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IgY: A Key Isotype and Promising Antibody for the Immunoprophylaxis Therapy of Infectious Bursal Disease Virus Infections

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The infectious bursal disease (IBD) is a highly contagious and acute poultry disease caused by Birnavirus. However, the vaccination is the only disease prevention, but several factors impeded vaccine development. Thus, a need for time to develop a novel technique for managing and treating respiratory diseases in poultry birds. Passive immunization is a hope and a possible alternative used in birds to meet this need. The current research attempted to produce egg yolk-based polyclonal antibodies against the IBD virus. The benefits of IgY include ease of extraction, lack of reaction with mammalian Fc receptors, and low production cost. Commercial layers were immunized with inactivated IBD virus subcutaneously according to the treatment regimen. The eggs were gathered daily, and yolk antibodies were extracted with the ammonium sulfate precipitation technique. The use of an indirect hemagglutination test demonstrated that IgY was IBD-specific. Until the end of the experiment, the specific IgY immunoglobulins did not lose activity when stored at 4℃. The specific immunoglobulin (IgY) treated challenged birds were demonstrated 92% recovery in comparison to the control group. The study implies that the IBDV specific IgY is an easily prepared and rich source of antibodies and offers an alternative therapeutic agent to cure IBD-infected birds.

Keywords: **Immunoglobulin-Y, Passive Immunization, IBDV, Immunoprophylaxis, Vaccine**

Introduction

In intensive poultry farming, immunosuppression is a persistent problem rising with the growing demand for poultry production. The leading cause of immunosuppression are the viruses that primarily infect and destroy the immune cells in birds. In immunosuppressive flocks, the factors that lowered the expected growth and production are the chronic disease situations, susceptibility to opportunistic pathogens, and the suboptimal response to a vaccine. Immunosuppressive viruses have economic and societal effects because of direct and indirect losses provoked by these viruses. Besides this, the significant concern to human health is drug residues, and antibiotic resistance increases with the use of antibiotics to cope with secondary infections [1].

The infectious bursal disease (IBD) virus is very resistant and stable to chemical and physical agents, ultraviolet radiations, and heat. Thus, it persists for several weeks in poultry houses even after disinfection and cleaning. No specific treatment exists, but palliative treatment is practiced to control IBD virus in poultry birds. The principal means is the administration of attenuated and killed vaccines to prevent IBD. Still, vaccines' development faces various challenges, including short-term immune responses or low efficacy and anti-

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genic variations in viral strains. In the case of disease outbreaks, the rapid development of vaccines may not be possible to control disease spread. Hence, it is a need for time to develop novel methodologies for managing and treating respiratory diseases in poultry birds [2].

In immunocompromised individuals, an alternative approach to treat the infections is passive immunization avoiding the vaccination side effects. Antibodies are mainly polyclonal, derived from the immunized animal sera for the passive immunization approaches. However, the safety and standardization of the polyclonal antibodies are the challenges considered in the effective use of antibodies. The suggestive alternate to these polyclonal antibodies is the Monoclonal antibodies (mAbs), but the high production cost (USD 100/g) limited its utilization [3].

Egg yolk immunoglobulins (IgYs) are produced by amphibians, reptiles, chickens, and other avian species, and their function is similar to the mammalian IgG. IgYs are present in the sera of chicken and are passed to the embryo through egg yolk. This finding suggests that IgY-based therapy could have a longer circulating half-life, increasing its efficacy against infections. The infectious bursal disease is the primary cause of mortality in the poultry industry, despite significant advances in poultry research. There is one death every three seconds in the poultry industry, most of which are caused by infectious diseases. Antibodies (IgY) are a good option in treating this disease, and the treatment option with antibodies to protect against contagious diseases is known as passive immunity. IgY is a potential alternative and has a promising role in treating such diseases [4].

The production of IgY is a non-invasive alternative to the current methods of repetitive bleeding in laboratory animals. The production of IgY is very cheap and shows high stability over a wide range of pH and temperatures. This study aims to highlight the use of IBD-specific IgYs in immunotherapy to control infectious bursal disease virus infections.

Materials and Methods

Ethical statement

All the processes used in the trial were approved by the Institutional Biosafety Committee, University of Agriculture Faisalabad, Pakistan.

Isolation of IBD virus

The infectious bursal disease virus was procured from the infected bursa of Fabricius from field outbreaks in the Faisalabad district. The bursa samples were chopped in phosphate buffer saline (PBS) to prepare 10% suspension using a homogenizer. Homogenized bursae were subjected to ultrasonication in a jacketed vessel using 600 rapids at an intensity of 75 watts/cm with a titanium probe (15 cm diameter) for 5 min to disintegrate the virus particles. The temperature was kept under 20℃ by placing on the ice blocks. The sonicated antigen was centrifuged at 5000 rpm for 15 min and the supernatant was collected. The IBD virus was characterized in the previous study by [5].

