

High immune efficacy against different avian influenza H5N1 viruses by oral administration of a *Saccharomyces cerevisiae*-based vaccine in chickens

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Research article

Keywords: EBY100/pYD1-HA, Oral administration, Cross-protection

Posted Date: June 15th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-33780/v1>

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Abstract

Background

A safe and effective vaccine is the best way to control large-scale highly pathogenic avian influenza virus (HPAI) A (H5N1) outbreaks. *Saccharomyces cerevisiae* (*S. cerevisiae*) is an ideal mucosal delivery vector for vaccine development, and we have previously shown that conventional injection administration with a *S. cerevisiae*-based vaccine (EBY100/pYD1-HA) was protective against homologous H5N1 virus in a mouse model. Due to the diameter of *S. cerevisiae* is around 10 μm which results in a severe inflammation by injection route, therefore, oral administration is a more suitable approach for EBY100/pYD1-HA conferring cross-protection in poultry.

Results

We extended our work by evaluating the immunogenicity and cross-protective efficacy of oral vaccination with EBY100/pYD1-HA in the chicken model. Oral immunization with EBY100/pYD1-HA could induce robust serum IgG, mucosal IgA and cellular immune responses. Importantly, EBY100/pYD1-HA provided complete cross-protection against different H5N1 viruses challenge.

Conclusions

These findings suggest EBY100/pYD1-HA, a promising H5N1 oral vaccine candidate, can avoid potential reassortment of other avian influenza viruses in oral administration of live virus vaccines and overcome the drawbacks of conventional injection route. Importantly, this platform will be able to provide opportunities for broader applications in poultry during HPAI A (H5N1) outbreaks.

Background

The emergence and spread of highly pathogenic avian influenza (HPAI) A (H5N1) viruses have fueled concerns of a potential zoonotic poultry pandemic [1], and spurred efforts towards developing vaccines against A (H5N1) influenza viruses, as well as improving vaccine production methods [2]. Current licensed vaccines including adjuvanted formulation are predominately inactivated whole avian influenza H5N1, and have been available for the control of outbreaks in poultry [3]. These vaccines have limitations since they require intramuscular injection and the efficacy of these vaccines have not been fully elucidated [4]. In addition, some of these vaccines were poorly immunogenic and may require higher concentration of the immunogen to achieve protective immunogenicity [5]. Further, the egg-based manufacturing processes of these vaccines also have safety and production issues. A live-attenuated A (H5N1) vaccine had been generated by reverse genetics, but the risk of generating virus reassortment in the field prohibits the use of this vaccine in most instances [6]. Thus, there is a clear need for new vaccine formulation and delivery strategies that can provide increased efficacy and safety.

In attempts to develop more efficacious A(H5N1) vaccines, several strategies, such as mammalian cell-based vaccines [7], recombinant protein-based vaccines [8], recombinant virus-like particle (VLP) vaccines [9], DNA vaccines [10], bacteria or yeast vectored vaccines [11, 12] and viral vectored vaccines [13, 14] have been extensively explored as alternative approaches. Included in the list of alternative strategies are the recombinant yeast-based A (H5N1) vaccines, which are promising candidates that meet the requirement of vaccine production in a timely manner and would be able to induce robust protective immunity against A(H5N1) virus infection [15].

S. cerevisiae is a representative strain of yeast and widely applied in industries performing fermentation, particularly for food industry. *S. cerevisiae*-based vaccines as a novel strategy in the fight against infectious diseases provide a ray of hope for both in public health and for domestic poultry [16]. Mucosal delivery administration of vaccine is superior to conventional methods of injection in terms of operative ease and safety concerns [17]. Oral administration is an economical approach in enhancing mucosal immunity for the control of infection from influenza virus while reducing the cost of vaccine delivery [18]. Further, compared to intracellular expression of recombinant viral proteins, the display of viral proteins on carrier cell surface can facilitate their recognition by the host mucosal immune system, thereby enhancing their capability of eliciting protective immunity [17].

We have previously shown that EBY100/pYD1-HA could provide protection by injection route in a mouse model [15]. However, there has an important issue that EBY100/pYD1-HA induce a serious inflammation in the injection site since the diameter of *S. cerevisiae* is around 10 μm . Furthermore, Chickens are used by default for pathogenicity and vaccine efficacy studies for poultry [19]. To address this issue, we hypothesize that oral vaccination with unadjuvanted EBY100/pYD1-HA can produce cross-protective immunity in the chicken model and consider an effective platform for the development of A (H5N1) vaccine for a mass vaccination in poultry.

In the present study, we extended our previous work by evaluating the immunogenicity of EBY100/pYD1-HA in the chicken model. Oral vaccination with EBY100/pYD1-HA induces strong humoral, cell-mediated and mucosal immunity, and confers cross-protection against different H5N1 viruses challenge. Importantly, production of EBY100/pYD1-HA usually requires approximately two weeks and have great potential for mass production in a short period of time for poultry during A (H5N1) outbreaks.

