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Serological Evaluation of H9-RBD-Pichia, a Novel Recombinant Influenza Vaccine, in BALB/c Mice.

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Abstract

Background: The H9N2 influenza virus is one of the most important subtypes of influenza that has caused irreparable damage to the poultry industry, and many efforts are underway to develop its recombinant vaccines.

Objectives: The aim of this study was to evaluate the effectiveness of H9-RBD-Pichia, a novel recombinant subunit vaccine, in stimulating the immune system and producing hemagglutinin-inhibiting antibodies in mice.

Methods: 24 female BALB/c mice were purchased and randomly divided into six groups. Group 1: Oral administration of lysed yeast twice. Group 2: One injection of lysed yeast. Group 3: Two

injections of lysed yeast. Group 4: One injection of pure recombinant protein. Group 5: Two injections of pure recombinant protein, and Group 6: Negative control. Prime and booster immunizations were performed on days 14 and 28, respectively. Sera samples were collected on day 42. Hemagglutination Assay (HA) and Hemagglutination Inhibition (HI) assays were performed on antigen and sera samples, respectively.

Results: HA titers of 1:4, 1:4, and 1:32 were seen in the positive control, intact, and bead-treated yeasts, respectively, which shows the functionality of the expressed H9-RBD recombinant protein. By performing the HI assay, it was observed that this vaccine could stimulate the immune system and produce anti-hemagglutinin antibodies in mice. The highest antibody titer (1:512) was observed when yeast lysate was injected twice on days 14 and 28.

Conclusions: The H9-RBD-Pichia vaccine has been produced based on genetic engineering technology and could produce high titers of hemagglutinin-inhibiting antibodies in mice.

Keywords: H9N2, influenza, *Pichia pastoris*, recombinant vaccine.

Introduction

The most common subtype of low-pathogenicity avian influenza (LPAIV) in poultry is H9N2. In the 1960s, the first isolation of the H9N2 virus was conducted from turkeys in the United States of America (Iqbal *et al*., 2013). This subtype has been recognized in a number of mammalian species. Humans, swine, dogs, weasels, and mink are just a few examples in which the H9N2 viruses have been identified. In most cases, the H9N2 virus or its antibodies have been detected

during regular surveillance in healthy mammals, but in certain cases, they have been linked to clinical illness (Song and Qin, 2020).

H9N2 viruses have raised concerns about zoonotic infection; hence, numerous animal models, especially mice, have been employed to assess their propensity to infect humans (Pusch and Suarez, 2018). In Iran, H9N2 avian influenza virus serological evidence has been found among poultry workers, veterinarians, and occupations associated with poultry (Hadipour, 2010; Heidari *et al*., 2016).

Nili and Asasi, (2003) showed that the mortality rates on H9N2-infected farms typically ranged from 20% to 60% in Iran. Although the isolate in the current H9N2 AI outbreak in Iran was classified as having low pathogenicity (LP), mortality in the field on several farms reached 65%. Such a significant fatality rate could be attributed to coinfection with other respiratory diseases. Clinical symptoms included anorexia, decreased water consumption, depression, sneezing, coughing, dyspnea, and body weight loss in both field and experimental investigations. In field cases, sinus swelling and ocular-nasal discharge were frequent (Nili and Asasi, 2003).

The major antigen in the activation of a protective immune response against the influenza virus is Hemagglutinin (HA), making it a crucial vaccine target. In addition to mediating the fusion of the viral envelope with the endosomal membrane, HA is expressed on the viral surface as trimeric glycoproteins that bind to sialic acid on target cells to facilitate host cell entrance (Wu and Wilson, 2020).

One of the primary objectives for the health of humans and animals is immune protection against infectious diseases (Lopez and Legge, 2020; Mascola and Fauci, 2020). It has been shown that immunization against influenza can diminish viral shedding and tissue spread in challenged birds (Abdi Haji *et al*., 2021). Today, in addition to the scientific literature, aspects of certain immunization tactics and vaccine-induced immune responses are extensively investigated and widely debated in the media. It is typically difficult to rapidly include the entire infectious agent

in a safe and protective vaccine formulation, whether in inactivated or attenuated versions, especially in cases of influenza pandemics. This is also supported by the biological risks that are inevitably present when pathogens are chemically inactivated on a large scale or when attenuated strains have the potential to revert to their virulence (Delrue *et al*., 2012; Lee *et al*., 2012).

