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Short Communication

Immunogenicity of Concentrated and Purified Inactivated Avian Influenza Vaccine Formulation

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ABSTRACT

Avian influenza (AI) H9N2 is a low pathogenic virus subtype belonging to Orthomyxoviridae family. Given the prevalence of this subtype as an infectious agent in poultry industry, special attention has been always directed toward the development of vaccine production against this infection. The vaccine of this infection is produced by killing the virus and using a mixture of inactivated antigen and oil phase. Egg-based viral antigens have high levels of unwanted proteins that may adversely affect the vaccine formulation. In addition, it is required to raise the antigen concentration for the production of combination vaccines, especially in low doses. This underscores the need to the improvement of the downstream purification process and concentration of antigens. The optimization of downstream processing would decrease the cost of vaccine procurement and maintenance. Regarding this, the present study was conducted to evaluate a downstream procedure for the concentration and purification of avian influenza virus (H9N2) and investigate the immunogenicity of the vaccine containing these antigens. To this end, after harvesting and clarifying virus-containing allantoic fluid, it was concentrated and purified using ultrafiltration and chromatography, respectively. The concentrated and purified samples were checked for their ovalbumin level and emulsified with oil adjuvant to access their immunogenicity. The results showed that one dose of both formulated antigens (i.e., concentrated and purified) was effective in raising the immune response in the vaccinated chicks for a long time. The applied formulation had a one-year stability in the refrigerator. Furthermore, the concentrated antigen showed a high hemagglutination activity through a year when storing in the refrigerator. Based on the findings, the optimization of downstream process of influenza (H9N2) vaccine production and use of new technologies could be considered in the large-scale preparation of a sustainable vaccine without any unwanted risk factors.

Keywords: Avian H9N2 Influenza Virus, Vaccine, Immunogenicity, Ultrafiltration

Immunogénicité de la Formulation de Vaccin contre la Grippe Aviaire Inactivée Concentrée et Purifiée

Résumé: Grippe aviaire (AI) H9N2 est un sous-type de virus faiblement pathogène appartenant à la famille des Orthomyxoviridae. Compte tenu de la prévalence de ce sous-type en tant qu'agent infectieux dans l'industrie de la volaille, une attention particulière a toujours été accordée au développement de la production de vaccins contre cette infection. Le vaccin contre cette infection est produit en tuant le virus et en utilisant un mélange d'antigène inactivé et de phase huileuse. Les antigènes viraux à base d'œufs contiennent des taux élevés de protéines indésirables qui peuvent avoir un effet défavorable sur la formulation du vaccin. En outre, il est nécessaire d'augmenter la concentration en antigène pour la production de vaccins combinés, en particulier à faible dose. Cela souligne la nécessité d'améliorer le processus de purification en aval et la concentration

d'antigènes. L'optimisation du traitement en aval réduirait les coûts d'achat et de maintenance des vaccins. À cet égard, la présente étude visait à évaluer une procédure en aval pour la concentration et la purification du virus de la grippe aviaire (H9N2) et à étudier l'immunogénicité du vaccin contenant ces antigènes. À cette fin, après avoir recueilli et clarifié le liquide allantoïque contenant le virus, celui-ci a été concentré et purifié par ultrafiltration et chromatographie, respectivement. Les échantillons concentrés et purifiés ont été contrôlés pour leur taux d'ovalbumine et émulsifiés avec un adjuvant d'huile pour accéder à leur immunogénicité. Les résultats ont montré qu'une dose des deux antigènes formulés (à savoir concentrée et purifiée) était efficace pour augmenter la réponse immunitaire chez les poussins vaccinés pendant une longue période. La formulation appliquée avait une stabilité d'un an dans le réfrigérateur. En outre, l'antigène concentréprésentait une activité hémagglutinante élevée pendant un an lorsqu'il était conservé au réfrigérateur. Sur la base des résultats, l'optimisation du processus en aval de la production de vaccin antigrippal (H9N2) et l'utilisation de nouvelles technologies pourraient être envisagées dans la préparation à grande échelle d'un vaccin durable sans aucun facteur de risque indésirable.

