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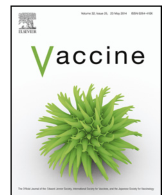

Practical aspects in the use of passive immunization as an alternative to attenuated viral vaccines

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ABSTRACT

Passive immunization as a method to protect birds has been tested for many years and shown to be effective. Its advantages over active vaccination include no use of partially virulent viruses, overcoming the gap in the level of protection at young age due to interference of maternal antibodies to raise self-immune response following active vaccination and the possible immunosuppressive effect of attenuated vaccine viruses. However, a major obstacle to its implementation is its relatively high cost which is dependent, among other things, mainly on two factors: the efficacy of antibody production, and the use of specific pathogen-free (SPF) birds for antibody production to avoid the possible transfer of pathogens from commercial layers. In this study we show efficient production of immunoglobulin Y (IgY) against four different pathogens simultaneously in the same egg, and treatment of the extracted IgY with formalin to negate the need for SPF birds. Formalin, a common registered sterilization compound in vaccine production, was shown not to interfere with the Fab specific antigen binding or Fc-complement activation of the antibody. Following injection of 1-day-old broilers with antibodies against infectious bursal disease virus, protective antibody levels were acquired for the entire period of sensitivity to this pathogen (35 days). Passive vaccination with formalin-sterilized IgY against multiple antigens extracted from one commercial egg may be a cost-effective and advantageous complementary or alternative to attenuated vaccines in poultry.

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1. Introduction

Birds are protected against pathogens in the first days of their life by maternal antibodies passed from the hen via the egg yolk to the developing embryo [1]. To obtain efficient protection against the major pathogenic viral diseases, including Newcastle disease virus (NDV), influenza virus and infectious bursal disease virus (IBDV), maternal flocks are hyper-immunized to induce high levels of immunoglobulin Y (IgY) antibodies in the sera that will be transferred to the progeny [2]. The half-life of antibodies in the newly hatched chicks is approximately 3 days [3], considerably shorter than the 2- to 4-week half-life of mammalian maternal IgG [4]. As a consequence, full protection by maternal antibodies lasts for part of the growth period, after which the rate of neutralizing antibodies decreases below protective levels [5–8]. To protect broiler flocks for their entire growth period, 35–42 days, active vaccinations, comprised mostly of attenuated viruses, are administered.

IBDV is one of the major avian pathogens that cause severe immunosuppression and a high rate of morbidity and mortality in broilers. In most cases, outbreaks arise after the decline in the level of maternal antibodies [9], with symptoms of reduced immunocompetence and a decrease in weight gain or feed-conversion performance [10]. Disease severity is influenced by the degree of passive immunity [5,11] and a close correlation exists between titers of maternal neutralizing antibodies and protection against immunosuppression, bursal lesions or mortality [5,12,13]. Passive vaccination with IgY extracted from eggs of layers that were vaccinated with IBDV capsid viral protein 2 (VP2) or whole virus has been found to confer protection [1,14,15]. Egg-extracted IgY has been shown to protect against other pathogens as well [16].

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Antigen-specific IgY can be extracted on a large-scale from eggs of immunized layers at levels of 2–10% of the total antibodies in the eggs' antibodies [17]. The use of IgY has been studied extensively, demonstrating its effectiveness in preventing and treating infectious diseases. Using specific pathogen-free (SPF) birds as source of IgY can conceivably avoid the transfer of pathogens in farm layers to the vaccinated birds. However, the high cost of raising SPF birds significantly raises the price of this treatment.

The aim of this study was to investigate practical aspects of the use of passive immunization as an addition or alternative to active vaccination. We determined efficient production of specific anti-pathogen antibodies in laying hens, attempted to extend the half-life of IgY, investigated large-scale use of non-SPF IgY, and determined the rate of decline of externally administered IgY in the vaccinated bird.

2. Materials and methods

2.1. Antibody production against specific antigens in laying hens

Layers were vaccinated with inactivated NDV (Nectiv, strain VH, Phibro Vaccines, Israel), or a mixture of inactivated NDV strain VH and recombinant VP2 subunit of IBDV (Bursativ), which also contains inactivated NDV (Phibro Vaccines), or a combination of vaccines against NDV, IBDV (VP2), influenza virus and reovirus (Shapativ and Tri-Reo, respectively, Phibro Vaccines Israel), according to the manufacturers' instructions. In Addition, one group was vaccinated with influenza virus and another by reovirus. Two additional groups of birds were vaccinated with VP2 (10 μg/bird) injected intramuscularly with adjuvant or administered orally together with heat-labile enterotoxin (LT) from *Escherichia coli* (50 μg/bird) [18]. Negative control birds were vaccinated with 0.5 mg *E. coli* protein extract [19].