Nowadays, recombinant subunit vaccines are being considered an alternative to conventional vaccine strategies because they are safe and there is no need for large quantities of eggs or live viruses. In addition, the production of these vaccines is economical and can be done swiftly during a pandemic (Athmaram *et al*., 2011; Lei *et al*., 2016; Ghadimipour *et al*., 2014). Additionally, tampering with subunit vaccinations might eliminate the need for biosafety level 3 laboratories and high-biosafety facilities.

There are several types of systems for the production of recombinant vaccines. As shown in Figure 1, one of the most interesting systems that has been widely used by researchers is the production of new recombinant subunit vaccines by using an expression host system. The most prevalent cell factories utilized for recombinant protein production with appealing characteristics include Escherichia coli, a few yeast species, insect cells, mammalian cells, and plant cells (Wang *et al*., 2019). The yeast cells have been extensively used for the production of different proteins of animal or human origin (Karbalaei *et al*., 2020). It should be mentioned that there are differences among studies and the process of producing a recombinant vaccine depends on the type of host, pathogen, and laboratory technique.

P. pastoris has become a highly successful expression system, due to its increasing popularity, which can be attributed to several factors such as high growth rate, the ease of genetic manipulation, high yield expression of heterologous proteins and the capability of performing eukaryotic post-translational modifications (Balamurugan *et al*., 2010). The *P. pastoris* system is much easier to perform and cheaper in cost compared with bacterial expression systems (Liu *et al*., 2013). *P. pastoris* usually produces higher yield of recombinant proteins and is less

demanding in terms of time and effort relative to complex eukaryotic systems (Maccani *et al*., 2014).

The H9-RBD-Pichia is a novel recombinant subunit vaccine that has been produced based on the surface display of the receptor binding site (RBD) of the hemagglutinin antigen in *Pichia pastoris* yeast using genetic engineering technology. The aim of this study was to evaluate the effectiveness of this vaccine in stimulating the immune system and producing hemagglutinin-inhibiting antibodies in mice.

Material and methods

Preparation of the recombinant vaccine

The new H9 subunit vaccine produced in our laboratory was used for the immunization of mice. Transgenic *P. pastoris* cells, containing the *H9-RBD* gene were cultured, precipitated, and disrupted using the glass bead lysis method as described previously (Zhang *et al*., 2020). In addition, the purification of pure H9-RBD antigen was performed using the HisPur™ Ni-NTA Resin (Thermo Scientific, Catalog Number: 88221) according to the manufacturer's instructions.

Culture condition

Specific media for the culture of *Pichia pastoris* and the expression of the recombinant antigen, including yeast extract peptone dextrose (YPD), buffered complex glycerol medium (BMGY), and buffered methanol-complex medium (BMMY), were prepared according to the pichia expression kit manual (Thermo Fisher Scientific, Catalog Number: K171001).

Mice immunization

Six to eight-week-old healthy female BALB/c mice were purchased from the Mashhad University of Medical Sciences and randomly divided into six groups, with four mice in each group.

- **Group** 1: Oral administration of lysed yeast twice.
- **Group** 2: One subcutaneous injection of lysed yeast.
- **Group** 3: Two subcutaneous injections of lysed yeast.
- **Group** 4: One subcutaneous injection of pure recombinant protein.
- **Group** 5: Two subcutaneous injections of pure recombinant protein
- **Group** 6: Negative control (administration of untransformed yeast)

The animals were housed in a controlled environment at standard room temperature with a 12-hour day/night cycle of light and received a commercially formulated diet and water *ad libitum* until the end of the experiment.

P. pastoris cells expressing the H9-RBD and untransformed yeast cells, as negative control, were bead-treated, diluted in 200 µl of sterile endotoxin-free PBS (1×10^9 CFU/mouse), and injected subcutaneously in each group in the neck region. The purified recombinant protein (100µg/ mouse) was also injected without adjuvant. In another group, *P. pastoris* cells were precipitated after expression, washed twice with PBS, resuspended in PBS, and orally administered using a stainless steel mouse gavage needle. Prime and booster immunizations were performed on days 14 and 28, respectively. Sera samples were collected on day 42. In order to determine the potential adverse effects of the vaccine, water consumption, food intake, and mice weight were monitored in all groups until the end of the experiment. It should be mentioned that the in vivo experiment was performed according to the Animal Research Reporting in Vivo Experiments (ARRIVE) guidelines.

Hemagglutination Assay

The recombinant protein expressing *P. pastoris* (OD600 of 10) was washed twice with PBS and used as antigens in an HA assay, along with bead-treated yeast cells, to assess the ability of the expressed recombinant protein to hemagglutinate chicken RBCs. A two-fold serial dilution of antigens was made using PBS in a microtitre plate, followed by the addition of 25 µl of 1% chicken RBCs and incubation at room temperature for 30 minutes (Sano and Ogawa, 2014).

