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Short Communication

Protective Efficacy of the Commercial Vaccine against H9N2 Avian Influenza and Newcastle Disease in Rare Wild Birds in Shanghai Zoo

Yingying Wang[1], Sufen Zhao[2]* and Qunxiu Liu[3]*

[1]*Shanghai Zoo, Shanghai, China, 200335*
[2]*Beijing Key Laboratory of Captive Wildlife Technologies, Beijing Zoo, Beijing, China, 100044*
[3]*Shanghai Urban Construction Vocational College, Shanghai, China, 201415*

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QL and SZ designed the experiments. YW and QL collected the samples. YW performed the majority of the experiments and wrote the paper.

Key words
Shanghai Zoo, wild birds, Avian influenza, Newcastle disease, Antibody protection efficiency

ABSTRACT

Avian influenza (AI) and Newcastle disease (ND) are the two diseases which most threaten the health of birds and poultry. To prevent these two diseases, poultry immunization procedures have been rigorously standardized. Wild birds in Shanghai Zoo are currently immunized against both diseases with commercial vaccines for birds, but their immune status and the immune effect are not very clear. In order to determine the protective effect of the current AI (H9 subtype) and ND vaccines on wild birds in Shanghai Zoo and to explore a more reasonable and effective immunization scheme, the hemagglutination inhibition (HI) test was used to detect antibodies in some rare wild birds in the zoo six months after vaccination and to investigate the protection efficiency of the two vaccines. The results show that the H9 subtype AI immunization programs adopted by Shanghai Zoo can enable wild birds such as pheasants, waterfowl, and waders to produce antibody titers that meet national standards. The current immunization program against ND can provide sufficient protection for pheasants and waterfowl, but in some wading birds fails to reach 100% due to different species, which needs to be verified in future work. Therefore, if necessary, the vaccination interval between administration of AI vaccines (H9 subtype) can be appropriately extended to reduce the stress reaction caused by capture and vaccination and reduce the economic cost of disease prevention and control. In regard to the ND vaccine, different immunization programs should be formulated for different bird species; immunization procedures can be optimized, and the antibody level of wading birds should be monitored after vaccination.

Avian influenza (AI) and Newcastle disease (ND) are the two most serious viral infectious diseases for rare wild birds (Dimitrov *et al*., 2010). Wild birds are the natural host of avian influenza virus (AIV) and the gene repository for all AIVs, and various HA and NA subtypes have been found in wild birds. Although H9 subtype AIV is a low-pathogenic AIV, which means it cannot directly cause morbidity or death in wild birds, but it can be latent in this population and effectively replicate and spread (Wang *et al*., 2016). In addition, it can form new highly pathogenic subtypes of AIV through gene reassortment, such as the emergence of H7N9 and H10N8, that can cause human infection and death (Centers for Disease and Prevention, 2013; Chen *et al*., 2014; Gao *et al*., 2013). Therefore, the replication and transmission of H9 subtype AIV in wild birds must not be ignored. Newcastle disease virus (NDV) can infect more than 240 species of birds in 27 orders, mainly infecting chickens, pigeons, quail, turkeys, etc. (Brown and Bevins, 2017). In recent years, its host range has been expanding, and waterfowl such as geese and ducks can also be infected (Dimitrov *et al*., 2016). Therefore, the threat of these two viruses to wild poultry and human health must not be ignored.

There are nearly 20 orders and more than 200 kinds of zoo birds in Shanghai Zoo, including waterfowl, wading birds, and ornamental birds. Every autumn and winter, there are also exotic wild birds that inhabit here. Thediversity can lead to an increased risk of AIV and NDV transmission and outbreak. Therefore, we should attach

* Corresponding author: liuqunxiu@126.com, zhaosufen111@126.com
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great importance to the prevention and control of these two diseases. At present, vaccine immunization is the main measure to prevent and control AI and ND (Lozano-Dubernard *et al*., 2010). Shanghai Zoo immunizes captive wild birds against these two diseases in spring and autumn every year. However, the protective effect of the current commercial poultry vaccine on wild birds under the Zoo's current immunization program is still unclear. In order to determine the protective effect of H9 subtype AI and ND vaccines on wild birds, this paper evaluates the immunization effect of these vaccines on this population. The results of this study can be useful for designing management programs for captive wild birds.

