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Primary survey of avian influenza virus and Newcastle disease virus infection in wild birds in some areas of Heilongjiang Province, China

Yu-Ping Hua*, Hong-Liang Chai, Si-Yuan Yang, Xiang-Wei Zeng, Ying Sun

College of Wildlife Resources, Northeast Forestry University, Harbin 150040, P. R. China

Two hundred thirty specimens of wild birds were collected from some areas in Heilongjiang Province during the period of 2003~2004, including two batches of specimens collected randomly from a same flock of mallards in Zhalong Natural Reserve in August and December, 2004, respectively. Primary virus isolation and identification for avian influenza virus (AIV) and Newcastle disease virus (NDV) were performed. The results showed that only two specimens of young mallards collected from Zhalong Natural Reserve in August, 2004 were positive to AIV (isolation rate 0.9%), and one strain (D57) of these two virus isolates was identified to be H9 subtype by hemagglutination inhibition test. Meanwhile, the two batches of blood serum samples of mallards from Zhalong were also examined for antibodies against AIV and NDV. Among 38 blood serum samples collected in August, antibodies against the hemagglutinin of H1, H3, H5, H6 and H9 subtypes of AIV were found in 1, 0, 2, 0 and 8 samples, respectively; and 11 samples were found with antibody against NDV. Whereas the NDV isolation in both two batches of specimens of mallard was negative, all of the 32 blood serum samples collected in December were negative for antibodies against AIV and NDV.

Key words: AIV, China, Heilongjing Province, NDV, wild birds

Introduction

The virus causing avian influenza belongs family Orthomyxoviridae. The virus could be divided into several subtypes based on the antigenic relationships of the virus glycoproteins, hemagglutinin (H) and neuraminidase (N). Presently, 15 H subtypes (H1-H15) and 9 neuraminidase subtypes (N1-N9) have been recognized [1]. Newcastle disease virus (NDV) is a member of Rubulavirus genus subfamily Paramyxovirinae in the family Paramyxoviridae. Avian paramyxovirus has nine serological subtypes, and NDV is formally recognized as avian paramyxovirus 1 (APMV-1) [1]. Avian influenza (AI) and Newcastle disease (ND) are two serious infectious viral diseases which disserve poultry birds. At present, all subtypes of avian influenza virus (AIV) and some serological subtypes of APMV have been isolated in wild waterfowl, so it has been believed that wild birds, especially wild waterfowl, are the natural reservoirs of AIV and APMV virus , and as the transmission agents they play an important role in spread of these diseases to domestic poultry [2,4,7]. Once the pathogenetic stain is infected into domestic poultry, the epidemic disease will break out and lead a great loss to the economy [11].

Heilongjiang Province in China is vast in territory and diverse in natural environments. It has a variety of habitats such as forests, lakes, rivers, plains and swamps, which supply optimal places for the inhabiting and breeding of birds, especially for birds from subtropical zone and cool temperate zone. There have been 371 species of wild birds recorded in Heilongjiang Province. Amongst them, 149 species are Passerine birds, 222 species are non-Passerine birds including 144 species of waterfowl. Within the waterfowls, 65 species are natatorial birds such as geese, ducks and gulls; and 79 species are wading birds such as herons, storks, granes and some shorebirds. The Zhalong Natural Reserve, located at the Song-neng Plain in the western part of Heilongjiang Province, has widespread reed swamps. So, it is one of the biggest National Natural Reserves in China, which mainly focuses on the conservation of crane and other huge waterfowls and swamp ecological system. Due to the good natural environments, the reserve has become one of important breeding and migrate-rest places for those migrating birds that live in the south area through the winter and breed in the north in spring.

In this study, the primary examination was performed for understanding the situation of AIV and NDV infection or virus carrying in the wild birds in Heilongjiang Province. The examination and analysis on the specimens of young wild ducks obtained from Zhalong Natural Reserve were a

*Corresponding author
Tel: +86-451-82190745; Fax: +86-451-82190745
E-mail: yuping_hua@126.com

part of central work. The objective of this research was to understand more about the role of wild birds in spread of AIV or NDV and provide some basic information for adopting efficient prevention and control measures to reduce and avoid the transmission of AIV and NDV from wild birds to poultry.

Materials and Methods

Collection of the specimens

Source of the specimens: During 2003 and 2004, 230 specimens of wild birds which belong to 63 birds species were obtained from Zhalong Natural Reserve, Sanjiang Natural Reserve, Maoershan National Forestry Park, Archeng, Mishan, Baoquanling, Haerbin, Pingshan, Daqing and some other places in Heilongjiang Province. Among the 230 samples, 122 were collected from wild waterfowls which accounted for 53% in total number. Most samples were bowels and entire ashes. Tracheal and cloacal swabs and blood serum samples of 38 individuals were gathered randomly from a flock of about 200 young wild ducks captured in Zhalong Natural Reserve in August 2004 and raised in an outdoor cage together. And the tracheal and cloacal swabs and blood serum samples were collected randomly again from 32 individuals of this flock in December, 2004.

