METHOD FOR PREPARING POSITIVE SERUM AGAINST LOW-PATHOGENIC AVIAN INFLUENZA VIRUS AND USE THEREOF

Field of the Invention

[01] The present invention pertains to the technical field of veterinary biological products, and in particular, to a method for preparing positive serum against low-pathogenic avian influenza virus and applications thereof.

Background to the Invention

[02] Avian influenza (AI) is an acute infectious avian disease caused by the avian influenza virus (AIV). Based on pathogenicity, avian influenza viruses are classified into highly pathogenic avian influenza virus (HPAIV) and low pathogenic avian influenza virus (LPAIV). Among these, HPAIV is restricted to H5 and H7 subtypes. Although LPAIV exhibits lower pathogenicity, infection in poultry often leads to immunosuppression, reducing the flock's resistance to various pathogens and increasing susceptibility to concurrent or secondary infections. Vaccination is one of the key measures for preventing LPAIV. Currently, inactivated vaccines represent the primary form of low-pathogenic avian influenza vaccines. Quality monitoring and evaluation of the virus strains used in the production and testing of these vaccines are critical to ensuring overall vaccine efficacy. However, there is a limited availability of national reference standards for evaluating production and testing strains of different LPAIV subtypes. Furthermore, existing methods face challenges such as lengthy preparation times and the need for improved positive serum titers.

Statement of Invention

[03] The objective of the present invention is to provide a preparation method for low pathogenic avian influenza virus positive serum and its application. The positive serum prepared by the method of the present invention exhibits good specificity and high sensitivity.

[04] The present invention provides a preparation method for low pathogenic avian influenza virus positive serum, comprising the following steps: performing a primary immunization on an animal using a seed virus containing live low pathogenic avian influenza virus; after the primary immunization, performing a single immunization using an inactivated vaccine of the low pathogenic avian influenza; after the single immunization, separating the animal serum.

[05] Preferably, the viral titer of the seed virus is from $10^{4.0}$ EID_{50}/0.1 ml to $10^{6.0}$ EID_{50}/0.1 ml; the low pathogenic avian influenza virus includes H9, H3, or H6 subtype avian influenza virus.

[06] Preferably, the immunization method for the primary immunization is intranasal administration.

[07] Preferably, the inactivated vaccine of low pathogenic avian influenza is used for a single immunization 13 days after the primary immunization, and serum is harvested 7 days after immunization; the method of the single immunization comprises intramuscular injection.

[08] Preferably, the preparation method of the inactivated vaccine of low pathogenic avian influenza comprises the following steps: inactivating a seed virus containing live low pathogenic avian influenza virus to obtain an inactivated antigen; mixing the inactivated antigen with Tween-80 to obtain an aqueous phase; mixing the aqueous phase with an oil phase to obtain an inactivated water-in-oil vaccine.

[09] Preferably, the volume ratio of the inactivated antigen to Tween-80 is 97:3; the volume ratio of the aqueous phase to oil phase is 2:3; said oil phase comprises white oil and Span-80; the volume ratio of the white oil to Span-80 is 95:5.

[10] Preferably, the animal includes avian animals; the avian animal comprises a chicken; the age of immunized chickens is from 14 days to 21 days at the time of the primary immunization.

[11] The present invention also provides the low pathogenic avian influenza virus positive serum prepared by the preparation method described in the above solutions, the

low pathogenic avian influenza virus includes H9, H3, or H6 subtype avian influenza virus;

[12] The HI titer of the H9 subtype avian influenza virus positive serum is ≥ 12 $\log_2$, the neutralization titer is ≥ 1:4096, and the H9 subtype avian influenza virus positive serum only shows a positive reaction with H9 subtype avian influenza virus or antigen, and shows negative reactions with Newcastle disease virus, Egg Drop Syndrome virus, H3, H5, H6, and H7 subtype avian influenza viruses;

[13] The HI titer of the H6 subtype avian influenza virus positive serum is ≥ 12 $\log_2$, the neutralization titer is ≥ 1:2048, and the H6 subtype avian influenza virus positive serum only shows a positive reaction with H6 subtype avian influenza virus or antigen, and shows negative reactions with Newcastle disease virus, Egg Drop Syndrome virus, H3, H5, H7, and H9 subtype avian influenza viruses;

[14] The HI titer of the H3 subtype avian influenza virus positive serum is ≥ 11 $\log_2$, the neutralization titer is ≥ 1:1024, and the H3 subtype avian influenza virus positive serum only shows a positive reaction with H3 subtype avian influenza virus or antigen, and shows negative reactions with Newcastle disease virus, Egg Drop Syndrome virus, H5, H6, H7, and H9 subtype avian influenza viruses.

[15] The present invention also provides the use of the low pathogenic avian influenza virus positive serum described in the above solutions, comprising at least one of the following aspects: 1) preparation of products for detecting, identifying, or diagnosing low pathogenic avian influenza virus; 2) preparation of products for research on the pathogenesis and transmission mechanisms of low pathogenic avian influenza virus; 3) preparation of products for evaluating or monitoring the quality of live vaccines against low pathogenic avian influenza virus; 4) preparation of a standard for low pathogenic avian influenza virus positive serum.

[16] Preferably, the products are constructed based on at least one of the following detection techniques: indirect immunofluorescence assay, enzyme-linked immunosorbent assay, immunoblotting, and immunohistochemistry.

[17] The present invention provides a preparation method for low pathogenic avian influenza virus positive serum, comprising the following steps: performing a primary

immunization on an animal using a seed virus containing live low pathogenic avian influenza virus; after the primary immunization, performing a single immunization using an inactivated vaccine of the low pathogenic avian influenza; after the single immunization, separating the animal serum.