Propagation of virus in embryonated eggs

Specific pathogen-free (SPF) embryonated eggs (09 days old) were obtained from the poultry farm, University of Agriculture Faisalabad, Pakistan. A small hole was drilled using an egg puncher in the shell of SPF eggs of nine days old. With a sterilized 1 ml syringe, the virus suspension (500 µl) was injected into the Chorioallantoic membrane (CAM). The hole was closed with the help of molten wax, and eggs were incubated in an egg incubator at 37℃ for 96 h. The embryo was inspected every day for survival by candling. After 96 h, lesions in CAM, death of the embryo, and stunting growth of the embryo were examined for the signs of the viral infection. The IBDV was passaged three times through the CAM route [6].

Identification of virus

The hyperimmune sera were raised in rabbits against the IBD virus propagated in embryonated eggs. The virus was confirmed using the agar gel precipitation test (AGPT). The agar overlay method was adopted for this experiment; a layer of 3 mm with 4% agar was covered with a layer of 4 mm with 0.9% agarose gel (Sigma-Aldrich, USA) prepared in borate buffer (pH 8.8) with the 2% addition of sodium azide (Sigma-Aldrich ≥ 99.5%). Five agar wells were formed at an equal distance of 4 mm in the agar plate surrounding the central well. The IBD virus was added to the central well while the antiserum raised in rabbits was added in all four wells, keeping the fifth well as control. The agar plate was incubated for 48 h at 37℃, and observed results [2, 7].

Immunization of layer birds with IBDV

Thirty, 20-week-old, healthy White Leghorn hens (layers) were housed in an animal house facility, Institute of Microbiology, University of Agriculture Faisalabad, Pakistan. The virus antigen was inactivated with 0.3% formalin. The layer birds were immunized five times in two weeks with the IBD virus preparation containing 0.5 ml of the inactivated IBDV antigen subcutaneously. The egg collection was started after the initial immunization. Collected eggs were stored at 4℃ until sufficient numbers were obtained [8, 9].

Isolation and purification of IgY

The eggs collected from the immunized layer birds were used to separate IgY protein. The egg yolk was separated from the white and washed twice with distilled water at 4℃. The yolk was separated from the membrane and nine-fold diluted with distilled water at 4℃. 1 N HCl was used to adjust the pH to 5.0. Charcoal (0.01%) was added and pH re-adjusted to 4.0, and the diluted yolk was subjected to freezing at -20℃ overnight. The frozen yolk was thawed at 4℃ and centrifuged at 10,000 ×*g* for 10 min at 4℃. The supernatant was collected and added with 0.24 g/ml of ammonium sulfate and incubated at 25℃ for 30 min with continuous stirring. The samples were centrifuged under 4℃ for 12 min at 10,000 ×*g*, and the supernatant was discarded. The pellet was dissolved in a small volume of 2M ammonium sulfate solution and re-incubated at 25℃ for 30 min. The solution was centrifuged at 10,000 ×*g* for 12 min under 4℃, and the supernatant was discarded. The pellet was resuspended in a small fraction of PBS and stored at -20℃ till further processing [10].

Antibody titration through indirect hemagglutination test

A 5.0 ml human blood group "O-" was aseptically collected in a disposable syringe and transferred to a vacutainer coated with EDTA as an anticoagulant. The blood was centrifuged for 10 min at 1500 rpm, and the buffy coat was pipetted off. The double volume of PBS (pH 7.2) was added to the vial to mix the red blood cells (RBCs) and centrifuged thrice. Packed RBCs were collected and sensitized using antigen.

Test procedure. An equal volume of PBS and the infectious bursal disease virus was taken and sonicated under an ultrasonifier at 600 cycles/second for three minutes. The suspension was centrifuged at 1500 ×*g* for 10 min to collect the supernatant for sensitization. The sensitization of the RBCs with antigen was achieved by mixing 1 ml of the washed RBCs, 2 ml of the antigen, and 2 ml of the PBS, placed in an incubator at 37℃ for 2 h with gentle mixing after 1 h. The treated RBCs were washed thrice with PBS to remove the excessive antigen, and 1% suspension of the sensitized erythrocytes was used in Indirect Hemagglutination (IHA). Briefly, after making a two-fold serial dilution of the IgY antibodies, an equal quantity of sensitized human O-negative RBCs (1%) was added to each well of the microtitration plate. The plate was tapped gently to ensure the even dispersion of the RBCs and incubated for half an hour at 37℃. The level of agglutination in each well of the row was recorded. Egg yolk antibodies (IgY) caused a distinct RBCs agglutination were recorded as positive. The samples with button-shaped settling of erythrocytes in the center of the well were considered negative. The indirect hemagglutination titer of each sample was described as the reciprocal of its endpoint dilution [11].