Process of specimens: Viscus tissues, mainly included larynx, trachea, lungs and rectum were grinded and treated with antibiotics. Tracheal and cloacal swabs were put into the glycerol-physiological saline with antibiotics, then put in 37°C for 2 hours. Afterward the swabs were pressed adequately on the wall of cuvettes1, the suspension was centrifuged in the speed of 3,000 rpm for 30 min and the topper clear liquid was collected, then the liquid was filtrated with filter membrane (0.22 μm). The filtrated liquid was taken as the inoculated material after aseptic examination. Blood was collected and serum was isolated immediately, then stored in –20°C.

Virus isolation and identification

Embryo inoculation: 0.2 ml inoculated material was inoculated into the allantoic cavity of 9~11 days old specific-pathogen-free (SPF) chicken embryonated egg. Each sample inoculated three eggs. After 72~96 hrs of incubation at 37°C, the eggs were chilled and allantoic fluids were harvested in axenic and the undiluted allantoic fluids were tested for H activity. We continually passed the allantoic fluids for 3 generations on embryonated eggs, then harvested the allantoic fluids for virus detection by hemagglutination (HA) test and RT-PCR.

Hemagglutination assay: Allantoic fluids of embryonated eggs in each generation were examined by HA test.

RT-PCR: A pair of primers, DLp1: 5'-ATC ACT CAC TGA GTG ACA TC-3' and DLp2: 5'-CCT CCA GTT TTC TTA GGA TC-3', was designed based on the comparison of the conserved sequences of the nuclear protein (NP) gene of AIV published in GenBank database (National Center of Biotechnology Information, NCBI, USA). Un12: 5'-AGCA AAAGCAGG-3', an universal primer, was used in reverse transcription. The total RNA was extracted from the third passage of inoculated allantoic fluid by using TRIZOL RNA extraction kit (Invitrogen, USA) according to the manufacturer's instructions, and was stored at –70°C. The procedure of the reverse transcription was as follows: mix 8 μl RNA and 2 μl Un12 primer (10 pmol/μl). This mixture was heated to 70°C for 10 min in a water bath and then cooled immediately in an ice-water bath for 5 min, then 5 μl buffer, 4 μl dNTP, 0.5 μl Rnasin, and 1 μl M-MLV was added, finally the DEPC water was added making the entire volume up to 25 μl. The reaction mixture was incubated at 42°C for 60 min in a water bath, then was kept at 95°C for 5 min. The cDNA production was stored at –30°C. PCR was performed in a reaction mixture containing 5 μl of 10-times reaction buffer, 4 μl dNTP, 2 μl of each primer, 1 μl Taq DNA polymerase (5U/μl), and 5 μl cDNA. Then water was added to make the entire volume up to 50 μl. The PCR condition for the amplification of NP was 95°C for 5 min, 30 cycles consisting of 45 s at 94°C, 45 s at 52°C, 45 s at 72°C, and 7 min at 72°C. PCR product was detected by electrophoress in 1% gels with ethidium bromide staining.

Identification of hemagglutinin subtype: AIV positive allantoic fluids specimen by HA and RT-PCR were further characterized for H subtype by hemagglutination inhibition (HI) test. Standard AIV positive sera and antigens of H1, H3, H5, H6 and H9 subtypes were supplied by Harbin Veterinary Research Institute, China.

Detection of specific antibody in sera

The blood sera of mallards collected from Zhalong Natural Reserve were examined for antibodies against AIV and NDV by HI test. NDV V4 antigen was supplied by Animal Medical Laboratory of College of Wildlife Resource in Northeast Forestry University, China.

Results

Virus isolation and identification

Virus isolation from all 230 wild birds specimen was performed. The result showed that only two AIV isolates numbered D57 and D58, within the 38 young mallards specimen collected in Zhalong Natural Reserve in August, were obtained. Hemagglutinin titers of allantoic fluids of first generation in D57 and D58 were all 2^6, and amplification of AIV NP gene by RT-PCR also gave the positive results. Not like the first batch of 38 specimen, the second batch of

Table 1. Detection results of serum antibodies against AIV and NDV by HI in young mallards gathered in Zhalong Natural Reserve in August, 2004

		Blood serum samples																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
AIV	H1																			
	H3																			
	H5	2^3																		
	H6																			
	H9								2^3			2^3	2^6							
NDV									2^3				2^5				2^3			2^3
		Blood serum samples																		
		20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
AIV	H1							2^3												
	H3																			
	H5														2^2					
	H6																			
	H9										2^6			2^8	2^6		2^6			2^5
NDV									2^4		2^5		2^6	2^7	2^6		2^6			2^5

32 specimen collected 4 months later from the same block of young wild ducks fed in Zhalong Natural Reserve were all negative for AIV isolation. At present, the D57 isolate was identified to be the H9 subtype of AIV by HI. All of the 230 specimens were negative for NDV isolation.