Hemagglutination inhibition (HI) assay

After heat inactivation at 56°C for 30 min, sera samples were serially diluted twofold and incubated with four HA units of commercially available H9 antigen (Pasouflu, Pasouk, Mahdasht, Iran) in a 96-well U-bottom microtiter plate for 30 minutes at room temperature. Then, 25 µL of 1% PBS-washed chicken erythrocytes were added to each well and incubated for 30 minutes. The HI titer was determined as the maximum serum dilution that could inhibit the hemagglutination reaction (Sano and Ogawa, 2014).

Statistical analysis

The data were statistically analyzed by the one-way analysis of variance (ANOVA) followed by tukey test for post hoc comparisons using the SPSS software version 22 (SPSS Inc, Chicago, Illinois, USA). *P*-value<0.05 was considered significant for all tests.

Results

Growth parameter:

There was no significant difference between the control and immunized groups in terms of food intake or the mice's weight ($P > 0.05$). In addition, no mortality was observed in the control or immunized groups.

Hemagglutination Assay

HA assay was carried out to indirectly ensure the functionality of the expressed H9-RBD recombinant protein. A functional antigen prevents chicken erythrocytes from settling by agglutination. Therefore, the settling of RBCs at the bottom of the well indicates the lack of hemagglutinin activity in the sample. Settling patterns were observed in negative control wells containing PBS or untransformed *P. pastoris.* HA titers of 1:4, 1:4, and 1:32 were seen in the positive control, intact, and bead-treated yeasts, respectively. The HA titer in the bead-treated yeast was higher than in intact yeast cells. It is plausible that some of the recombinant protein is retained in the cell or periplasmic space and released after cell lysis (Buckholz and Gleeson, 1991; Ferrara *et al*., 2006).

Hemagglutination inhibition (HI) assay

In this experiment, it was found that injection and oral administration of the recombinant vaccine candidate produced in this study could stimulate the immune system and produce anti-hemagglutinin antibodies in mice. The highest antibody titer (1:512) was observed when yeast lysate was injected twice on days 14 and 28. Administering the booster vaccine increased the antibody titer significantly compared to the groups that received just one dose of the vaccine ($P < 0.05$). The antibody titer in injection groups was significantly higher than in oral vaccine groups. No hemagglutination-inhibiting activity was observed in the control serum.

Discussion

P. pastoris is a safe organism whose genome has been completely sequenced and widely used for the expression of endogenous, secreted membrane proteins and the production of recombinant subunit vaccines (Juturu and Wu, 2018). For instance, Wasilenko *et al*. (2010) expressed the HA protein from the influenza H5N1 subtype in the *P. pastoris* GS115 strain and reported that oral administration of the transformed yeast resulted in the production of neutralizing antibodies in white leghorn chickens. In another study, Pietrzak *et al*. (2016) transformed a recombinant pPICZαC vector containing H5N1 hemagglutinin into the *P. pastoris* KM 71 strain. Their study showed that the recombinant protein produced in the yeast expression system could be used as an effective vaccine and protect layer chickens from the lethal challenge. Furthermore, a published paper by De Sá Magalhães and Keshavarz-Moore, (2021) showed that *P. pastoris* is a strong system and cost-effective tool for the production of recombinant vaccines in low- and middle-income countries.

As mentioned before, surface-display technology has been used in the production of the H9-RBD-Pichia vaccine. Therefore, the main epitopes of influenza HA antigen have been placed on the external surface of *Pichia pastoris* yeast, and the resultant vaccine can be administered orally or injected subcutaneously.

In this study, the transgenic yeasts were treated with glass beads. Although some researchers have used the sonication cell lysis method for the disruption of yeast cells, the lysis of *P. pastoris* cells with 0.5 mm glass beads was a more effective method in this study, which is in agreement with Zhang *et al*. (2020) who showed that bead lysis is a more productive method in comparison to sonication and high-pressure homogenization approaches.

Adjuvants are chemical or biological substances that boost the immune system. According to a study performed by Radmehri *et al*. (2021), a combination of the ISA70VG adjuvant and nano-

selenium could be used to enhance the immunological response of chickens to the bivalent ND+AI vaccine. Analysis of serological tests revealed that the antibody titer in mice injected with bead-treated transgenic yeast is higher than in mice injected with pure antigens. This may be caused by the natural adjuvant activity of *P. pastoris* cell wall components. It has been previously demonstrated that the administration of recombinant protein accompanied by yeast cell wall components is more immunogenic than administering pure protein (Rios-Hernandez *et al*., 1994; Stubbs *et al*., 2001).

