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Evaluation of Specific Pathogen-Free Ducks Infected with the Highly Pathogenic Avian Influenza Virus H5N1 Subtype Derived from Wild Birds

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Abstract.- Wild birds of the *Anseriformes* and *Gharadriiformes* are the natural hosts and reservoirs of avian influenza virus (AIV). These birds play a key role in the transmission of the highly pathogenic avian influenza virus (HPAIV) of the H5N1 subtype. The pathogenicity of H5N1 HPAIV in waterfowl appears to have increased. In this study, specific pathogen-free (SPF) ducks (Shaoxing sheldrake) of different ages were used as a model to imitate the natural infection with the H5N1 HPAIV. The results showed that 1–2 weeks old SPF ducks are not sensitive to the virus. However, the virus can replicate in the digestive and respiratory tracts of 3–6-weeks old SPF ducks. There was an inverse ratio between mortality and age (3 and 6 weeks old) of ducks. Although the amount of virus shed by 6-week-old SPF ducks was highest,the mortality rate was the lowest. The disease in ducks shows a range of degree of severity from mild clinical signs to mortality. But the virus can replicate to a high level in some vital organs (brain, pancreas and kidneys) which causes death directly. The high virus titer in the pancreas may be related to the carriage, distribution and spread of the virus within ducks. This study has confirmed that there is a correlation between the pathogenicity of the H5N1 HPAIV and age of waterfowl. The data suggested that the degree of pathogenicity of the virus may be closely related to the development status of the duck's immune system.

Keywords: Pathogenicity, highly pathogenic avian influenza virus (HPAIV), H5N1, SPF ducks, wild birds.

INTRODUCTION

The disease syndrome of avian influenza (AI) is caused by influenza A virus infection. Avian influenza virus (AIV) can be divided into sixteen hemagglutinin (HA) subtypes and nine neuraminidase (NA) subtypes, according to differences in the HA and NA proteins. All the subtypes have already been isolated from waterfowl and other wild birds (Olsen *et al*., 2006). Research showed that wild aquatic birds, especially from the orders *Anseriformes* and *Gharadriiformes* are the natural hosts and reservoirs of AIV (Stallknecht, 1998; Swayne and Halvorson, 2003; Chai *et al*., 2011). Highly pathogenic (HP) AIV belongs to the A class of infectious diseases and causes great losses. Some researchers suggested that the pathogenicity of HPAIV is related to a particular host and virus gene segments (Jiao *et al*., 2005; Li *et al*., 2005; Zhu *et al*., 2008). Pathognicity is also a polygenetic trait and is affected by various other factors. Earlier studies (prior to 2002) showed that the H5N1 HPAIV was not pathogenic in waterfowl (Chen *et al*., 2004), and its evolution was maintained at a relatively stable or static status in the host (Sturm-Ramirez *et al*., 2005). Since its first outbreak in Hong Kong in 2002, when large numbers of wild and domestic waterfowl were infected with the H5N1 HPAIV, there have been increasing numbers of reports of this disease (Ellis *et al*., 2004; Liu *et al*., 2005). It appears that the pathogenicity of the H5N1 HPAIV in waterfowl has been enhanced in recent years. The infection has changed from producing no clinical signs to systemic infection and high mortality. In addition, previous studies have shown that the rate of infection, pathogenicity and mortality associated with the H5N1 HPAIV in ducks may be related to the routes of exposure, types and ages of the ducks (Kishida *et al*., 2005; Kwon *et al*., 2005; Pantin-Jackwood *et al*., 2007; Kwon and Swayne, 2010; Cagle *et al*., 2012). This study is aimed to use specific pathogen free (SPF) ducks as a model to imitate natural infection

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of waterfowls with the H5N1 HPAIV in order to examine its pathogenicity and investigate the relationship between the pathogenicity and the age of infected waterfowl.

MATERIALS AND METHODS

Virus and experimental animals

The Avian Influenza Virus (AIV) A/Bar Headed Goose/Qinghai/3/05 (H5N1) strain was isolated, identified and stored by the National Avian Influenza Reference Laboratory of Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The experimental SPF ducks were raised separately in an isolator, ventilated under negative pressure with high efficiency particulate air (HEPA)-filtered air, and maintained under continuous lighting. Appropriate food and water were provided *ad libitum* access. All experiments using H5N1 viruses, including the test animals (SPF ducks), were performed in biosecurity level-3 Ag (BSL-3-Ag) facilities at Harbin Veterinary Research Institute. All test animals were provided by the animal center of Harbin Veterinary Research Institute.

