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Prior infection with antigenically heterologous low pathogenic avian influenza viruses interferes with the lethality of the H5 highly pathogenic strain in domestic ducks

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ABSTRACT. Low and highly pathogenic avian influenza viruses (LPAIVs and HPAIVs, respectively) have been co-circulating in poultry populations in Asian, Middle Eastern, and African countries. In our avian-flu surveillance in Vietnamese domestic ducks, viral genes of LPAIV and HPAIV have been frequently detected in the same individual. To assess the influence of LPAIV on the pathogenicity of H5 HPAIV in domestic ducks, an experimental co-infection study was performed. One-week-old domestic ducks were inoculated intranasally and orally with phosphate-buffered saline (PBS) (control) or 10^6 EID_{50} of LPAIVs (A/duck/Vietnam/LBM678/2014 (H6N6) or A/Muscovy duck/Vietnam/LBM694/2014 (H9N2)). Seven days later, these ducks were inoculated with HPAIV (A/Muscovy duck/Vietnam/LBM808/2015 (H5N6)) in the same manner. The respective survival rates were 100% and 50% in ducks pre-infected with LBM694 or LBM678 strains and both higher than the survival of the control group (25%). The virus titers in oral/cloacal swabs of each LPAIV pre-inoculation group were significantly lower at 3–5 days post-HPAIV inoculation. Notably, almost no virus was detected in swabs from surviving individuals of the LBM678 pre-inoculation group. Antigenic cross-reactivity among the viruses was not observed in the neutralization test. These results suggest that pre-infection with LPAIV attenuates the pathogenicity of HPAIV in domestic ducks, which might be explained by innate and/or cell-mediated immunity induced by the initial infection with LPAIV.

KEY WORDS: co-infection, duck, H5, influenza

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The influenza virus is a negative sense, single-stranded segmented RNA virus belonging to the *Orthomyxoviridae* family and classified into the four types A, B, C, and D based on antigenicity [1, 10]. Influenza A virus is antigenically and genetically classified by its surface glycoproteins hemagglutinin (HA; H1–H18) and neuraminidase (NA; N1–N11) [7, 36, 37]. Influenza A viruses of the H1–H16 and N1–N9 subtypes have been isolated from wild waterfowl, which are considered natural hosts [1, 40]. Avian influenza virus (AIV), mainly isolated from wild waterfowl and poultry, is classified as either highly pathogenic avian influenza virus (HPAIV) or low pathogenic avian influenza virus (LPAIV) depending on its pathogenicity to chickens [2].

After an H5N1 HPAIV caused a highly pathogenic avian influenza outbreak in a goose farm in China in 1996, its virus progenies (Gs/Gd-like viruses) have experienced antigenic mutations and genetic reassortment [42]. These viruses have spread to Asia, Europe, North America, and Africa, inflicting extensive damage to the poultry industry [3]. Particularly in Asia, HPAIVs

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have been continually circulating since 2003 and have caused major problems in poultry flocks [3, 8]. Gs/Gd-like viruses are classified into 10 clades ranging from 0 to 9 according to the phylogenetic lineage of H5 subtype HA [41]. Since clade 2.3.4.4 H5N8 HPAIV was first isolated from ducks in a live bird market in the Zhejiang Province, China at the end of 2013, H5 HPAIVs belonging to this clade have been isolated in Asia, Europe, North America, and Africa [21].

In Southeast Asia, China, and Egypt, the H6 and H9 subtype LPAIVs have been maintained in poultry in parallel with HPAIVs [6, 15, 35]. H6 LPAIVs have been isolated from wild waterfowl and poultry worldwide since it was first isolated from turkeys in the United States in 1965 [39]. H9 LPAIVs have spread in poultry populations worldwide since the 1990s [27]. Notably, H9N2 subtype LPAIVs have been circulating in poultry in Asia and the Middle East, causing decreases in egg-laying rates and feeding efficiency, and are sometimes associated with high mortality especially in chickens due to co-infection with other viruses and bacteria [9, 18, 29]. Furthermore, the N1 NA and the internal protein gene segments of the first human H5N1 HPAIV isolate in Hong Kong in 1997 were derived from H6N1 and H9N2 LPAIVs, which were prevalent in poultry at that time [28]. Taken together, circulating H6 and H9 LPAIVs cause not only reduced productivity of poultry flocks but also genetic reassortment with other LPAIVs or HPAIVs, thereby giving emergence of viruses with novel gene constellations, posing a further risk to the poultry industry and public health.

