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Protocols

Preparation and evaluation of IgY against human papillomavirus

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ABSTRACT

Human papillomavirus (HPV) infection is a major global health challenge and is closely related to the occurrence of diseases such as cervical cancer. Unfortunately, effective treatments are still lacking. In view of the advantages of antibody drugs, antibody-targeted therapy may become one of the means of treatment and prevention of HPV infection. This study explores the potential of antibody-targeted therapy using immunization with HPV nine-valent vaccine in Leghorn chickens. The resulting egg yolk antibodies (IgY) was extracted from eggs using the bitter-ammonium sulfate method and confirmed through SDS-PAGE analysis. The neutralizing titer was performed by pseudovirus-neutralizing antibody experiments, which could reach 1:2000 (18.2 μg/mL). This successful preparation of IgY against HPV 6/11/16/18/31/33/45/52/58-L1 protein showed its potential as a therapeutic agent, particularly post-HPV16 infection. This work lays the groundwork for HPV-specific IgY preparation and contributes to advancing targeted therapies for cervical cancer, prompting further research in HPV-related therapeutic approaches.

1. Introduction

Cervical cancer poses a significant global health challenge. It ranks as the fourth most prevalent cancer worldwide, leading to substantial morbidity and mortality among women, particularly in developing countries such as China (Sung et al., 2021; Viveros-Carreno et al., 2023). Human papillomavirus, characterized by its circular double-stranded DNA structure, spherical particles, icosahedral symmetry, and absence of an envelope with a diameter of 60 nm, is a key player in cervical cancer development (Harden and Munger, 2017). HPV types are categorized into high-risk and low-risk, with persistent infection of high-risk types being the primary driver of cervical cancer (Burd, 2003; Schiffman et al., 2016). According to the World Health Organization (WHO), high-risk HPV includes types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 73, among which types 16 and 18 are identified as the principal carcinogenic types, collectively responsible for approximately 70 % of cervical cancer cases (Molina et al., 2020).

HPV protein L1 is a pivotal component of the HPV capsid protein, demonstrating a remarkable ability to spontaneously assemble in vitro into virus-like particles (VLP) with structural similarities to natural viruses (Ashique et al., 2023; Kirnbauer et al., 1992). Given the high immunogenicity of HPV capsid protein L1 and its status as the primary target of current HPV prophylactic vaccines (Williamson, 2023), it holds paramount importance in the HPV infection process. The multifaceted roles of HPV L1, encompassing viral receptor recognition, genome delivery, and organism protection induction, underscore the significance of preparing highly specific antibodies against the HPV L1 protein for medical clinical applications (Oyouni, 2023).

Currently, immunoglobulin G (IgG) antibodies used for diagnosis and treatment are mainly monoclonal antibodies (mAbs) (Aghbash et al., 2022). MAbs are studied for targeted therapy due to their good specificity. However, in targeted therapy, there are problems such as activation of autoantigen immune response leading to non-tumor specific immune response, and its large-scale preparation and storage face major challenges (Scott et al., 2012). In addressing this issue, IgY emerges as a promising solution (da Silva and Tambourgi, 2010). IgY is a polyclonal antibody produced by B-lymphocytes in laying birds, derived from poultry egg products, stimulated by specific external antigens. These antibodies are selectively transferred to the yolk through the bloodstream, accumulating in eggs (Schade et al., 2005). IgY is an effective preventive or therapeutic antibody with two light chains and two heavy chains, and its molecular size is about 180KDa (Carlander

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et al., 1999). The advantages of IgY as an antibody-drug over mAbs include non-invasive production, high cost-effectiveness, and no interaction with complement and mammalian Fc receptors (Hadi et al., 2024). It is reported that IgY antibody can effectively prevent or treat a variety of viral and bacterial infections, such as dengue virus, influenza A virus, Helicobacter pylori, and Escherichia coli diarrhea (Karthikeyan et al., 2022; O'Donnell et al., 2020; Yang et al., 2014; Zhang et al., 2021). Therefore, IgY holds promising potential for its application in preventing and controlling HPV.