Animal infection test

Fifty-six SPF ducks (Shaoxing sheldrake) were separated into seven virus-inoculated groups, eight SPF ducks in each group, which comprised ducks of 1, 2, 3, 4, 5, 6 or 12 weeks old age group. All birds were challenged with 10^5 EID_{50} H5N1 HPAIV via ocular, nasal, oropharyngeal and cloacal routes. One day post challenge, five ducks (of the same age as each of the seven virus-inoculated groups) were included as the cohabitation infection animals. In total, thirty-five SPF ducks (not virus-inoculated) were used in the cohabitation infection experiment in this study.The ducks were observed for 21 days and their clinical signs and mortality were recorded daily.

Virus replication and excretion in ducks

On 3rd, 5th, 7th, 10th and 14th day post infection (dpi), swab specimens were collected from the trachea and cloaca from all ducks, and used for virus isolation and titration. At 3 dpi, three virus-inoculated ducks from each group were euthanized. Samples of the brain, trachea, thymus, skeletal muscle, spleen, pancreas, cecal tonsil, bursa of fabricius, lung, kidney and heart were collected and weighed. The organ samples were put into 1mL PBS with antibiotics (penicillin and streptomycin sulfate, Roche) for viral isolation and titration. SPF chicken embryos, 9–11 days old, were used for titration of swabs or isolated virus, and the EID_{50} was calculated by the Reed–Muench method (OIE, 2002).

RESULTS

The clinical signs and mortality

SPF ducks (3 to 5 weeks old) in the challenged and contact groups showed morbidity with obvious clinical signs. At 3 dpi, the ducks exhibited diarrhea, anorexia, torticollis, trembling, ataxia, constant circling, paddling and collapse with neurological signs. Twelve weeks old SPF ducks showed no apparent clinical signs, but had only mild diarrhea and recovered quickly, and they all survived (Table I). The data showed that H5N1 HPAIV had different pathogenic effects in ducks of different ages, which varied from nearly 100% mortality (in 3–5 weeks old ducks) to 100% survival in four groups of ducks (1, 2, 6 and 12 weeks old ducks) (Table I).

One to two weeks old SPF ducks showed mild clinical signs with 100% survival rate, and the virus load in the tissue samples was relatively low. The result showed that 1–2 weeks old ducklings are not sensitive to the highly pathogenic virus. But the 3–5 weeks old SPF ducks showed significant clinical signs. In addition, the mortality rate and time to death were inversely proportional to age in the three groups of 3, 4 and 5 weeks old ducks. In contrast, 6 and 12 weeks old SPF ducks showed no obvious clinical signs with survival rate of 100% in both two groups. This data suggested that a well-developed immune system can inhibit virus replication and prevent the occurrence of death.

Shedding of H5N1 HPAIV

The results showed that no virus was detected in tracheal and cloacal swabs from 1, 2 and 12 weeks old ducks in the infection and contact groups (Table I). At 3rd and 5th dpi, the virus was detected in

Table I.- Mortality, mean time of death and virus isolation in infected or contact ducks with A/Bar Headed Goose/Qinghai/3/05 (H5N1) virus (expressed as log10EID_{50}/0.1mL).

