Biologicals xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Preparation and immunological evaluation of inactivated avian influenza virus vaccine encapsulated in chitosan nanoparticles

Samah H. Mohamed[a], Abdel Satar Arafa[b], Wesam H. Mady[b], Hanan A. Fahmy[c], Lamiaa M. Omer[d], Rania E. Morsi[e,*]

[a] Immunology Department, Animal Health Research Institute, Dokki, Giza, Egypt
[b] Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP), Animal Health Research Institute, Dokki, Giza, Egypt
[c] Biotechnology Department, Animal Health Research Institute, Dokki, Giza, Egypt
[d] Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abbassia, Cairo, Egypt
[e] Analysis & Evaluation Department, Egyptian Petroleum Research Institute, 11727, Cairo, Egypt

ARTICLE INFO

Keywords:
Avian influenza virus
Chitosan nanoparticles
Humoral and cell mediated immunity

ABSTRACT

Efficacy maximization of inactivated avian influenza vaccine using safe adjuvants was investigated. Chitosan nanoparticles were prepared by ionic gelation method with average size of 150 nm and their Zeta potential was 11.5 mV. After encapsulation of avian influenza vaccine, the average size was 397 nm and Zeta potential was 4.29 mV. The highest HI antibody titer results were shown in chicken group vaccinated with inactivated avian influenza virus AIV-chitosan followed by the group vaccinated with inactivated AIV-chitosan nanoparticles then the group vaccinated with oil inactivated AIV vaccine, on using chicken antigen at 2 weeks post second vaccination. Upon using duck antigen, the highest HI antibody titers were shown in chicken group vaccinated with inactivated AIV oil emulsion vaccine followed by chicken group vaccinated with AIV-chitosan nanoparticles then the group vaccinated with AIV–chitosan. Chicken in the group vaccinated with AIV-chitosan nanoparticles induced the best results of lymphocyte proliferation assay. The results of phagocytic activity percentage and phagocytic index of AIV-chitosan nanoparticles and AIV–chitosan groups at 3 days post first vaccination were increased significantly in comparison with other groups, whereas at 14 days post first vaccination, group vaccinated with AIV-chitosan nanoparticles showed significant increase in phagocytic activity percentage and phagocytic index.

1. Introduction

Influenza A viruses are member of the family Orthomyxoviridae, that have been classified into 18 diverse Haemagglutinin (HA) antigens (H1 to H18) and 11 distinctive Neuraminidase (NA) antigens (N1 to N11) [1,2]. The virus is transmitted by direct contact between infected and susceptible birds or indirect contact through airborne droplets or exposure to virus contaminated fomites [3]. Severe outbreaks were recorded in different areas worldwide including Egypt since 2006 due to infections with a highly virulent influenza A virus H5N1 subtype, causing substantial morbidity, and mortality in chickens and turkeys [2,4]. Epidemiological analysis shows that the virus is still circulating and becomes endemic in Egypt causing severe economic losses for poultry production sectors as well as poses a threat to human health [5,6].Vaccination is one option of influenza prevention and the principle strategy to control virus infection. Non adjuvanted whole inactivated influenza virus vaccine is of low immunogenicity [7].

Natural polymers have garnered considerable attention for vaccine delivery owing to their biocompatibility, low cost, continuous and prolonged antigen release [8]. Chitosan is a natural biopolymer produced by the deacetylation of chitin, a main component of the shells of crustaceans, for example crab, shrimp, and crawfish [9,10]. Chitosan has gotten significant consideration for its applications as an adjuvant and as a delivery platform [11]. This can be ascribed to its peculiar properties which make it a promising material for vaccine delivery [12]. It is non-toxic, biocompatible, biodegradable and cost effective. In addition, chitosan can easily form nanoparticles possessing high loading capacity for several proteins and different antigens [13]. Chitosan nanoparticles can be formed without exposure of the antigen to hostile conditions of heat or use of organic solvents [11]. They are spontaneously prepared using ionic gelation method with the guide of tripolyphosphate as a multivalent anion species [14].

