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Egg immunoglobulins - an alternative source of antibody for diagnosis of infectious bursal disease

Shanmugasamy Malmarugan[1]*, Muthusamy Raman[2], Sreenivasan Jaisree[3], and Palanisamy Elanthalir[3]

[1]*Department of Veterinary Microbiology, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Namakkal, Tamil Nadu, India*

[2]*Department of Veterinary Parasitology, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Namakkal, India*

[3]*Final Year B.V. Sc Students, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Namakkal, India*

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ABSTRACT

The current trends for pursuing research programmes warrant abundant, non-invasive experimentation techniques avoiding the use of laboratory animals have led to a search for alternate sources of commercial antibodies for various immunodiagnostic and prophylactic assays. The present experiment exploited the diagnostic property of egg immunoglobulins (IgY) against infectious bursal disease (IBD) as a novel, alternative approach. The anti-IBD -IgY was harvested by the water dilution method, purified by salt precipitation, characterized by SDS - PAGE and assessed for its purity by immunoelectrophoresis. An average 200 - 250 mg. of immunoglobulins were harvested from an egg with 57-60% recovery of specific immunoglobulins. The anti - IBD-IgY thus harvested was found to possess immunodiagnostic potency as assessed by agar gel precipitation test and counter immunoelectrophoresis for replacing the use of conventional antibody.

Key words: egg immunoglobulins, diagnostic property, infectious bursal disease, chicken

Introduction

Current diagnostic assays for viral infections utilize monoclonal and polyclonal antibodies obtained from laboratory animals using highly invasive and fastidious procedure.

* Contact address:
Dr. S. Malmarugan, Assistant Professor, Department of Veterinary Microbiology, Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Namakkal-637 001, India, Phone: +91 4286 266 374/ 266 491; E-mail: micromals@rediffmail.com

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Since the modern scientific community insists on reduced, refined or replacement of laboratory animals, there is a need for alternative source of antibodies that could be produced without distress to the animals (LEE et al., 2002). During the last decade, avian egg yolk has received much attention due to its potential polyclonal antibody source, possessing several advantages over conventional mammalian antibodies due to its low cost, abundance and non-invasive qualities (LARSSON et al., 1992; SRIRAM and YOGEESWARAN, 1999). Overruling the conventional bleeding procedure the egg immunoglobulins have a long shelf life and no risk of interference by Fc receptors (LOSCH et al., 1986; LARRSON et al., 1993). The preliminary studies in immunoprophylaxis against IBD (ETTERRADOSSI et al., 1997) and diagnosis of viral pathogens such as rabies (THANGAMEENAKSHI and MICHAEL, 2002) using the egg immunoglobulins revealed its vast potential of IgY as a viable alternative source of antibody. In the present study an attempt has been made for production and purification of anti-IBD-IgY as an alternative source for conventional antibody for the diagnosis of IBD.

Materials and methods

Experimental birds. Six 18-week-old, single-comb white leghorn chickens (BV 300) purchased from a registered breeder farm in Namakkal, and with good nutritional and known vaccination status, were used for production of anti-IBD-IgY. The birds were maintained in an intensive management system using elevated individual cages and were fed with standard layer mash and feed supplements.

Antigen. Live freeze-dried culture of the non-pathogenic strain D78 of IBD-virus containing at least 10^4 PFU per dose was used as antigen for production of anti-IBD-IgY.

Inoculum. The inoculum suspension was prepared by mixing one ml. of antigen containing 10^4 PFU per dose with equal volume of Freund's adjuvant (Gibco Life Technologies, BRL, USA) using a three-way adapter and administered for six birds intramuscularly at multiple sites in thigh and breast muscles.