Methods

Ethics statement

All experiments were performed in strict accordance with the Guidelines for Use and Care of Experimental Animals in Southwest Jiaotong University (Approval number: 7762). All virus challenge experiments were performed under the enhanced animal biosafety level – 3 (BSL – 3) facilities. More than 25% body weight loss was used as criterion for euthanasia. all survived chickens were euthanized using CO₂ inhalation for 5 minutes.

Vaccine Preparedness

EBY100/pYD1-HA was generated as described previously [15], but without quantification of EBY100/pYD1-HA expressing HA.

EBY100/pYD1-HA was inactivated at 60 °C for 1 h and then used for subsequent oral immunization. The final concentration of EBY100/pYD1-HA was adjusted to 0.5 optical density (OD)_{600nm}/μL phosphate buffer saline (PBS) and EBY100/pYD1 were served as controls.

Quantification Of EBY100/pYD1-HA Expressing HA Protein

Quantification of HA protein was determined by indirect enzyme-link immunosorbent assay (ELISA) as described previously [20]. In a brief, 5 OD_{600nm} of EBY100/pYD1-HA pellets were re-suspended in 100 μL of a monoclonal mouse anti-HA antibody (0, 15, 30, 45, 60, 75, 90, 105, 120 μg/mL) in PBS containing 2% bovine serum albumin (BSA) and incubated at room temperature for 2 h. Followed by incubation with Goat anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) (1 mg/ml) at room temperature for 1 h. After washing with sterile PBS, the cells were re-suspended in 100 uL of HRP substrate 3,3',5,5'-tetramethylbenzidine (TMB) in the darkness for 25 min and then 100 uL of 2 mol/l H₂SO₄ was added to stop the reaction. OD_{450nm} value of the supernatant was measured using a microplate reader. EBY100/pYD1 was used as a negative control.

Chickens, Dosage And Oral Immunization

Specific pathogen-free (SPF) white Leghorn chickens (7 days old) were purchased from SLC laboratory center (Shanghai, China) and housed with sterile food and water.

Groups of chickens (19 per group) were orally immunized with 200 uL of PBS, 100 OD_{600nm} of EBY100/pYD1 and 100 OD_{600nm} of EBY100/pYD1-HA, respectively, on day 1 for the prime immunization and day 14 for the boost immunization.

Sera, intestine washes and spleen were collected from vaccinated chickens on days 13 and 28 after the initial immunization.

Measurement Of Antibody Responses

HA-specific serum IgG and mucosal IgA antibodies were determined separately by ELISA as described previously [15]. Briefly, 2 μg of recombinant HA protein (kindly provided by NIH Biodefense and Emerging Infections Research Resources Repository, Manassas, VA, USA) was used as a coating antigen on the 96-well ELISA plate with overnight incubation at 4 °C. The wells were washed three times with Tris buffered PBS containing 0.05% Tween 20 (TBS-T) and blocked with TBS-T containing 1% BSA at room

temperature for 2 h. Serially diluted chickens sera or 1:50 intestine washes were added to the plates and incubated at 37 °C for 1 h, followed by biotinylated goat anti-chicken IgG or biotinylated goat anti-chicken IgA and alkaline phosphatase (AP) labeled-streptavidin at 37 °C for 1 h, respectively. The plates were washed three times with TBS-T, and then incubated with 100 μ L of p-Nitrophenyl Phosphate (PNPP) substrate. The reaction was developed at room temperature for 25 min and then was stopped with 50 μ L of 2 M sodium hydroxide (NaOH). OD value was measured at 405 nm using ELISA plate reader. The IgG and IgA titers were determined to be the lowest dilution with an OD_{405nm} greater than the mean OD_{405nm} of naïve controls plus 2 standard deviations.

Lymphocyte Proliferation Assay

Splenocytes isolated from the vaccinated chickens on days 13 and 28 after the initial vaccination (2×10^5 cells/well) were cultured on the 96-well plate with 2 μ g/mL of HA-specific peptide (ISVGTSTLNQRLVP) was used as a stimulus. After 36 h incubation, 10 μ L of the CCK-8 solution was added to each well. The following procedures were performed according to the manufacturer's instructions. The plates were measured at 450 nm using a microplate reader.