In this study, we utilized the human papillomavirus nine-valent vaccine as the immunogen for immunizing Leghorn chickens. The extraction of IgY from eggs was carried out using the bitter-ammonium sulfate method, and its confirmation was subsequently validated through SDS-PAGE analysis. We determined a notable neutralizing titer of 1:2000 (18.2 μg/mL) through pseudovirus-neutralizing antibody experiments. Based on the above results, the IgY against HPV 6/11/16/18/31/33/45/52/58-L1 protein was prepared successfully and showed the potential of the antibody as a therapeutic agent after HPV16 infection. Our work not only establishes a theoretical basis for the development of HPV-specific IgY but also introduces a new strategy for the prevention and treatment of HPV.

2. Material and methods

2.1. Animals and vaccine

Leghorn chickens (20 weeks of age) were obtained from the Guangdong Medical Laboratory Animal Center. Taconic Laboratories (Guangdong, China). All procedures were performed by Guangdong Provincial Animal Resources Committee approved protocols for animal use.

Human papillomavirus nine-valent vaccine (Recombinant protein, containing 30 μg HPV6 L1 protein, 40 μg HPV11 L1 protein, 60 μg HPV16 L1 protein, 40 μg HPV18 L1 protein, 20 μg HPV31 L1 protein, 20 μg HPV33 L1 protein, 20 μg HPV45 L1 protein, 20 μg HPV52 L1 protein, 20 μg HPV58 L1 protein) was manufactured by Merck Sharp & Dohme (State of New Jersey, USA), lot number 70082398/00–1/4121.

2.2. Cells and viruses

The 293FT cells were purchased from BeNa Culture Collection (Beijing, China) and cultured in high sugar DMEM containing 10 % fetal bovine serum (FBS) and 1 % Penicillin-Streptomycin Solution. HPV16/18 pseudovirus with green fluorescent protein (GFP) reporter gene was generously provided by Suzhou Biodragon Immunotechnologies Co. Ltd (Jiangsu, China).

2.3. Reagents and apparatus

DMEM high glucose medium, Penicillin-Streptomycin Solution, trypsin, fetal bovine serum (FBS), and phosphate buffered salt solution (PBS) were purchased from GIBCO (USA). Pre-stained protein marker, SDS-PAGE Gel Kit, and ECL Ultra Sensitive Chromatography Kit were purchased from Beyotime Biotech Inc (Shanghai, China). Tetramethylbenzidine (TMB) was purchased from Sigma Co. Ltd (St. Louis, USA). All the chemicals used in this study were analytical reagent grade.

Multi-function imager was conducted on a UVPchemstudio plus (Jena, Germany). Multi-Function Measuring Instrument for measuring fluorescence intensity and absorbance (Massachusetts, USA). The cells were observed with the Axio Observer7 fluorescence microscope (Oberkochen, Germany).

The complete culture medium was high sugar DMEM containing 10 % FBS and 1 % Penicillin-Streptomycin Solution.

2.4. Preparation of anti-HPV-specific IgY

HPV nine-valent vaccine (0.05 mg) in 0.5 mL of PBS was a subcutaneous multipoint injection and intramuscular injection to immunize the Leghorn chicken (20 weeks). The immunized procedure was modified from Duan et al. with minor modifications (Duan et al., 2009). The immunized protocol was repeated every seven days, and a total for three times. After six months of the strengthened immunization, the egg was collected. The IgY was extracted by bitter-ammonium sulfate law, with minor modifications (Polson et al., 1980). Briefly, the yolk was added to the PBS containing 3.5 % PEG 6000 (3/1, v/v) and stirred for 30 min. The solution was allowed to stand until the mixed solution was stratified and then filtered to remove insoluble impurities. The solid powder of PEG 6000, which can account for 12 % of the volume of the filtrate, was added. Stirring was continued for 40 min after sufficient dissolution, and the precipitate obtained after standing and settling was the crude extract of lg Y. Salting out was performed using saturated ammonium sulfate. The collected precipitate was dissolved in a small amount of phosphate buffer and dialyzed for a three-day maintenance period. In the end, antibody purity was analyzed using 10 % SDS-PAGE.

