Journal of Immunological Methods xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Immunological Methods

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

Research paper

Production and application of anti-nucleoprotein IgY antibodies for influenza A virus detection in swine

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ARTICLE INFO

Keywords:
Chicken IgG
Immunodiagnostic
Immunocytochemistry
Immunohistochemistry
Swine influenza

ABSTRACT

Influenza A virus (IAV) causes an important respiratory disease in mammals and birds leading to concerns in animal production industry and public health. Usually, antibodies produced in mammals are employed in diagnostic tests. However, due to animal welfare concerns, technical advantages and the high cost of production, alternatives to the production of antibodies in mammals have been investigated. The aim of this study was to produce egg yolk immunoglobulin (IgY) in laying hens against a highly conserved protein (nucleoprotein- NP) of IAV and to evaluate the application of anti-NP IgY antibodies in virus detection by immunocytochemistry (ICC) and immunohistochemistry (IHC). Three laying hens of the White Leghorn line were inoculated seven times with a recombinant NP protein and their eggs collected seven days after the 3rd, 5th and 7th inoculations. Immunoglobulin Y antibodies were purified from egg yolk through precipitation with ammonium sulfate. The titers and specificity of the purified antibodies were determined by ELISA, western blotting, ICC and IHC. High levels of specific anti-NP antibodies were detected by ELISA after the 5th inoculation, reaching a peak after the 7th inoculation. The mean yield of total protein in yolk after the 7th inoculation was 13.5 mg/mL. The use of western blotting and ICC demonstrated that anti-NP IgY binds specifically to NP protein. Moreover, the use of anti-NP IgY antibody in ICC test revealed positive staining of MDCK cells infected with IAV of the three subtypes circulating in swine (H1N1, H1N2, and H3N2). However, no staining was observed in lung tissues through the IHC test. The data obtained showed that anti-NP IgY antibodies bound specifically to influenza virus NP protein, detecting the main virus subtypes circulating in swine, reinforcing their usefulness in the influenza diagnosis.

1. Introduction

The influenza viruses belong to the *Orthomyxoviridae* family and are characterized by a genome composed of single-strand, negative-sense, segmented RNA (Zheng et al., 2015). The influenza A virus (IAV) can infect several animal species such as swine, avian, equine, as well marine mammals and human (Brown, 2000). H1N1, H3N2, and H1N2 are the main virus subtypes circulating endemically in swine populations worldwide (Lewis et al., 2016; Van Reeth et al., 2012). The disease is economically important for swine production due to increased production costs and reduced performance of the affected animals (Brown, 2013).

Surveillance and characterization of IAV in pigs are essential for the swine industry, as they can provide information on genetic and antigenic alterations in viruses with potential impact on human health (Choi et al., 2002). With the increasing demands for reliable tests for the diagnosis of influenza in swine, the validation of trials that provide rapid results in the analysis of multiple samples, which are easy to perform and low cost is very important (Swenson et al., 2001). For the definitive diagnosis of the disease, it is necessary to detect the viral nucleic acid and/or viral antigen in tissues or other clinical samples or to perform viral isolation (Detmer et al., 2013; Torremorell et al., 2012, Van Reeth et al., 2012). Detection of antibodies against the virus in the serum indicates previous infection, and for determination of a recent infection, paired serum samples collected at intervals of 3–4 weeks need to be analyzed (Van Reeth et al., 2012). Internal proteins such as nucleoprotein (NP) and matrix protein (M) are the most genetically conserved proteins among different subtypes of IAV (Van Reeth, 2007), with a low mutation rate during the evolution of the virus (Zheng et al., 2015). Thus, anti-NP antibodies can be used to detect all virus subtypes, and can be useful in the initial screening of clinical samples for subsequent virus characterization (Detmer et al., 2013).

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https://doi.org/10.1016/j.jim.2018.06.023
Received 4 April 2018; Received in revised form 16 May 2018; Accepted 29 June 2018

Usually, antibodies employed in diagnostic tests are produced in mammals (Lee et al., 2017), and may be polyclonal or monoclonal (Narat, 2003). The procedure for obtaining these antibodies involves immunization and subsequent repeated blood collections or euthanasia of the animals (Chalghoumi et al., 2009). A non-invasive alternative method for antibody production is the use of poultry (Schade et al., 2005). Laying hens produce IgY antibodies that are transported from the blood to the egg yolk (Carlander et al., 2003) and then subsequently purified from the yolk. An important advantage of using IgY antibodies in diagnostic tests is that these antibodies do not cross-react with Fc receptors, nor activate the complement system, thus reducing the occurrence of false-positive results (Karlsson et al., 2004). In addition, large amounts of antibodies are produced in laying hens (1 to 2.8 g of IgY per month) when compared to antibody production in rabbits (200 mg of IgG) (Schade et al., 2005). Therefore, fewer animals are utilized for this purpose, reducing production costs (Carlander et al., 2003; Narat, 2003).

Considering the importance of the diagnosis of influenza in swine, this study aimed to produce IgY antibodies in laying hens against the NP protein and to evaluate their application in the diagnosis of influenza in swine.

2. Material and methods

2.1. Production of NP antigen for inoculation of laying hens

The nucleoprotein gene segment (NP) of A/swine/Brazil/12A/2010 (H1N1) strain (GenBank accession number: KM507541) was cloned into pET23d expression vector (Novagen, Tokyo, Japan). The recombinant NP (rNP) was expressed in *E. coli* BL21 using 1 mM of IPTG (Fermentas, Waltham, MA, USA) at 25 °C for 2 h at 180 rpm and purified by Ni^{2+} affinity chromatography column HisTrap FF using 500 mM of imidazole in a phosphate buffer (NaH_2PO_4 and Na_2HPO_4, pH 7.4) (GE, Healthcare Life Sciences, Madison, WI, USA) followed by anion exchange chromatography using HiTrap Q HP eluted with 1 M of NaCl, as indicated by manufacture's protocol (GE, Healthcare Life Sciences, Madison, WI, USA). Western blot was used to confirm the rNP expression and identity.