Experimental design. The method adapted by SRIRAM and YOGEESWARAN (1999) was followed with slight modification to the immunization regimen. The primary injection was given on day 0 and followed by a booster dose on day 14 using Freund's incomplete adjuvant and on day 21 without adjuvant. The birds were bled a week after the last dose was given to assess the development of antibody against IBD and were examined using agar gel precipitation test. Following positive reaction, the eggs were collected daily for 60 days and stored at 4 ^{0}C until further processing. Hyper-immune serum was also collected from the immunized birds for three consecutive weeks as conventional antibody source to compare the diagnostic potential of IgY.

Harvesting of IgY. The anti-IBD-IgY was separated from eggs as per the water dilution method described by AKITA and NAKAI (1992) with the following modifications.

Egg yolk free of egg white obtained from pre-warmed refrigerated eggs was rinsed in triple distilled water and rolled in tissue paper for complete removal of the albumen. After several such washes, the yolk membrane was punctured, allowing the yolk to flow into a graduated measuring cylinder holding the membrane.

The yolk was diluted by adding 9 volumes of pre-cooled triple glass distilled water and the pH was adjusted to 5-5.2 with 1M HCl and incubated at 4 ^{0}C for 6 to 8 hrs. Following incubation the supernatant was harvested and centrifuged at 3000 x g for 25 min in a refrigerated centrifuge (Remi, C-24, India). The resulting immunoglobulins (supernatant) containing filtrates (water-soluble fraction) were collected and estimated for protein concentration by the Modified Biuret and Dumas method (DUMAS, 1971).

Purification of IgY . The IgY containing water-soluble fractions were purified by the salt precipitation method by titrating against 33% ammonium sulphate solution as described by HANSEN et al. (1998) in three cycles. The precipitate from the last cycle containing IgY was dissolved in 0.1M PBS, pH 7.2, dialysed against the same buffer until ammonium sulphate was completely removed. The Protein concentration of the final suspension containing purified immunoglobulins was estimated using the Modified Biuret and Dumas method (DUMAS, 1971).

Purity of the immunoglobulin harvested from egg yolk was tested using immunoelectrophoresis (GRABER and WILLIAMS, 1953) against normal and anti-chicken sera.

Characterization of IgY. The partially purified immunoglobulin was characterized by SDS-PAGE on a 1.5 mm. large gel (80 mm. x 50 mm.) using a discontinuous system in a vertical slab gel electrophoresis with 10% separating gel and 5% stacking gel (LAEMMLI, 1970) keeping IgY (Genei, India, Bangalore) as a reference immunoglobulin.

Diagnostic evaluation of IgY. The diagnostic potential of the immunoglobulin harvested in the present study was assessed using Agar Gel Precipitation Test (AGPT) and Counter Immuno Electrophoresis (CIE).

Agar gel precipitation test . The method described by OUCHTERLONY (1958) was followed, with slight modifications.

Four ml. of 1% agarose solution (with 8% NaCl) was poured onto a clean glass slide and allowed to solidify. The wells were made as one central well and two peripheral wells of diameter 4 mm., with 2 mm. interface. For testing the efficacy of IgY the IBDV antigen was placed in the central well and the peripheral wells were filled with purified IgY and hyper-immune serum. The slide was kept at 37 ^{0}C in a humid chamber for 24 hrs. The slide was then viewed for the presence of precipitation lines.

Counter immuno electrophoresis. The method described by SOMVANSHI et al. (1985) was followed to test the efficacy of purified anti-IBD-IgY with hyper-immune serum.

Results

In the present study the sera collected on days 14, 21, 28 from immunized hens were tested against IBD antigen for the development of humoral immune response using AGPT. Following the positive reaction the eggs were collected from day 30 until the end of observation (day 60) and stored at a refrigerated temperature until further analysis.