ELISpot For Determination Of T Cell Responses

HA-specific secreting Interferon gamma (IFN- γ) and Interleukin-4 (IL-4) secreting cells were determined using commercial ELISpot assay Kits according to the manufacturer's instructions. Briefly, splenocytes (1.0×10^6 cells/well) isolated from the vaccinated chickens were cultured on the 96-well plates containing chicken IFN- γ or IL-4, and stimulated with 10 μ g/ml of HA-specific peptide (ISVGTSTLNQRLVP) for 36 h in a humidified 37 °C, 5% CO₂ incubator. The plates were washed with sterile PBS, and incubated with biotinylated goat anti-chicken IFN- γ or IL-4 antibody overnight at 4 °C, followed by AP conjugated streptavidin at room temperature for 2 h. The plates were washed and then developed with BCIP/NBT, and then counted using an ImmunoSpot ELISpot reader.

Hemagglutination Inhibition (HI) Assay

The HI titer was determined as described previously [15]. Briefly, Receptor-destroying enzyme (RDE) treated sera were serially diluted (2-fold) in v-bottom 96-well microtiter plates followed by the addition of 4 hemagglutination units of A/Vietnam/1203/2004 (H5N1) or A/Chicken/Henan/12/2004 (H5N1) whole inactivated virus, and then incubated with 1% (v/v) chicken red blood cells (RBCs) for 30 minutes at room temperature. HI titer was determined by the reciprocal value of the last dilution of sera that completely inhibited hemagglutination of chicken RBCs. A negative titer was defined as less than 10.

H5N1 Viruses Challenge

Two weeks after the final vaccination, chickens were anesthetized and infected intranasally with 20 μ L of 5 \times 50% lethal dose (LD₅₀) of A/Vietnam/1203/2004 (H5N1) or A/chicken/Henan/12/2004 (H5N1). Challenged chickens were monitored daily for body weight changes and survival rate for 14 days. Lungs (n = 3 chickens / group) were isolated at day 3 post-challenge for determining viral titers [28].

Statistical analysis

All data is represented as mean \pm standard deviation. To determine the statistical significance, the Kaplan-Meier survival analysis was performed using Graphpad Prism. Two tailed Student's *t* test and one-way analysis of variance (ANOVA) were used to determine for differences between groups. The level of statistical significance was expressed as $p < 0.05$.

Results

Quantification of EBY100/pYD1-HA expressing HA protein

As shown in Fig. 1, the optical density was stable at the concentration of monoclonal anti-HA antibody beyond 60 μ g / mL, which suggested that 5 OD_{600nm} of EBY100/pYD1-HA expressed 60 μ g of HA protein.

Determination Of HA-specific Antibody Responses

To evaluate the antibody responses induced by EBY100/pYD1-HA, serum IgG and IgA of intestine washes were separately measured by ELISA on days 13 and 28 after the initial vaccination. The group that received EBY100/pYD1-HA was able to respond with effective and significant HA-specific serum IgG and mucosal IgA antibody levels compared to control groups (PBS and EBY100/pYD1) (Fig. 2). Therefore, these results indicate that oral administration with EBY100/pYD1-HA can induce a robust humoral and mucosal immune responses in chicken model.

Determination Of T Cell Proliferation

To determine T cells proliferation, splenocytes were separated from the vaccinated chickens and stimulated with HA-specific peptide. After 36 h incubation, T cell proliferation was measured by CCK-8 kit. As compared with control groups, a rapid and significant proliferation of T cells was observed in the EBY100/pYD1-HA group (Fig. 3A). Thus, these results reveal that EBY100/pYD1-HA can elicit T cell proliferation and immune responses.

Cellular Immune Responses Induced By EBY100/pYD1-HA

To further examine cellular immunity induced by EBY100/pYD1-HA, we determined IFN- γ and IL-4 secreting splenocytes by the ELISpot kits. Splenocytes were isolated from the vaccinated chickens on

days 13 and 28 after the initial immunization, and stimulated with HA-specific peptide. The levels of IFN- γ and IL-4 secreting cells in the EBY100/pYD1-HA group were significantly higher than those in the control groups (Fig. 3B). The levels of IFN- γ secreting cells were higher than the IL-4 level in the EBY100/pYD1-HA group (Fig. 3B). Taken together, these results demonstrate that EBY100/pYD1-HA can induce both Th1 and Th2 type immune responses with preference of the Th1 type immune responses as evidenced by higher levels of IFN- γ production.

HI Titers

To assess the induction of functional antibody responses by EBY100/pYD1-HA, sera from chicken orally administrated with PBS or EBY100/pYD1 regardless of doses showed only background levels of HI titers. However, EBY100/pYD1-HA could elicit meaningful HI titers of 50 ± 1.21 and 48 ± 1.25 against A/Vietnam/1203/2004 (H5N1) or A/Chicken/Henan/12/2004 (H5N1) on days 28, respectively (Table 1). Therefore, EBY100/pYD1-HA is more immunogenic and elicits higher levels of functional antibody responses compared to PBS and EBY100/pYD1 groups.