Mots-clés: Virus H9N2 de la Grippe Aviaire, Vaccin, Immunogénicité, Ultrafiltration

INTRODUCTION

Avian influenza (AI) H9N2 as a low pathogenic virus belongs to influenza type A virus from the family of *Orthomxoviridae,* which is a more common infection in birds (Lee and Saif, 2009). However, the observation of this virus in other animals and several humans is indicative of the evolvement of this virus in human hosts (Butt et al., 2005; Butt et al., 2010), which urges the consideration of H9N2 control (Li et al., 2005). This subtype was isolated for the first time in 1966 in the USA, and then in China, Pakistan, Iran, and South Korea in poultry industry (Fusaro et al., 2011). H9N2 has been also isolated from chicken farms in Tehran, Iran, and other provinces of this country (Vasfi Marandi and Bozorgmehri Fard, 2002; Alizadeh et al., 2009). This influenza virus subtype has been accepted as an infection in poultry since mid-1990 (Alexander, 2000). Consequently, the prevention of the viral spreading of H9N2 subtype is critical to protect animal and public health. Vaccination maintains a noteworthy role in protecting poultry industry against avian influenza H9N2 infections. Killed vaccines are generally produced by the combination of inactivated crude viral antigens with an adjuvant. This kind of vaccine preparation has been carried out for a variety of influenza subtypes, such as H5/H7 (Hwang et al., 2011; OIE, 2015) and H9N2 (Moghaddam Pour et al., 2006; Lee et al., 2011). Downstream processing is used for preparing cell- or egg-derived human vaccine (He et al., 2011); however, currently, just egg-based process is applied for the procurement of avian influenza killed vaccine in a completely crude format. The crude antigen itself has many other impurities that may affect the quality of final vaccine. In this study, we presented concentrated and purified avian influenza H9N2 vaccine formulation as a new procedure. Our aim was to launch a new process for the production of highly pure or semi-pure low-volume poultry vaccine in a large scale, which could be a basis for other combination formulations.

MATERIAL AND METHODS

Egg incubation and antigen preparation. Influenza virus strain A /chicken /Iran /259/1998 (H9N2), confirmed by the central veterinary laboratory of Weybridge, Surrey, UK, was propagated in 10-day-old specific pathogen-free (SPF) embryonated chicken eggs. Allantoic fluid was harvested after 48 h at 37 °C and was checked for its hemagglutination (HA) activity (OIE, 2015). Clarification was performed by centrifugation at 5000 rpm for 30 min at 4 °C using a centrifuge (Sorvall, USA); in addition, microfiltration was accomplished

using a 2-µm paper filter (Millipore Tripod Steel Housing, USA). The clarified antigens were inactivated by 0.2% formalin for 48 h at 37 °C. Totally, 10 batches (10 L) of inactivated virus were prepared.

Virus concentration and purification. Inactive antigens were concentrated and purified as previously described (Shirvan et al., 2016). Briefly, 10 batches (10 L) of viral inactivated H9N2 antigens were concentrated by cross flow filtration through a 50 kDa cutoff cassette (slice 200, Sartorius, Germany) with a maximum flow rate of 10 ml/min. Then, the flow through samples and retentate antigens were checked for their HA activity. The resultant sample after cross flow filtration was loaded on a 100×2. 5 cm Sephadex G-100 column (GE Healthcare) equilibrated with 0.2 M phosphate buffer, 0.1 M NaCl, and pH of 7 at 4 °C. All fractions with HA activity were collected and analyzed for their ovalbumin (Seramun Diagnostica GmbH, Heidesee, Germany) and protein concentration (Bradford method, BioRad kit). Furthermore, they were subjected sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (SDS-PAGE; Laemmli, 1970).

Antigen formulation and vaccination. Both inactive concentrated and purified influenza antigens were emulsified through homogenization with Montanide ISA-70 (3:7 (v/v; Seppic, Puteaux, France) according to the manufacturers' instruction. The amount of antigen in aqueous phase was equal to $10^{8.3}$ EID50/dose (Moghaddam Pour et al., 2006). All 21-day-old SPF chickens received one dose of 0.2 ml emulsified inactivated H9N2 influenza subcutaneously in the dorsal interior region of the neck. This was performed in two separate groups with concentrated and purified antigens, which were prepared in two formulations. All animals, along with the control group, were housed in the same area.