2.5. The determination of anti-HPV-specific IgY titers

The titer of IgY against HPV was determined by ELISA, with minor modifications(Wen et al., 2012; Yu. et al., 2024). Briefly, (a) 100 μL HPV nine-valent vaccine (50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.19, 0.098, 0.049, 0.024 μg/mL) in carbonate buffer was added into each well of microplates and incubated at 4 ℃ overnight; (b) 270 μL skim milk powder (30 mg/mL) was added to block at 37 ℃ for 1 h after the plates were washed three times with PBST (PBS containing 0.05 % Tween-20); and (c) Wash again with PBST for 3 times, and dilute IgY with PBS to different concentrations (36.38, 18.19, 9.10, 4.55, 2.27, 1.14, 0.57 mg/mL) and Set non-immune IgY (1 mg/mL) as the control group, add 100 μL/well to the corresponding wells of the plate, and let stand at 37 ℃ for 1 h; (d) after further washing, add diluted 0.2 μg/mL sheep anti-chicken HRP secondary antibody to the corresponding wells of the plate, and leave it at 37 ℃ for 1 h. (e) after another washing step, 100 μL substrate solution (H_2O_2 and TMB solution) was added and incubated at 37 ℃ for 15 min; (f) before measuring the absorbance at 450 nm, the reaction was stopped by 50 μL 2 M H_2SO_4. After TMB color development, the absorbance value at OD= 450 nm was detected using the Multi-Function Measuring Instrument. Different curves were plotted using the antigen dilution as the horizontal coordinate and the absorbance values at different antibody concentrations as the vertical coordinate.

2.6. The specificity of IgY

The specificity of IgY against HPV was determined by western blot, with minor modifications(Krähling et al., 2021; Towbin et al., 1979). Briefly, HPV16/18 pseudoviral diluent (Titer=143/357) and pseudoviral lysate (pseudoviral diluent after 2 %Triton 100 cleavage) were electrophoretic together with SDS-PAGE. Finally, Coomassie brilliant blue staining and Western blotting analysis were performed, respectively. Anti-HPV-L1 IgY diluted four thousand times (9 μg/mL) was used as the detection antibody for Western blotting, and 0.2 μg/mL rabbit anti-chicken antibody connected with horseradish peroxidase was used as the secondary antibody, and the color was developed by using ECL Ultra Sensitive Chromatography Kit, and then placed in a Multi-function Imager for exposure imaging.

2.7. Identification of neutralizing antibodies to pseudoviruses

The identification of IgY against HPV has neutralized antibodies to pseudoviruses, with minor modifications(Bao et al., 2022; Krähling et al., 2021). Briefly, the 293FT cells were cultured to reach the

logarithmic growth phase, counted using the hemocyte counting plate counting method, diluted to 1.5 × 10^5 cells/mL with complete medium and inoculated in 96-well cell plates, 100 μL/well, and used for pseudoviral infections after 6 h of incubation in an incubator containing 5 % carbon dioxide. The IgY of different concentrations (0, 1.1, 2.2, 4.5, 9, 18.2, 36.4, 72.8 μg/mL in complete culture medium, among them, those without IgY were positive control group) of 55 μL were mixed with HPV16/18 pseudovirus (titer=143/357) placed at 4 ℃ for 1 h. The mixture of IgY and pseudovirus was slowly attached to the corresponding hole of 293FT cell culture plate, 100 μL/well, and incubated in a cell incubator containing 5 % carbon dioxide and 37 ℃ for 72 h. The expression of green fluorescent protein by pseudovirus-transfected cells was observed under an inverted fluorescence microscope, and the fluorescence intensity was detected using the Multi-Function Measuring Instrument. Evaluation of the effect of IgY on pseudovirus transfection according to the experimental results.

2.8. Statistical analysis

The results were analyzed by analysis of variance. In testing the significance of the difference in inhibition of the activity of IgY against the HPV16 pseudovirus and HPV18 pseudovirus, respectively, Student's *t*-test was used. A probability level of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Preparation of egg yolk antibodies

After four immunizations, egg yolk antibodies were extracted from eggs and then purified. The IgY was confirmed by SDS-PAGE. As shown in Fig. 1, an obvious band at a molecular weight of 65 kDa corresponded to the heavy chain band of IgY, while an inconspicuous band at a molecular weight of 25 kDa corresponding to the light chain of IgY occurred, which agreed with Syahruni et al. (Syahruni et al., 2021). There is an inconspicuous band at the molecular weight of about 35–40 kDa, which may be due to the degradation of a few IgY under high-temperature conditions or the formation of light chain dimers(Li et al., 1996). The purified protein still contained few impurity proteins between 70 and 25 kDa, but IgY was the main protein, and impurity proteins had no significant effect on interfering IgY and antigen binding (Santos et al., 2014). The concentration of purified IgY was measured as 36.38 mg/mL by BCA concentration assay.

