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Passive immunization using purified IgYs against infectious bursal disease of chickens in Pakistan

Muhammad Wasif Malik, Najma Ayub, Irfan Zia Qureshi*

Department of Biological Sciences, Quaid-i-Azam University, Islamabad P.O. Box 45320, Pakistan

Infectious bursal disease (IBD) is an acute and highly contagious disease of young chickens caused by Birnavirus. Mortality of infected birds can be best prevented if injected with antibodies. The present study was an attempt to raise specific hyper-immune polyclonal antibodies against IBD virus in Pakistan. Commercial layers divided into four groups were injected with IBD vaccine subcutaneously according to four different treatment regimens. Eggs were collected daily and antibodies were purified from yolk with dextran sulphate. Titers of antibodies in serum and yolk were evaluated with enzyme linked immunosorbant assay and agar gel precipitation test. Antibody titers were significantly higher in yolk than serum. Eggs collected at 28 days post-vaccination had maximum antibody titers. Of treatment regimens, T_3 was found to be most effective for hyperimmunization. Lyophilized antibodies stored at 4°C did not lose their activity till the end of experiment. IBD virus infected birds were injected with purified antibodies which induced 92% recovery as compared to control birds. The study implicates that the purified antibodies may be useful as a therapeutic agent to cure IBD infected birds.

Key words: immunoglobulin Y, immunoglobulin therapy, passive antibody transfer, passive immunotherapy

Introduction

Infectious bursal disease (IBD) is an acute highly contagious, viral disease of growing chickens with worldwide occurrence. IBD was observed in chickens in 1957 in Gumboro district of Delaware, USA. Birds that survive the disease are permanently immunosuppressed, therefore more susceptible to other disease causing agents and do not respond adequately to vaccinations. The bursa of Fabricius (BF) is the primary target organ of IBD virus (IBDV) where it replicates in immature bursa-derived lymphocytes (B-lymphocytes) of chickens. IBD can be characterized by sudden onset, short course, and extensive destruction of lymphocytes in BF and profuse watery diarrhea followed by death or rapid recovery [3].

There is no specific treatment available for this disease but palliative treatment may be undertaken for its control. Passive hyperimmune therapy (PHT) is another alternative to standard vaccination. Passive immunization with antibodies derived from blood is widely used to prevent or treat infections like measles, hepatitis A, hepatitis B, tetanus, varicella, rabies, and vaccinia etc. [5].

Rabbits and other animals are normally used for production of polyclonal antibodies; however, by 1962 it was found that immunoglobulin concentration in the yolk was equal to or greater than that found in hen serum [9]. According to an increasing number of publications, the antibodies produced in hens are useful in many applications, including immunotherapy and immunodiagnostics [11,1,2]. In some cases, hens being distant relatives of mammals; offer a good alternative to rabbits in producing antibodies against mammalian antigens [12]. Fischer *et al.* [4] investigated extraction of immunogloblin Y (IgY) from egg yolk using polyacrylamide gel electrophoresis (PAGE) and densitometric analysis and concluded that dextran sulfate was very effective, quick and simple method to extract antibodies.

The present study was designed to raise and isolate specific hyper immune polyclonal antibodies against IBD. Quantification of antibodies was done through enzyme linked immunosorbant assay (ELISA) and AGPT and different antibody titers were prepared. Furthermore the antibody titers were applied to infected birds for evaluation as effective therapeutic agent.