[18] After natural infection with avian influenza virus, the body is stimulated to produce various immune responses, including cellular immunity and humoral immunity. B cell-mediated humoral immunity plays an important role in the host's clearance of viral infection and the antibody production process. However, when the antibody level reaches a certain degree in the infected host, the produced antibodies may reduce B cell activation through mechanisms such as rapid antigen clearance, formation of antigen-antibody immune complexes, and epitope masking, thereby affecting the production of specific antibodies. During immunization, inactivated avian influenza vaccines primarily bind to the antigenic epitopes of the viral HA protein, causing the body to produce specific antibodies against HA. However, inactivated avian influenza vaccines require multiple immunizations to enable the body to produce high-titer antibodies.

[19] Through preliminary experiments, the present invention found that infection of SPF chickens with different subtypes of low pathogenic avian influenza virus can produce specific antibodies with high HI levels as early as 11 days post-infection. When inactivated vaccine immunization is performed on day 13 post-infection, specific antibodies with high HI titers and high neutralization titers can be harvested 7 days after a single immunization with the inactivated vaccine. Therefore, the present invention combines the natural infection characteristics of low pathogenic avian influenza virus, the humoral immune mechanism of inactivated vaccines, and rationally sets the infection time of the live low pathogenic avian influenza virus and the immunization time of the inactivated vaccine, enabling the preparation of neutralizing antibodies against low pathogenic avian influenza virus with high titer, good specificity, and sensitivity within a short time. Additionally, the preparation method of the present invention is not limited to a single subtype of low pathogenic avian influenza virus. The prepared positive serum can be used for detection by techniques such as indirect immunofluorescence, Western blot, and immunohistochemistry. It can also be used for the detection of extraneous viruses in

poultry viral live vaccines, isolation and identification of clinical viruses, differential testing of live vector vaccines, clinical monitoring and epidemiological investigation of low pathogenic avian influenza virus, and evaluation of the immune effect of related vaccines. The preparation method of the present invention requires only one immunization after the primary immunization to quickly prepare positive serum with high HI titer and neutralization titer in a short time, which is of great significance for enriching the sample panel for quality evaluation of avian influenza vaccines and for the monitoring and control of avian influenza virus.

Brief Description of the Drawings

[20] In order to more clearly illustrate the technical solutions in the embodiments of the present invention or the prior art, the drawings required for describing the embodiments will be briefly introduced below. Obviously, the drawings in the following description are merely some embodiments of the present invention. For those of ordinary skill in the domain of the present study, other drawings may also be obtained from these drawings without any creative effort.

[21] FIG 1 shows the determination of the preparation method for the low pathogenic avian influenza virus positive serum; wherein, DPI stands for days post live virus inoculation; DPV stands for days post inactivated vaccination; 28 DPV and 35 DPV represent 7 days and 14 days after the second inactivated vaccination, respectively.

[22] FIG 2 shows the HI titers of positive serum prepared using the method of the present invention for different subtypes of low pathogenic avian influenza virus; wherein, DPI stands for days post live virus inoculation; DPV stands for days post inactivated vaccination.

[23] FIG 3 shows the results of detecting the expression of H9 subtype avian influenza virus HA protein in CEF cells by a recombinant live vector vaccine, using the avian influenza virus (H9 subtype) positive serum.

[24] FIG 4 shows the results of RT-PCR detection; wherein, A shows the RT-PCR detection results of a mixed sample of IBV and H9 AIV; B shows the RT-PCR detection

results of the mixed sample of IBV and H9 AIV after neutralization with H9 subtype avian influenza virus positive serum; wherein, M is DNA marker DL2000; Lane 1: Mixed allantoic fluid of IBV and H9 AIV; Lane 2: IBV positive control (Strain CVCC AV1514); Lane 3: H9 AIV positive control (Strain CVCC AV1563); Lane 4: Negative sample (Nuclease-free water); Lane 5: Allantoic fluid sample after neutralization with H9 subtype avian influenza virus () positive serum.

[25] FIG 5 shows the Western blot results of detecting H9 subtype avian influenza virus HA protein expression using H9 subtype avian influenza virus positive serum; wherein Lane 1: Empty plasmid; Lane 2: Plasmid pCAGGS-H9HA1; Lane 3: Plasmid pCAGGS-H9HA2; Lane 4: Plasmid pCDNA-H9HA1; Lane 5: Plasmid pCDNA-H9HA2.

Detailed Description

[26] The present invention provides a method for preparing low pathogenic avian influenza virus positive serum, comprising the following steps: performing a primary immunization on an animal using a seed virus containing live low pathogenic avian influenza virus; after said primary immunization, performing a single immunization using an inactivated vaccine of the low pathogenic avian influenza; after the single immunization, separating the animal serum.

[27] The preparation method of the present invention requires only one immunization after the primary immunization to rapidly prepare positive serum with high HI titer and neutralization titer in a short time. This facilitates the implementation of unified quality control for similar veterinary biological products produced by different manufacturers, and provides an important material basis for the quality assurance of low pathogenic avian influenza virus vaccines and disease prevention and control.

[28] In the present invention, the low pathogenic avian influenza virus is not limited to a single subtype low pathogenic avian influenza virus strain. Different subtypes of low pathogenic avian influenza virus can also obtain positive serum with high HI titer and neutralization titer through the preparation method of the present invention. As one embodiment, the live low pathogenic avian influenza virus includes H9, H3, or H6 avian

influenza virus; the present invention imposes no special restrictions on the specific strains of the H9, H3, or H6 subtype avian influenza virus, and any known H9, H3, or H6 subtype avian influenza virus can be used.

[29] As one embodiment, the number of the primary immunizations is one.

[30] As one embodiment, the viral titer of the seed virus is from $10^{4.0}$ EID_{50}/0.1 ml to $10^{6.0}$ EID_{50}/0.1 ml; the present invention imposes no special restrictions on the preparation method of the seed virus, and conventional methods in this research area may be used.

[31] As one embodiment, the immunization method for the primary immunization is intranasal administration; the dosage of the seed virus containing live low pathogenic avian influenza virus used for the primary immunization is 0.1 ml (containing $10^{6.0}$ EID_{50}) per chicken.

