Isolation and Identification of Bird Viruses in Wetland Ecology

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Abstract. In order to study the occurrence of Newcastle disease virus (NDV) in wild birds of Honghu wetland in Hubei, we collected wild bird feces samples in the wild bird habitat of Honghu in Hubei in the winter and spring of 2017 ~ 2019. By SPF chicken embryo isolation, hemagglutination-positive samples were identified by RT-PCR. A total of 38 NDV and 3 mixed viruses including NDV and avian influenza virus (AIV) were detected. The results showed that the NDV positive rate in wild birds was about 1%. These monitoring results lay the foundation for further understanding the status of NDV carried by wild birds and formulating corresponding preventive measures.

1. Introduction

The vast and numerous wetlands have a variety of ecological functions and are rich in natural resources. They are known as the "kidneys of the earth", species reservoirs, climate regulators, and water purifiers. They are important immune systems on the planet. It plays an irreplaceable role in protecting the ecological environment, maintaining biodiversity, and developing the economy and society. [1-5] Wetland ecosystems are water ecosystems. Its biological community is composed of aquatic and terrestrial species, which has active material circulation, energy flow, and species migration and evolution, and has high ecological diversity, species diversity, and biological productivity. Wild birds are the most important and active species in wetlands. Their species and quantity are important indicators of the quality of wetlands. Wild birds often carry unpredictable viruses during migration, which directly threaten animal safety, ecological safety, and affect to human production and life. Based on the fecal samples collected from Honghu Wetland during the wild bird migration and wintering period in 2017 ~ 2019, this paper uses biological laboratories to isolate and identify, and uses monitoring data to study the change rule of Newcastle disease virus positive rate, and to explore the trend about infection of NDV and pathogenic host range.

With the gradual immunization of vaccines and the evolution of strains, clinical necropsy symptoms of Newcastle disease are becoming increasingly atypical, and the diagnosis needs to rely on laboratory tests. The routine laboratory testing methods include fluorescent RT-PCR, RT-PCR, HI tests, virus isolation and identification, and virus gene sequence determination, etc. [6] In this experiment, chicken embryo isolation and RT-PCR were used to study the carrying of NDV from wild bird droppings collected in Honghu wetland.
2. Materials and Methods

2.1. Main reagents and instruments

2.1.1. Main reagent. SPF Chicken Embryo (Beijing Merial Vital Laboratory Animal Technology Co., Ltd.), RNA extraction kit, proteinase K, M-MLV reverse transcriptase, RRNA inhibitor, DEPC water, dNTP Mixture, 2×Tsingke Master Mix (Xi'an Tianlong Technology Co., Ltd.)

2.1.2. Main instrument. Keyu brand microcomputer incubation equipment, eppendorf Centrifuge 5810R, G100 gene amplification instrument, nucleic acid extraction instrument (Xi'an Tianlong Technology Co., Ltd.), Autoclave (HIBAYAMA HVE-50), Vortex QL-901, ElectrophoresisApparatus (DYY-6B Type constant voltage and steady flow ElectrophoresisApparatus), FR-980A biological electrophoresis image analysis system.

2.1.3. Buffer and solution formulations.
(1) Sampling solution: 15% glycerol, 425mL of 1×PBS, 1.5g of 0.3% BSA, make up to 500mL.
(2) TAE buffer: 48.4g of Tris, 11.42mL of glacial acetic acid, 6.624g of EDTA, make up to 10L.
(3) Anticoagulant: 5g of sodium citrate, 0.9g of sodium chloride, make up to 100mL with distilled water, filter and sterilize.
(4) Physiological saline: 0.9g of sodium chloride, make up to 100mL with distilled water, and autoclave at 121℃ for 15min.
(5) 1% red blood cell: the mixed blood of SPF or non-immune healthy chicken is collected, injected into a 50mL centrifuge tube with an appropriate amount of anticoagulant, equilibrated on a balance and centrifuged in a centrifuge, with 1500rpm, and centrifuged for 15min. Then we separate out the upper serum, and repeat the washing 4 ~ 5 times by adding normal saline, and finally make up to 1% red blood cells stored at 4℃ for later use [7].

2.2. Primer synthesis
Designing primers with reference to the full sequence of NDV strains published in GenBank, as follows:

ClassI_NDV_F: CACCAAGCTGGAGAAAGGGCATAC,
ClassI_NDV_R: CAGTATGTTTGCAGCATTCTGGTTGG,
ClassII_NDV_F: CCATTGCTAAATACAATCCTTTCA,
ClassII_NDV_R: CTGCCACTGCTAGTTGTGATAATCC.

Class I NDV product size is 727bp, and Class II NDV is 708bp.

2.3. Experimental method

2.3.1. Sample processing. The collected cotton swabs were thawed on ice, centrifuged at 8000rpm for 5 min at 4℃ after mixing with vortex, and 200μL of the upper serum was taken. 20μL of 1000×dual antibody (penicillin: streptomycin = 1:1) was added to the upper serum. Finally inoculating chicken embryos after shaking and mixing.