Detection of specific antibodies in sera

The blood sera of young mallards from Zhalong Natural Reserve in August and December were examined respectively for AIV and NDV specific antibodies by HI. Table 1 showed the result of the sera obtained in August. The sera obtained in December were all negative for AIV and NDV.

Discussion

AIV isolation and HA subtypes primary identification was done from 230 specimens of wild birds gathered in Heilongjiang province during 2003 and 2004. As a result only two specimens of young wild ducks gathered in Zhalong Natural Reserve in August, 2004 were positive for AIV, the total isolation rate was about 0.9%. H subtype of the D57 isolate was H9 identified by HI test.

The results in this study were consistent with the reports before, of that wild waterfowl especially wild ducks were much easier to be infected and to harbor AIV, whose frequency of harboring AIV was higher than that of other species. Moreover, the risk of young mallards to be infected was higher, and the rate of virus isolation was also higher [3,10].

According to the primary results of AIV isolation and serum antibody detection, that D57 isolate was identified to be H9 subtype and the highest proportion of serum antibody for H9 subtype, it could be deduced H9 may be a dominant AIV subtype carried by wild birds in the area of Zhalong Natural Reserve.

It is believed that the isolating rate of AIV in wild birds is conspicuous lower in winter than in summer [5]. When the second batch of specimens was collected in Zhalong, the flock of wild ducks had been fed for 4 months in a cage after been captured, so it could greatly reduce the chances of being infected again by outside source of virus. Which may be the main reason that, unlike in the specimens collected in August, virus isolation in all the specimens gathered in December 2004 were negative, even though they were from the same flock of ducks.

As the case of the lower isolating rate of AIV (0.9%) in this study, we think the fresh extent of the specimen should have very important influence on the isolating rate of virus. And sampling wild birds is very difficult and the specimens are very hard to store or keep in the standard condition after sampling in the wild, which is the problem we must pay attention to in collecting samples in the wild field.

The two batches of blood serum samples collected randomly from a same block of mallards in Zhalong in August and December, 2004 were examined for antibody against AIV. As a result the data of two batch of samples differed greatly. Among 38 blood serum samples collected in August, antibodies against the hemagglutinin of H1, H3, H5, H6 and H9 subtypes of AIV were found in 1, 0, 2, 0 and 8 samples separately and the influenza antibody frequency was 2.63, 0, 5.26, 0, 21.05% respectively. Whereas all the 32 blood serum samples collected in December were negative for antibodies against the AIV.

The detection results of blood serum samples gathered in

August could reflect,in some extent, the situation of AIV infection in wild waterfowl in this area. The antibody positive frequency for H9 subtype was conspicuous higher than for any other subtypes, which may indicate H9 subtype AIV was more common at least in Zhalong or in the migration and living areas of this group of wild ducks.

Avian influenza broke out in some countries and also in China in 2004. There are many places in Heilongjiang Province to be the migrate-rest and breeding places of many migratory birds. In this study AIV of H5 subtype was not found, but two serum samples showed positive in antibody against H5, which was consistent with the situation of outbreak and epidemic of AIV in 2004. It also indicated the case of H5 subtype AIV infection was really existed in wild waterfowl and it was possible to them to spread this virus.

Analyzing the detection result that 32 blood serum samples gathered in December were all negative in antibodies against AIV, it is considered that this flock of wild ducks had been freely living in wild and would have the chances to contact closely with virus infected poultry or wild birds or contaminated environment before being captured in August. However after being put in cage, the specific antibodies in body decreased gradually and lost almost completely or they were lower than the detectable level when being detested in December, because of the loss of new infection sources resulted from isolation from environment.

APMV was not found in both of two patch of specimens of wild ducks gathered in Zhalong by viral isolation, but there were 11 serum samples gathered in August shown positive in specific antibody against NDV. It imported that NDV or APMV exist extensively in natural environment. As a susceptible host, wild waterfowl especially wild duck and goose play an important role in carrying and spreading virus. The previous studies revealed that some NDV strains existing in migratory bird population had latent virulence, once these strains spread and passaged in poultry they could gain high virulence to poultry [8].

Most wild birds do not appear noticeable clinical symptom after infected by AIV, but they can egest virus persistently through alimentary canal polluting ambient water resource and habitats. So they are virus resource of poultry. Because wild birds do not fall ill after infected or their outward appearance is healthy, this virus resource is always ignored. Many data indicate the outbreak of AIV in poultry have spatial and transient relationship with wild birds [6,9], and the veneniferous migratory birds can spread AIV all over the world. Thus investigating the situation of AIV and NDV infection and carrying of wild birds has very important values in theory and economy.

Because of some difficulties in sampling in wild birds, the limited quantity of samples, and the preservation and transportation conditions being not enough to meet the requirements of viral isolation, in this study the detection of AIV and specific antibodies was only limited to parts of the serotypes, and the detection of APMV infection status was primarily done in the examination of NDV specific antibody. But we believe that the results obtained in this study could in a certain extent reflect the status of AIV and APMV infection or carrying in wild birds in parts of Heilongjiang Province. To gain overall and profound recognition and comprehension should rely on long-term and extensive monitoring works.

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