The reason why HPAIVs, which generally cause acute and lethal infection in chickens, have been maintained for long periods might be explained by the presence of waterfowl in wet markets such as domestic ducks (*Anas platyrhynchos* var. *domesticus*). HPAIVs often cause subclinical infection accompanied by viral shedding in domestic ducks, depending on the virus strain and age at infection [14, 17, 25]. Domestic ducks may play a role in the source of HPAIV infection within flocks, particularly in wet markets where multiple species of live poultry, including ducks, are bought and sold [30]. Previous reports have suggested that superinfection with LPAIV and HPAIV in poultry might be another factor involved in the maintenance of HPAIVs in poultry populations [16, 24, 32]; experimental pre-infection with H9N2 LPAIV in chickens reduced the mortality rate and viral shedding associated with subsequent H5N1 HPAIV infection. Co-circulating LPAIV and HPAIV in poultry flocks in the field, such as wet markets and farms, might cause asymptomatic infection in some poultry, which subsequently function as viral reservoirs.

Since 2011, we have carried out avian-flu surveillance in Vietnamese poultry and continuously isolated H5 HPAIVs from apparently healthy domestic waterfowl, such as domestic ducks and Muscovy ducks (*Cairina moschata*), in wet markets (data not shown). In parallel, a number of LPAIVs, including H6N6 and H9N2 subtype viruses, have been isolated; 12 H6N6 (isolation rate: 4.2%) and 11 H9N2 (3.9%) were isolated from domestic waterfowls in Hanoi, Vietnam (unpublished data). In some cases, HA genes of both LPAIV and HPAIV were detected in the swab material from a single individual. These results imply that ducks co-infected with both LPAIV and HPAIV also function as viral reservoirs of HPAIV, as shown in previous studies using chickens [16, 24, 32]. However, the pathogenesis of LPAIV and HPAIV during superinfection in domesticated waterfowls remains unclear. The present study examined the impact of LPAIV pre-infection in domestic ducks on subsequent lethal H5 HPAIV infection to provide useful information for the eradication of AIVs in poultry populations. We applied H6N6 and H9N2 LPAIVs for primal inoculation and an H5N6 HPAIV, classified into the globally prevalent clade 2.3.4.4 [43], for secondary inoculation. These three viruses were isolated from domesticated ducks in our surveillance in Hanoi, Vietnam in 2014–2015; in other words, they were actually co-circulating in poultry flocks in Northern Vietnam. We set the timing of HPAIV challenge in the superinfection experiment at 2 weeks of age in line with previous reports [14, 25]: domestic ducks were more vulnerable to H5 HPAIV infection at 2 weeks of age than older ducks. To ensure the induction of sufficient immune responses, prior inoculation with the LPAIVs was conducted 7 days before (1 week of age).

MATERIALS AND METHODS

Virus specimens

Two LPAIVs (LBM678 and LBM694) and one H5 subtype clade 2.3.4.4 HPAIV (LBM808) were used in this study: A/duck/Vietnam/LBM678/2014 (H6N6) (accession number: AB983227–983234), A/Muscovy duck/Vietnam/LBM694/2014 (H9N2) (LC000593–LC000600), and A/Muscovy duck/Vietnam/LBM808/2015 (H5N6) (LC168618–168619 and LC279755–279760). These isolates were collected as part of our virus surveillance in Vietnam. The viruses were inoculated into 10-day-old embryonated chicken eggs (Aoki Breeder Farm, Nasu, Japan). After incubation, allantoic fluid from these eggs was collected, stored at −80°C as the seed virus stock, and used in subsequent experiments.

Measurement of 50% egg infectious dose (EID_{50})

Samples were serially diluted 10-fold in phosphate-buffered saline (PBS; Takara, Kusatsu, Japan) supplemented with 1,000 U of penicillin G potassium (Meiji Seika, Tokyo, Japan) and streptomycin sulfate (Meiji Seika) (PS-PBS) and were inoculated at a volume of 100 µl into 10-day-old embryonated eggs, with 3–4 eggs per sample. The eggs were incubated for 48 hr at 35°C. A hemagglutination test [11, 33] was performed using allantoic fluid, and the EID_{50} was calculated using the method of Reed and Muench [31].