The protein concentration of the water-soluble fraction following the water dilution method, assessed by the biuret method, was in the range of 35-40 mg/ml. The biuret

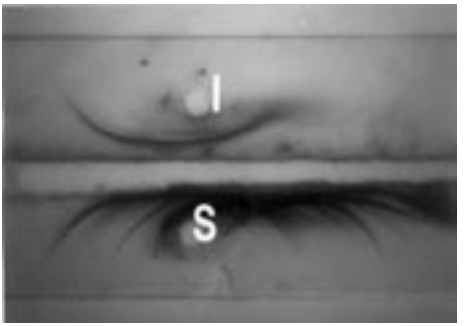

Fig. 1. Immunoelectrophoresis revealing a single, sharp precipitin arc of IgY (I) and many precipitin arcs of serum (S) against anti-chicken serum

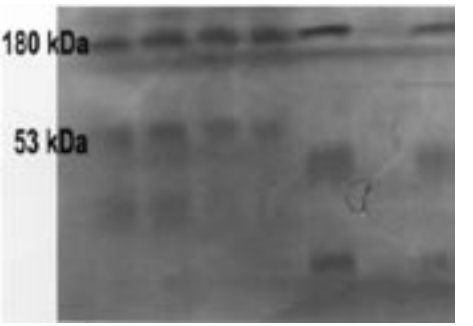

Fig. 2. SDS-PAGE analysis of anti-IBD-IgY revealing two protein bands at 180 kDa and 53kDa

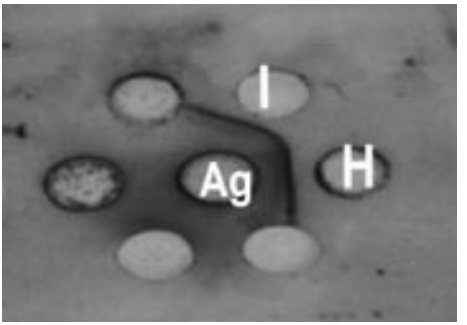

Fig. 3. Agar gel precipitation test revealing a sharp precipitin line of IgY(I) and hyper-immune serum (H) against IBD antigen

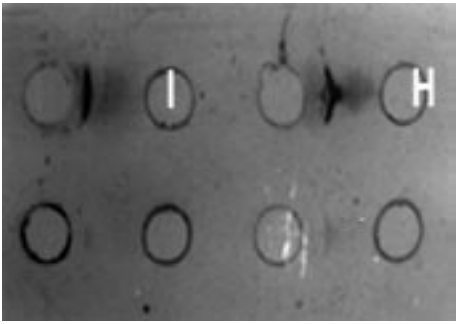

Fig. 4. Counter-immuno-electrophoresis revealing a sharp precipitin line of IgY(I) and hyper-immune serum (H) against IBD antigen

estimation of 60% ammonium sulphate precipitated anti-IBD-IgY harvested from the egg yolk was in the range of 20-25 mg/ml, indicating a moderately higher recovery of IgY (57-60%) from the egg yolk.

The purity of the egg immunoglobulins harvested from egg yolk tested by immunoelectrophoresis revealed one sharp precipitin arc between the antigen well and antigen trough extending towards the anode. Two additional arcs were also visible as being very faintly parallel to the first sharp line (Fig.1).

The protein profile of the IgY using SDS-PAGE revealed two bands at 180 kDa and 53 kDa (Fig. 2).

The IgY, evaluated for its comparative efficacy with conventional antibody (serum) for its diagnostic value against IBD, showed very similar precipitin lines in AGPT (Fig. 3) and CIE (Fig. 4).

Discussion

In the present study birds immunized during 16-18 wks of age before the start of lay responded well and produced immunoglobulins as suggested by ANON (1996). As indicated otherwise, the stress induced by handling them after the 20th week (start of lay) could have an adverse effect on egg and immunoglobulin production (SRIRAM and YOGEESWARAN, 1999).

Collection of immunized eggs from day 30 after the first injection was compared with concurrent antibody titre as observed in AGPT against hyper-immune serum. JENSENSIUS et al. (1981) similarly reported collection of immunized eggs containing specific antibodies from day 35 of primary injection that remained higher for approximately one to six months.