Hemagglutination and hemagglutination-inhibition titration. At this stage, 50 µL of phosphate-buffered saline was added to all wells of a round-bottomed 96-well plate (Nunc, Pasadena, TX, USA). Subsequently, 50 µL of the sample was added to the first row and mixed well. Twofold serial dilutions were made of 50 µL virus suspension crossways the plate. A volume of 1% chicken's red blood cells was added to dilute the virus suspensions. The microplate was kept at room temperature for 30 min. The red blood cells that were not bound by influenza virus sunk to the bottom of the well, and the aforementioned well was used to determine the HA activity (OIE, 2015). Individual serums were prepared after 21, 42, 70, 99, and 120 days from vaccination of 10 SPF chickens (21 days old). For more than one year, a new group of 10 SPF chickens (21 days old) was vaccinated every 3 months, and their serums were collected. All prepared serums were checked by the hemagglutinin inhibition test (HI) with 4 units of influenza virus to determine the elevation of antibody (OIE, 2015).

RESULTS AND DISCUSSION

Harvesting, Concentration, and Purification. A total of 10 batches of allantoic fluid, containing H9N2 influenza virus, were clarified by microfiltration and centrifugation. The ratio of HA activity to total protein was increased much more in centrifugation than in filtration. Clarification with centrifugation was significantly more efficient and easy to handle than that with microfiltration; however, it seems that in volumes more than the one adopted in our experiments, it would be controversial. Results of concentrating 10 L batches showed that during the enhancement of HA activity in influents, no measurable HA titers were observed in permeated fluids. Based on SDS-PAGE analysis, cross flow filtration led to a major decrease in total protein, mostly ovalbumin and other ingredients (Figure 4). The best separation of virus through column chromatography was observed in 18% of void volume. Totally, HA activity in all purification steps were seen at the first fraction, while column flow was monitored at 280 nm (Figure 3). Ovalbumin impurities in the concentrated and purified samples were 68 and 0.7 µg/ml, respectively (Shirvan et al., 2016).

Immunogenicity and duration of immunity. In order to determine the HI antibody in immunized chickens, serum was collected from vaccinated animals every 21-30 days and tested by the HI assay. The analysis of the HI titers of vaccinated chickens vaccinated with the concentrated and purified antigens revealed a strong immune response above the limitation level (HI=5) during 4 months, with a peak on the 99th day just for one dose. Based on the pattern of immunity duration, antibody level would be sufficient possibly for 6 months to cover the limitation level. Although both vaccines had a similar immunity duration pattern, the purified antigen showed a faster response and higher level of antibodies (Figure 1). The HI titers of the serums obtained from the vaccinated chickens revealed that the prepared vaccine could stimulate immune response for a period of more than 1 year while stored at 4 °C (Figure 2). The vaccinated chickens had no respiratory symptoms or clinical signs during and after the experiments indicating that the formulation was safe.

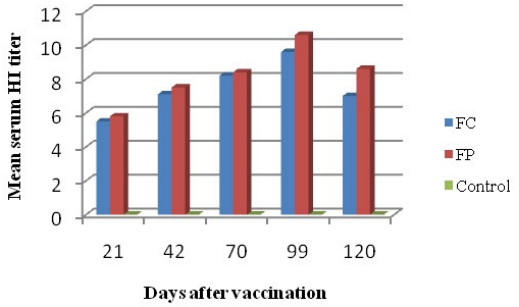

Figure 1. Duration of the immunity of inactivated H9N2 vaccine in specific pathogen-free chickens (They were inoculated intramuscularly with one dose of oil-emulsion concentrated antigen (FC) or oil-emulsion purified antigen (FP). Serum antibodes were tittered by hemagglutination-inhibition test.)

Stability of emulsified antigens. The prepared vaccines were checked for their appearance, phase separation, and thermal stability. The results demonstrated that both formulated antigens were stable at 37 °C for 1 month and at 4 °C for more than a year. Furthermore, no phase separation was observed at the mentioned time and temperature. The H9N2 influenza subtype is a low pathogenic avian influenza virus.

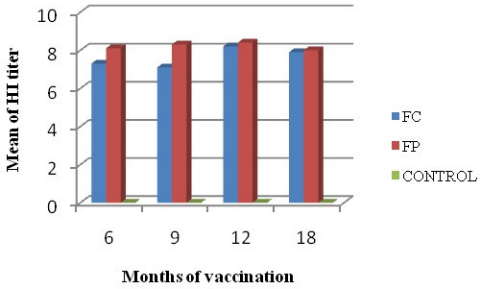

Figure 2. Immunogenicity of prepared vaccine with concentrated (FC) and purified (FP) antigens (Serums were collected from vaccinated chickens at 3 months interval, and their antibody was measured by hemagglutination-inhibition test.)