Materials and methods

The commercial H9 subtype AI inactivated vaccine (SD696 strain) was produced by Ck/SD/6/96 (Veken Biotechnology, Harbin, China). The vaccine contained the inactivated AIV A/chicken/Shandong/6/96 (H9N2) strain (abbreviated SD696 strain), and the virus content before inactivation was more than $1.0\times10^{8}EID_{50}$/ 0.2mL. The ND-inactivated chicken vaccine (CLONE30 strain) was produced by Interwell International Co., Ltd.; MA5+CLONE30 new-branch double-attenuated vaccine is produced by Interwell International Co. Ltd.

For wild birds weighing less than 1.5kg, 0.25mL of H9 subtype AI inactivated vaccine was injected intramuscularly, and for birds weighing more than 1.5kg, 0.5ml was injected intramuscularly. Inactivated Newcastle disease vaccine (CLONE30 strain) was used for intramuscular injection, and the immune dose of Newcastle disease was the same as that of avian influenza vaccine. At the same time, 2 doses/feather were used to immunize MA5+CLONE30 new double attenuated vaccine. All birds were manually restrained for vaccine administration.

Serology samples were obtained by jugular venipuncture from all birds during manual restraint for management purposes (health check-up or tagging). Sterile blood samples were collected six months after immunization. The serums obtained were frozen at -20℃ and transported to the laboratory for storage until testing. The sampling and vaccination procedures were performed under the conditions of manual restraint.

The hemagglutination inhibition (HI) test antigen (Ck/SD/6/96) was produced by Harbin Veken Biotechnology Co., Ltd. (Harbin, Heilongjiang, China). The antibody titers were determined by HI tests using 1% chicken red blood cells according to GB/T 18936-2003, the highly pathogenic avian influenza diagnostic method for the determination of serum antibody levels. Ultimately, it was found that the highest diluted multiples of the serum which completely inhibited four coagulation units of antigen as HI titer were valid with the titer of negative control ≤ 2 log2 and the titer error of positive control ≤ 1 log2. According to the Chinese Ministry of Agriculture's standards, HI titers of AI ≥5 log2 are appropriate.

ND HI antibody detection was determined according to the method specified in GB/T 16550-2008 Newcastle disease diagnostic technical standard. The highest dilution of serum that completely inhibits 4 hemagglutination units of antigen was used as the HI titer. The test result was only valid when the titer of the negative control well was no more than 2log2 and the error of the titer of the positive control well was no more than 1 titer. According to the relevant standards of the Ministry of Agriculture, the HI titer of ND ≥ 4log2 was determined to meet the immune standard.

Results and discussion

Samples of fresh feces of test birds were collected for nucleic acid detection of subtype AIV and NDV, and the results were negative. Which reduce the risk of non-immune antibodies produced by test birds infected with these two viruses and that maybe affect the test results. which was conducive to the study of immune antibodies.

The immunization procedures of H9 subtype AI and ND in Shanghai Zoo were carried out every spring and fall. The blood samples were collected after immunization. Post-immunization stress response was one important indicator for vaccine effect evaluation. Years of practice indicated that H9 subtype AI vaccine had greater side effects on spotted-billed penguins. Therefore, we did not vaccinate Spheniscus demersus against such subtypes. We normally vaccinated other birds with the two vaccines. The detailed immunization is available in Table I.

After vaccination, the living conditions of the wild birds in the project were observed. After three consecutive weeks of observation, it was found that H9 subtype AI and ND vaccine injection did not have a great impact on the diet, exercise, and other behaviors of wild birds, and no deaths occurred among the population.

The H9 subtype AI immunization programs adopted by Shanghai Zoo could enable wild birds such as pheasants, waterfowl, and waders to produce antibody titers that meet national standards. The presence of undetectable H9 subtype AI antibodies in brown horse chickens may be due to the failed vaccination.