The protein concentration of water-soluble fraction obtained using the water dilution method was higher than that reported by LEE et al. (2002). However, the findings were in complete conformity with AKITA and NAKAI (1993) and SRIRAM and YOGEESWARAN (1999) who obtained equal protein concentration in water soluble fraction against enteropathogenic *Escherichia coli* and Asialo GM1 antigens, respectively. This variation in the recovery of IgY in the water soluble fraction might be due to the dilution factor, pH, variations in incubation temperature and time that would affect the settling down of lipids and thereby affecting the recovery of immunoglobulins in the water soluble fraction (AKITA and NAKAI, 1992)

The recovery of IgY (62%) from the water soluble fraction obtained using the salt precipitation method (60%) was found to be higher than that of SVENDSEN et al. (1995) and HARIKOSHI et al. (1993) using 60% ethanol precipitation and 60% ammonium sulphate precipitation, respectively.

It may be presumed that the fast precipitation and solubilization of lipoprotein caused by addition of 60% ammonium sulphate might have led to minimal lipid contamination with higher recovery of IgY from the water soluble fraction, indicating the superiority of the salt precipitation method (SRIRAM and YOGEESWARAN, 1999) over the several earlier methods of purification (ABBASSI et al., 1999).

In the present study the reported recovery of 20-25 mg of IgY/ml of yolk was much higher than the conventional antibody obtained from hyper-immune serum. An average 200 mg. of antibodies containing 5% of antigen specific antibodies can be collected per month through 40 ml. of blood collected over a period of one month in the conventional antibody production following invasive methods, while, 1500 mg. of antibody containing 2-10% of antigen specific antibodies can be easily harvested from 5-7 eggs per week through non-invasive methods (ANON., 1996) indicating egg immunoglobulins as a viable alternative source of antibody production.

Immunoelectrophorogram of egg immunoglobulins indicated the purity of antibodies (IgY) as anti-chicken serum recognizes only two arcs against IgY compared to many arcs against normal chicken sera.

The protein profile analysis of immunoglobulins using SDS-PAGE confirmed that the antibody isolated from the egg yolk was electrophoretically pure and specific, coinciding with the standard IgY (CHEN et al., 1999)

Immuno-reactive assays such as AGPT and CIE conducted in the study have shown similarly sharp precipitin lines for both IgY and hyper-immune serum raised against IBD. Since the egg immunoglobulins are abundant, non invasive, pure and viable replacement for conventional antibody, it could be an alternative for use in routine diagnostic assays, either in primary binding assays such as ELISA (THANGAMEENAKSHI and MICHAEL, 2002), immunoperoxidase test (LEE et al., 2002) and secondary binding assays.

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SAŽETAK

Sadašnje smjernice u provođenju istraživačkih projekata zahtijevaju neinvazivne tehnike u provođenju pokusa, izbjegavanje korištenja laboratorijskih životinja te pronalazak alternativnih izvora komercijalnih protutijela za različite imunodijagnostičke i profilaktičke postupke. Ovaj pokus temeljio se na dijagnostičkim osobinama imunoglobulina jajeta (IgY) protiv zarazne bolesti burze (ZBB) kao novom alternativnom pristupu. Anti-ZBB-IgY dobiveni su metodom razrjeđivanja vodom, pročišćeni pomoću precipitacije solima, obilježeni pomoću SDS-PAGE i pretraženi na čistoću imunoelektroforezom. Prosječna količina od 200-250 mg imunoglobulina dobivenih po jednom jajetu sadržavala je 57-60% specifičnih imunoglobulina. Na ovaj način dobiveni anti-ZBB-IgY posjeduju imunodijagnostičku sposobnost i mogu zamjeniti konvencionalna protutijela, što je i potvrđeno GDP testom i imunoelektroforezom.

Ključne riječi: imunoglobulini jajeta, dijagnostičko svojstvo, zarazna bolest burze, pilići