Animal infection experiments

One-day-old ducks (Cherry Valley var.; Takahashi Artificial Hatchery, Osaka, Japan) were purchased and reared in the Tottori University Animal Experimentation Building until the age of 1 week. We selected one duck from the flock, placed it under isoflurane (FUJIFILM Wako Pure Chemical Corp., Tokyo, Japan) anesthesia, and obtained a blood sample via cardiac puncture

because venous blood collection from 1-week-old domestic ducks is technically difficult. The blood sample was incubated at 35°C for 1 hr, allowed to stand at 4°C for 12 hr, and then centrifuged to collect the supernatant. The hemagglutinin inhibition (HI) test [33] was performed to confirm that serum antibodies to each challenge virus were negative (within the limits of detectability). In the present study, if the selected duck was seronegative, all ducks in the same flock were also considered naïve. To prevent bias, animals were separated into flocks according to their weights and subjected to the following virus inoculation experiments. All animal infection experiments were carried out in self-contained isolator units (CLEA, Tokyo, Japan) at a biosafety level 3 facility of the Avian Zoonosis Research Center, Tottori University, Japan. The experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of Tottori University (approval number: 19-T-3). Throughout the present study, any birds unable to feed or drink were euthanized, and recorded as dead at the following day's observation time.

Each isolator accommodated three 1-week-old domestic ducks. The ducks were intranasally inoculated with 100 µl of allantoic fluid containing each virus at 10^6 EID_{50} and then observed for clinical signs at 24-hr intervals for 14 days. Oral and cloacal swabs were collected in 2 ml of PS-PBS at 1, 2, 3, 5, 7, 10, and 14 days post inoculation (DPI) to assess viral shedding. At the end of the 14-day period, the ducks were also checked for specific antibodies against challenged strains in serum via the HI test before euthanasia by isoflurane. The ducks that had died of infection were dissected, and their tissues (brain, trachea, lung, kidney, and colon) were aseptically sampled. The tissue samples were homogenized using a Micro Smash^{TM} MS-100R (3,000 rpm, 30 sec; Tomy Seiko, Tokyo, Japan) to create a 10% (w/v) organ emulsion in PS-PBS. Serially ten-fold diluted samples were inoculated into 10-day-old chicken embryos, and the EID_{50} was calculated. To assess the replicability of LPAIVs in domestic ducks in the acute phase of infection, three ducks were inoculated with LBM678 (H6N6) or LBM694 (H9N2), as described above. The ducks were euthanized at 3 DPI, and virus titers of the tissues were determined.

For the superinfection study, four 1-week-old ducks were housed in each isolator. The ducks were inoculated intranasally and orally with PBS or an LPAIV, LBM678 (H6N6) or LBM694 (H9N2), at a titer of $10^{6.0}$ EID_{50}/ 0.2 ml. Seven days later, LBM808 (H5N6) was inoculated in the same way, and clinical symptoms were observed for an additional 10 days. At 0, 1, 2, 3, 5, 7, and 10 DPI with LBM808 (H5N6), oral and cloacal swabs were collected, and virus titers were determined. The differences in viral titers compared to the PBS pre-inoculated ducks were statistically evaluated using the unpaired *t*-test (*P<0.05, **P<0.01). The serum samples were collected at 10 DPI before euthanasia with isoflurane. Serum antibodies against the first and second challenge viruses were assessed using the HI test. Ducks that died during the experiment or were euthanized at 10 DPI were dissected, and the virus titers of tissues were examined as described above.

Antigenic analyses

Chickens were hatched from fertilized eggs (White Leghorn, Aoki Breeder Farm) and reared exclusively in our laboratory. The viruses were inactivated with formalin (FUJIFILM Wako Pure Chemical Corp.; final concentration, 0.1%) and purified by sucrose density-gradient ultracentrifugation [17]. Chickens were immunized at both 4 and 6 weeks old of age with the purified virus and Complete Freund's Adjuvant (FUJIFILM Wako Pure Chemical Corp.). Two weeks after the second immunization, whole blood was sampled via cardiac puncture under isoflurane anesthesia. Serum samples were retrieved in the same way as described above and stored as a polyclonal antibody for subsequent antigenic analyses, the HI test, neuraminidase inhibition (NI) test [4], and neutralization assay. For the neutralization assay, polyclonal chicken antiserum that was serially 2-fold diluted and $10^{2.0}$ EID_{50} of the virus were mixed and incubated for 1 hr at room temperature. This mixture was inoculated into four 10-day-old embryonated eggs and cultured for 48 hr at 35°C. The HA test was performed using the collected allantoic fluid, and the neutralizing titer was expressed as the reciprocal of the highest dilution of the serum that showed 50% or more inhibition of viral replication.