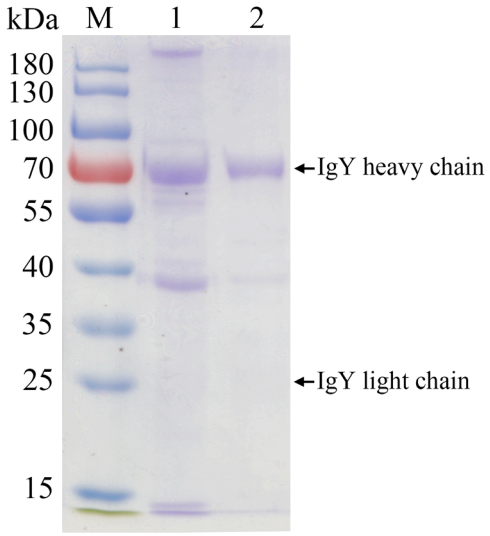

Fig. 1. 10 % SDS-PAGE patterns of IgY purified from egg yolk. The sample concentration for SDS-PAGE was 90 μg/mL. Lanes: M, molecular size marker, 1, Egg yolk, 2, IgY obtained after salting out.

3.2. The determination of anti-HPV-specific IgY titers

By indirect ELISA, various HPV L1 proteins in the nine-valent vaccine were used as envelope antigens, IgY with different concentrations as capture antibodies, and TMB staining, as shown in Fig. 2. When the concentration of antibody was higher than that of 18.19 mg/mL, the absorbance increased with the increase of antigen concentration. However, when the antibody concentration is less than or equal to 9.1 mg/mL, with the increase of antigen concentration, the absorbance value will decrease after increasing to a certain extent. Non-immune IgY was a control group, and indirect ELISA detection showed that the prepared IgY had an excellent binding effect with the HPV-L1 protein.

3.3. The specific IgY against HPV

SDS-PAGE as well as Western blotting were performed using HPV16/18 pseudoviruses as samples, as shown in Fig. 3 that a band of the target pseudovirus capsid protein can be seen at a mass of about 70 kDa, and the size matches the theoretical size of the HPV16/18-L1 proteins (Uniprot reference: VL1_HPV16/ VL1_HPV18). Western blotting showed that the antibody could specifically bind to HPV16/18 pseudoviral capsid protein L1 and did not bind to any other proteins, both in untreated pseudoviruses and 2 %Triton 100-treated pseudoviruses. In contrast, the binding of the pseudovirus to the IgY was significantly less after lysis with the same volume of 2 % Triton 100, probably due to the degradation of part of the L1 protein after treatment with Triton 100. However, the specific binding bands were still clearly visible on the PVDF membrane.

3.4. Identification of neutralizing antibodies to HPV pseudoviruses

Different concentrations of IgY were mixed with pseudovirus and then incubated with 293FT cells for three days before observing the cells expressing the green fluorescent protein in an inverted fluorescence microscope. As depicted in Fig. 4, the experimental group with a high concentration of IgY exhibited a lower expression of the pseudovirus green fluorescent protein gene within the cells compared to the positive