Cross-protective efficacy induced by EBY100/pYD1-HA

In order to support the cross-protective potential of EBY100/pYD1-HA in chickens against different H5N1 viruses, the vaccinated chicken were challenged with a lethal dose ($5 \times LD_{50}$) of homologous A/Vietnam/1203/2004 (H5N1) or heterologous A/Chicken/Henan/12/2004(H5N1) virus at two weeks after the final immunization (Fig. 4). The conditions of chickens relating to body weight change and survival rate were monitored daily for 14 days. As shown in Fig. 4, Chickens that received PBS or EBY100/pYD1 showed significant body weight loss and high viral titers in lung on day 3 after A (H5N1) virus infection, and died from lethal infection within 8 days. In contrast, chicken that were orally administrated with EBY100/pYD1-HA presented with slight weight loss and low virus titer in lung, and completely survived at post challenge (Fig. 4E, F). These results provide reliable evidence that EBY100/pYD1 is an effective immunogen to confer cross-protective immunity with high efficacy against highly pathogenic H5N1 avian influenza viruses.

Discussion

Our laboratory has previously reported the construction and characterization of EBY100/pYD1-HA without quantification of HA protein. Conventional injection administration of EBY100/pYD1-HA in mice provided effective immune protection against A (H5N1) virus challenge [15]. Furthermore, we generated another yeast-based H7N9 oral vaccine could also protect mice from A (H7N9) challenge [20]. In the present study, we extend our previous work by investigating the immunogenicity of EBY100/pYD1-HA with oral administration and evaluating the cross-protective immunity of EBY100/pYD1-HA in chicken model. Our results demonstrate that chickens orally vaccinated with EBY100/pYD1-HA elicited significantly higher serum IgG and mucosal IgA antibodies responses, as well as IFN- γ /IL-4 secreting cells

and HI titers. Also, oral administration of EBY100/pYD1-HA in the absence of mucosal adjuvant would be able to confer cross-protection against lethal challenge with different H5N1 viruses. Collectively, these results highlight the potential of yeast-based vaccine as an alternative approach which represents an important direction for developing an oral vaccine in poultry against different H5N1 viruses without the uses of injection needles and mucosal adjuvant.

Due to serious limitations of manufacturing platform, currently available avian influenza H5N1 vaccines are generally poorly immunogenic, and have safety concerns [6, 21, 22]. Furthermore, most of influenza A (H5N1) vaccines require administration by intramuscular or subcutaneous injection, which have been shown to be insufficient for the generation of protective immunity at the mucosal surface [23]. Mucosal delivery route not only can induce effective systemic immune responses, but also elicit mucosal immune responses which are helpful for controlling virus replication in the respiratory tract. As an alternative strategy for vaccine development, an edible nonpathogenic vector that carries the recombinant H5N1 HA protein, such as yeast, provides the potential of preventing and controlling H5N1 virus infection via oral administration.

Oral administration has many advantages over injected parenteral immunization as shown for A (H5N1) vaccines [11], as well as other pathogens such as rotavirus, typhoid fever for human [17]. Considering vaccine design, antigen presentation on the surface of yeast is more effective because it is easier to be recognized and presented by M cells in the small intestine [24]. Further, it is important that a mucosal delivery system by oral administration of A (H5N1) vaccine is required to protect vaccine material from gastric degradation and promote uptake of the antigen in the gastrointestinal tract and stimulate adaptive immune responses, rather than the tolerogenic responses that are seen in studies done with the administration of soluble antigens [25]. Therefore, the present yeast-based vaccine developed for oral immunization against different H5N1 viruses is an optimal delivery technology that antigen presented on the surface of yeast that represents future direction of mucosal vaccine development via oral administration.

Regarding the T cell responses, previous studies provided evidence that Th1 cells are superior Th2 cells in providing protection against viral infection by secreting IFN- γ , stimulating B cells, and directing CD8 + T cells mediated cytotoxicity [26]. Oral administration of EBY100/pYD1-HA was found to be more effective in generating T cells secreting IFN- γ indicating Th1 type responses than secreting IL-4 cytokine representing Th2 type responses. These observations were verified by our results on HI assay and virus challenge. It should be noted that the presence of HI antibodies with titers of $\geq 1:40$ is considered as an efficacy endpoint [27]. Further, survival rate is more than 60% against different H5N1 viruses challenge highlighting the cross-protective potential of the vaccines. Taken together, this novel platform technology based on recombinant H5 HA in surface displayed *S. cerevisiae* is a promising approach for developing A (H5N1) vaccines that effectively generates strong cellular immune responses and provides significant cross-protection.

Conclusions

In summary, the present study has shown to evaluate the immune protective efficacy of *S. cerevisiae*-based H5 vaccine (EBY100/pYD1-HA) in chicken model. Therefore, this system can be used as a platform for the development of oral vaccines against multiple influenza viruses and offer significant advantages for vaccination especially in poultry-originated zoonotic infections in human. Considering the current platforms of approved vaccines, it is important to investigate detailed comparative immunogenicity and protective efficacy of EBY100/pYD1-HA in comparison with inactivated whole virus and attenuated live H5N1 vaccines.