RESULTS

Pathogenicity of the HPAIV and LPAIVs in 1-week-old domestic ducks

The pathogenicity of LBM808 (H5N6), LBM678 (H6N6), and LBM694 (H9N2) in 1-week-old ducks was assessed (Tables 1 and 2). All three ducks inoculated with LBM808 (#1–#3) showed no clinical sign until 3 DPI (data not shown), then died suddenly at 4 DPI (Table 1). These ducks shed the virus via oral and cloacal routes at a titer of $\leq 10^{4.5}$ EID_{50}/ml after 1 DPI. A higher titer of the virus than the inoculum (10^6 EID_{50}) was recovered from all tested tissues of the dead ducks (Table 2). All ducks inoculated with LBM678 (#4–#6) and two of the three ducks with LBM694 (#7 and #8) survived for 14 days (Table 1). One duck (#9) with LBM694 suddenly died at 11 DPI without showing any specific symptoms of avian-flu (data not shown). This duck showed mild neck injury at 9 DPI, and no virus was recovered from its swabs (Table 1) and tissues (data not shown). We concluded that the duck accidentally died. The surviving ducks with LPAIVs showed weak HI antibody responses against each challenge virus (HI titers 2–16 HI, Table 1). Some ducks (#4–#6 and #8) temporally and slightly shed the virus via the oral route. No virus was recovered from the tissues of the ducks euthanized at 3 DPI with each LPAIV (#10–#15, Table 2).

Prior infection with LPAIV interfered with HPAIV pathogenesis in domestic ducks

In order to examine whether prior infections with LPAIV affect the pathogenicity of HPAIV in domestic ducks, 1-week-old ducks were intranasally and orally inoculated with LBM678, LBM694, or PBS. Seven days after LPAIV priming, allowing the induction of sufficient immunity following the initial infection, LBM808 was similarly inoculated. Clinical signs were observed for an additional 10 days.

Six of the eight ducks pre-inoculated with PBS (#16–21) showed the first clinical signs such as loss of appetite and depression

Table 1. Viral shedding and antibody responses of domestic ducks intranasally inoculated with avian influenza viruses at 1 week of age

Virus	ID		Viral titers in oral / cloacal swabs (log EID_{50}/ml)								HI titers against challenged virus at 14 DPI
			0	1	2	3	5	7	10	14 (DPI)	
LBM808 (H5N6)	#1	4d dead	- / -	3.1 / 1.5	4.5 / 3.4	3.4 / 2.4	NA	NA	NA	NA	NA
	#2	4d dead	- / -	1.5 / 2.7	3.3 / 4.0	4.1 / 3.3	NA	NA	NA	NA	NA
	#3	4d dead	- / -	3.4 / 3.4	3.6 / 3.7	1.5 / 1.9	NA	NA	NA	NA	NA
LBM678 (H6N6)	#4	14d survived	- / -	- / -	- / -	2.5 / -	- / -	- / -	- / -	- / -	2
	#5	14d survived	- / -	≤0.7 / -	- / -	- / -	- / -	- / -	- / -	- / -	2
	#6	14d survived	- / -	1.7 / -	- / -	- / -	- / -	- / -	- / -	- / -	2
LBM694 (H9N2)	#7	14d survived	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	2
	#8	14d survived	- / -	1.3 / -	- / -	- / -	- / -	- / -	- / -	- / -	16
	#9	11d dead	- / -	- / -	- / -	- / -	- / -	- / -	- / -	NA	NA

EID_{50}, 50% egg infectious dose; HI, hemagglutinin inhibition; DPI, days post inoculation; -, ≤0.5; NA, not applicable.

Table 2. Viral titers in tissues of domestic ducks intranasally inoculated with avian influenza viruses at 1 week of age

Virus	ID	DPI	Viral titers (log EID_{50}/g)				
			Brain	Trachea	Lung	Kidney	Colon
LBM808 (H5N6)	#1	4†	10.0	8.2	7.2	6.9	6.2
	#2	4†	8.9	8.9	7.3	7.1	7.1
	#3	4†	9.8	7.9	7.4	6.2	6.6
LBM678 (H6N6)	#10	3	-	-	-	-	-
	#11	3	-	-	-	-	-
	#12	3	-	-	-	-	-
LBM694 (H9N2)	#13	3	-	-	-	-	-
	#14	3	-	-	-	-	-
	#15	3	-	-	-	-	-

† Died of infection. DPI, days post inoculation; EID_{50}, 50% egg infectious dose; -, ≤1.5.