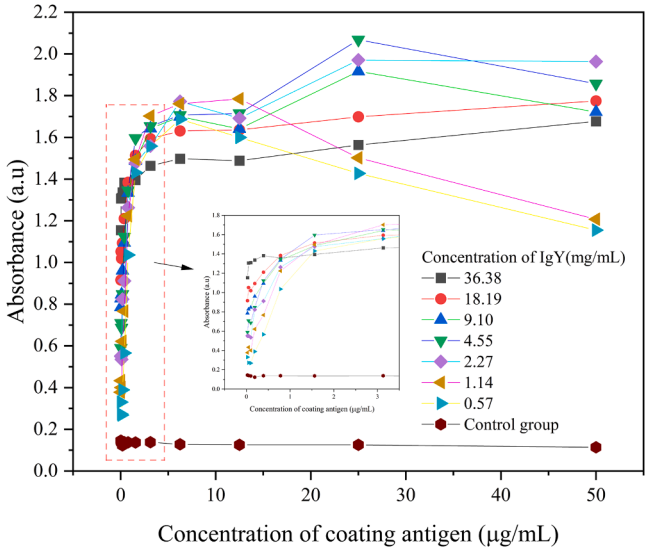

Fig. 2. Determination of egg yolk antibodies titer by indirect ELISA. Changes in absorbance when different concentrations of nine-valent vaccine coated antigen and different concentrations of IgY were used as detection antibodies. The absorbance value data were detected by Multi-Function Measuring Instrument at OD= 450 nm.

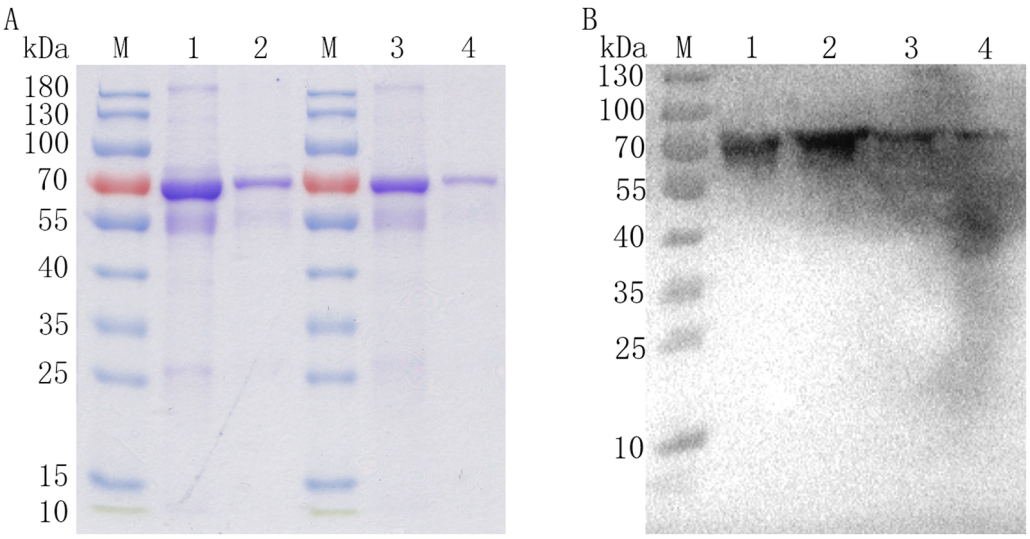

Fig. 3. A: Analysis of HPV pseudoviruses by SDS-PAGE; B: Determination of specificity of IgY by Western blot. Four thousand times (9 μg/mL) of IgY was used as the primary antibody, and ten thousand times (0.2 μg/mL) of diluted HRP was used as the secondary antibody for western blot analysis. M: Marker; lane 1: HPV 16 pseudovirus; lane 2: HPV 16 pseudovirus after 2 % Triton 100 cleavage; lane 3: HPV 18 pseudovirus; lane 4: HPV 18 pseudovirus after 2 % Triton 100 cleavage.

