Characterization of IgY antibodies, developed in hens, directed against camel immunoglobulins

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Abstract: **Chicken egg yolk antibodies (IgY) against camel immunoglobulins were generated and their specificity was shown by double immunodiffusion, immunoelectrophoresis, and Western blotting. Anti camel antibodies specifically react to camel antibodies in immunodiffusion and immunoelectrophoresis were demonstrated. Western blotting revealed that IgY antibodies recognize the heavy chain in a number of mammalian species. Anti-camel antibodies bound camel IgG1, IgG2, IgG3 subclasses like the subclasses of sheep, but no reactivity to all subclasses chains in cattle and horse antibodies were observed. Polyclonal anti-camel IgY was specifically light chain reactive. Overall, the potential of using egg yolk immunoglobulins as a convenient source of anti antibodies to camel immunoglobulins was demonstrated.**

Key words: IgY, camel, immunodiffusion, SDS-PAGE, western blotting.

Introduction

The yolk of eggs laid by immunized chickens has been recognized as an excellent source of polyclonal antibodies. Using chicken as the immunization host for producing egg yolk antibodies (IgY) instead of IgG from mammalian species brings a number of advantages: (1) the animal suffering is reduced (no bleeding), as antibodies are obtained directly from the egg and only egg collection is required upon immunization, (2) antibody isolation is fast and simple; (3) very low quantities of antigen are required to obtain high and long-lasting immunoglobulin titers in the egg yolk from immunized hens, and (4) a single egg contains as much antibodies as an average bleed from a rabbit (Davalos-Pantoja *et al.,* 2000; Schade *et al.,* 1996; Tini *et al.,* 2002).

With regard to function, there are important differences between IgY and IgG. IgY does not bind to protein A or G; an important feature of IgG that allows simple IgG isolation (Akerstrom *et al*., 1985). However, there are several procedures for equally simple IgY isolation to compensate (Akita *et al*., 1993; Schwarzkopf *et al*., 1996). Chicken egg-yolk immunoglobulins (Igs) do not interfere with mammalian IgG and they do not activate mammalian complement (Gee *et al*., 2003). Moreover, since chicken IgY does not cross-react with mammalian IgG and does not bind bacterial or mammalian Fc receptors, non-specific binding is reduced, and the need for cross-species immunoabsorptions is also eliminated (Carlander *et al.,* 2000). Using chickens for the production of anti mammals' Igs might yield a higher percentage of specific antibodies due to the phylogenetic distance between birds and mammals (Gassmann *et al.,* 1990). This makes production of antibodies against conserved Ig epitops that does not cross-react with mammalian Ig. These facts bring great advantages to the application of IgY technology in many medical areas, such as diagnostics (Cipolla *et al.,* 2001; Davalos-Pantoja *et al.,* 2001; Du Plessis *et al.,* 1999; Gross *et al*., 1996) and immune therapy (Carlander *et al.,* 2000).

Anti-camel Igs can be used in most immunological assays and diagnostic methods, such as solid phase assays, immunoelectrophoretic techniques, and affinity chromatography. The

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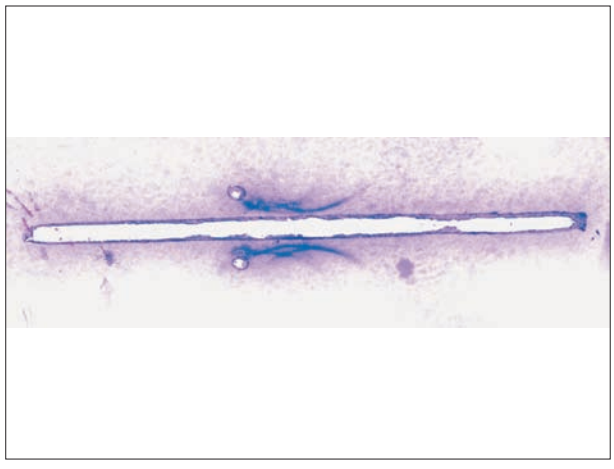

Fig. 1: Pattern on Grabar- Williams immunoelectrophoresis of camel serum. Egg yolk Anti-camel IgY immunoglobulins preparation was utilized in the trough.

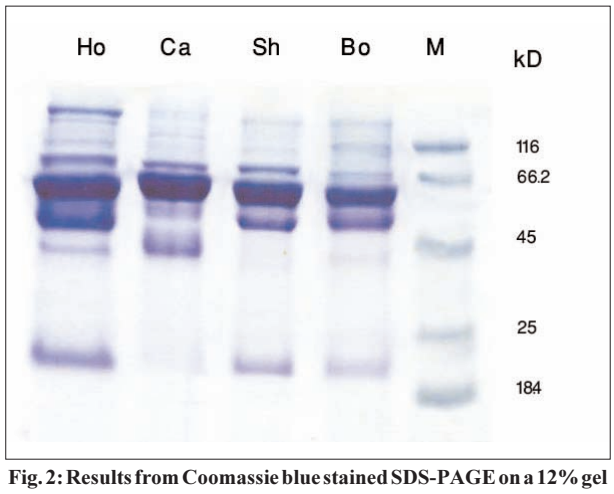

Fig. 2: Results from Coomassie blue stained SDS-PAGE on a 12% gel of horse, camel sheep and cattle normal serum under reducing condition. Lane Ho,horse serum; lane Ca, camel serum; lane Sh, sheep serum; lane Bo, cattle serum and M, protein molecular weight marker.

objectives of this study were to produce and evaluate chicken anti-camel IgG as a source of antibodies for routine diagnostic immunoassays.