The current immunization program against ND could provide sufficient protection for pheasants and waterfowl, but the production of ND antibodies in some wading birds failed to reach 100%; these birds include flamingos, Eurasian spoonbills, red-crowned cranes, and hooded cranes. The reason for the difference seemed to lie in the different breeds, which needs to be verified in future work.

Table I. Investigation of antibody 6 months after Newcastle disease (ND) antibody of H9 subtype avian influenza (AI) vaccination.

Common name (Scientific name)	Number tested	Immune information after ND vaccination		Immune information after AI vaccination	
Species		Positive number	Protection rate (%)	Positive number	Protection rate (%)
Waders	**42**				
Siberian crane (*Grus leucogeranus*)	3	3	100	3	100
Red-crowned crane (*Grus japonensis*)	3	2	67	3	100
Blue crane (*Anthropoide sparadiseus*)	2	2	100	2	100
Hooded crane (*Grusmonacha*)	4	3	75	4	100
White-naped crane (*Grus vipio*)	3	3	100	3	100
Oriental white stork (*Ciconiaboyciana*)	13	12	92	13	100
Crowned crane (*Balearica pavonina*)	3	3	100	3	100
Flamingo (*Phoenicopterus ruber*)	4	2	50	4	100
Wattled crane (*Bugeranus carunculatus*)	1	1	100	1	100
Marabou stork (*Leptoptilos javanicus*)	1	1	100	1	100
Common crane (*Grus grus*)	1	1	100	1	100
Eurasian spoonbill (*Platalea leucorodia*)	4	2	50	4	100
Pheasants	**51**				
Blue eared pheasant (*Crossoptilon auritum*)	3	3	100	3	100
Brown eared pheasant (*Crossoptilon mantchuricum*)	2	2	100	0	0
Black curassow (*Crax alector*)	3	3	100	2	0.67
Golden pheasant (*Chrysolophuspictus*)	3	3	100	3	100
Lady Amherst's pheasant (*Chrysolophus amherstiae*)	1	1	100	1	100
Silver pheasant (*Lophuranycthemera*)	11	10	0.91	11	100
Taiwan blue pheasant (*Lophura swinhoii*)	3	3	100	3	100
Pea fowl (*Pavonini*)	22	22	100	22	100
Reeves's pheasant (*Syrmaticus reevesii*)	3	3	100	3	100
Columbiformes	**2**				
Victoria crowned-pigeon (*Goura victoria*)	2	2	100	2	100
Waterfowl	16				
African penguin (*Spheniscus demersus*)	10	10	100	/	/
Black swan (*Cygnus atratus*)	6	6	100	6	100

The protective effect six months after immunization of ND in wading birds and pheasants showed that the efficacy of pheasants was much better than that in wading birds, this indicated that there were some differences in the vaccines immune effects used in different kinds of wild birds.

The ND antibody level of wading birds was significantly lower than that of pheasants. The reasons for this outcome should be investigated, and the ND vaccination procedures for wading birds should be optimized and adjusted according to the findings.

The effective protection period of H9 subtype AI vaccine in wild birds is one year. Six months after vaccine immunization, the H9 subtype AI antibody of wading birds, pheasants, and waterfowls remained at a high level and an effective protection rate. It is suggested that the immunization interval of H9 subtype AI vaccine can be appropriately extended to reduce the stress response caused by wild bird capture and vaccination, which can also reduce the economic cost of disease prevention and control.

At present, the vaccination interval of NDV in pheasants and waterfowls can also be appropriately extended. However, for wading birds, we should strengthen

the monitoring of ND antibody level and pay attention to proper guidance during immunization.

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IRB approval

Shanghai Zoological Park Approval was obtained for research involving animals (No.SZP006).

Ethical statement

All procedures performed in accordance with the Principles of Code for Zoo Management of Chinese Association of Zoological Park (CAZG), and guidelines of the Scientific Research Committee of Shanghai Zoological Park.

Statement of conflict of interest

The authors have declared no conflicts of interest.

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