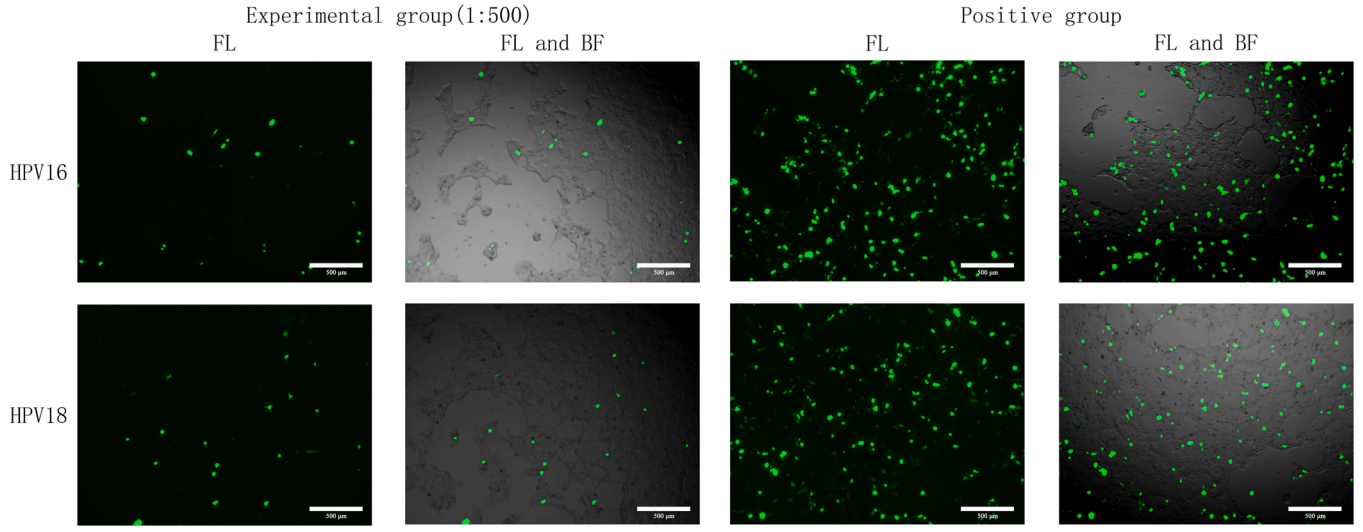

Fig. 4. The expression of green fluorescent protein (GFP) in 293FT cells was observed under fluorescence microscope 72 h later. FL: Fluorescent lamp; BF: bright field; Experimental group (1:500): IgY concentration was 72.8 μg/mL; Positive group: Complete medium (no IgY added).

control group, where high content expression was evident. This observation underscores the outstanding inhibitory effect of a high concentration of IgY on pseudovirus infection.

The fluorescence intensity (Arbitrary Unit, A.U.) of the cell-expressed green fluorescent protein spots was read by a Multi-Function Measuring Instrument. The average of the data from three sets of replicate experiments was taken and compared with the positive control 50 % without IgY, which in turn was used to evaluate the effect of the IgY on inhibition of pseudovirus entry into the cells. The experimental results showed that the fluorescence inhibition rate decreased with the continuous dilution of the antibody concentration. The IgY had a higher inhibitory effect on HPV16 pseudoviruses than HPV18 pseudoviruses. Inhibition rate (IR) was counted based on the following equation:

IR%= {[A.U.(Positive group)-A.U.(Experimental group)]/A.U.(Positive group)}* 100%

The titer value is based on the neutralizing antibody criterion, i.e., the antibody dilution at which the intensity of the fluorescent signal presented by the antibody sample to be examined does not exceed 50 % of that of the positive control (Mazzini et al., 2021; Pastrana et al., 2004). As shown in Fig. 5, the IgY had a 2000-fold (18.2 μg/mL) neutralizing antibody titer to the pseudovirus of HPV type 16. The inhibition rate of HPV18 pseudovirus transfection was low, the 500-fold (72.8 μg/mL) dilution only reached nearly 40 % inhibition rate, and the inhibition rate of HPV16 at the same concentration reached 60 %, with the increase of the dilution rate, the inhibition rate of the IgY for the 16/18 pseudoviruses gradually decreased. The inhibition rate of the relative fluorescence of HPV18 pseudoviruses was less than 10 % at the 32,000-fold (1.1 μg/mL) dilution rate, which is far lower than the judgment standard of the neutralizing antibody and is consistent with the observation of fluorescence microscopy.