[32] After the primary immunization, the present invention performs a single immunization using the inactivated vaccine of low pathogenic avian influenza.

[33] In the present invention, the live low pathogenic avian influenza virus and the virus used to prepare the inactivated low pathogenic avian influenza vaccine are the same virus.

[34] As one embodiment, the inactivated vaccine of low pathogenic avian influenza virus is used for a single immunization 13 days after the primary immunization; the method of the single immunization comprises intramuscular injection; the single immunization is a multi-point immunization, further comprising multi-point injection of the vaccine in areas such as the leg and breast; the dosage for intramuscular injection is 1 ml per chicken.

As one embodiment, the inactivated LPAIV vaccine is a water-in-oil emulsion vaccine.

[35] As one embodiment, the preparation method of said inactivated vaccine of low pathogenic avian influenza comprises the following steps: inactivating a seed virus containing live low pathogenic avian influenza virus to obtain an inactivated antigen; mixing the inactivated antigen with Tween-80 to obtain an aqueous phase; mixing the aqueous

phase with an oil phase to obtain a water-in-oil vaccine.

[36] As one embodiment, the volume ratio of the inactivated antigen to Tween-80 is 97:3; the oil phase comprises white oil and Span-80, and further consists of white oil and Span-80, wherein the volume ratio of the white oil to Span-80 is 95:5; the white oil is injectable white oil; the injectable white oil is high-quality injectable white oil; the volume ratio of said aqueous phase to oil phase is 2:3. The inactivated vaccine prepared with this ratio has optimal properties, stability, safety, and immunogenic efficacy, with suitable viscosity for easy injection. By rationally adjusting the ratio of inactivated antigen to adjuvant, the present invention can relatively prolong the continuous stimulation time of the inactivated antigen, reduce the antigen dose and number of inoculations, and also protect the antigen from binding by pre-existing serum antibodies, enabling the body to produce high-titer antibodies.

[37] As one embodiment, the reagent used for the inactivation comprises 0.1% formaldehyde, and the inactivation involves adding a formaldehyde solution to the seed virus containing live low pathogenic avian influenza virus; the inactivation time is 36 hours; the inactivation temperature is 37°C; and shaking is performed approximately every 12 hours. After mixing the aqueous phase and oil phase, homogenization is also included to complete emulsification; the homogenization is performed using a homogenizer; the homogenization program comprises: stirring at 15,000 r/min for 2 minutes, then stirring at 3,000 r/min for 1 minute, repeated 2-3 times.

[38] The adjuvant added to the inactivated vaccine of the present invention is a water-in-oil adjuvant, and an appropriate ratio of aqueous phase to oil phase is adopted. After local injection of the antigen into the body, the adjuvant, through mechanisms such as antigen recruitment, storage, and slow continuous release, prolongs the continuous stimulation time of the antigen on the body, thereby increasing the antibody titer, prolonging the antibody persistence, and consequently improving the body's immune response effect.

[39] After the single immunization, the present invention separates the animal serum. As one embodiment, the time for separating the animal serum is 7 days after the single immunization. The present invention imposes no special restrictions on the method

for separating the animal serum, and conventional methods in the art may be used.

[40] As one embodiment, the animal includes avian animals; the avian animals include chickens; the chickens include SPF chickens, and further include young SPF chickens; the age of immunized chickens is from 14 days to 21 days at the time of primary immunization.

[41] After separating the animal serum, the present invention further includes subjecting the serum to sterility testing and mycoplasma testing; after passing the sterility testing and mycoplasma testing, the serum is vacuum freeze-dried directly without adding any protectant.

[42] The present invention also provides the low pathogenic avian influenza virus positive serum prepared by the preparation method described in the above solutions; the low pathogenic avian influenza virus includes H9, H3, or H6 subtype avian influenza virus;

[43] The HI titer of the H9 subtype avian influenza virus positive serum is ≥ 12 $\log_2$, the neutralization titer is ≥ 1:4096, and the positive serum only shows a positive reaction with H9 subtype avian influenza virus or antigen, and shows negative reactions with Newcastle disease virus, Egg Drop Syndrome virus, H3, H5, H6, and H7 subtype avian influenza viruses;

[44] The HI titer of the H6 subtype avian influenza virus positive serum is ≥ 12 $\log_2$, the neutralization titer is ≥ 1:2048, and the positive serum only shows a positive reaction with H6 subtype avian influenza virus or antigen, and shows negative reactions with Newcastle disease virus, Egg Drop Syndrome virus, H3, H5, H7, and H9 subtype avian influenza viruses;

[45] The HI titer of the H3 subtype avian influenza virus positive serum is ≥ 11 $\log_2$, the neutralization titer is ≥ 1:1024, and the positive serum only shows a positive reaction with H3 subtype avian influenza virus or antigen, and shows negative reactions with Newcastle disease virus, Egg Drop Syndrome virus, H5, H6, H7, and H9 subtype avian influenza viruses.

[46] As one embodiment, the neutralization titer of the H6 subtype avian influenza

virus positive serum is 1:5754.

[47] As one embodiment, the neutralization titer of the H3 subtype avian influenza virus positive serum is 1:2884.

[48] The LPAIV positive serum of the present invention features good specificity and strong sensitivity. It can be used for detection by techniques such as indirect immunofluorescence, Western blot, and immunohistochemistry. It can also be used for extraneous virus testing in poultry viral live vaccines, isolation and identification of clinical viruses, differential testing of live vector vaccines, clinical monitoring and epidemiological investigation of LPAIV, and evaluation of the immune effect of related vaccines. It is of great significance for evaluating different subtypes of LPAIV vaccine strains and further improving the relevant standard system for veterinary biological products.

[49] The present invention also provides the use of the LPAIV positive serum described in the above solutions, said use comprising at least one of the following aspects:

[50] 1. preparation of products for detecting, identifying, or diagnosing LPAIV;

[51] 2. preparation of products for research on the pathogenesis and transmission mechanisms of LPAIV;

[52] 3. preparation of products for evaluating or monitoring the quality of LPAIV live vaccines;

[53] 4. preparation of an LPAIV positive serum standard.