2.3.2. Chicken embryo inoculation. Take 9 to 11-day-old chicken embryos to streaking in a dark room, avoiding large blood vessels, and line drawing between the two blood vessels along the junction of the air chamber and the liquid surface. After streaking, we place the chicken embryos on an egg tray and take them into the biosafety cabinet, then wipe the scribe line with an alcohol cotton ball. With burning the hole punch on the alcohol lamp, the egg shell can be poked gently at the upper part of the scribe line, then burn the hole punch again, and beat the chicken embryos in turn. hole. After punching, we use a syringe to aspirate the treated sample, then inject it into the allantoic cavity of the chicken embryo, and seal the pinhole with paraffin to mark it, and place it in a 37℃ incubator.
2.3.3. **Chicken embryo breeding and allantoic fluid collection.** Observed with lighted eggs every day, the dead chicken embryos were taken out and placed in refrigerator at 4℃, and all chicken embryos were placed in refrigerator at 4℃ for 8h or overnight after 72h. Then collect the chicken embryo, and wipe the eggshell with an alcohol cotton ball. Then break the eggshell with one end of the tweezers, and remove the membrane, and use a pipette tip with a filter element to suck 50μL of allantoic fluid onto the hemagglutination plate, and then add 50μL of 1% chicken erythrocytes, waiting for 20 minutes, to observe positive blood clotting, then collect the allantoic fluid of hemagglutination-positive chicken embryos, and store it at -80℃.

2.3.4. **Extraction and reverse transcription of viral RNA.** Melt the hemagglutination-positive allantoic fluid on ice. Use a RNase-free pipette tip to add 200μL to the corresponding well of the kit in a biosafety cabinet. Then add 20μL proteinase K respectively. After the addition, put them into the nucleic acid extraction instrument. When the procedure is completed, we collect RNA in the corresponding well (approximately 90μL per well), and insert it into the ice box quickly after collection. Take 10μL of the extracted RNA, then add 1μL each of the corresponding 10umol/l primers Tuni-12 and Tuni-13, and mix and centrifuge in the PCR machine. After heating at 70℃ for 5 minutes, then place them in ice bath for 2 minutes. Add the following reagents to each tube: 10μL of 5×RT buffer, 25μL of DEPC, 1μL of dNTP Mixture, 1μL of RRI, 1μL of M-MLV, gently mix them with 42℃ 1h, 95℃ 2min, 4℃ hold. Then use the reverse transcription product as a template for PCR.

2.3.5. **PCR identification of NDV virus.** Take the above cDNA and add them into the reaction system on ice, including 9.5μL of ddH2O, 12.5μL of 5×Tsingke Master Mix, 0.5μL each of the forward and reverse primers, and 2μL of the template. Put them in the PCR instrument and run the program at 95℃ for 3min, then at 95℃ for 1 min, at 51℃ for 45s, at 72℃ for 2min and 30s, for 35 cycles, and finally at 72℃ for 10mins. After the procedure is completed, 5μL of the reaction product is tested on 1.2% agarose gel electrophoresis.

3. **Results and discussion**

Chicken embryo samples collected from Honghu Wetland in Hubei from 2017 to 2019 were used for chicken embryo isolation, hemagglutination detection, RNA extraction, reverse transcription and PCR and agarose gel electrophoresis. (the PCR product has 727bp band, indicating NDV CLASS I positive. A band of 708bp indicates a positive NDV CLASS II.) The following results were obtained.

| Sampling year | Sampling month | Number of sampling swabs (parts) | NDV positive | NDV positive rate (%) |
|---------------|----------------|---------------------------------|--------------|----------------------|
| 2017          | 12             | 250                             | 0            | 0                    |
| 2018          | 01             | 974                             | 1            | 0.10%                |
|               | 02             | 908                             | 15           | 1.65%                |
|               | 11             | 390                             | 0            | 0                    |
|               | 12             | 1128                            | 25           | 2.22%                |
| 2019          | 01             | 491                             | 0            | 0                    |
|               | 02             | 650                             | 0            | 0                    |

Table 1. Surveillance statistics of Honghu wild bird Newcastle disease virus from 2017 to 2019.
4. Summary
The specific sampling information from 2017 to 2019 is shown in Table 1. The positive rate in January 2018 was 0.10% in the months tested positive. The positive rate in February 2018 was 1.65%, and the positive rate in December 2018 was 2.22%. It can be seen that the separation rate of NDV is slightly different in different months. The above data remind us that we should strengthen the detection of NDV to detect and control its spread as soon as possible. In recent years, the range of NDV infection and pathogenic hosts has expanded. Some wild bird NDV and wild bird type A bird flu virus attenuated strains can be transformed into virulent strains after passaging in chickens [8–9], thus endangering animal safety and the development of poultry breeding. Studies have shown that the evolution rate of the NDV genome and the frequency of recombination between different genotypes and different gene fragments are gradually increasing [10]. Some NDV isolated in this study may have cross infection between wild birds and poultry. Therefore, strengthening the monitoring of wild bird-sourced NDV is essential to protecting animal safety and ecological safety, promoting the healthy development of poultry farming, and preventing and controlling the large-scale epidemic of Newcastle disease.

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