Table 3. Viral shedding and antibody responses of domestic ducks intranasally inoculated with H5N6 highly pathogenic avian influenza virus at 2 weeks of age

Pre-inoculation materials at 1 week of age	ID	Outcome (Duration of symptom onset)	Viral titers in oral / cloacal swabs (log EID_{50}/ml) after LBM808 (H5N6) HPAIV challenge							HI titers at 10 DPI against	
			0	1	2	3	5	7	10 (DPI)	Pre-inoculated LPAIV	LBM808 (H5N6)
PBS	#16	4d dead (3–4 DPI)	- / -	2.8 / 1.4	2.8 / 2.8	3.5 / 2.5	NA	NA	NA	NA	NA
	#17	4d dead (4 DPI)	- / -	- / 1.5	3.3 / 3.3	4.0 / 2.8	NA	NA	NA	NA	NA
	#18	5d dead (3–5 DPI)	- / -	1.4 / 2.8	1.8 / 2.8	3.8 / 3.5	3.8 / 3.5	NA	NA	NA	NA
	#19	5d dead (4–5 DPI)	- / -	1.2 / 2.8	2.3 / 2.8	3.5 / 2.5	2.5 / 4.3	NA	NA	NA	NA
	#20	5d dead (4–5DPI)	- / -	1.6 / 1.6	2.8 / 2.4	2.8 / 4.3	3.5 / 3.3	NA	NA	NA	NA
	#21	6d dead (4–6 DPI)	- / -	1.6 / ≤0.8	1.7 / 2.3	3.3 / 2.8	3.7 / 3.5	NA	NA	NA	NA
	#22	10d survived	- / -	1.4 / -	2.3 / 1.5	3.0 / 5.0	- / -	- / -	- / -	NA	64
	#23	10d survived	- / -	- / ≤0.9	2.8 / 1.5	3.0 / 4.3	- / -	- / 3.3	- / 1.4	NA	128
LBM678 (H6N6)	#24	5d dead (5 DPI)	- / -	- / 1.6	2.5 / 3.5	2.8 / 2.8	1.8 / 3.5	NA	NA	NA	NA
	#25	5d dead (5 DPI)	- / -	1.6 / ≤0.8	2.0 / 2.8	3.8 / -	1.2 / 2.0	NA	NA	NA	NA
	#26	10d survived	- / -	- / -	- / -	- / 2.5	- / -	- / -	- / -	32	64
	#27	10d survived	- / -	- / -	- / -	1.2 / -	- / -	- / -	- / -	32	64
	Siginificant differences				* /	* / *					
LBM694 (H9N2)	#28	10d survived (4–10 DPI)	- / -	1.4 / -	1.6 / 2.0	≤0.8 / 3.8	- / -	- / -	- / -	64	32
	#29	10d survived	- / -	2.5 / ≤0.6	2.0 / -	1.4 / 2.5	- / -	- / -	- / -	128	256
	#30	10d survived (5 DPI)	- / -	1.4 / 1.5	1.8 / 1.4	1.0 / 2.8	- / -	- / -	- / -	32	64
	#31	10d survived (4–6 DPI)	- / -	1.8 / 1.4	2.3 / 1.8	1.5 / 3.0	- / -	- / 1.4	- / 1.5	64	128
	Siginificant differences				/ *	** /	* / **				

*P<0.05, **P<0.01: significantly lower titers compared to pre-inoculation group with PBS. EID_{50}, 50% egg infectious dose; HPAIV, highly pathogenic avian influenza virus; HI, hemagglutinin inhibition; DPI, days post inoculation; LPAIV, low pathogenic avian influenza virus; -, ≤0.5; NA, not applicable.