In the present invention, the product can be used for LPAIV extraneous virus testing or cell neutralization testing, to evaluate the effect of preventing and controlling LPAIV.

[54] As one embodiment, the product is constructed based on at least one of the following detection techniques: indirect immunofluorescence assay, enzyme-linked immunosorbent assay, immunoblotting, and immunohistochemistry.

[55] As one embodiment, the product includes a reagent or a kit.

[56] To further illustrate the present invention, the preparation method and application of a low pathogenic avian influenza virus positive serum provided by the

present invention are described in detail below with reference to the accompanying drawings and examples. However, these descriptions should not be construed as limiting the scope of protection of the present invention.

[57] Example 1

[58] A Preparation Method for Low Pathogenic Avian Influenza Virus Positive Serum

[59] The steps are as follows:

[60] 1. Preparation of Seed Virus and Antigen for Immunization

[61] 1.1 Preparation of Seed Virus for Immunization

[62] The H9 avian influenza virus strain CVCC AV1551 (identified, preserved, and supplied by the National Center for Veterinary Culture Collection (CVCC)) was diluted 1000-fold with physiological saline and inoculated into the allantoic cavity of 10-day-old SPF chicken embryos, 0.1 ml per embryo. The inoculated embryos were incubated at 37°C for 96 hours, after which the allantoic fluid was harvested aseptically. Virus suspensions that passed sterility testing and had a viral titer ≥ $10^{6.0}$ EID_{50}/0.1 ml were stored at -70°C for future use.

[63] 1.2 Preparation of Antigen for Immunization

[64] Formaldehyde solution was added to the virus suspension prepared in step 1.1 to a final concentration of 0.1%. The mixture was then inactivated at 37°C for 36 hours, with shaking approximately every 12 hours. After inactivation, the inactivated virus suspension was inoculated into the allantoic cavity of 10-day-old SPF chicken embryos, 0.2 ml per embryo. The inoculated embryos were incubated at 37°C for 96 hours, then the allantoic fluid was harvested and tested for hemagglutination activity. Samples showing negative hemagglutination activity were blindly passaged once. If the allantoic fluid from the blind passage also showed negative hemagglutination activity, it confirmed complete inactivation.

[65] 2. Optimization of Inactivated Vaccine Preparation Conditions

[66] This invention conducted screening and comparative experiments on the water-to-oil ratio during the preparation of the inactivated vaccine to identify the optimal ratio and thereby determine the best preparation process for the inactivated vaccine.

[67] The completely inactivated virus suspension was mixed with sterile Tween-80 at a ratio of 97:3. The mixture was stirred thoroughly until the Tween-80 was completely dissolved to form the aqueous phase. The aqueous phase was then mixed with the pre-prepared oil phase (made by mixing high-quality injectable white oil (by weight) and Span-80 (by weight) uniformly at a ratio of 95:5, followed by autoclaving) at volume ratios of 1:1, 1:2, 1:3, and 2:3 (V/V), respectively. The mixtures were homogenized using a homogenizer at 15,000 r/min for 2 minutes, then at 3,000 r/min for 1 minute, repeating this cycle 2-3 times. A sample of the emulsified vaccine was taken and dropped into water. Emulsification was preliminarily considered complete if the first drop dispersed but subsequent drops did not. The emulsified vaccines were then stored in a 4°C refrigerator and observed for demulsification.

[68] The four prepared inactivated vaccines were tested for stability and viscosity. The results showed that Vaccine A had unstable formulation and high viscosity; Vaccines B, C, and D were all homogeneous emulsions of the water-in-oil type, with low viscosity, and no aqueous phase separation was observed at the bottom of the tubes after centrifugation at 3,000 r/min for 15 minutes. Detailed results are shown in Table 1.

[69] **Table 1 Physical Property Test Results of the Four Vaccines**

Group	Appearance	Formulation Type	Stability	Viscosity
Vaccine A (1:1)	Homogeneous emulsion	-	Layering	92.6 cP
Vaccine B (1:2)	Homogeneous emulsion	Water-in-oil	0 ml aqueous phase separation	44.5 cP
Vaccine C (1:3)	Homogeneous emulsion	Water-in-oil	0 ml aqueous phase separation	34.8 cP
Vaccine D (2:3)	Homogeneous emulsion	Water-in-oil	0 ml aqueous phase separation	40.6 cP

[70] The four prepared inactivated vaccines were each administered to 21-day-old SPF chickens (1 ml per chicken) for safety testing. The results showed that Vaccine C caused local inflammatory reactions in some test chickens, while the other three vaccines did not. Detailed results are shown in Table 2.

[71] **Table 2 Safety Test Results of the Four Vaccines**

Group	Local Inflammation	Comprehensive Evaluation
Vaccine A (1:1)	Not found in 10/10	Qualified
Vaccine B (1:2)	Not found in 10/10	Qualified
Vaccine C (1:3)	Not found in 7/10	Unqualified
Vaccine D (2:3)	Not found in 10/10	Qualified

[72] The four prepared inactivated vaccines were each administered to 21-day-old SPF chickens (0.3 ml per chicken) for potency testing. The results showed that Vaccine A produced antibodies with high dispersion and the lowest average HI antibody titer (4.3 $\log_2$); Vaccine C produced relatively low antibodies, with an average HI titer of 5.6 $\log_2$; Vaccine B produced an average HI antibody titer of 7.4 $\log_2$; Vaccine D produced the highest antibody titer, with an average HI titer of 8.5 $\log_2$. Detailed results are shown in Table 3.