2.2. Animals

Three laying hens of the White Leghorn line of 20 weeks of age were used for NP protein inoculation. The animals were kept in individual cages in facilities at the School Farm of the Universidade Estadual de Londrina (UEL), and provided with water and feed *ad libitum*. The execution of this experiment was approved by the Ethics Committee on the use of animals of UEL (5217.2017.39).

2.3. Inoculation of animals

The laying hens were inoculated with seven doses (20 μg/dose) of recombinant NP protein on polyacrylamide gel (Harlow and Lane, 1988) intramuscularly at four points of the pectoral muscle on days 1, 14, 28, 42, 84, 126, and 168 of the experiment. The eggs were collected one week after the 3rd, 5th, and 7th inoculation (harvests during seven days) and stored at 4 °C for subsequent antibody extraction and comparison of antibody levels over time. In order to evaluate non-specific IgY antibodies, to use as a negative control, eggs from non-inoculated hens were also collected.

2.3.1. Purification of IgY antibodies from egg yolk

For purification of IgY antibodies, the ammonia sulfate precipitation protocol was used, as described by Akita and Nakai (1992) with modifications. The yolk of the collected eggs was separated from the white, freed of the protective membrane, diluted at 1:7 in acidic water pH 2.5 and the resulting suspension was maintained at 4 °C overnight. Subsequently, the suspension was filtered on Whatmann n° 1 for removal of the lipid portion. Next, 1:3 saturated ammonium sulfate was added and the filtration was maintained under stirring at room temperature (RT) for 30 min. The solution was then centrifuged at 3000 × *g* for 15 min and the precipitation was resuspended in 18% sodium sulfate and kept under stirring at RT for 20 min. After this period, the solution was centrifuged again at 3000 × *g* and the precipitation resuspended in 14% sodium sulfate at RT under stirring. The solution was centrifuged again for 20 min, resuspended in PBS pH 7.4, dialyzed and stored at −20 °C.

The concentration of IgY was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard and its purity analyzed by electrophoresis in 10% polyacrylamide gel (SDS-PAGE) stained with Coomassie Blue.

2.4. Immunochemical characterization of IgY antibodies by western blotting

For the western blotting technique, NP protein (10 μg/mL) was mixed in sample buffer (50 mM Tris, pH 6.8, 1% SDS, 0.025% blue bromophenol, 10% glycerol, 20 mM DTT) and subjected to 12.5% electrophoresis polyacrylamide gel with SDS. The antigen separated by electrophoresis was transferred to the nitrocellulose membrane at 150 mA, which was held overnight at 4 °C. The membrane was subjected to Ponceau solution to confirm protein transfer, and then washed with distilled water and incubated with PBS/5% skimmed milk at 37 °C for 2 h. Thereafter, three washes were performed with PBS pH 7.4 and the membrane was incubated with anti-NP IgY antibodies or non-specific IgY (1 mg/mL), diluted in PBS/1% milk for 1 h in RT under agitation. After incubation, the membrane was washed three times with 0.05% Tween 20-PBS, before adding the goat anti-chicken IgG conjugated with peroxidase (A30-104P-Bethyl Laboratories) diluted in PBS/1% milk (1: 1000), for 1 h at RT under stirring. The membrane was then washed three times with 0.05% Tween 20-PBS, and the revelation was performed using 5 mg 3,3′-diaminobenzidine in 30 mL PBS plus 150 μL hydrogen peroxide. The revelation was interrupted with distilled water. For estimation of the molecular mass of the antigens, a standard molecular weight was used (Benchmark ™ Protein ladder, 10,748–010, Invitrogen, Carlsbad, CA, USA).

2.5. Determination of antibody reactivity

The anti-NP IgY antibody levels were determined by indirect Enzyme-Linked Immunosorbent Assay (ELISA). For this, microtiter plates (Corning Costar Corporation, Acton, MA, USA) of 96 wells were coated with 0.2 μg/well of NP protein in sodium carbonate-bicarbonate buffer solution, pH 9.6 and then incubated at 4 °C overnight. After this time, the plates were washed three times with PBS pH 7.4 and blocked with PBS/skimmed milk powder (150 μL/well) for 2 h at 37 °C. Thereafter, three washes were performed with 0.05% Tween 20-PBS. Two-fold serial dilutions (from 2 mg/mL to 0.0312 mg/mL) of the anti-NP IgY antibodies from inoculated hens (3rd, 5th, and 7th inoculation) were used for determination of antibody reactivity. IgY antibodies were diluted in PBS/1% milk, added 100 μL/well and incubated for 1 h at 37 °C. In the negative control, IgY antibodies from non-inoculated hens were utilized. Afterwards, plates were washed three times with 0.05% Tween 20-PBS and 100 μL/well of goat anti-chicken IgG conjugated with peroxidase (A30-104P-Bethyl Laboratories) was added, in the concentration 1:40000 (diluted in PBS/1% milk), and incubated for 1 h at 37 °C. Plates were washed with 0.05% Tween 20-PBS and 100 μL/well of the sodium acetate substrate solution buffer in 0.1 M pH 5.2 containing 0.1 mg of tetramethylbenzidine and 0.005% of hydrogen peroxide added, followed by incubation at 37 °C for 15 min. Finally, 50 μL/well of the 2 N sulfuric acid solution was added to interrupt the reaction, and the optical density reading was performed with a 450 nm filter.