4. Discussion

Cervical cancer, a leading cause of mortality, is intricately linked to

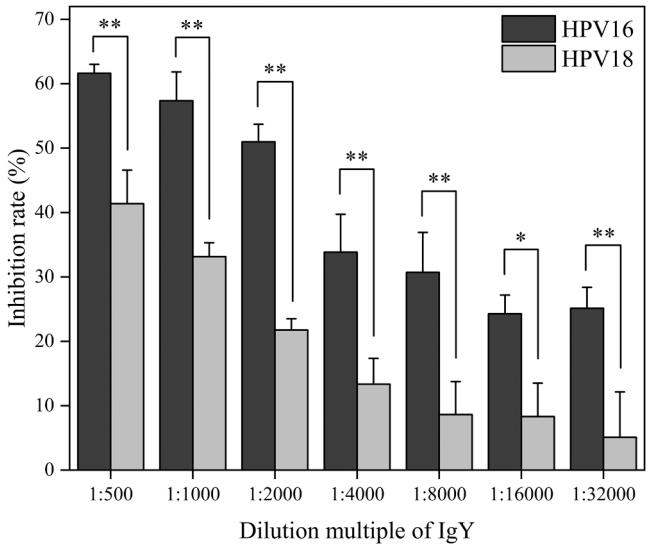

Fig. 5. Typical results of relative fluorescence inhibition rates of different dilution ratios of IgY. Different concentrations of mixed IgY or PBS and pseudovirus were pre-incubated at 37 ℃ for 1 h and then added into pre-cultured 293FT cells (cell density: 1.5 $\times 10^5$ cells/mL) for 72 h. The fluorescence intensity was measured with the excitation wavelength of 485 nm and the emission wavelength of 520 nm and the inhibition rate was calculated. Values are the mean of triplicate samples. Vertical bars indicate the standard deviation. * and * * indicate statistically significant differences between HPV16 and HPV18 ($P < 0.05$ and $P < 0.01$, respectively).

persistent HPV infections, particularly high-risk types 16 and 18. Prophylactic vaccination pre-infection and prompt testing and treatment post-infection play crucial roles in halting cervical cancer development. HPV viruses are difficult to culture in vitro and require more stringent culture environments that are difficult for the average laboratory to achieve. HPV VLPs are viral structures assembled only from the viral capsid protein L1, which do not contain viral nucleic acids and are non-infectious, as well as retaining antigenically similar structures to natural viruses (Xiang et al., 2022), and are the most used regional fragments in HPV vaccine applications and research, such as the Merck Sharp & Dohme Corp. Nine-valent vaccine (Brown et al., 2014). HPV prophylactic vaccines currently provide good protection against HPV infection, but continued exploration is needed for methods of treating post-infection antivirals. Antibody-dependent enhancement (ADE) can lead to an increase in viral load and aggravate secondary viral infection and is one of the factors to be considered in the research and development of antibody drugs (Wan et al., 2020). Studies have shown that IgY can neutralize infection by dengue virus and Zikavirus without inducing ADE, confirming its therapeutic potential (O'Donnell et al., 2020). However, it is worth noting that the synergistic effect of multiple specific IgY may lead to ADE phenomenon due to the receptor having similar epitopes (Jahangiri et al., 2021). The treatment of antibody drugs relies on binding to capsid protein antigens on viruses or bacteria (Pastrana et al., 2004). In a report on the IgY against HPV16 E7 oncoprotein, it was shown that IgY antibodies recognized more different epitopes than IgG antibodies, forming a unique antibody repertoire (Di Lonardo et al., 2001). The production process of IgY does not require bleeding and is, therefore, non-invasive (Polson et al., 1980). In commercial production, it is simpler and less costly than IgG antibody production and has the advantages of good specificity, good thermal stability, and the inability to bind to or activate the mammalian complement system (El-Kafrawy et al., 2023; Wang et al., 2023). It is a potential therapeutic antibody. When preparing anti-HPV6/11/16/18/31/33/45/52/58-L1 antibodies, it is necessary to ensure that the antibody has high specificity and affinity, properties that are essential for the neutralizing activity of the antibody. In this study, hens were immunized with subcutaneous multipoint injections and intramuscular injections a total of four times to ensure that high-affinity IgY could be prepared. Enzyme-linked immunosorbent assay (ELISA) utilizes highly specific recognition and binding of antigen-antibody to achieve analysis and detection, with high sensitivity, simplicity, and other characteristics, and has been widely used in the detection and analysis of bacteria, viruses, small molecule antigens, and other samples (Lazcka et al., 2007). In this study, the good affinity of IgY was identified by ELISA, and it was observed that the absorbance value decreased with the continuous dilution of antibody when the antigen concentration of the nine-valent vaccine was lower than 0.78 μg/mL. In addition, in the same concentration of coated antigens, higher concentrations of IgY will show a lower light absorption value, possibly due to the tight arrangement of IgY Fab arms, resulting in increased steric hindrance of binding antigens (Lee et al., 2002).