Age of Duck	Route of exposure [a]	Virus isolation [b] (log10(EID_{50}/0.1mL)±SD)						Mortality [c]	MDT [d]
		Tracheal titer 3 dpi	Cloacal titer 3 dpi	Tracheal titer 5dpi	Cloacal titer 5 dpi	Tracheal titer 7 dpi	Cloacal titer7 dpi		
1w	Inoculation	0(0/5) [e]	0(0/5)	0(0/5)	0(0/5)	0(0/5)	0(0/5)	0(0/5)	-
	Contact	0(0/5)	0(0/5)	0(0/5)	0(0/5)	0(0/5)	0(0/5)	0(0/5)	-
2w	Inoculation	0(0/5)	0(0/5)	0(0/5)	0(0/5)	0(0/5)	0(0/5)	0(0/5)	-
	Contact	0(0/5)	0(0/5)	0(0/5)	0(0/5)	0(0/5)	0(0/5)	0(0/5)	-
3w	Inoculation	1.74±0.03 (5/5)	0.97±0.02 (5/5)	0	0	0	0	5/5	3-5
	Contact	0.98(2/5)	0.95(2/5)	0(0/2)	0(0/2)	0	0	5/5	3-6
4w	Inoculation	1.18(5/5)	1.06(5/5)	1.35(2/4)	1.22(2/4)	0(0/3)	0(0/3)	2/5	5-7
	Contact	1.48±0.04 (4/5)	0.95 (4/5)	1.58±0.14(3/5)	1.48±0.04 (3/5)	0 (0/3)	0 (0/3)	2/5	6-7
5w	Inoculation	1.38±0.04 (5/5)	1.31±0.06 (5/5)	1.32 (2/5)	1.08±0.13 (2/5)	0 (0/4)	0 (0/4)	1/5	5-7
	Contact	1.53±0.06 (5/5)	1.21±0.02 (5/5)	1.43 (2/5)	1.17 (2/5)	0 (0/4)	0 (0/4)	1/5	6-7
6w	Inoculation	2.03±0.18 (5/5)	1.65±0.02 (5/5)	1.22 (2/5)	0.95 (2/5)	0 (0/5)	0 (0/5)	0/5	-
	Contact	2.17±0.01 (5/5)	1.47±0.04 (5/5)	1.32±0.09 (5/5)	0.98 (2/5)	0 (0/5)	0.95 (1/5)	0/5	-
12w	Inoculation	0(0/5)	0(0/5)	0(0/5)	0(0/5)	0(0/5)	0(0/5)	0/5	-
	Contact	0(0/5)	0(0/5)	0(0/5)	0(0/5)	0(0/5)	0(0/5)	0/5	-

[a]Ducks were inoculated via ocular, nasal, oropharyngeal and cloacal routes with 10^5 EID_{50} of the A/Bar Headed Goose/Qinghai/3/05 (H5N1) virus or exposed by direct contact with the virus infected ducks.

[b]Mean log10 titers expressed as EID_{50}/0.1mL from oropharyngeal and cloacal swabs were sampled from five individual ducks on the days indicated. The limit of detection was $10^{0.95}$ EID_{50}/0.1mL.

[c] Number of dead ducks/number of inoculated or exposed ducks.

[d] Mean time to death, in days.

[e] Number of positive ducks/number of inoculated or exposed ducks.

Table II.- Virus isolation in organs of ducks infected with A/Bar Headed Goose/Qinghai/3/05 (H5N1) virus (expressed as log10EID_{50}/0.1mL)

Virus titer in organs on day 3 [a]	Age of duck (Weeks)						
	1	2	3	4	5	6	12
Brain	1.11±0.10 (3/3)[b]	0.96±0.01 (3/3)	4.17 (2/3)	3.38±0.18 (2/3)	2.59±0.08 (3/3)	1.83 (3/3)	0.99 (1/3)
Trachea	0 (0/3)	2.17±0.01 (3/3)	2.89±0.24 (3/3)	2.80±0.05 (3/3)	2.71±0.07 (3/3)	2.69±0.08 (2/3)	2.06±0.20 (3/3)
Thymus	0 (0/3)	1.22 (2/3)	1.29±0.06 (3/3)	1.25±0.06 (3/3)	0.95 (2/3)	0.95 (2/3)	0 (0/3)
Muscle	0.95 (2/3)	0.98 (1/3)	2.21±0.06 (2/3)	1.81±0.04(2/3)	0 (0/3)	0 (0/3)	0 (0/3)
Spleen	0.98 (2/3)	1.75 (1/3)	1.68 (2/3)	1.63 (2/3)	1.72±0.05 (2/3)	2.17±0.01 (3/3)	3.09±0.23 (3/3)
Pancreas	0.96±0.01 (3/3)	2.17 (2/3)	3.57±0.09 (2/3)	3.38±0.10 (2/3)	3.32 (3/3)	3.06±0.20 (3/3)	0.95 (1/3)
Cecal tonsil	0.98 (1/3)	0.95 (1/3)	1.68±0.01 (2/3)	1.63 (2/3)	0.95 (1/3)	1.11±0.10 (3/3)	1.94±0.20 (3/3)
Bursa of Fabricius	0.95 (1/3)	0.98 (1/3)	0.98 (2/3)	0.98±0.01 (2/3)	1.22 (2/3)	2.67±0.07 (3/3)	0.95 (1/3)
Kidney	1.40±0.03 (3/3)	2.06±0.20 (3/3)	3.80±0.03 (3/3)	3.65±0.03 (3/3)	3.54±0.08 (3/3)	3.44±0.10 (3/3)	2.94±0.20 (3/3)
Lung	1.05±0.11 (3/3)	2.67±0.07 (3/3)	3.06±0.20 (3/3)	2.94±0.20 (3/3)	2.83±0.01 (3/3)	2.71±0.07 (3/3)	2.5 (3/3)
Heart	0.98 (1/3)	1.63 (2/3)	1.81±0.04 (2/3)	1.64±0.02 (2/3)	1.27±0.07 (2/3)	1.08±0.13 (1/3)	0.98 (1/3)