Vaccination regimen to obtain high and long-lasting antibody titers against various antigens and their combinations in egg yolk was as follows: 5-month-old laying Leghorn layers that were vaccinated prior to the experiment as recommended by the Israeli Ministry of Agriculture from day 1 to 3 months of age were additionally vaccinated in this experiment with the above-listed vaccines at 3-week intervals between the first, second and third vaccinations, and a fourth vaccination 2 months after the third vaccination. Blood was drawn 2 weeks after each vaccination and 4 months after the fourth vaccination. Eggs were collected 2 weeks after the third vaccination and kept at 4 °C for up to 10 days before IgY extraction. Sera were kept at −20 °C until examination.

2.2. Antibody extraction and purification from egg yolk

IgY was purified from egg yolk following a previously reported protocol [20] and the concentration was determined by Nano-Drop spectrophotometry (Thermo, Waltham, MA, USA) at 280 nm. Egg-extracted IgY was kept at −20 °C until examination.

2.3. Enzyme-linked immunosorbent assay (ELISA)

The presence of antibodies against NDV, LT or VP2 in chicken serum and yolk was determined by ELISA. Plates (Nunc, Waltham, MA, USA) were incubated overnight at 4 °C with antigen diluted in carbonate-coating buffer (pH 9.6). VP2 and LT were added to a concentration of 1 μg/well and the lyophilized Newcastle Tabic V.H. vaccine (Phibro Vaccines) was suspended according to manufacturer's instruction then diluted 1:50,000. Plates were washed following each step using washing buffer (0.05% (v/v) Tween-20 in phosphate buffered saline (PBS)) then drying on a paper towel. Plates were blocked for 1 h at 37 °C. with 150 μl/well of blocking buffer (5% (w/v) skim milk dissolved in washing buffer). Series of serum or yolk dilutions were added and incubated for 1 h at 37 °C. Plates were then incubated (1 h in 37 °C) with horseradish peroxidase (HRP) conjugated Rabbit anti-Chicken-IgY secondary antibodies (Sigma–Aldrich, Israel) which were diluted 1:5000 in blocking buffer. Substrate solution of o-phenylenediamine dihydrochloride (Sigma–Aldrich) was added and optical density was determined at 450 nm (OD_{450}) by ELISA reader. The titer was determined as the dilution at which the tested optical density reached that of the negative control.

The level of antibodies against VP2 in chicken sera following passive immunization was also tested with the Synbiotics ELISA kit test ProFLOK® IBD *Plus* (Kansas City, MO) according to the manufacturer's instructions.

The titer of anti-reovirus of chicken vaccinated by reovirus vaccine or reovirus in combination with three other vaccines was determined by a kit test (avian reovirus antibody test kit, IDEXX, Westbrook, Maine) according to the manufacturer's instructions. Titer is determined as the dilution ($\log_2$) that has an O.D. as the negative control.

2.4. Hemagglutination inhibition (HI) test

Anti-NDV and anti-influenza antibody levels in the serum of Leghorn layers following vaccination and in their laid egg yolk were determined by HI test, performed according to the OIE (World Organization for Animal Health) Terrestrial Manual 2012 [21] (for detailed protocol, see [20]).

2.5. Formalin conjugation to IgY

Purified IgY (16 mg/ml) was incubated with formaldehyde (CH_2O, M_W = 30.03 g/mol, Sigma–Aldrich) to a final concentration of 0.12% or 0.25% (v/v) with shaking for 1 h at room temperature, resulting in reversible conjugation of formalin to protein. For covalent conjugation, the IgY and formalin mixture was reduced by addition of sodium cyanoborohydride ($NaBH_3CN$, M_W = 62.84 g/mol, Sigma–Aldrich) (1:2 protein-w/w) and incubated with shaking for an additional 2 h at room temperature, followed by overnight dialysis against PBS in 3.5-kD-cutoff cassettes (Thermo).

To assess the efficiency of reversible and covalent IgY–formalin conjugation, the number of unbound amine residues in the protein was determined according to Gefen et al. [19] with some modifications. Briefly, the formalin-conjugated IgY was diluted to a concentration of 0.25 mg/ml with 0.1 M sodium tetraborate buffer pH 9.3 (Sigma–Aldrich), then reacted with 0.01 M trinitrobenzenesulfonic acid (Sigma–Aldrich). Following incubated with shaking for 30 min at 37 °C the absorption of the solution was measured at OD_{405}. The number of unbound amine residues was calculated from a calibration curve of glycine.

2.6. Evaluation of in vitro biological activity of antibody covalently conjugated to formalin

2.6.1. Antigen-binding efficiency

The binding of anti-NDV IgY–formalin conjugate to NDV was compared to that of non-conjugated IgY by HI test.

2.6.2. Activation of complement

E. coli (strain NCIMB 702070) was grown in tryptic soy broth (Bacto) with shaking at 37 °C to an OD_{492} of 0.5. Then, 150 μl of the solution was mixed with chicken sera or chicken sera inactivated at 56 °C (700 μl) and 160 μl anti-*E. coli* IgY (31 mg/ml). Samples were incubated with shaking at 37 °C and OD_{492} was measured in 15 min intervals starting from 1 h. Optical density levels were calculated by subtracting the optical density obtained in a solution of *E. coli* and IgY without sera. The experiment was repeated three times.

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