High antibody titers were seen in the groups that received the booster vaccine, and this is in agreement with previous studies. Lei *et al*. (2020) used surface display technology to produce an oral vaccine against H7N9 influenza. *Saccharomyces cerevisiae* yeast expressing recombinant HA antigen was administered orally to BALB/c mice on days 1 and 14. A single administration of the vaccine led to the production of low levels of detectable antibodies. But a significant increase in the level of antibodies was observed after booster immunization.

At the end, it should be pointed out that although vaccination of susceptible species, particularly poultry, is an effective approach for avian influenza control, it should not be considered as a single strategy and a combination of different measures, including biosecurity, management, diagnosis of influenza infections, Active and passive surveillance, and reducing the susceptibility of the host, must be used (Swayne, 2012). Besides, veterinarians, farmers, and poultry-related careers should receive a proper education since teaching is one of the most important factors in AI control. Various aspects of this recombinant vaccine and its protective effects in chickens will be investigated in our next study.

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ارزیابی سرولوژیکی H9-RBD-Pichia، یک واکسن نوترکیب جدید آنفلوانزا، در موش BALB/c

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چکیده:

زمینه: ویروس آنفولانزای H9N2 یکی از مهمترین تحت تیپ‌های آنفلوانزا است که خسارات جبران‌ناپذیری به صنعت طیور وارد کرده است و تلاش‌های زیادی برای تولید واکسن‌های نوترکیب آن در حال انجام است.

هدف: هدف از این مطالعه بررسی اثربخشی واکسن جدید زیرواحد نوترکیب H9-RBD-Pichia در تحریک سیستم ایمنی و تولید آنتی‌بادی‌های مهارکننده هماگلوتینین در موش است.

روش کار: 24 موش ماده BALB/c خریداری و به طور تصادفی به شش گروه تقسیم شدند. گروه 1: دوبار تجویز خوراکی مخمر لیز شده. گروه 2: یکبار تزریق مخمر لیز شده. گروه 3: دوبار تزریق مخمر لیز شده. گروه 4: یکبار تزریق پروتئین نوترکیب خالص. گروه 5: دوبار تزریق پروتئین نوترکیب خالص و گروه 6: کنترل منفی. واکسیناسیون اولیه و یادآور به ترتیب در روزهای 14 و 28 انجام شد. نمونه های سرم در روز 42 جمع‌آوری شدند. آزمایشات هماگلوتیناسیون (HA) و ممانعت ازهماگلوتیناسیون (HI) به ترتیب بر روی نمونه های آنتی ژن و سرم انجام شد.

نتایج: تیترهای HA 1:4، 1:4 و 1:32 به ترتیب در مخمرهای کنترل مثبت، دست نخورده و تیمار شده با گوی شیشه ای مشاهده شد که نشان دهنده ی عملکرد پروتئین نوترکیب H9-RBD بیان شده است. با انجام سنجش HI، مشاهده شد که این

واکسن می تواند سیستم ایمنی را تحریک کرده و آنتی بادی های ممانعت کننده از هماگلوتینین را در موش تولید کند. بالاترین تیتر آنتی بادی (1:512) زمانی مشاهده شد که مخمر لیز شده، دو بار در روزهای 14 و 28 تزریق شد.

نتیجه گیری: واکسن H9-RBD-Pichia بر اساس فناوری مهندسی ژنتیک تولید شده است و می تواند تیتر بالایی از آنتی بادی های مهارکننده هماگلوتینین را در موش تولید کند.

کلیدواژه‌ها: H9N2، آنفولانزا، پیکیا پاستوریس، واکسن نوترکیب، نمایش سطحی

Figure legends

Fig. 1. The general process of recombinant vaccine production using the expression system. The main antigen of a pathogen that stimulates the immune system and results in the production of neutralizing antibodies should be detected and its nucleotide sequence determined. After that, the sequence of this specific gene must be codon optimized, and cloned into an expression vector through biotechnological methods such as restriction enzyme digestion and ligation processes. The recombinant plasmid can be transformed into an expression host system. After transcription and translation processes that occur inside the transformed cells, the recombinant antigen can be secreted into the culture medium or attached to the cell surface. In the case of attachment on the surface, the whole cell might be used as a source of antigen, either with or without an adjuvant.