[a] Tissue samples were obtained from the ducks 3 days after challenge with 10^5 EID_{50} of the A/Bar Headed Goose/Qinghai/3/05 (H5N1) virus (expressed as log10EID_{50}/0.1mL±SD).
[b] Number of positive ducks / Number of sampled ducks.

tracheal and cloacal swabs from 3, 4, and 5 and 6 weeks old ducks in both infected and cohabitated groups; the viral load reached $10^{2.17}$ EID_{50}/0.1 mL and $10^{1.65}$ EID_{50}/0.1 mL. At 7th dpi, one 6-week-old duck was detected to have a low level of virus in a cloacal swab, at $10^{0.95}$ EID_{50}/0.1 mL. There was no significant relationship between the quantity of virus shed by 3–6 weeks old ducks and age. However, there was an obvious inverse correlation between age and mortality: the mortality rate decreased with an increase in age.For instance, the ducks in the 6 weeks old group showed no deaths although the amount of virus shedding in this group was high.At 10th and 14th dpi, no virus was detected in tracheal and cloacal swabs from either group, data were not shown.

Replication of H5N1 HPAIV in ducks

Although this virus strain could not cause disease and death in ducks of all ages, virus was detected in the brain, trachea, thymus, muscle, spleen, pancreas, cecal tonsil, bursa of fabricius, kidney, lung and heart of the ducks tested (at 2–4 weeks) in the challenged group,the virus load in the brain reached $10^{4.17}$ EID_{50}/0.1mL (Table II). This data showed that this highly virulent virus can replicate to a high level in many organs of the duck. When the viral replication was compared at different ages, the viral load in the brain, pancreas, kidneys, lung and trachea in 3–6 weeks old ducks was significantly higher than at 1, 2 and 12 weeks old ducks in which the most significant differences were found for the brain, kidney and pancreas. It is speculated that the rapid death of ducks is directly related to the high dose of virus in these organs.

DISCUSSION

Almost all subtypes of AIV have been isolated from wild waterfowls in the world. The high density of ducks and the poor biosafety measures in the duck farms play an important role in the evolution and prevalence of avian influenza. It was reported that infection with H5N1 AIV of earlier isolates under natural conditions did not cause disease and death in ducks (Chen *et al*., 2004). However, recent studies indicated that the pathogenicity of H5N1 HPAIV in waterfowls has shown considerable differences

(Hulse-Post *et al*., 2005; Kishida *et al*., 2005; Sturm-Ramirez *et al*., 2004, 2005). The waterfowls could serve as the carriers of HPAIV, allowing the virus replication in the intestine and the virus excretion without systemic infection and death (Hulse-Post *et al*., 2005; Kishida *et al*., 2005). On the other hand, the infection of certain virus in ducks could result in the shedding of virus via the throat and cloaca routes with obvious clinical signs and the occurrence of death (Sturm-Ramirez *et al*., 2004; Kishida *et al*., 2005). Epidemiological investigation and experimental studies have shown that wild and domestic ducks are susceptible to AIV under different conditions (Chen *et al*., 2004; Cooley *et al*., 1989; Shortridge *et al*., 1998). However, the relationship between the H5N1 virus pathogenicity and the age of infected ducks was still unclear.

After SPF ducks of different ages had been infected with virus, obvious disease and deaths appeared mainly in 3, 4 and 5 weeks old groups. The clinical signs seen in the ducks were similar to those in previous reports in which the effect of HPAIV in ducks can range from mild disease to death, with different degrees of severity of clinical signs (Ellis *et al*., 2004; Hulse-Post *et al*., 2005; Kishida *et al*., 2005; Nguyen *et al*., 2005; Zhou *et al*., 2006). The results showed that the virus load in 3–5 weeks old infected SPF ducks was correlated with age, but the degree of clinical signs and mortality, and even the time of death, showed an inverse ratio. The mortality rate of the infected ducks reduced gradually along with the increase in age among which the 5-weeks old ducks had the lowest mortality rate. The data suggested that the pathogenicity of the H5N1 HPAIV is correlated with the developmental status of the duck immune system. As the immune system develops, the susceptibility of ducks to the virus infection and the severity of the diseases it caused gradually decrease.