[73] **Table 3 Immunogenicity Test Results of the Four Vaccines in SPF Chickens**

Group	HI Antibody Titers ($\log_2$) (Individual values)	GMT ($\log_2$)
Vaccine A (1:1)	4,5,4,5,6,5,4,5,0,5	4.3
Vaccine B (1:2)	7,7,8,7,7,7,7,8,8,8	7.4
Vaccine C (1:3)	6,6,5,6,5,6,6,5,6,5	5.6
Vaccine D (2:3)	8,9,8,8,9,9,9,9,8,9	8.5
Control	<1, <1, <1, <1, <1, <1, <1, <1, <1, <1	<1

[74] Based on the above results, considering the physical properties, stability, safety, and immunogenic efficacy of the prepared inactivated vaccines, the optimal water-to-oil ratio for preparing the inactivated vaccine was determined to be 2:3.

[75] **3. Optimization of Serum Preparation Conditions**

[76] The diluted H9 subtype avian influenza virus CVCC AV1551 strain suspension, with a viral content of $10^{6.0}$ EID_{50}/0.1 ml, was used to inoculate 21-day-old SPF chickens via intranasal administration, 0.1 ml per chicken. Blood was collected on days 5, 7, 9, 11, and 13 post-inoculation to separate serum for HI titer determination. Based on the HI titers, on day 13 post live virus inoculation, the prepared vaccine was administered via intramuscular injection at multiple sites such as the leg and breast, 1 ml per chicken. Blood was collected on days 7, 14, and 21 post-vaccination to separate serum for HI titer determination. On day 21 after the first inactivated vaccination, a second immunization was performed using the same vaccine and method. Blood was collected 7 and 14 days after the second immunization to separate serum for HI titer determination.

[77] The research results showed that a high level of HI antibodies was reached by day 13 after H9 subtype avian influenza virus infection. A high HI antibody level (average HI titer of 13 $\log_2$) was achieved by day 7 after the first inactivated vaccination. Subsequent secondary inactivated vaccination did not significantly increase the antibody level compared to day 7 after the first inactivated vaccination (see Figure 1). Based on these results, it was determined that high HI titer low pathogenic avian influenza virus positive serum can be prepared using one immunization with live low pathogenic avian influenza virus followed by one booster immunization with inactivated vaccine, with a total interval of 20 days between the two immunizations.

[78] **Example 2**

[79] **HI Titer Determination**

[80] The H9 subtype avian influenza virus positive serum was prepared using the method described in Example 1. The prepared serum was aliquoted into ampoules within a biological safety cabinet and then vacuum freeze-dried using a freeze dryer.

[81] The freeze-dried serum was reconstituted to its pre-freeze-drying volume with physiological saline, and the serum HI titer was determined using the hemagglutination inhibition (HI) test.

[82] The detailed operational steps of the hemagglutination inhibition test are as follows:

[83] 1. Preparation of the 4 HA Unit Antigen for Testing

[84] 1.1. Determination of Antigen Hemagglutination (HA) Titer

[85] Both the Avian Influenza Virus H9 Subtype Hemagglutination Inhibition (HI) Test Antigen (batches Z319–Z328) and the H9 avian influenza viruses (CVCC AV1534, CVCC AV1551, CVCC AV1552, CVCC AV1562, CVCC AV1563, and CVCC AV1571 strains) were obtained from the CVCC. The H9 subtype avian influenza virus HI test antigen was purchased from Harbin Weik Biotechnology Co., Ltd.

[86] A 96-well V-bottom microtiter plate was used. 25 μl of PBS (0.1 mol/L, pH 7.0–7.2, same below) was added to each well. 25 μl of antigen was added to the first row, with 2–4 replicate wells for each antigen. The antigen was then subjected to two-fold serial dilutions. After dilution, 25 μl of PBS was added to each well, followed finally by 25 μl of a 1% chicken red blood cell suspension. The plate was mixed using a microplate shaker and left undisturbed at room temperature for 30 minutes before reading the results. The highest dilution of the antigen that caused 100% agglutination of the red blood cells was taken as the endpoint.

[87] 1.2. Preparation of the 4 HA Unit Antigen

[88] Based on the determined HA titer of the HI antigen, the 4 HA unit antigen was prepared using PBS. The prepared 4 HA unit antigen was diluted with PBS to dilutions of 1:2, 1:3, 1:4, 1:5, 1:6, and 1:7. To 25 μl of antigen at each dilution, 25 μl of PBS was added, followed by 25 μl of a 1% chicken red blood cell suspension. The mixture was mixed thoroughly and left undisturbed at room temperature for 30 minutes before reading the results. If the 1:4 dilution was the endpoint for 100% red blood cell agglutination, it indicated that the prepared antigen was indeed 4 HA units. If the endpoint for 100% agglutination was 1:5 or 1:6, it indicated the prepared 4 HA unit antigen was actually higher than 4 units. If the endpoint for 100% agglutination was 1:2 or 1:3, it indicated the prepared 4 HA unit antigen was actually lower than 4 units. Adjustments should be made appropriately based on the test results to ensure the working antigen solution is 4 HA units.

[89] 2. HI Test

[90] **2.1.** A 96-well V-bottom microtiter plate was taken, and 25 μl of PBS was added to each well.

[91] **2.2.** 25 μl of the test serum was aspirated and added to the corresponding wells in the first row of each plate. Standard positive serum and negative serum controls were included on each plate. Then, two-fold serial dilutions were performed.

[92] **2.3.** 25 μl of the antigen containing 4 HA units was added to each well, and the plate was left undisturbed at room temperature for 30 minutes.

[93] **2.4.** 25 μl of a 1% (V/V) chicken red blood cell suspension was added to each well, mixed gently, and the plate was left undisturbed at room temperature for 30 minutes.

[94] **2.5. Result Judgment** The reaction plate was tilted. Any serum reaction well where the red blood cells flowed from the bottom of the well at the same speed as in the red blood cell control well was judged as hemagglutination inhibition. The highest serum dilution that completely inhibited the 4 HA unit antigen was taken as the HI titer.

[95] **3. Results**

[96] The prepared avian influenza virus (H9 subtype) positive serum had an HI titer of ≥ 12 $\log_2$. The detailed results are shown in Table 4.