Materials and Methods

Immunization of hens

Hundred commercial layers (Bab-Cock breed) of 72 weeks of age were obtained from suppliers and were housed in individual cages to avoid mixing of the eggs. ELISA titer of IBD antibodies before vaccination was 3,083. Oil based vaccine against IBD virus (Bursine-K; Forte Dodge, USA)

*Corresponding author
Tel: + 92-51-9219809; Fax: +92-51-9219888
E-mail: irfanzia@qau.edu.pk, irfan_qureshi_pk2002@yahoo.com

was injected subcutaneously into chickens according to four treatment regimens. In the first treatment program (T_1), birds were vaccinated twice with 0.5 ml vaccine seven days parted. In the second treatment program (T_2), birds were vaccinated daily starting from 0.1~1 ml for 10 days. In the third treatment program (T_3), birds were vaccinated daily starting from 0.1~1 ml in ten days and 0.5 ml afterwards weekly for two times. In the fourth treatment program (T_4), birds were vaccinated twice; at start of experiment and then after two weeks with dose rate of 0.5 ml each time. The eggs were collected daily, labeled accordingly and were stored in a refrigerator at 4°C until isolation of the immunoglobulin [6]. All experimental procedures were conducted according to ethical guide lines provided by the Ministry of Food, Agriculture and Livestock, Government of Pakistan.

Purification of immunoglobulins by dextran sulphate

The procedure was essentially as described in Koko *et al*. [6]. Reagents were obtained from Sigma and Pharmacia USA. Briefly, egg yolks were separated from the white. Phosphate buffered saline (PBS) was added in the yolk to bring the volume to 50 ml and mixed. Diluted yolk suspension was centrifuged for 10 min at 2,000 *g*. Pellet was discarded and supernatant was saved. Supernatant was mixed with 3 ml of 10% dextran sulphate solution (W/V in dH_2O) and 7.5 ml of 1 M calcium chloride solution; incubated for 30 min and then centrifuged as above. Clear supernatant was saved. Solid sodium sulphate (a total of 20 g/100 ml) was slowly added to the supernatant and incubated for 20 min, and centrifuged for 10 min at 2,000 *g*. The pellet was saved and dissolved in 10 ml of 1 × PBS. Dissolved immunoglobulins were separated from non-dissolved material by centrifugation as above. Clear supernatant was saved. 6.2 ml of 36% sodium sulphate solution was added to supernatant and centrifuged for 10 minutes at 2,000 *g*. Pellet was saved and immunoglobulin pellet was dissolved in 5 ml of 1 × PBS. The immunoglobulin solution was divided into small aliquots and stored at 4°C.

Antibody titration

ELISA: An ELISA kit was obtained from IDEXX Laboratories (USA) and standard ELISA procedures were followed. Values were recorded at 650 nm absorbance. ELISA plate was read using an ELISA plate reader (SLT Labinstruments, Austria). The IBD ELISA titer was calculated by using following formula: Log_{10} Titer = 1.09 (Log_{10}S/P) + 3.36.

Agar gel precipitation test (AGPT) was performed according to procedures defined by Thayer and Beard [14]. AGPT unit was calculated from the last precipitation line shown by highest antibody dilution.

Lyophilization of antibodies

Water was removed from frozen samples mainly by sublimation. Approximately 90% of the total water in the sample (essentially all of the free water and some of the bound water) was removed by sublimation. Bound water was removed by de-sorption, resulting in a product that has <1-3% residual water. This step required 1/3~1/2 the time needed for primary drying.

Field trials of antibodies

Field trials were made to check the efficacy of antibodies. Farms with outbreak of IBD were visited. The diseased birds of different breeds were searched from commercial poultry farms on the basis of symptoms including dullness, depression, off feed, onset of white or greenish diarrhea. Postmortem examination confirmed bursitis. A total of 250 birds (mixed breeds of chicken) were taken and divided into five groups of 50 birds each. Commercial broilers, commercial layers, broiler breeders and indigenous birds were placed in groups 1, 2, 3 and 4, respectively. The purified antibodies of different dilutions containing different AGPT units were injected intraperitoneally to IBDV infected birds. Group 5 was maintained as control and was given normal saline. After performing passive immunization, all birds were examined for ten days for any mortality or recovery.

Statistical analysis

All the data for the experiment was analyzed using the Sigma Stat (USA). Significant differences among different treatments were analyzed with one-way ANOVA and Student-Newman-Keul's Test. The statistical model was completely randomized design with six replicates. The differences were considered significant where $p < 0.001$ or 0.05.