Challenge studies

SPF chickens (n = 20) were housed in an animal house facility, Institute of Microbiology, University of Agriculture Faisalabad, for challenge/ protection studies using IgY. Birds were fed with commercial starter feed and ad-libitum water until the administration of the treatment. The birds were inoculated with 10^4 Embryo Infectious Dose (EID_{50}/ml) of very virulent IBD virus via the intraocular route to stimulate a natural infection. Following the commencement of the experiment, the cages were not cleaned for 4 days, and from the 5th day of infection, the cages were cleaned, and birds were offered 5 ml of the yolk in drinking water. Birds were observed for clinical signs and mortality, and the serum samples were collected at 0, 4 and 10 days post-exposure (DPE). Serum antibody titers were measured through ELISA, and the S/P ratios were calculated according to the manufacturer's instructions [12].

Results

IBD virus inoculation in embryonated eggs

Embryonated chicken eggs were observed daily, and

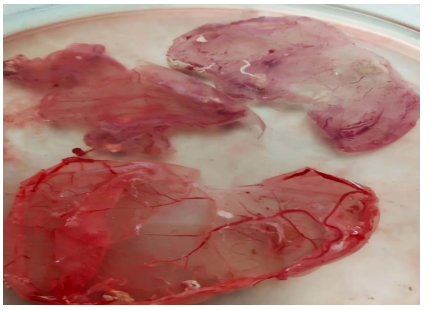

Fig. 1. Gross lesions on the Chorioallantoic membrane after inoculation of IBDV in embryonated chicken eggs.

the dead embryos after the first 24 h of post-inoculation were discarded in every passage. After 96 h, eggs were chilled at 4℃, and CAM was harvested in a sterilized petri dish (Fig. 1). The mortality of 90 to 100% in embryos was observed during the 1st and 2nd passages, while it was reduced to 70% in the 3rd passage. Grossly, the chicken embryos showed edematous distention of the abdomen. Hemorrhages were visible on the cerebrum, skin, toe joint, and CAM. The CAM was separated and centrifuged for 20 min at 1500 rpm to collect the virus in the supernatant. The presence of the IBD virus in the supernatant was confirmed through AGPT using known antiserum raised in rabbits.

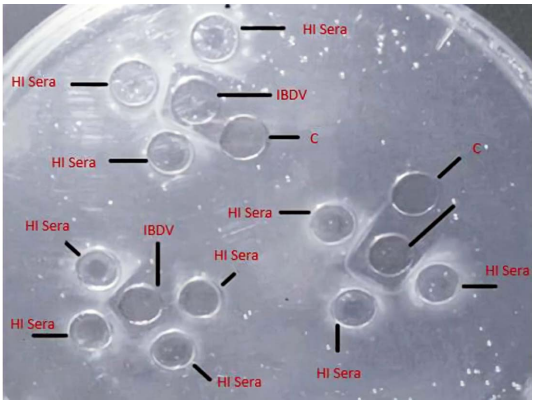

Fig. 2. A white precipitation line on the plate was observed between IBD virus in the central well and antiserum in the surrounding wells after 48 h incubation on a 0.9% agarose gel plate.

Confirmation of IBDV through AGPT

The presence of the IBD virus inoculated in embryonated eggs was authenticated through an Agar gel Precipitation test. A very clear precipitation line was observed within 24 h of incubation, while in negative control wells, a line of precipitation was not observed. A white precipitation line appeared against a positive control well containing hyperimmune sera raised in rabbits against infectious bursal disease virus (Fig. 2).

Determination of immunoglobulin-Y antibodies in the egg yolk

Egg yolk antibodies (IgY) were detected through the IHA both quantitatively and qualitatively. After immunization of the layers with infectious bursal disease virus, 65 eggs were collected in four weeks. The attachment of RBCs sensitized IBD virus with specific IgY antibodies recorded the hemolysis of red blood cells, and the maximum dilution was 1:128, at which the efficacy was recorded (Table 1). In contrast, hemolysis RBCs were

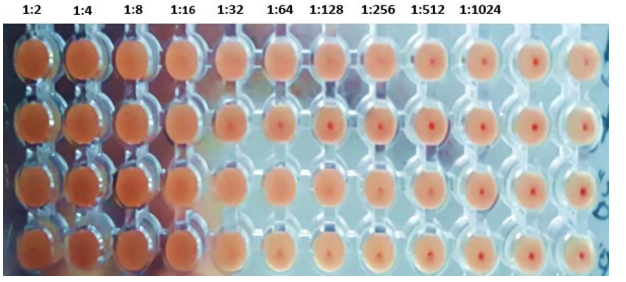

Fig. 3. Hemagglutination pattern of IBDV sensitized RBCs after the addition of anti-IBDV specific egg yolk antibodies (IgY).