[97] **Table 4 Determination Results of HI Titer for the Positive Serum**

Virus/Antigen	HI Titer ($\log_2$)
CVCC AV1534	13
CVCC AV1551	13
CVCC AV1552	13
CVCC AV1562	13
CVCC AV1563	12
CVCC AV1571	12
Z319	13
Z320	13
Z321	13
Z322	13

Virus/Antigen	HI Titer (log_2)
Z323	13
Z324	13
Z325	13
Z326	12
Z327	12
Z328	13
H9 AIV HI Test Antigen (from Harbin Weike Biotechnology Co.LTD)	12

[98] Example 3

[99] Serum Specificity

[100] The Avian Influenza Virus H9 Subtype Hemagglutination Inhibition (HI) Test Antigen, Newcastle Disease Virus (NDV) HI Test Antigen, Avian Egg Drop Syndrome Virus (EDSV) HI Test Antigen, H3 subtype avian influenza virus (Strain 212), and H6 subtype avian influenza virus (Strain 642) were all obtained from the CVCC. The Avian Influenza Virus H7 Subtype and H5 Subtype HI Test Antigen were purchased from Harbin Weik Biotechnology Co., Ltd.

[101] The serum specificity was determined according to the HI test steps described in Example 2. The test results are detailed in Table 5. The results showed that the prepared positive serum only showed a positive reaction with H9 subtype avian influenza virus antigen, and showed negative reactions with H5 subtype and H7 subtype avian influenza virus antigens, Newcastle disease virus antigen, Egg Drop Syndrome virus antigen, H3 AIV, and H6 AIV. This indicates that the prepared positive serum has good specificity.

[102] Table 5 Specificity Results of Avian Influenza Virus (H9 Subtype) Positive Serum

Serum	HI Titer (log_2)						
	H9 AIV Antigen	H5 AIV Antigen	H7 AIV Antigen	NDV Antigen	EDSV Antigen	H3 AIV	H6 AIV
H9 AIV	12	<1	<1	<1	<1	<1	<1

Positive Serum						

[103] Example 4

[104] Neutralization Titer Determination

[105] H9 avian influenza viruses (CVCC AV1534, CVCC AV1551, CVCC AV1552, CVCC AV1562, CVCC AV1563, and CVCC AV1571 strains) were all obtained from the CVCC.

[106] The prepared avian influenza virus (H9 subtype) positive serum was subjected to two-fold serial dilution. Four dilutions (1:2048, 1:4096, 1:8192, 1:16384) were separately mixed with an equal volume of H9 subtype avian influenza virus strains CVCC AV1534, CVCC AV1551, CVCC AV1552, CVCC AV1562, CVCC AV1563, CVCC AV1571, each containing 200 EID_{50} per unit dose. The mixtures were allowed to react at 37°C for 60 minutes. Then, each dilution was inoculated via the allantoic cavity into five SPF chicken embryos, 0.2 ml per embryo. The inoculated embryos were incubated at 37°C for 120 hours. The number of surviving, dead, and infected embryos in each group was recorded. The 50% protective dose (PD_{50}) was calculated using the Reed-Muench method, and then the neutralization titer of the serum was calculated.

[107] The results are detailed in Table 6. The results showed that the neutralization titer of the prepared avian influenza virus (H9 subtype) positive serum was ≥ 10^-3.76 /0.1 ml.

[108] Table 6 Determination Results of Neutralization Titer for Avian Influenza Virus (H9 Subtype) Positive Serum

Serum	CVCC AV1534	CVCC AV1551	CVCC AV1552	CVCC AV1562	CVCC AV1563	CVCC AV1571
H9 AIV Positive Serum	$10^{-3.96}$ /0.1 ml	$10^{-3.96}$ /0.1 ml	$10^{-3.96}$ /0.1 ml	$10^{-3.76}$ /0.1 ml	$10^{-3.76}$ /0.1 ml	$10^{-3.76}$ /0.1 ml

[109] Example 5

[110] Cross-Neutralization Test

[111] The seed viruses of NDV, Infectious Bronchitis Virus (IBV), H3 AIV, and H6 AIV were subjected to 10-fold serial dilution and placed into two sets of test tubes. For the first set, appropriate dilutions were mixed with an equal volume of the prepared positive serum. The other set was mixed with an equal volume of negative serum. After incubation at room temperature for 1 hour, the mixtures were inoculated via the allantoic cavity into 9-11-day-old SPF chicken embryos. After incubation at 37°C for an appropriate time, the neutralization index was calculated.

[112] The results are detailed in Table 7. The results showed that the cross-neutralization titers of the prepared positive serum against NDV, IBV, H3 AIV, and H6 AIV were all less than 1:10, proving the good specificity of the prepared serum.

[113] Table 7 Determination Results of Cross-Neutralization Titer for the prepared positive serum

Virus	Serum	EID_{50}	Neutralization Index
NDV	H9 AIV Positive Serum	$10^{8.3}$	2.0
	Negative Serum	$10^{8.0}$	
IBV	H9 AIV Positive Serum	$10^{5.6}$	2.5
	Negative Serum	$10^{5.2}$	
H3 AIV	H9 AIV Positive Serum	$10^{8.4}$	6.3
	Negative Serum	$10^{7.6}$	
H6 AIV	H9 AIV Positive Serum	$10^{8.5}$	7.9
	Negative Serum	$10^{7.6}$	

[114] Example 6 Application of the Serum Preparation Method in Preparing Positive Sera for Different Subtypes of Low Pathogenic Avian Influenza Virus

1. Preparation of the Virus and Antigen (Using H3 and H6 Avian Influenza Viruses as Examples)

[115] 1.1 Preparation of the Virus for Immunization

[116] The clinical strains H3 avian influenza virus (Strain 212) and H6 avian influenza virus (Strain 642) (identified and preserved by the CVCC) were diluted 1000-fold

with physiological saline and inoculated via the allantoic cavity into 10-day-old SPF chicken embryos, 0.1 ml per embryo. The inoculated embryos were incubated at 37°C for 96 hours, after which the allantoic fluid was harvested aseptically. Virus suspensions that passed sterility testing and had a viral content ≥ $10^{6.0}$ EID_{50}/0.1 ml were stored at -70°C for future use.