Abbreviations

ANOVA: Analysis of variance; AP: Alkaline phosphatase; BSA: Bovine serum albumin; BSL-3: Biosafety level -3; ELISA: Enzyme-linked immunosorbent assay; HI: Hemagglutination inhibition; HRP: Horseradish peroxidase; HPAI: Highly pathogenic avian influenza; IFN- γ : Interferon gamma; IL-4: Interleukin-4; LD₅₀: 50% lethal dose; NaOH: Sodium hydroxide; OD: Optical density; PBS: *Phosphate buffer saline*; PNPP: *p-Nitrophenyl Phosphate*; RBCs: Chicken red blood cells; RDE: Receptor-destroying enzyme; *S. cerevisiae*: *Saccharomyces cerevisiae*; SPF: Specific pathogen-free; TBS-T: Tris buffered PBS containing 0.05% Tween 20; TMB: 3,3',5,5'-tetramethylbenzidine.

Declarations

Ethics approval and consent to participate

All animal studies complied with the Guidelines for Use and Care of Experimental Animals and were approved by the Animal Committee of the Institute of Southwest Jiaotong University consent to participate (Approval number: 7762).

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests associated with this study.

Funding

This material is based on study supported by the National Natural Science Foundation of China (No. 31360225), Sichuan Science and Technology Program (No. 2019YFN0134) and the Fundamental

Research Funds for the Central Universities (No. 2682018CX73). The funders did not participate in study design, data collection, analysis and interpretation or preparation of the manuscript.

Authors' contributions

HL conceived and designed the study. QC, TG, YR, XL contributed to animal study and data interpretation. All contributed to data analysis and results interpretation. HL wrote the manuscript and produced all figures. All authors reviewed and approved the manuscript.

Acknowledgements

The authors gratefully acknowledge for Dr. Dominic Man-Kit Lam and John S. Tam for technical assistance and editorial improvements.

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Table

Due to technical limitations, Table 1 is provided in the Supplementary Files section.