Table 1. Determination of egg yolk antibodies (IgY) through Indirect Hemagglutination (IHA) test.

Days	Total Egg yolks	Antibody titers using IHA test								
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	GMT
0-7	12	4	5	3	-	-	-	-	-	3.7
8-14	13	-	-	2	7	4	-	-	-	17.1
15-21	16	-	-	1	3	6	2	4	-	39.4
22-28	24	-	-	-	1	2	4	12	5	104

prevented from the eggs of non-immunized control hens (Fig. 3).

Exposure/protection model

The challenge model was carried out to determine the therapeutic and prophylactic efficacy of IBDV specific IgY in the infected poultry birds. The challenged birds received daily treatment with IgY for six days starting from the 5th-day of the virus challenge, and the bursae collected from infected birds were positive for IBD virus antigen using AGPT. Birds recovered very quickly after the treatment with specific IgY antibodies. The pre-challenge ELISA titer was 750 with an SD value of 155. However, after the treatment with hyperimmune yolk (IgY), the surprising surge of geometric mean titer reached 7750 with an SD value of 200 evident at 10 DPE. AT 1 and 2 DPE, no clinical signs of the disease were observed, but a sharp peak was seen at 3 DPE. The total mortality was 8% throughout the experiment. However, 92% of birds were recovered by 10-day post-exposure.

Discussion

The first evidence of maternal transfer of IgY from chicken to egg yolk for embryo was reported 100 years ago. The immunized hens produce egg yolk IgY antibodies could efficiently produce large quantities of antibodies (≈ 100 mg of total IgY/egg). These antibodies may provide practical biological alternatives for specific antiviral therapy in the case of infectious diseases. In aqueous conditions, immunoglobulin-Y are highly stable at 65℃ and pH range of 4 to 9, even retaining the antigen-binding activity in the presence of pepsin at pH 4 to 6. These attributes make IgY excellent candidates for several types of applications and processing. The large-scale but quite simple production, together with the ease of storage and transportation, makes IgYs a suitable candidate among global therapeutic agents that may be used in times of a pandemic [13].

Immunoglobulin-Y has various advantages: IgY is the naturally produced antibody and is non-synthetic in nature; these are directed against multiple antigen targets, so they do not induce resistance and are nontoxic as a natural component of eggs. These antibodies do not affect the microbial population of the host due to targeting the particular pathogen [8]. In the treatment regime of IgY, unlike the antibiotics, egg yolk immunoglobulins neither induce any side effects nor deposit in the muscles. Hence, specific IgY treatment therapy avoids the potential violation of regulations in countries that prohibit antibiotics for livestock and poultry. Furthermore, specific IgYs can be lyophilized in powder form and stored for months without refrigeration at low moisture levels and are faster and cheaper to produce than vaccines. The collection of eggs for the production of IgY is an animal-friendly and pain-free procedure, which is valuable from the animal welfare perspective [12, 14].

As a potential complementary defense against the IBD virus, we have been using IgY to control seasonal IBD. This study has shown that the IBDV is immunogenic in poultry and induced high IgY titers and long-lasting humoral immune response. Thus, this study demonstrated the feasibility of producing large quantities of specific IgY to prevent infectious bursal disease virus. This study observed a continual increase of IBD-specific IgY in egg yolks starts in the 2nd week after the initial immunization. IBD-specific antibodies reached the highest in yolk by the fourth week; during this period, 65 eggs were collected from thirty immunized chickens. Producing an equal amount of antibodies would require 100–125 guinea pigs assuming that 15–20 ml of sera can be collected from each animal. Strikingly, high levels of antigen-specific egg yolk IgY can be produced for up to 2 years when the bird is continuously boosted every three months. Furthermore, the collection of yolk antibodies is non-invasive without affecting the immunized chickens [9].

The antigenic specificity of IBD-specific IgY was determined in IHA using the IBD virus, and hemolysis of erythrocytes were demonstrated at a dilution of 1:128. Of particular interest was the finding that specific IgY raised against the IBD virus could neutralize the virus and showed a sustainable titer demonstrating the inhibitory invitro effect of the virus in the challenge/response model. The oral specific IgY treatment results in the reduction of infection in 92% of the birds.

Concludingly, specific IgY may be used to prevent the infectious bursal disease in the poultry. A single egg contains approximately 100 mg of IgY. Each hen produces almost 150 eggs during the laying period, so substantial quantities of IgY can be produced at a low cost; thus, a

chicken is referred to as a small "factory" for antibody production. The specific IgY may be raised against infectious disease and formulated as oral, nasal, and aerosol sprays to provide protection and stockpiled for its use in times of disease outbreaks. Thus, this approach has great potential to control infectious diseases, specifically infectious bursal disease in poultry.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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