Materials and Methods

Animals and Sera: 3 White Leghorn (Hi-line W-36) laying hens were used for immunization by camel immunoglobulin. Blood samples were obtained by jugular venipuncture from several camels that were brought into slaughter house. After coagulation of the blood, serum was separated by centrifugation and stored at -20°C.

Purification of Camel Immunoglobulins: Camel Immunoglobulins were purified using ammonium

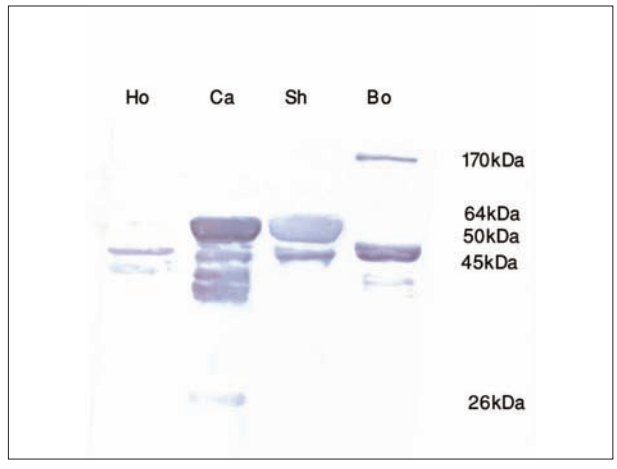

Fig. 3: Reactivities of chicken anti -camel IgY with cattle, sheep, camel and horse serum in western blotting. Lane Ho,horse serum; lane Ca, camel serum; lane Sh, sheep serum; lane Bo, cattle serum

sulfate precipitation as described by Hey and Westwood, 2002. Briefly, camel serum samples were pooled, the globulin was precipitated with 40% saturated ammonium sulfate and dialyzed exhaustively against at least three changes of PBS.

Generation of IgY Anti-camel Antibodies: Approximately 80-100 mg of camel Igs was resuspended in 500 ml of phosphate-buffered saline (PBS) and emulsified with an equal volume of complete Freund's adjuvant. The antigen-adjuvant mixture was injected into the pectoral muscle of hens at days 0, 7, 14, 21, and 28. Isolation of polyclonal chicken antibodies, collected daily from eggs, was performed by separation of the water-soluble fraction upon dilution with water at pH 5.2, followed by ammonium sulfate precipitation method (Gross *et al.,* 1996). Briefly, after separation from the egg white, the yolk was brought to 5 fold of initial volume with HCl 3mM, vigorously mixed and remained for 4h at 4 °C. Following centrifugation at 1200g for 10 min, the supernatant was decanted and chloroform (equal volume) was added. The mixture was shaken for 1-2 min and placed at 4 °C for 12h. After centrifugation at 1200g for 15 min, the supernatant was decanted and IgY was precipitated with 45% saturated ammonium sulfate. Pellet containing the IgY was resuspended in 2 ml of PBS and dialyzed against at least three changes of PBS. Antibodies stored at -20 °C until used.

Double immunodiffusion was done essentially by

the method described by Hey and Westwood, 2002. Camel, bovine, sheep, and horse serum were used in double immunodiffusion test.

Immunoelectrophoresis. For electrophoresis, an Electrophoresis Cell and 300 Power Supply (Bio-Rad Laboratories) were used. Agarose gel (0.8 %), prepared in sodium barbital buffer (.005 M, pH=8.2), was used in this experiment. Electrophoresis buffer was the same as agarose buffer. Immunoelectrophoresis experiment was done according to established procedures (17-Hey *et al*. 17). The gel was deproteinized by washing several times in 0.3 M NaCl for 18 h and visualized by staining with Coomassie blue dye.

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blots. Serums of different species including camel, cattle, sheep, and horse were resolved in discontinuous polyacrylamide gels (Ausubel *et al.,* 2002). Samples and molecular mass markers (Fermentas, Laboratories) were boiled for 3 min in sample preparation buffer, with 2-mercaptoethanol, before being loaded in the gels. Samples were loaded onto preparative SDS-12% PAGE Mini-PROTEIN 3 Cell gel (Bio-Rad) and electrophoresed in Tris-glycine buffer (0.025 M Tris base, 0.192 M glycine, 0.1% SDS) at 120 V/gel for 2 h. Gels were stained with Coomassie blue or were blotted onto the nitrocellulose membranes.