To further assess the specificity and antiviral capacity of IgY, Western blot and pseudovirus neutralization assays were utilized to identify and determine the neutralizing activity of the antibodies, which is important for evaluating the efficacy of IgY as well as for the development of future strategies for their application in therapy. SDS-PANG electrophoresis using pseudoviruses that have been lysed with 2 % Triton100 and those that have not been lysed allows the molecular weight of the HPV L1 capsid protein to be determined and to determine the presence or absence of other proteins. By Western blot, it was found that the IgY specifically recognized HPV type 16/18 shell proteins and did not recognize and bind to other proteins, indicating that the IgY has strong sensitivity and specificity. Pastrana et al. (2004) established a method for neutralizing antibodies to HPV pseudoviruses based on secreted alkaline phosphate (SEAP) as a reporter gene, but the method is more complex and time-consuming. In this study, HPV16/18 pseudovirus containing a green fluorescent protein (GFP) reporter tag was used to infect 293FT cells, and the green fluorescent protein was expressed after infection of the cells, and the results of the antibody-neutralization assay were observed under a fluorescence microscope 72 h later. As shown in Fig. 5, the prepared IgY had different pseudoviral inhibitory effects on the two subtypes of HPV16 and HPV18. The IgY was more effective in inhibiting pseudoviral transfection of HPV type 16 compared to HPV type 18 subtypes, possibly because the HPV type 16 subtype, as a culprit responsible for 50 % of cervical cancers (Schiffman et al., 2007) should be the first virus to be considered for prevention, and thus may be associated with the highest amount of HPV16-L1 protein (60 μg) in the nine-valent vaccine.

Till now, there has been a lot of research on the potential anti-HPV effects of IgY. Di Lonardo et al. (2001) prepared yolk antibodies against HPV16 E7 protein and compared them with rabbit polyclonal antibodies. They found that IgY was more active than IgG, and the amount of antibodies produced by chickens was 5–10 times that of rabbits. Cheng et al., 2022 immunized hens with a peptide fragment of HPV16 E6 to obtain highly affinable IgY. Our results are consistent with these findings. However, various factors, such as different immunization procedures, test animals, or antibody collection times, can lead to differences between study groups. Compared with the IgG neutralizing titer previously reported by Kim et al. (2007), our prepared IgY neutralizing antibody had a relatively high titer (1:2000). Taken together, these findings and the work of IgY against HPV presented here strengthen the potential application of IgY antibodies in the control of cervical cancer occurrence.

The preparation of anti-HPV6/11/16/18/31/33/45/52/58-L1 antibodies and their evaluation in terms of pseudovirus neutralization titer holds promise as a novel strategy and a crucial tool for specific antibody therapy targeting HPV-associated infections and cancers. Future investigations could delve into the potential synergies of IgY treatment alone or in conjunction with other modalities, such as radiation or chemotherapy, aiming to amplify treatment efficacy based on the HPV infection type and cancer progression. These avenues may pave the way for innovative multimodal treatment pathways, providing diverse therapeutic regimens and strategies. However, further research and

clinical trials are needed to determine the safety and efficacy of antibodies in clinical therapeutic applications to improve public health and cancer treatment.

CRediT authorship contribution statement

Weiguang Chen: Conceptualization, Methodology, Data Curation, Writing - Original Draft. **Huanxin Xiao:** Conceptualization, Methodology, Writing - Review & Editing. **Mingxia Lin:** Conceptualization, Investigation, Data Curation. **Jiqing Zhou:** Conceptualization, Resources, Supervision. **Qiancheng Xuan:** Methodology, Data Curation. **Xiping Cui:** Conceptualization, Methodology, Supervision. **Suqing Zhao:** Funding acquisition, Investigation, Project administration, Conceptualization, Methodology, Resources, Writing - Review & Editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Suqing Zhao reports financial support was provided by Guangdong Basic and Applied Basic Research Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Data availability

Data will be made available on request.

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