

## ***In vivo* Inhibition of NAS Preparation on H9N2 Subtype AIV\***

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**Abstract:** NAS preparation, a kind of Chinese herbal medicine found by the Yunnan Eco-agricultural Research Institute, has potential antiviral activity. In this paper, the inhibiting effect of NAS preparation on H9N2 subtype Avian influenza virus (AIV) was investigated *in vivo*. Chickens infected with H9N2 virus were treated with NAS preparation for 4 days. The virus was then detected by hemoagglutination (HA) test and reverse transcription polymerase chain reaction (RT-PCR). The results showed that no H9N2 virus could be detected at the 7th day when the chickens were treated with 0.2g/kg/d or 0.1g/kg/d of NAS preparation. However the virus could be detected in other chickens without NAS preparation treatment. This result suggested that NAS preparation may be a potential drug candidate to control infection of H9N2 subtype AIV in chickens.

**Key words:** NAS preparation; H9N2 subtype AIV; HA; RT-PCR

Avian Influenza (AI) is a syndrome of avian infections and/or diseases. H9N2 subtype Avian Influenza Virus (AIV), known as lowly pathogenic Avian Influenza Virus (LPAIV), causes great losses in the poultry industry by causing avian eggs laying decrease, complex respiratory diseases, and successive breakouts of diseases such as Newcastle, colibacillary diseases or others which lead to high death rates<sup>[11]</sup>. In China, the first outbreak of AI which was attributed to the H9N2 subtype AIV happened in Guangdong

province in 1994. Since then the H9N2 subtype AIV has spread worldwide in poultry and can also infect human beings<sup>[1, 13, 14]</sup>.

At present vaccination is still one of the most effective measures to prevent AI but there are some difficulties in the use of vaccines to prevent the disease, because virus antigenic variations often take place, which are caused by the change of surface glycoproteins (mainly the haemagglutinin (HA) and neuraminidase (NA) proteins) of AIV<sup>[8]</sup>. Administration with chemical drugs to cure AI is limited by its lower curative effect and higher toxicity compared to Chinese herbal medicines. Therefore researchers have now changed the focus of the work of developing new anti-virus drugs to Chinese herbal medicines. Many Chinese

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herbal medicines have already been clinically used as anti-virus drugs, especially for treatment of human influenza. Because of their ability to directly inhibit virus replication, regulate the immune system, relieve pain and antiinflammation, as well as the merits of lower toxicity and fewer side effects, wide resource and low price, those drugs have obvious advantages and wide potential for the development of treatments for curing viral diseases<sup>[7, 16]</sup>.

NAS preparation was proved by many clinical tests to have a definite effect on animals' viral diseases. The purpose of this study was to apply the HA test and RT-PCR to determine the inhibiting effect of NAS preparation on H9N2 subtype AIV in chickens.

## MATERIALS AND METHODS

### Drugs

NAS preparation, provided by the Yunnan eco-agricultural research institute, was made up of *Hedyotis diffusa*, *Common stonecrop herb*, *Abrotani herba*, *Folium isatidis*, etc.. All of the herbs were collected from Yunnan, Guizhou, or Xizang province in China. The preparative method of 50mg/mL solution of NAS preparation was as follows: Exactly 50.0g of raw NAS preparation was dissolved in 800mL drinking water from which chlorine was removed by solarization. The mixture was boiled for 3-5 min with slow heating, transferred to a 1 000 mL flask and diluted with the chlorine-free water to volume.

### Virus

The H9N2 subtype AIV (A/Chicken/GuangXi/KM I /1999) was provided by the Institute of Avian Disease, South China Agricultural University. The virus (diluted 1 000 times) was inoculated into 10-day-old SPF (Specific Pathogen Free) embryonated chicken eggs at a dosage of 0.2 mL per egg. After the

eggs were incubated at 37 °C for 72h, allantoic fluids were harvested and used for infecting chickens. The determination of EID<sub>50</sub> was 10<sup>-10.25</sup>/0.2mL.

### Embryonated chicken eggs

10-day-old SPF embryonated chicken eggs, purchased from the Merial Vital Laboratory Animal Technology Co., Ltd. Beijing, were used to harvest and multiply virus *in vitro* and determine the agglutinogen.

### Animals

28-day-old Shiqi chickens, weighing 650-680g, were purchased from a chicken farm that was administered by Guangdong Academy of Agricultural Sciences. To ensure that the chickens were AIV-free, the Micr-HI Test was used to detect the antibody of AIV at 15 days and 25 days old respectively. The chickens with antibody or clinical signs of disease or abnormalities were not used in the study<sup>[11]</sup>. All the chickens were tagged, allowed free access to water, and fed non-medicated rations.