Chitosan has been found to enhance both cell-mediated and humoral immune responses [15,16]. Chitosan has been shown to possess

* Corresponding author.
E-mail address: raniaelsayed@yahoo.com (R.E. Morsi).

http://dx.doi.org/10.1016/j.biologicals.2017.10.004
Received 4 June 2017; Received in revised form 13 October 2017; Accepted 17 October 2017

macrophage activation ability and cytokine initiation [13]. It additionally activates dendritic cells through binding to Toll-Like receptor (TLR4) and mannose receptors [16,17]. Its positive charge confers it with superior uptake by Antigen Presenting Cells (APCs) due to ionic interactions with the negatively charged cell membranes [8]. The positive charge also permits electrostatic binding with negatively charged antigens and DNA for vaccine delivery [11]. Chitosan based nanoparticles possess higher stability than other vaccine delivery stages such as liposomes and immune stimulating complexes [18].

The commercially used inactivated avian influenza vaccines were formulated either by using mineral salts or oil emulsions as adjuvants. Although these adjuvants enhanced the immunogenicity of inactivated vaccines [19], many problems have been emerged including tissue damage and necrosis at the site of injection which could produce carcinogenic transformation and could potentially decrease the commercial value of meat from vaccinated animals [20,21]. Existing influenza vaccines have elicited humoral immunity which is useful in protection against homologous viral strain infection, but they are deficient in cellular immunity which is successful in protection against heterogeneous viral strains [22,23].

From all the previous, it is hypothesized that chitosan, as a safe biopolymer, may have the adjuvant potential to amplify the immune response against vaccination by stimulating the innate and adaptive the immune system. So the present study was designed to assess the adjuvant effect of chitosan and its nanoparticles on the humoral and cell mediated immune responses induced by inactivated avian influenza H5N1 virus vaccines in broiler chickens.

2. Material and methods

2.1. Materials

Virus strains: Local Egyptian AIV H_5N_1reassortant strains, S_1 strain (A/Chicken/Egypt/Q1995D/2010) and S_2 strain (A/duck/Egypt/M 2583A/2010) were provided to the Veterinary Serum and Vaccine Research Institute (VSVRI) by the National Research Center (NRC).

Embryonated chicken eggs **(ECEs)**: were obtained from specific pathogenic free (SPF) farm Khomoshem, El Fayoum and were used for virus propagation and titration.

SPF chickens: were obtained from SPF farm Khomoshem, El Fayoum were maintained in isolators at CLEVB for using in safety test and immunization with the prepared vaccines.

Chitosan: was extracted from marine shrimp waste materials and was found to have a degree of deacetylation of 85% as determined by potentiometric titration and molecular weight of 2122 kDa as determined from the intrinsic viscosity measurement.

Sodium tripolyphosphate (TPP): was purchased from Sigma-Aldrich.

Thioglycolate broth and nutrient agar: were used for detection of any anaerobic bacterial contaminants (Oxford LTD, England).

Sabourauds agar: was used for detection of any fungal contaminants (Disco laboratories, Detroit, Michigan, USA).

XTT cell proliferation assay kit: was obtained from ATCC cat. No. 301011K and used for lymphocytic proliferation assay.

(RPMI-1640), Fetal Bovine Serum (FBS) and lymphocyte separation medium 1.077 were purchased from Lonza, USA.

Heparin (20 IU/ml) was purchased from Nile Pharmaceutical Company, Egypt.

***Candida albicans* (c. *albicans*)** was obtained from mycology department, animal health research institute AHRI, Egypt.

Formalin and methanol were obtained from ADWIC laboratory chemical, Egypt. **Giemsa stain** was obtained from (MERCK), Germany.