[117] 1.2 Preparation of Inactivated Vaccine for Immunization

[118] Formaldehyde solution was added to the virus suspension prepared in step 1.1 to a final concentration of 0.1%. The mixture was then inactivated at 37°C for 36 hours, with shaking approximately every 12 hours. After inactivation, the inactivated virus suspension was inoculated via the allantoic cavity into 9-day-old SPF chicken embryos, 0.2 ml per embryo. The inoculated embryos were incubated at 37°C for 96 hours, then the allantoic fluid was harvested and tested for hemagglutination activity. Samples showing negative hemagglutination activity were blindly passaged once. If the allantoic fluid from the blind passage also showed negative hemagglutination activity, it confirmed complete inactivation.

[119] The completely inactivated virus suspension was mixed with sterile Tween-80 at a ratio of 97:3 and stirred thoroughly until the Tween-80 was completely dissolved. This aqueous phase was then slowly added to the oil phase (made by uniformly mixing high-quality injectable white oil (by weight) and Span-80 (by weight) at a ratio of 95:5, followed by autoclaving) at a volume ratio of 2:3 (V/V). The mixture was homogenized using a homogenizer at 15,000 r/min for 2 minutes, then at 3,000 r/min for 1 minute, repeating this cycle 2-3 times. A sample of the emulsified vaccine was taken and dropped into water. Emulsification was preliminarily considered complete if the first drop dispersed but subsequent drops did not. The emulsified vaccine was then stored at 4°C and observed for 2 days. If no demulsification occurred, the water-in-oil vaccine preparation was considered complete.

[120] 1.3 Serum Preparation

[121] The diluted H3 and H6 subtype avian influenza virus suspensions, with a viral content of $10^{6.0}$ EID_{50}/0.1 ml, were used to inoculate 21-day-old SPF chickens via

intranasal administration, 0.1 ml per chicken. Blood was collected on days 5, 7, 9, 11, and 13 post-inoculation to separate serum for HI titer determination. Based on the HI titers, on day 13 post live virus inoculation, the prepared vaccine was administered via intramuscular injection at multiple sites such as the leg and breast, 1 ml per chicken. Blood was collected on days 7 and 14 post-vaccination to separate serum for HI titer and neutralization titer determination.

[122] The research results showed that using the preparation method of the present invention, one immunization with live H3 or H6 subtype avian influenza virus followed by one booster immunization with the inactivated vaccine within 20 days could prepare positive serum with high HI titer (average HI titers of 11.1 $\log_2$ and 12.1 $\log_2$, respectively) (Figure 2) and neutralization titer (The neutralization titer of H6 AIV positive serum against its homologous virus was $10^{-3.76}$/0.1 ml; the neutralization titer of H3 AIV positive serum against its homologous virus was $10^{-3.46}$ /0.1 ml). This proves that the preparation method is not limited to a single subtype of low pathogenic avian influenza virus.

[123] Example 7 Application of Low Pathogenic Avian Influenza Virus Positive Serum in Research on Recombinant Live Vector Vaccines Expressing Avian Influenza Virus HA Protein

[124] 1. Virus Inoculation

[125] The recombinant turkey herpesvirus expressing the H9 subtype avian influenza virus HA protein (HVT-H9 strain) was diluted 100-fold with M199 medium and then inoculated onto a 48-well cell plate containing confluent CEF secondary cells. Four wells were inoculated, with 0.1 mL per well (containing 100 PFU). A blank control (uninfected cells) was also set up. The plate was placed in a 37°C, 5% CO_2 incubator for 72 h, then removed for the indirect immunofluorescence assay.

[126] 2. Indirect Immunofluorescence Assay (IFA)

[127] The medium was removed from the cultured 48-well plate. The cells were washed once with PBS (pH 7.2–7.4, same below) and the liquid was discarded. 300 μL of cold methanol was added to each well and left at room temperature for 15 min. The methanol was discarded, and the plate was air-dried. After washing once with PBS, 100 μL

of a 1:2000 dilution of the avian influenza virus (H9 subtype) positive serum was added to each well. The plate was incubated at 37°C for 1 h. The serum was then discarded, and the cells were washed three times with PBS containing 0.05% Tween-20, followed by two washes with PBS, with gentle shaking for 1 min during each wash interval. After washing, 100 μL of a 1:200 dilution of Anti-Chicken IgY (IgG) (whole molecule)-FITC antibody (Product No.: F4137, Brand: Sigma-Aldrich) was added to each well. The plate was incubated at 37°C for 1 h. The antibody was discarded, and the cells were washed three times with PBS containing 0.05% Tween-20, followed by two washes with PBS, with gentle shaking for 1 min during each wash interval. After washing, the plate was observed and photographed under an inverted microscope.

[128] The experimental results are shown in Figure 3. The results indicate that the avian influenza virus (H9 subtype) positive serum prepared by the present invention can be applied in research on recombinant live vector vaccines expressing the H9 subtype avian influenza virus HA protein.

[129] Example 8 Application of Low Pathogenic Avian Influenza Virus Positive Serum in Clinical Virus Isolation

[130] 1. Neutralization

[131] Chicken embryo allantoic fluid identified as a mixed infection of IBV and H9 AIV (obtained by inoculating SPF chicken embryos with clinical samples) was diluted 10-fold and filtered through a 0.22 μm filter. The filtrate was mixed with an equal volume of a 16-fold diluted avian influenza virus (H9 subtype) positive serum. The mixture was placed in a 37°C water bath for 1 h, with one agitation during the incubation.