Figures

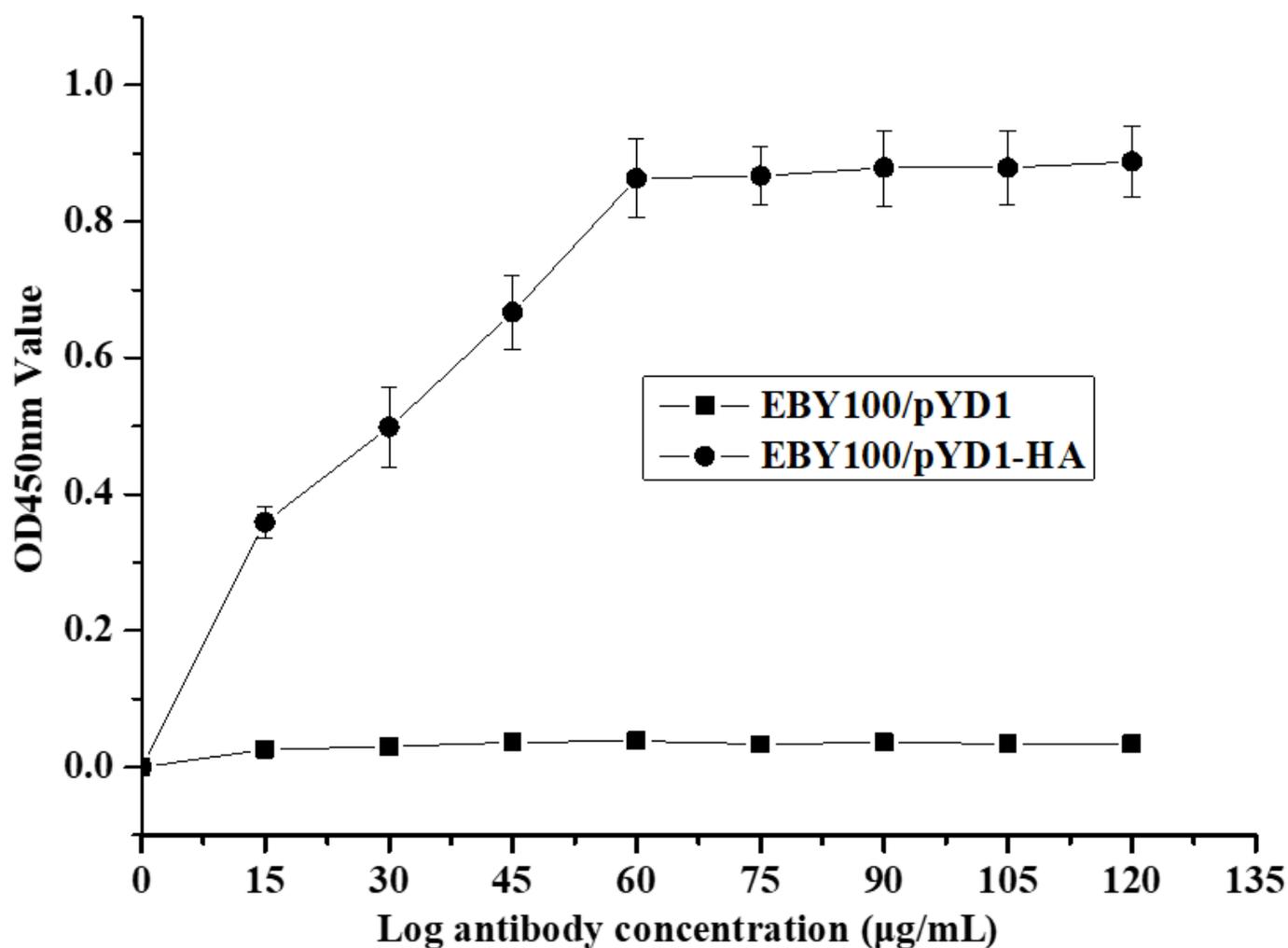


Figure 1

Determination of EBV100/pYD1-HA expressing HA protein by indirect ELISA. The OD450nm values were obtained from three independent experiments. Bar indicates means \pm SDs.