at 3–4 DPI and died by 6 DPI (Table 3). Half of the ducks that had been pre-inoculated with LBM678 suddenly died at 5 DPI. The remaining ducks (#26 and #27) showed no clinical signs and survived for 10 days. All ducks initially inoculated with LBM694 survived for 10 DPI with LBM808. Of these, one duck (#28) depressed with torticollis at 4 DPI; the torticollis remained for the observation period while recovered from the depression at 7 DPI. Two ducks (#30 and #31) also showed short-term depression. Surviving ducks with LPAIV pre-inoculation (#26–#31) seroconverted to both the challenge LPAIV and LBM808 at 10 DPI. Just before the second inoculation with LBM808 (0 DPI), no virus was recovered from the oral and cloacal swabs in any of the ducks. The ducks pre-inoculated with LBM678 or LBM694 shed significantly lower titers of the virus via oral and cloacal routes around 2–5 DPI. Throughout the superinfection study, the recovered viruses after 1 DPI were identified as secondary inoculated HPAIV by HA subtype-specific PCR [21, 38] (data not shown). Notably, almost no virus shedding from the surviving ducks with LBM678 (#26 and #27) was observed during the experimental period.

The virus titers in the tissues of the ducks that had died of infection or had been euthanized at 10 DPI were determined (Table 4). The virus was recovered from all tested tissues of the ducks that had died at 4–6 DPI. No virus was isolated from the tissues of the ducks that survived for 10 days after LBM808 inoculation.

Antigenic relationships among applied viruses

Antigenic relationships among LBM678, LBM694, and LBM808 strains were assessed by HI, NI, and neutralization assays (Table 5). The LBM808 strain showed more than 64-fold reduced reactivity to antisera against LBM678 or LBM694 in the cross-HI test. Similarly, no antiserum neutralized the heterologous strains. In the NI test, the antigenicity of N6 subtype strains, LBM808 and LBM678, was closely related; the antiserum against LBM678 showed only an 8-fold reduced NI titer, and *vice versa*.

Table 4. Viral titers in tissues of domestic ducks intranasally inoculated with H5N6 highly pathogenic avian influenza virus at 2 weeks of age

Pre-inoculation materials at 1 week of age	ID	DPI	Viral titers (log EID_{50}/g) after LBM808 (H5N6) HPAIV challenge				
			Brain	Trachea	Lung	Kidney	Colon
PBS	#16	4†	5.0	8.3	7.3	6.5	5.3
	#17	4†	7.0	7.8	7.3	5.4	5.5
	#18	5†	9.3	8.3	6.8	6.5	3.8
	#19	5†	3.8	6.8	6.3	5.4	6.3
	#20	5†	3.3	6.0	5.5	6.5	5.3
	#21	6†	8.8	7.3	5.8	5.5	4.3
	#22	10	-	-	-	-	-
	#23	10	-	-	-	-	-
LBM678 (H6N6)	#24	5†	8.5	8.7	4.4	4.5	3.3
	#25	5†	7.3	6.8	5.5	2.4	3.5
	#26	10	-	-	-	-	-
	#27	10	-	-	-	-	-
LBM694 (H9N2)	#28	10	-	-	-	-	-
	#29	10	-	-	-	-	-
	#30	10	-	-	-	-	-
	#31	10	-	-	-	-	-

† Died of infection. DPI, days post inoculation; EID_{50}, 50% egg infectious dose; HPAIV, highly athogenic avian influenza virus; -, ≤1.5.

Table 5. Antigenic relationships among LBM808 (H5N6), LBM678 (H6N6), and LBM694 (H9N2) strains

Antigens	HI test			NI test			Neutralization test		
	Antiserum against			Antiserum against			Antiserum against		
	LBM808	LBM678	LBM694	LBM808	LBM678	LBM694	LBM808	LBM678	LBM694
LBM808 (H5N6)	1,280[a]	10	<10	1,280	160	<10	2,560	<10	<10
LBM678 (H6N6)	40	640	<10	640	1,280	<10	<10	640	<10
LBM694 (H9N2)	<10	<10	10,240	<10	<10	10,240	<10	<10	2,560

[a] Homologous titer. HI, hemagglutinin inhibition; NI, neuraminidase inhibition.