### Infections and treatments

The 96 Shiqi chickens were divided randomly into 6 groups: a NAS preparation high-dose group, a NAS preparation middle-does group, a NAS preparation low-does group, a amantadine control group, a positive control group, and a negative control group. Except for the negative control group, the chickens in all the other groups were infected with AIV at a dosage of 0.2mL via intranasal and eye-drop routes. After 48 h of infection, the chickens of the NAS preparation high-dose group, NAS preparation middle-dose group and NAS preparation low-dose group were fed, by way of a drench, the NAS preparation at a dosage of 0.2g/kg/d, 0.1g/kg/d and 0.05g/kg/d, respectively, while the chickens of the amantadine control group drunk water freely which contained amantadine at a concentration of 0.05mg/mL.

Chickens in the above groups were administered daily for 4 days to ensure that all drugs were taken. The chickens in the positive and negative control group were not treated with any drug. All the chickens were free access to water during the treatments. After infection with AIV, the clinical manifestations of all chickens were recorded daily over the 14 day experiment.

#### **Collection of samples for virus detection**

The mucus of throats and cloacal chambers of all chickens was collected by cotton pledgets at 3d, 5d, 7d, 9d, 11d and 14d respectively after the chickens were infected with the virus. Cotton pledgets containing mucus were put into Hanke's reagent and mixed. The mucus were scattered and the mixture was centrifuged. The supernatant was separated and divided into two parts: one for inoculating chick embryos and the other for extracting viral RNA. For each group, the hearts, livers, lungs, spleens, and kidneys from the chickens that died during the experiment and the survival after 14 d experiment were collected and grounded. The grounded organs were centrifuged and supernatant was divided into two parts for detection virus with HA test and the method of RT-PCR respectively.

#### **Multiplication of virus and Determination of the hemagglutinin test**

After the mucus of throats and cloacal chambers was treated as above, 1mL sample from each group was added in a sterile centrifuge tube followed by mycillin. After incubation and filtration, the purified virus was made and injected into the allantoic cavities of eggs with a dosage of 0.2mL per egg to multiply the virus. 6 eggs were injected for each sample. The negative control eggs were injected with physiologic saline instead of the virus sample. All the eggs were incubated at 37°C. All the embryos that died were

discovered visually with an electric torch at the first day and discarded. The allantoic fluid of eggs that died within 24-96 h were collected under sterile conditions and the hemagglutination valence ( $\log_2$ ) was determined by HA test<sup>[3,20]</sup>. The arithmetic mean (99% confidence interval) of hemagglutinin valence for each group was calculated according the referenced formula<sup>[2]</sup>. For samples from the organs from chicks in each group which died during the experiment or the survived after the experiment, were treated and injected into eggs with the same procedure as for the mucus samples. The hemagglutination valence was also determined by HA test.

#### **RT-PCR**

RT-PCR was used to detect virus in the samples (the mucus and the organs). A conservative primer pair was designed based on HA gene sequences of H9N2 subtype AIV in the GenBank using the software package DNASTar (Version 5.0). The primers were synthesized by the Shanghai Sangon Biological Engineering Technology & Services Co., Ltd, with the sequences as follows, HA-U: 5'-TGTAAGTAACTAAGCATTTG-3', HA-L: 5'-CTCGCTGAATTCATGCTGAA-3'. The extraction of virus' RNA was performed using a Total RNA Kit (Sino-American Biotechnology Co.) according to the manufacturer's instructions. The RT-PCR were performed by routine methods. And the amplified PCR products were examined with 1.0% agarose gel electrophoresis.

## **RESULTS**

#### **Clinical symptoms of chickens**

At 3d after infection with H9N2 subtype AIV, 38 percent of chickens, except those from negative control group, showed clinical symptoms to different extents. At 5d after infection, all the chickens, except

Table 1. *In vivo* inhibition of AIV H9N2 subtype

Groups	HA valence ( $\log_2$ )					
	3d	5d	7d	9d	11d	14d
NAS high-does group <sup>a</sup>	4.83±0.753	5.83±0.753	0	0	0	0
NAS middle-does group <sup>b</sup>	5.00±1.095	7.00±1.414	0	0	0	0
NAS low-does group <sup>c</sup>	5.50±1.049	10.00±0.862	9.50±0.548	0	0	0
Adamantanamine control group	7.83 ±1.169	8.50±1.049	10.33±0.872	5.33±0.817	0	0
Positive control group	5.33±0.817	10.00±0.844	10.33±0.521	9.67±1.033	0	0
Negative control group	0	0	0	0	0	0

<sup>a</sup>0.2g/kg/d, <sup>b</sup>0.1g/kg/d, <sup>c</sup>0.05g/kg/d.

those from negative control group, showed typical clinical symptoms, such as feed intakes decreasing, feathers fluffing and mess, sinus nasalis swelling, conjunctiva flushing, lachrymation and excretion effusing from nasal cavity. But the symptoms of treatment group (especially the NAS preparation high-does group and middle-does group) were lighter than the positive control group. At 6 d after infection (the 4th day after treatment), the clinical symptoms disappeared gradually. At 11 d after infection, there was almost no clinical symptom in all groups. Throughout the whole experiment, no chicken died in any of the groups.