Results

ELISA titers in serum and yolk

ELISA titers of IBD antibodies in serum after 7 and 28 days of hyperimmunization demonstrated that in T_1, titer was 2,815 ± 62 and 10,398 ± 54, in T_2 3,247 ± 20 and 12,100 ± 27, in T_3 4,214 ± 56 and 15,591 ± 44, and in T_4 3,314 ± 18 and 12,448 ± 68, respectively. In contrast in case of yolk after 7 and 28 days of hyperimmunization; in group T_1 titers were 5,325 ± 12 and 14,009 ± 45, in T_2 6,143 ± 52 and 15,768 ± 60, in T_3 8,186 ± 15 and 18637 ± 13, in T_4 6,201 ± 75 and 14,552 ± 49, respectively.

Overall comparison within treatment groups of serum at 7 and 28 days using one-way ANOVA showed that the values were significantly different ($p < 0.001$) but pair-wise comparison within treatment groups with Student-Newman-Keul's Test

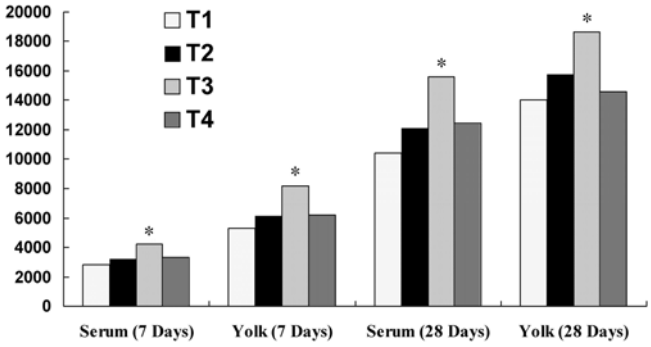

Fig. 1. ELISA titers of IBD antibodies in serum and yolk after 7 and 28 days of hyperimmunization. n = 6, * = $p < 0.05$.

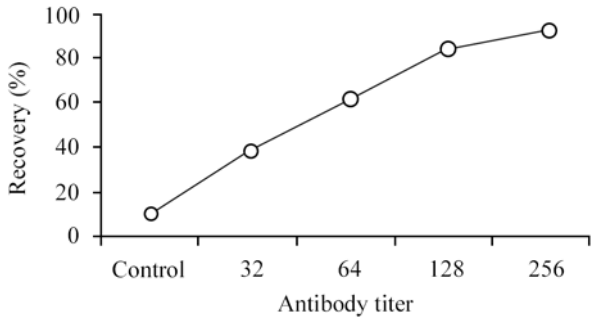

Fig. 2. Recovery rate of IBD antibodies infected birds.

showed that difference was statistically significant at $p < 0.05$ for groups other than T_2 and T_4. In case of yolk the difference was statistically significant ($p < 0.001$) within treatment groups but pair-wise comparison showed that the difference was statistically significant at $p < 0.05$, for groups other than T_2 and T_4 at 7 days and T_1 and T_4 at 28 days (Fig. 1).

Effect of different temperatures and lyophilization

For long term preservation and use of antibodies its proper storage is necessary. Different storage methods were employed and the efficacy of antibodies was checked using AGPT method. Purified antibodies were stored at room temperature (24°C), at 4°C and –20°C. Antibodies were lyophilized and stored at 4°C. AGPT was conducted after 0, 7, 14, 28, 42, 56, 70 and 90 days. It was observed that antibodies stored at room temperature lost their precipitating ability after 7 days, antibodies stored at 4°C showed positive AGPT until 56 days while antibodies stored at –20°C showed precipitating ability until 70 days. Lyophilized antibodies did not lose their precipitating ability until 90 days. These results revealed that lyophilization was most effective and efficient mean of storage for antibodies (Table 1).