DISCUSSION

In the present study, 1-week aged ducks died of a single HPAIV inoculation, which is earlier than 2-week aged ducks (Tables 1 and 3). These results were in agreement with previous findings, suggesting that younger domestic ducks were more vulnerable to H5 HPAIV infection [14, 25]. Prior infection with H6N6 and H9N2 LPAIVs contributed to an increase in the survival rate of domestic ducks following H5N6 HPAIV infection (Table 3). All ducks that survived with LPAIV pre-infection seroconverted to the homologous strain (Table 3), and the polyclonal antibody against each LPAIV did not neutralize the heterologous HPAIV *in vitro* (Table 5). These results suggest that the induced neutralizing antibody against the LPAIVs was not involved in the protection of the ducks from heterologous HPAIV infection. A previous study revealed that vaccination of Muscovy ducks with an inactivated H9N2 LPAIV adjuvanted with poly I:C did not induce effective immunity against antigenically heterologous H9 viruses [44]. Viral interference was also observed in the Pekin duck (*Anas platyrhynchos domesticus*) superinfected with completely heterologous LPAIV and Newcastle disease virus [26]. Innate immunity induced by live virus infection might play a key role in the cross-immunity of duck species. Activation of toll or RIG-I-like receptors and JAK-STAT signaling pathways was experimentally observed in Pekin ducks infected with H7 LPAIVs and H5N1 HPAIVs [5, 19, 22]. Effectiveness of pre-existing heterologous immunity to subsequently contracted influenza viruses was also observed in mammalian models, such as ferret [13, 20] and guinea pig [34]; mucosal immunity, $CD8^+$ T cell responses, and antibody antigenically stable regions (e.g., HA stalk region) induced by prior live virus infection were assumed to be contributing factors. Based on these previous findings, the study to elucidate how prior infection with H6 or H9 LPAIVs interfered with the pathogenesis of the H5N6 HPAIV is underway.

The LBM678, LBM694, and LBM808 strains were isolated from domesticated ducks in wet markets in Hanoi, Vietnam, during the same period (2014–2015). In this surveillance, HA genes of both LPAIV and HPAIV were detected in some individual ducks. Similarly, HA genes of both H9 and H5 AIVs were detected in individual chickens and ducks in wet markets in Cambodia [12]. These results suggest that co-infections with LPAIV and HPAIV actually occur in domestic duck flocks in the field, and that LPAIVs interfere with the pathogenicity of HPAIV in ducks, leading to subclinical infection with H5 HPAIV. Such phenomena have also been experimentally verified in chickens [16, 24, 32]. The LPAIVs used in the current study have already been confirmed to infect chickens (Supplementary Table 1). Therefore, the virus interference observed in ducks may also occur in chickens due to these LPAIVs and should be confirmed in further studies.

One duck (#8) and chickens inoculated with LBM694 (H9N2) showed relatively higher HI antibody responses than those exposed to LBM678 (H6N6) (Table 1 and Supplementary Table 1); thus, LBM694 might have higher immunogenicity to domestic poultry than LBM678. Similarly, the ducks (#28–#31) that were successfully infected with LBM694 may have been protected from subsequent lethal HPAIV infection via some effect other than humoral immunity (Table 3). Some ducks pre-inoculated with LBM694 (H9N2) developed clinical symptoms. We speculate that successful prior infection with LBM694 (H9N2) led to both positive and negative impacts after subsequent HPAIV infection: causing damage to epithelial cells in the respiratory and/or digestive tracts, contributing to clinical outcome after HPAIV inoculation, in parallel with rapid activation of immune responses and virus clearance. Two ducks that died after HPAIV infection (#24 and #25) were unlikely to be infected with LBM678 by initial inoculation. The remaining ducks that survived with LBM678 (#26 and #27) shed almost no virus after HPAIV inoculation. The LBM678 and LBM808 strains had the same NA subtype (N6), and their cross-reactivity was observed in the NI test, but not in the neutralization test (Table 5). It has been reported that prior inoculation with LPAIVs protected chickens from challenge with virulent viruses of the same NA subtype [23]. In the surviving ducks with LBM678, the induced antibody against N6 NA may have inhibited the NA enzyme activity of LBM808 and its multiple replications at viral entry or primary infection sites.

The present study revealed that pre-infection with LPAIV interferes with HPAIV pathogenicity in domestic ducks, as previously observed in chickens [16, 24, 32]. At present, LPAIV and HPAIV are co-circulating in poultry flocks in some countries [6, 15, 35], and pre-infection with LPAIV might play a similar role to a live vaccine and contribute to the maintenance of HPAIV. The emergence of AIVs with novel gene constellations via genetic reassortments of LPAIV and HPAIV has also been of concern. Our data suggest that the control of HPAIV in poultry requires also efforts to prevent the spread of LPAIV. In addition to AIV surveillance in poultry populations in each country, poultry distribution systems and wet markets that are usually densely populated with various birds should be hygienically improved.

POTENTIAL CONFLICTS OF INTEREST. The authors declare that they have no conflict of interest.

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