novel reassortants including CIV.

Animal challenge study

- Chicken:** H3 viruses from ducks have not adapted to chickens despite introduction of internal genes from H9N2 viruses circulating chickens. In contrast, H4 and H6 viruses from ducks seems to have potentials to infect chickens.
- Mouse:** Unlike H9N2 virus, replication of H3, H4, and H6 viruses in mice suggest that the AIV from ducks possess potentials to expand the host range to mammals.
- Quail:** Quails may play a role in the ecology of AIVs based on the susceptibility to multiple subtypes of AIVs.
- Dog:** AIVs circulating in ducks, particularly the H3 subtype, in LBMs have the potential of crossing species barriers.

CONCLUSIONS

- Korean LBM played an important role in extending genetic diversity of influenza viruses in Korea.
- The newly evolved AIVs have been continuously generated by reassortment events in ducks in LBMs with the potential of expanding the host range to mammals.
- Continued monitoring of poultry population, in particular quails and ducks, in LBM need to better understand the influenza ecology and interspecies transmission, as a component of pandemic preparedness.

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Corresponding Author Contact Information
E-mail: songs@konkuk.ac.kr
Phone: +82-2-450-3712

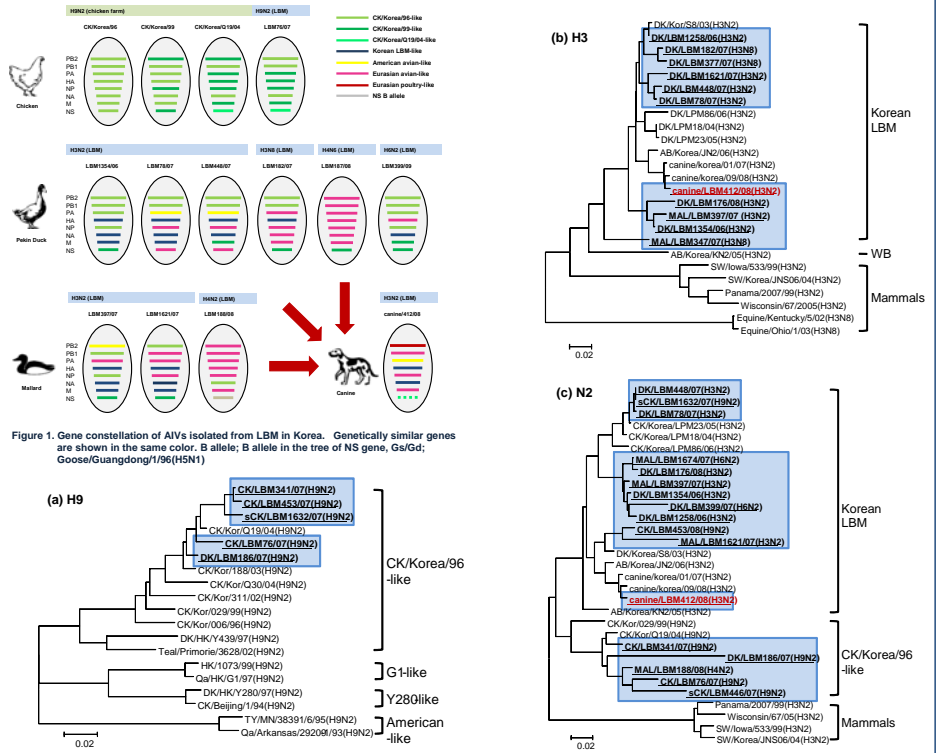


Figure 1. Gene constellation of AIVs isolated from LBM in Korea. Genetically similar genes are shown in the same color. B allele; B allele in the tree of NS gene, Gs/G; Goose/Guangdong/1196(H5N1)

Figure 2. Phylogenetic analysis of avian influenza viruses isolated from LBMs. (a) H9 HA gene; (b) H3 HA gene; (c) N2 NA gene. Bold type indicates the viruses examined in this study. CK, chicken; DK, duck; MAL, mallard; sCK, silky chicken; AB, aquatic bird; SW, swine; TY, turkey; QA, quail; HK, Hong Kong; Kor, Korea; MN, Minnesota.