#### Inhibiting effect of NAS preparation

Based on the results of hemagglutinin valence ( $\log_2$ ) in HA test (Table 1), the detection of virus for each group was as follows: At 3 d and 5 d after infection, the virus was detected in all chicken groups, except those of negative control. At the 7 d, virus was detected from the chickens in the NAS low preparation group, adamantanamine control group and positive control group. At 9 d the virus was detected in the chickens of the adamantanamine and positive control groups. At 11 d and 14 d, no virus was detected in any group. After 14 d experiment, all chickens were killed and the treated samples of organs were injected into embryonated eggs for the purpose of hemagglutinin valence determination. The results

showed that the hemagglutinin valence of all samples was zero.

#### Viral detection by RT-PCR

The samples used to detect virus were extracted for RNA and submitted to PCR amplification. The RT-PCR products were visualized by the Agarose Gel Electrophoresis. The gel image acquired by Image Acquisition and Analysis System (GDS8000PC, UVP Inc., America) were used to determine the presence of AIV. Samples containing H9N2 subtype AIV should give a fragment with size of about 306 bp.

The results of virus detection by RT-PCR were as follows: At 3 d and 5 d after infection, a fragment was amplified in the samples of all groups, except the negative control group, indicating the presence of the H9N2 subtype AIV in the samples. At 7 d after infection, positive results were obtained from the samples collected from NAS preparation low-dose group, the adamantanamine control group and the positive control group, while negative results were obtained for samples collected from the other groups. At 9 d after infection, only the samples collected from the adamantanamine control group and positive control group showed positive results, but not from the samples collected from the other groups. At 11 d and 14 d after infection, no virus was detected in all groups. Table 2 show the the results of PCR for the

Table 2 The viral detection of mucus collected from the throat and cloacal chamber.

Groups	3d	5d	7d	9d	11d	14d
NAS preparation high-does group	+	+	-	-	-	-
NAS preparation middle-does group	+	+	-	-	-	-
NAS preparation low-does group	+	+	+	-	-	-
adamantanamine control group	+	+	+	+	-	-
Positive control group	+	+	+	+	-	-
Negative control group	-	-	-	-	-	-

Note: “+” represent positive viral isolation; “-” represent negativ viral isolation.

detection of mucus which was collected from the throat and cloacal chamber. After 14d, all chickens in all experiment groups were killed. The hearts, livers, lungs, spleens and kidneys were collected from all the chickens and treated as above to detect virus with RT-PCR method, but no virus was found.

#### DISCUSSION

The widespread occurrence of AIV is an extreme threat to poultry cultivation and brings new pressure to food safety management [9, 10]. Recently, the outbreaks of AIV in Asia have caused a broad poultry infection in many countries and affected human health directly. But there are only a few anti-Influenza virus drugs commercially, such as amantadine, remantadine, ribavirin and ITFs. However the use of these drugs is confined by the problems such as viral resistance to drugs and toxicity. It is difficult to develop efficient and non-toxic anti-virus drugs because it is not easy to remove viral nucleic acids sequence when they integrate with those in the host. Traditional Chinese herb medicines, however, have a good application prospect for their merits of multi-target action mechanisms, less side effects and virus develop drug resistance more slowly. Thus there are many benefits to investigating the abilities of Chinese herbs as treatments for new or emerging disease [22].

Chinese herb medicines have two major methods to

inhibit the virus: One is the direct inhibition by killing or repressing the virus, the other is the indirect inhibition via regulating the organism's immune systems [22]. *Abrotani herba*, one of ingredients of the NAS preparation, has the functions such as reducing fever, promoting urination and detoxication that can repress or kill virus directly [5, 21]. *Hedyotic diffusa*, another NAS preparation ingredient, not only has the same functions as *Abrotani herba* but also has a noticeable immunological enhancement that can stimulate and enhance the production of antibody, and promote multiplication of homeocytes [4, 15]. *Common stonecrop herb*, the third NAS preparation ingredients, has the functions of clearing heat, cooling blood and hemostasis, as well as the advantages of lower toxicity and slower development of drug resistance [18, 6]. The results of the acute toxicity test of NAS preparation in mice showed that the LD<sub>50</sub> is only 9.0573±0.0309g/kg (95% confidence limits was 7.8777-10.4136g/kg). According to the classification of acute toxicity, it is a low toxic material [19]. Furthermore, the result of this study showed that the NAS preparation showed obvious inhibition effects on AIV *in vivo* when the chickens were treated at the dosages of 0.2g/kg/d and 0.1g/kg/d for 4 d after infection. In addition, the study of NAS preparation on anti-AIV *in vitro* which has been done in the Animal Influenza Lab of the Ministry of Agriculture, Harbin Veterinary Research Institute,

CAAS, showed that the medicine has obvious inhibition effects on AIV *in vitro*<sup>[17]</sup>. Based on the above results, NAS preparation has a certain effect not only on different strains of one subtype AIV but also on different subtypes AIV. NAS preparation, therefore, is a good candidate to be developed into a prospective anti-AIV drug.

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