Since the first report of this virus in 1998 in Iran (Vasfi Marandi and Bozorgmehri Fard, 2002), it is still circulating in poultry industry (Ghaniei et al., 2013), and also has an inter-species transmission to human *(Hosseini et al., 2013). This transmission to human was* also announced from other groups in China (Butt et al., 2010). Vaccination would be the best strategy to control avian influenza with pandemic strains and is used in different countries, such as Iran (Vasfi Marandi and Bozorgmehri Fard, 2002), China (Lee et al., 2007), Pakistan (Naeem et al., 1999), and Korea (Choi et al., 2008). Basically, all of these vaccines are prepared with inactive crude antigen and homogenized with oil; however, the use of new nano-adjuvant in this process is under study (Khalili et al., 2015). In the present study, concentrated and purified antigens, which were previously characterized for their lack of impurities (Shirvan et al., 2016), were separately used for oil emulsion vaccine preparation. Inconsistent with the results of another study performed by Kalbfuss et al. (2007), in the current study, formalin inactivated virus did not show any significant HA titer in both concentrated and purified antigens (data not shown). The reduction of total protein level against the elevation of HA titer revealed the high recovery of antigens from concentrated and purified batches (Shirvan et al., 2016), which is in line with other

techniques (Khalili et al., 2015). Based on the immunological studies, the prepared vaccines showed a high antibody response over time for one dose (0.2 ml) that could be decreased in volumes of interest, particularly in the combination vaccine formulations.

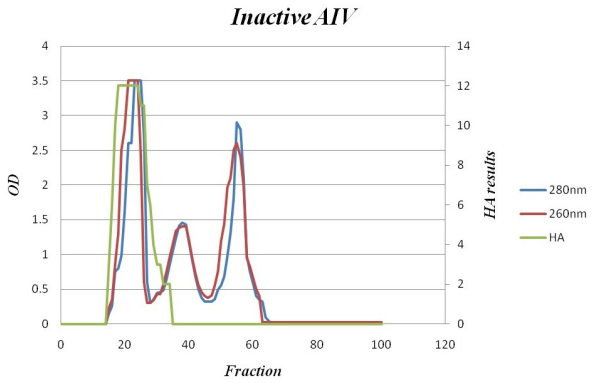

Figure 3. Fractionation of inactivated and concentrated H9N2 virus on sepharose resin loaded with 18% of bed volume (280 nm [blue line], 260 nm [red line], and hemagglutination-inhibition activity [green line])

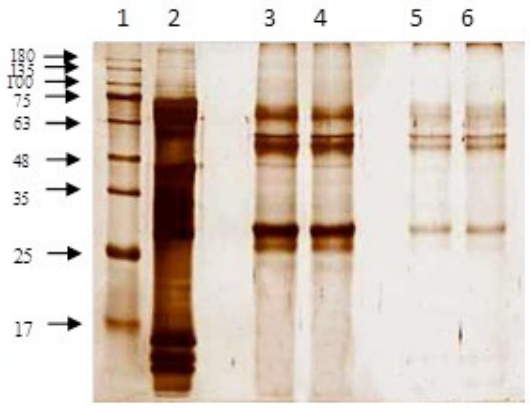

Figure 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of purified avian influenza virus samples.

Compared to other studies (Lee et al., 2011) reporting a dose of 0.5 ml, our results revealed that the reduction of vaccine dose volume could still induce high titers of antibodies. Because of using purified antigens, the prepared vaccine has a greater stability (i.e., around 2 years). Regarding the OIE requirements, this formulation has enough antigens per dose to protect chicken from mortality and shedding (OIE, 2015). The results of the present study demonstrated a high immunogenicity for the concentrated and purified H9N2 influenza low-volume formulation with no clinical signs. Under trial condition, the prepared vaccine would induce immunity for almost 6 months with one dose. The results obtained from multiple batches revealed that this process can be scaled up in the production of low volume of avian influenza vaccine, even less than 0.2 ml, that is significantly concentrated without any impurities. This platform aimed to take advantage of downstream technologies that are commercially available and cost-effective to produce pure and potent products, mostly multivalent low-volume vaccines with low logistic cost. To the best of our knowledge, this is the first report of preparing H9N2 influenza formulation vaccine with concentrated or purified antigen.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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