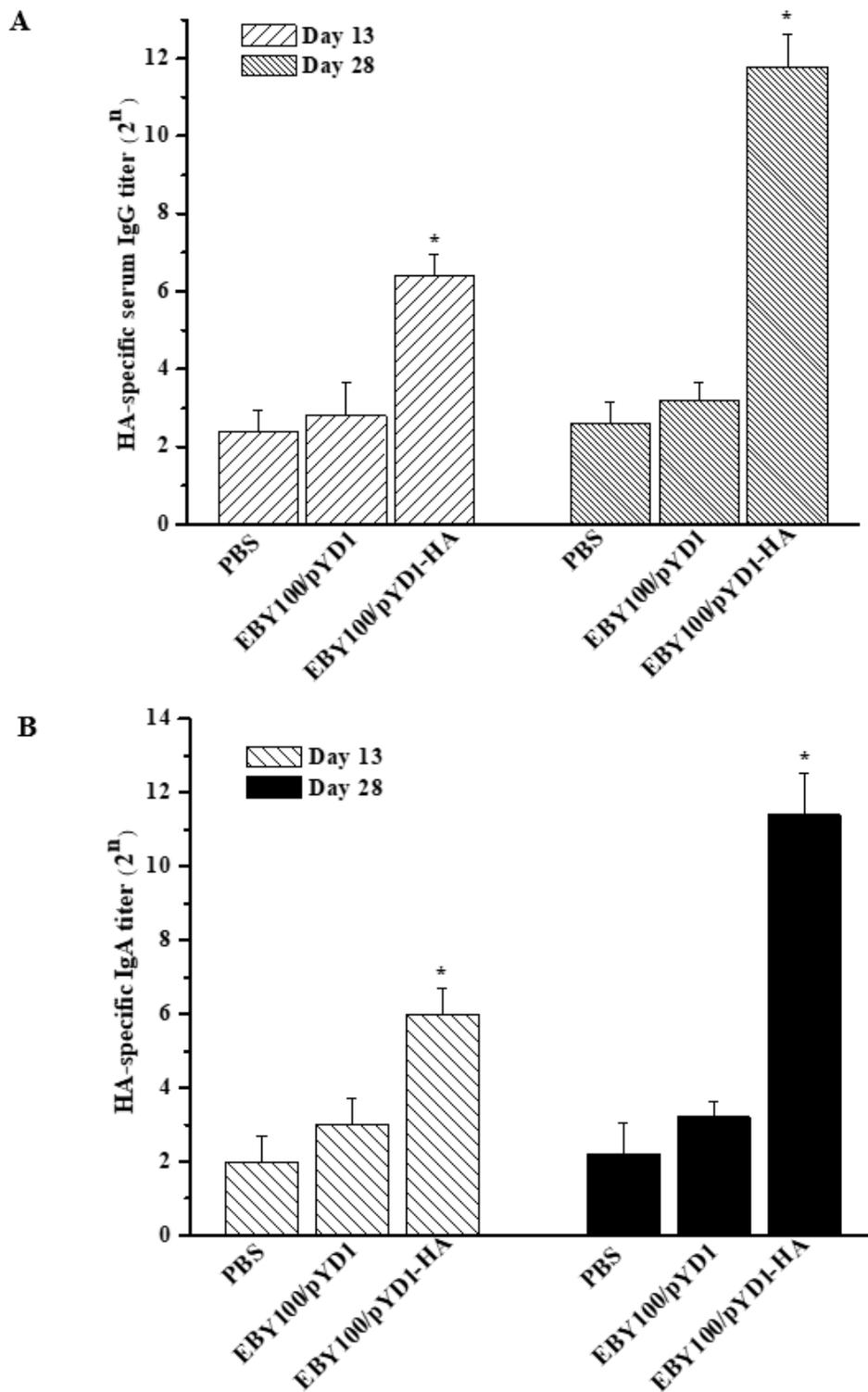


Figure 2

Antibody responses elicited by oral administration of EBV/pYD1-HA. (A) HA-specific serum IgG titer. (B) Secretory mucosal IgA titer in the small intestine washes. Asterisk represents statistically significant difference compare with the PBS- and EBV100/pYD1 controls.

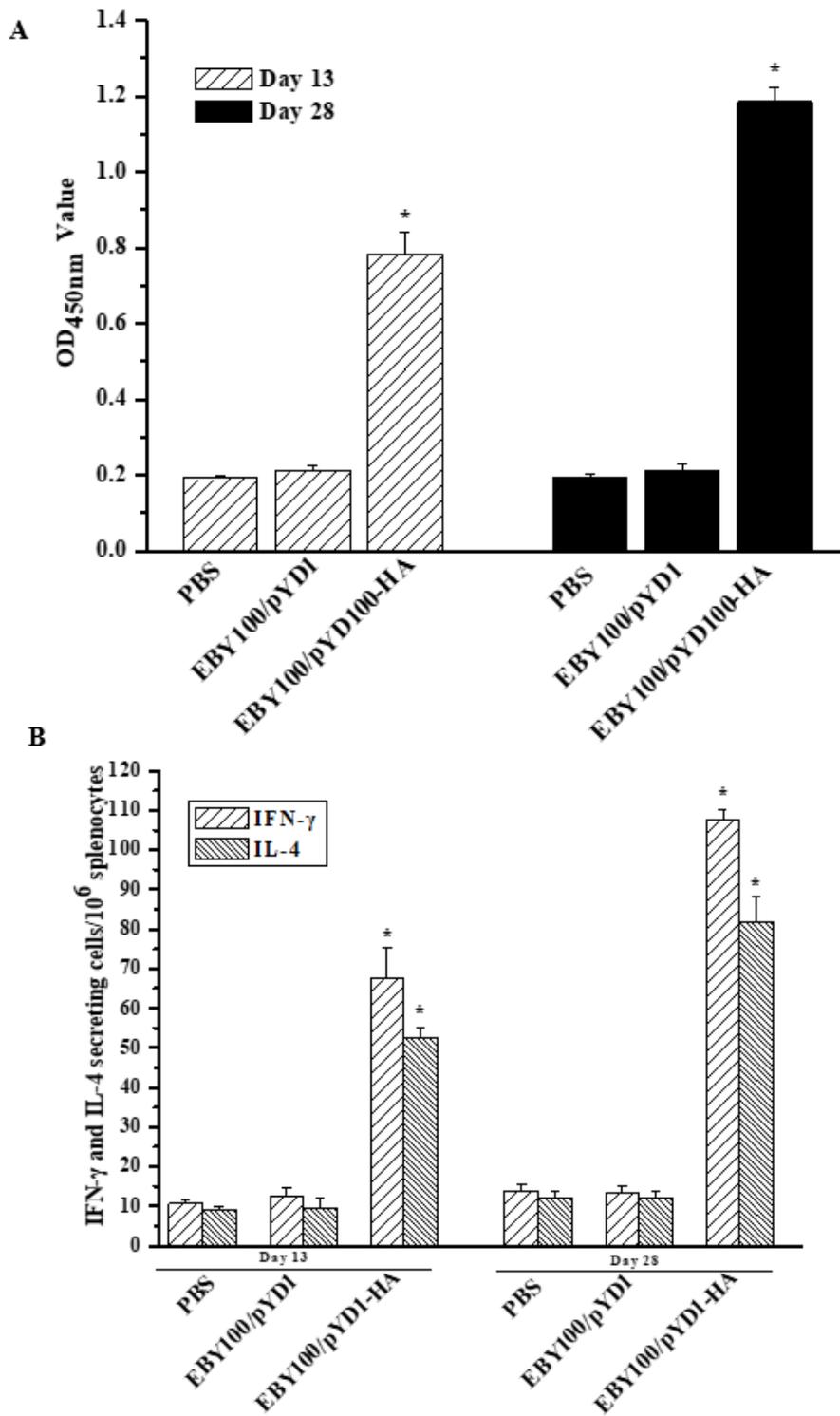


Figure 3

Cellular immune responses induced by oral administration of EBY/pYD1-HA. (A) Detection of lymphocyte proliferation. (B) IFN- γ and IL-4 secreting cells were separately determined by ELISpot assay. Asterisk indicates significant difference compare with the PBS- and EBY100/pYD1 controls. ($p \leq 0.05$), ($n=3$ chickens per group).