Fig. 2. In the HA experiment, the results showed that bead-treated yeasts (B) with 1:16 titer, intact transgenic yeast (C) with 1:4 titer, chimerical HA antigen (D) with 1:4 titer, have considerable hemagglutinin activity in chicken red blood cells. Negative control (A). Hemagglutinin activity in group

C indicated surface-display partial H9 antigen in transgenic yeast. In the HI experiment, the highest antibody titer was observed when yeast lysate was injected twice. Numbers 1-6 are representatives of groups 1-6, respectively. 7: Positive control 8: The second positive control (chicken serum with a titer of 1:128)

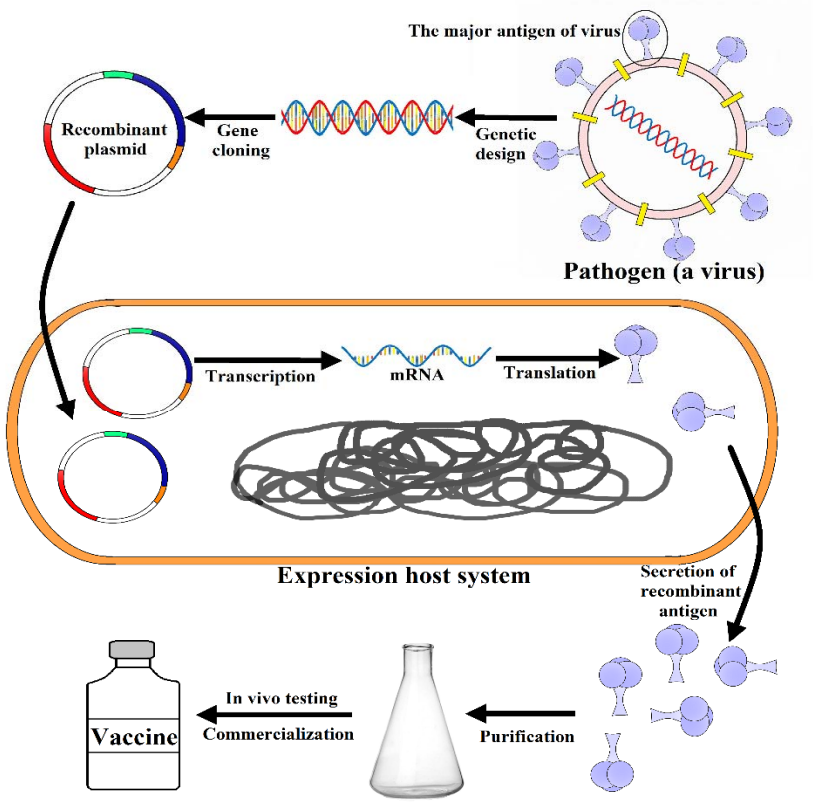

Figure. 1.

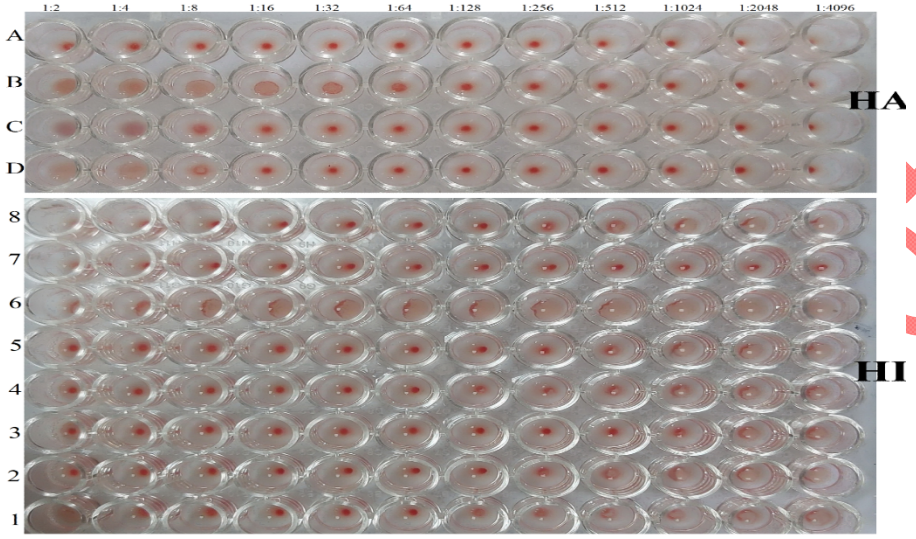

Figure. 2.