2.2. Methods

2.2.1. Vaccine preparation

Propagation of virus in ECE: The technique was done according to OIE Manual [2]. Briefly, the seeds of AI virus of 2 strains were diluted 10^4in sterile phosphate buffer saline (PBS) with antibiotic mix, the diluted virus suspension was inoculated into the allantoic sac of 10 day old SPF ECE and incubated at 37 °C for 5 days. Two control groups of ECE were included, one inoculated with saline solution and the other group left un-inoculated. The inoculated eggs were observed daily. Embryos that died were chilled until harvesting time. At the harvesting time, eggs with live and died embryos were used to collect the allantoic fluid which examined for haemagglutination activity using rapid HA test.

Sterility test: The harvested allantoic fluids were tested for any extraneous bacteria or viruses, according to OIE Manual [2].

Viral titration: According to OIE Manual [2], the harvested allantoic fluid containing AIV was titrated in 10 day old ECE. Serial 10 fold dilution from 10^{-1} to 10^{-12} of the virus was carried out using sterile phosphate buffer saline with antibiotic mix. 0.1 ml of each dilution starting from 10^{-6} till 10^{-12} was inoculated in allantoic cavity of 5 ECE. The eggs were incubated at 37 °C and candled daily for 7 days. The dead eggs were chilled at 4 °C until examined using HA test. The viral titer was expressed in terms of 50% end point and calculated according to Reed and Muench [24]. The titer of the avian influenza (Al) serotypes was adjusted to be 10^9 EID 50/dose for vaccine preparation.

Virus Inactivation: The harvested allantoic fluids of both AI serotypes (S1 and S2) were separately inactivated with formalin at a final concentration of 0.1% as described by VasfiMarandi and Bozorgmehrifard [25]. The inactivated viruses were tested for completion of inactivation using 10 day old SPF ECE. The allantoic fluid of each inoculated ECE was examined for HA activity, two successive blind passages were carried out before it was considered as completely inactivated.

Preparation of inactivated oil emulsion AI vaccine: Equal parts (v/v) of the inactivated fluid suspension of both S1 and S2 serotypes of AI virus were mixed. The concentration of each serotype was adjusted to contain at least 10^9 EID50/dose. The bivalent suspension was emulsified with Montanide ISA-70 oil (SEPPIC, Puteaux, CEDEX, France) at a ratio of 3:7 according to the manufacture.

Preparation of AIV–chitosan: The preparation was carried out according to Zhao et al. [26] and Sawaengsak et al. [27] with some modification. Chitosan solution was prepared by dissolving chitosan in an aqueous solution of 2% acetic acid until the solution was transparent. Once dissolved, the chitosan solution was diluted with deionized water. The solution was adjusted to pH 5.4 and filtered through a 0.22 μm filter to produce a final concentration of 1% chitosan. Then 1% chitosan solution was mixed with the inactivated AIV solution at a ratio of 3:1 under magnetic stirrer at room temperature and then kept in a refrigerator until use.

Preparation of chitosan nanoparticles: According to the principle of ionic cross-linking, nanoparticles can be formed by intra and inter molecular crosslinking between positively charged chitosan and negatively charged TPP. Chitosan solution (1% w/v) was prepared as previously described. TPP was dissolved in deionized water at the concentrations of (1% w/v). The chitosan nanoparticles were prepared by dropping TPP (Sodium tripolyphosphate) solution to the chitosan solution with the ratio 5:1 of chitosan: TPP using a dosing pump in a flow rate of 10 ml/h under magnetic stirring with 1000 rpm at room temperature [26].

Preparation of AIV-chitosan nanoparticles: Avian influenza antigen–chitosan nanoparticles were prepared using ionic gelation cross-linking method. Two and a half milliliters of inactivated AIV solution were added drop by drop to 5 ml of chitosan solution under magnetic stirring. Subsequently, 1 ml of TPP solution was added dropwise to the above solution under magnetic stirring at room temperature. The