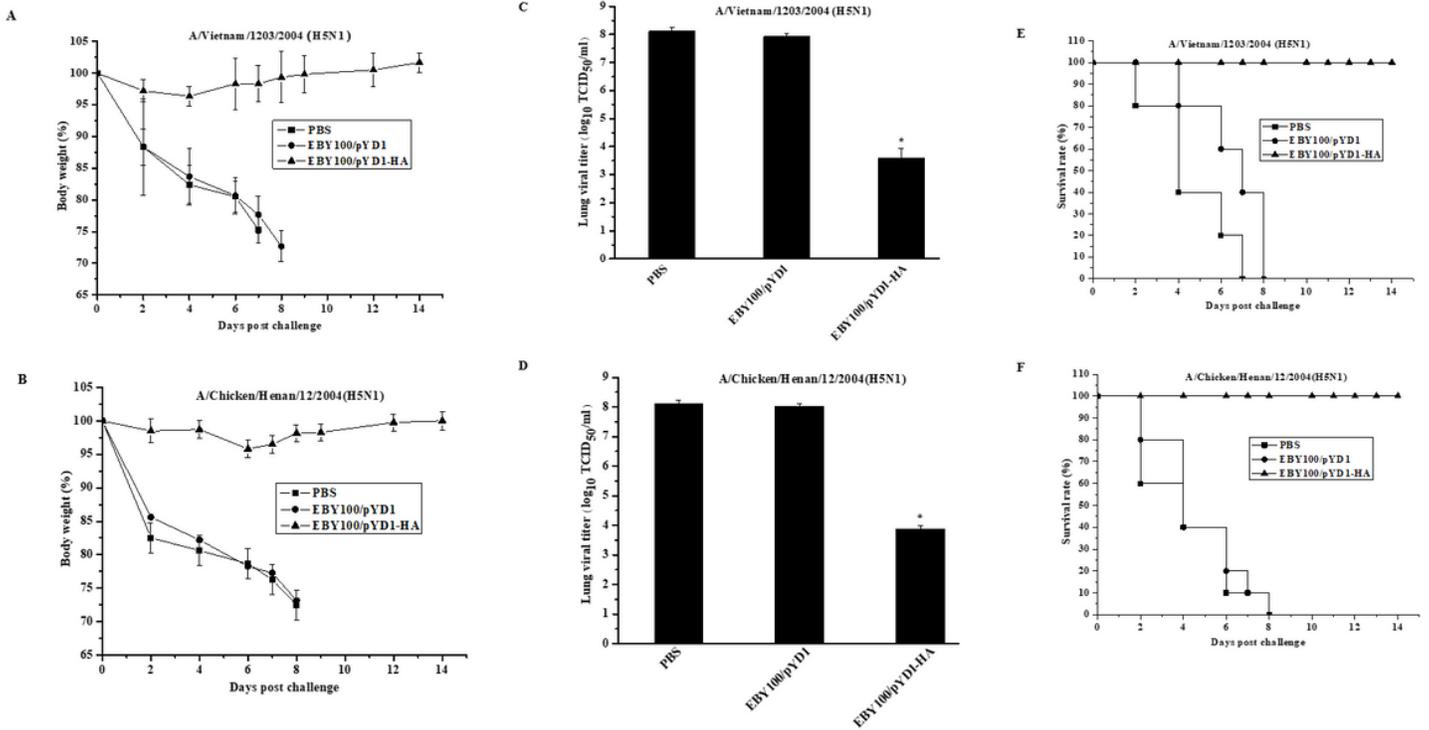


Figure 4

Cross-protection efficacy of EBV/pYD1-HA against different H5N1 viruses. Chickens were intranasally challenged with a lethal dose ($5 \times \text{LD}_{50}$) of H5N1 virus. (A) Weight change as a percentage at post A/Vietnam/1203/2004 (H5N1) challenge. (B) Weight change as a percentage at post A/Chicken/Henan/12/2004 (H5N1) challenge. (C) Lung viral titers at day 3 post A/Vietnam/1203/2004 (H5N1) challenge. (D) Lung viral titers at day 3 post A/Chicken/Henan/12/2004(H5N1) challenge. (n=3 chickens per group). (E) Survival rates at post A/Vietnam/1203/2004 (H5N1) challenge. (F) Survival rates at post A/Chicken/Henan/12/2004(H5N1) challenge. (n=10 per group). The data on the weight changes are presented as the means \pm SDs. Asterisk indicates that Asterisk indicates significant difference compare with the PBS- and EBV100/pYD1 controls. ($p < 0.05$).

Supplementary Files

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