Trials of purified antibodies in infected birds

A total dose of 1ml containing different AGPT units was injected. AGPT units of 256, 128, 64 and 32 were given to groups 1, 2, 3 and 4, respectively. Maximum recovery of 92% was shown by group 1. Percent recovery of 84%, 60%, and 38% was shown by groups 2, 3 and 4, respectively. Saline treated birds showed only 10% recovery (Fig. 2). Recovery state was judged by absence of dull or depressive behavior, active foraging and pecking and absence of greenish or whitish diarrhea.

Discussion

In a developing country like Pakistan the availability of antibodies for treatment of chicken infected with IBD virus is a major problem. The present investigation was therefore carried out to raise specific polyclonal hyper-immune antibodies against IBD. Our results show that antibody titers were significantly higher in yolk as compared to serum at both 7 and 28 days. These results are similar to Kuhlmann *et al.* [7] who showed that IgY produced by hen was 18 times higher than IgG produced in a rabbit. Moreover, chickens produce antibodies against highly conservative mammalian proteins too and the amount of antigen needed for immune response is very low. Furthermore, collection and storage of eggs are non-invasive and inexpensive [8].

Antibody titers produced in treatment regimen T_3 that contained a higher dose of antigen were greater as compared to other regimens. Thus treatment regimen T_3 was found to be an effective and efficient means of antibody production. This high level of antibody titer could have been due to consistent presence of antigen in the subcutaneous tissue of birds. It is therefore highly likely that the antigen continuously activated the immunocompetent cells over a long period of time. These results are in agreement with those of Tang *et al.* [13] who raised antibodies in parent flock and a similar high level of antibodies were obtained in egg yolk. The extraction

Table 1. Effect of temperatures and lyophilization on agar gel precipitation test (AGPT) titers of infectious bursal disease (IBD) antibodies

Storage temperature & lyophilization	AGPT titers of IBD antibodies							
	0	7	14	28	42	56	70	90 days
24°C	+	+	-	-	-	-	-	-
4°C	+	+	+	+	+	+	-	-
–20°C	+	+	+	+	+	+	+	-
Lyophilized antibodies	+	+	+	+	+	+	+	+

of IgY from egg yolk using dextran sulphate employed presently was simple with high yield. Our results are in line and supported by a study conducted by Fischer *et al.* [4] who compared eight extraction methods of IgY and demonstrated that dextran sulphate method was most effective, quick and simple for antibody purification. For long term storage, lyophilization proved a better means of antibody preservation which is analogous to Koko *et al.* [6].

Presently, testing of antibody on IBDV infected birds showed maximum recovery of 92% at 256 AGPT units. Antibody titer is related to viral pressure in a particular area or farm and accordingly high antibody titers are required to cope with high pressure of virus. For instance in Pakistan, the incidence of IBDV is enormously higher than other countries i.e. 5~20% and mortality is usually very high in Pakistan which is 25~100% in young broiler chicks [10]. In fact birds or flock need an optimal titer of IBD antibodies to remain prevented against the disease, which if drops birds are at increased risk of viral attack. It is implicated here that the birds can be cured better if provided with IBD antibodies equivalent to the difference of decreased antibody titers and preventive titers. The titers injected during present investigation were roughly equivalent to that difference and as such birds exhibited maximum recovery. As to why the remaining 8% birds did not exhibit any recovery was quite possibly due to a highly decreased level of their own antibody titers such that the amount of antibodies injected to sick birds remained unable to cure them. Yushen *et al.* [15] and several other investigators have used egg yolk as a source of antibodies for the control of IBD through passive immunization. However, to the best of our knowledge ours is the first report from Pakistan where purified antibodies against IBDV were used as a therapeutic agent.

In conclusion the study demonstrates that hyper-immunized yolk can be raised to purify antibodies which can then be used to control IBD infected commercial layers, commercial broilers, broiler breeders and indigenous birds.

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