All ducks in the 6-week-old group showed no obvious clinical signs or death, but high levels of virus shedding were detected. In addition, at 7^{th} dpi, the virus was detected in the cloaca of the cohabiting ducks. Migratory waterfowl can be long-term carriers, and can spread the virus over their migration routes and nesting areas (Hulse-Post *et al*., 2005). Infected ducks may carry, spread, reconstruct and shed the virus for long periods under certain conditions (Li *et al*., 2003, 2004). Although the results of this study did not show persistent virus shedding from infected ducks, no obvious clinical signs in 6 weeks old SPF ducks and low mortality in 5 weeks old SPF ducks were seen in certain groups, which may provide favorable conditions for the spread of the virus (Kwon *et al*., 2005; Songserm *et al*., 2006). In addition, all 6 weeks old ducks showed no obvious disease signs and death but all ducks showed high levels of virus shedding. This result is consistent with previous report that ducks could remain clinically healthy while carrying the H5N1 AIV, thus serving as the "Trojan horse" of H5N1 AIV and allowing the silent dissemination of the virus (Hulse-Post *et al*., 2005).

High titers of virus were detected in the trachea and cloaca at 3^{rd} and 5^{th} dpi in the 3–6-weeks old challenged and contact groups, which demonstrated that the virus can replicate in the digestive and respiratory tracts at this age. With an increase of age from 3 to 6 weeks, the number of infected cohabiting ducks increased gradually. The quantity of virus shed from the trachea increased gradually from 4 weeks old with a degree of regularity. Previous research has shown that the quantity of virus shed from the trachea is proportional to the pathogenicity. The sensitivity to the virus infection decreased when the ducks are beyond 6 weeks old in this study. In addition, the quantity of virus shed from the tracheas of contact group ducks was higher than in the challenged group. This indicated that, under natural conditions, and especially with close contact, the virus spreads easily among individuals. Compared with 3–5 weeks old groups, increased virus replication and shedding of the virus from trachea and cloaca were detected in the 6-weeks old group post challenge, but no deaths occurred in this group. It suggested that the specific immune response capable of restricting the virus pathogenicity strengthens as the immune system matures. Therefore, no deaths occurred in 6 weeks old ducks although the virus could still replicate to high titers. In this study, 7–11-week-old ducks were not challenged. However, the above results showed obvious virus shedding after infection of 3–6-weeks old ducks with AIV, which demonstrated that ducklings play a key role in the spread of AIV.

No deaths occurred in 12-weeks old ducks

after challenge, and no virus shedding was detected from the trachea and cloaca. Different viral loads were detected in most of the organs in the challenged group at 3 dpi. At about 12 weeks ducks are beginning to develop into adults. Although these older ducks can become infected with the highly pathogenic strain, there was no significant spread of the virus in this group.

The infection of H5N1 AIV could result in different degree of damage in which viruses of high virulence in ducks can replicate to a high level in many organs. However, the viral replication in ducks of different ages varied. The virus load in the brain, pancreas, kidneys, lung and trachea was obviously higher in 3–6-week-old than in 1-, 2-, and 12-week-old ducks. In particular, the brain, pancreas and kidney showed significant differences. It may be speculated that a high dose of virus in the organs can directly cause rapid death of the duck. Previous reports have suggested that the pancreas is an important site for the replication of influenza virus in ducks (Capua and Mutinelli, 2001; Perkins and Swayne, 2002; Liu *et al*., 2006). In this study, high doses virus could be obtained from the pancreas of the tested ducks, and these results are consistent with previous research.

The results showed that ducklings were more susceptible to AIV than older ducks, and that mortality varied with age. This indicates that the pathogenicity of the HPAIV H5N1 subtype in waterfowl is related to age. It may be speculated that the pathogenicity of the virus may be closely related to the development of the immune system of the duck. Ducklings showed obvious virus shedding after infection, which shows that they play a key role in the spread of AIV. The distinct biological characteristics and the open breeding methods of domestic waterfowl, which allows them to make frequent contact with poultry and other wild birds, provide convenient conditions for the spread of the avian influenza virus over wide areas and long distances. Therefore, it is necessary to monitor infection in ducklings in order to prevent outbreaks and reduce the prevalence of AI.

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Conflict of interest declaration

There is no conflict of interest in our research or paper.

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