[132] 2. Chicken Embryo Inoculation

[133] The neutralized allantoic fluid was inoculated via the allantoic cavity into 9-day-old SPF chicken embryos, 0.2 ml per embryo. The embryos were incubated at 37°C for 5–7 days. The embryos were then removed and placed at 4°C to cool for 4–6 h. The allantoic fluid from each embryo was harvested individually and tested for hemagglutination activity. Allantoic fluid samples with negative hemagglutination activity were blindly passaged once in chicken embryos, and then identified by RT-PCR.

[134] 3. Duplex RT-PCR Identification

[135] Viral nucleic acid was extracted according to the instructions of the TaKaRa MiniBEST Viral RNA/DNA Extraction Kit Ver.5.0 (Brand: Takara, Cat. #: 9766). Then, duplex RT-PCR was performed using the PrimeScriptTM One Step RT-PCR Kit Ver.2 (Dye Plus) (Brand: Takara, Cat. #: RR057A). The IBV-specific identification primers are listed in Announcement No. 717 of the Ministry of Agriculture and Rural Affairs of the People's Republic of China. The H9 subtype AIV-specific identification primers are listed in the Chinese Agricultural Industry Standard NY/T 772-2013 "Method of RT-PCR for Avian Influenza Virus". The IBV and H9 AIV specific identification primers were added to the duplex RT-PCR reaction system at a 1:1 ratio, as shown in Table 8.

[136] Table 8 Duplex RT-PCR Reaction System

Reagent	Volume (μl)
PrimeScript 1 Step Enzyme Mix	2
2×1 Step Buffer (Dye Plus)	25
IBV and AIV Forward Primer	0.5 each
IBV and AIV Reverse Primer	0.5 each
RNA Template	4
RNase free H_2O	17
Total Volume	**50**

[137] RT-PCR program: Stage 1: Reverse transcription at 50°C for 30 min; Stage 2: Pre-denaturation at 92°C for 2 min; Stage 3: PCR amplification: 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min; Final extension at 72°C for 5 min. After RT-PCR, 10 μL of the PCR product was taken for electrophoresis at 150V for 20 minutes. The gel was then analyzed using a gel imaging system.

[138] 4. Result Analysis

[139] The RT-PCR results (Figure 4) showed that the H9 avian influenza virus in the clinical mixed sample could be successfully neutralized by the avian influenza virus (H9 subtype) positive serum, and the IBV strain could be successfully isolated. Therefore, the prepared avian influenza virus (H9 subtype) positive serum can be applied in the isolation

of clinical viruses.

[140] Example 9 Application of Low Pathogenic Avian Influenza Virus Positive Serum in Research on the Pathogenesis of Avian Influenza Virus

[141] 1. Cell Transfection

[142] The constructed plasmids pCAGGS-H9HA and pCDNA-H9HA were transfected into A549 cells according to the instructions of Lipofectamine™ 3000 (Brand: Invitrogen, Cat. #: L3000015). 36 h after transfection, the cells were harvested for Western blot assay.

[143] 2. Western Blot Assay

[144] The cells were washed three times with 0.01 M, pH 7.2–7.4 PBS. Then, 1 ml of PBS was added, and the cells were scraped off and collected into a 1.5 ml centrifuge tube using a cell scraper. After centrifugation at 12,000 rpm for 5 min, the supernatant was discarded. The cell pellet was resuspended by flicking the tube. Then, 200 μL of High-Efficiency RIPA Lysis Buffer (Tissue/Cell) (Brand: Solarbio, Cat. #: R0010) was added, and the cells were lysed on ice for 10 min. After centrifugation at 12,000 rpm for 10 minutes, the supernatant was collected. LDS Sample Buffer (4×) (Brand: Invitrogen, Cat. #: NP0008) was added at a 3:1 ratio. The mixture was boiled at 100°C for 10 minutes. SDS-PAGE electrophoresis was performed using a precast gradient gel (4%–20%) (Brand: Solarbio, Cat. #: PG42010-S). After electrophoresis, the proteins were transferred to a membrane. The PVDF membrane with transferred proteins was moved to an incubation box containing blocking buffer (5% skim milk powder dissolved in 1× PBST) and blocked with shaking at room temperature for 2 h. After blocking, the membrane was removed from the blocking solution and washed three times with 1× PBST (Brand: Solarbio, Cat. #: P1031) at room temperature on a shaker to remove residual blocking solution, 10 minutes each time. The washed PVDF membrane was placed into a new incubation box, and avian influenza virus (H9 subtype) positive serum diluted 500-fold with 1× PBST was added. The membrane was incubated at 37°C for 1 h. After incubation, the membrane was washed three times with 1× PBST at room temperature on a shaker, 10 min each time. The washed PVDF membrane was placed into a new incubation box, and Rabbit Anti-Chicken IgY IgG (H+L)

(HRP) (Brand: Biodragon, Cat. #: BF03012) diluted 2000-fold with 1× PBST was added. The membrane was incubated at 37°C for 1 h. After incubation, the membrane was washed three times with 1× PBST at room temperature on a shaker, 10 min each time. The PVDF membrane was removed, developing solution (Brand: Thermo Scientific, Cat. #: 34577) was added, and the membrane was placed into a developer for development. The results are shown in Figure 5. The results show that the avian influenza virus (H9 subtype) positive serum prepared by the present invention can detect the transient expression of H9 subtype avian influenza virus HA protein in mammalian cells, indicating that this serum can be applied in research on the pathogenesis of H9 subtype avian influenza virus.

[145] In summary, the low pathogenic avian influenza virus positive serum prepared by the method of the present invention exhibits good specificity and sensitivity. It can be used for the detection of extraneous viruses in poultry viral live vaccines, the isolation and identification of clinical viruses, the differential testing of live vector vaccines, and the evaluation of the immune efficacy of related vaccines.

[146] Although the foregoing embodiments provide a detailed description of the present invention, they represent only a portion of the embodiments of the invention, not all possible embodiments. Those skilled in the present field of study can derive other embodiments from these examples without creative effort, and all such embodiments shall fall within the protection scope of the present invention.