Western blotting procedures were performed at room temperature (Ausubel *et al.,* 2002). The proteins were transferred onto a PVDF membrane (0.45-mm pore size) (Roche, Laboratories) with a Mini Trans-Blot apparatus (Bio-Rad) at 50 V for 50 min. The membrane was blocked with 3% bovine serum albumin in Tris-buffered saline (0.02 M Tris base-0.385 M NaCl-0.1%) and washed with Tris-buffered saline containing 0.05% Tween 20. Primary antibodies (IgY) were diluted in blocking solution. Then, the membrane was incubated with primary antibodies for one night at 37°C and washed three times for 5 min with Tris-buffered saline containing 0.05% Tween 20. Antibody binding was detected with a goat anti-chicken horseradish peroxidase conjugate (Biocheck, Laboratories). Membranes were developed by α-chloronaphthol (Sigma), scanned, and images were prepared using Adobe Photoshop and Microsoft PowerPoint.

Results

We tested the ability of IgY and serum from immunized and unimmunized hens to bind camel Immunoglobulins by double immunodiffusion test. Immune hens IgY had anti-camel Ig reactivity comparable to that of unimmune serum, whereas IgY from unimmunized hens had no significant anti-camel Ig reactivity. In further examination by double immunodiffusion, Immune IgY had no reactivity with immunoglobulins of other species, such as cattle, sheep, and horse.

To test IgY specificity, we analyzed whether IgY antibodies were capable of recognizing different classes of immunoglobulins in immunoelectrophoresis assay using camel serum. Figure 1 shows 3 typical precipitin lines in a pattern obtained for normal camel serum by immunoelectrophoresis technique.

Camel, cattle, sheep, and horse normal serum were resolved under reducing condition by SDS-PAGE and stained with Coomassie blue (Figure 2). Camel serum was resolved into an unclear light chain (26 kDa) and 3 heavy chains including IgG1=64 kDa, IgG2=45 kDa, and IgG3=50 with respect to data from Daley *et al.,* (2005) (Fig. 2, lane Ca2). Another band of about 66.2 kDa was also developed in all the species under study. The band of 45 kDa was clearly observed in camel serum and slightly in horse and cattle serum.

To determine which immunoglobulins, belonged to different species, and their subunits are recognized by the specific IgY, we tested the ability of antibody to react with camel, cattle, sheep, and horse polypeptides separated by SDS polyacrylamide gel and blotted onto nitrocellulose paper (Figure 3). Reactivity was observed with large subunits (64, 50 and 45 kDa) and a number of small subunits, as well as a 26-KDa light chain of camel immunoglobulin (Figure 3). Chicken anti- camel IgY bound camel, cattle, sheep and horse heavy chains Igs, but except for camel did not bind to all light chain fragments of

other species (Figure 3). Anti-camel antibodies specifically bound camel IgG1, IgG2, IgG3 subclasses, but except for sheep no reactivity to 64 kDa proteins in cattle and horse antibodies were observed. Reactivity to 45 kDa just developed in camel serum. The proteins of greater molecular weights (170 kDa) were bound by IgY, suggesting the occurrence of mild uncompleted reduction during the processing of samples for SDS-PAGE.

Discussion

Camels of the Old World and the New World have provided the indigenous human population with meat, milk, fiber, and fuel, they also serve as beasts of burden to carry loads, for millennia. Camels have specific pathogens; suffer from common diseases of ruminants, and are resistant to some pathogens (Dirie *et al.,* 2003; Rickard *et al.,* 1994; Rivera *et al.,* 1987). Little is known of the immunological and functional contributions of camel immunoglobulins to immune defense. This may be due to the poor availability of anti-camel antibodies for diagnostic immunoassays.

IgY has been produced and characterized specifically for conventional immunoglobulins of camel. The IgY antibodies are readily applied in serologic assays and should be useful in quantitative assessments of immunoglobulins in blood, milk, colostrum, and other body fluids. Its application in identifying antibodies induced during protective and nonprotective immune responses to different types of pathogens will improve the understanding of immune defense in camel and should aid in the design of effective vaccines.

Immunodiffusion and immunoelectrophoresis, using different mammalian sera, showed the anti-camel IgY specificity in precipitation tests. Western blotting demonstrated that anti-camel IgY antibodies specifically react to camel light chain and could bond to heavy chains of different mammalian sera. Cattle, sheep, and horse heavy chains antibodies were detected by the anti-camel IgY, suggesting identity of epitopes in these species. These data also indicate that the antibodies are directed against different fragments of Igs under reducing conditions, but do not effectively bind to native Igs to form precipitins. Alternatively, the determination of immunoglobulin by immunodiffusion with anti-camel specific IgY was less affected by common epitopes that are present among different sources of immunoglobulin. However, Anti-camel IgY in western blotting revealed that camel immunoglobulins have more identity to sheep Igs than cattle and horse Igs with respect to data from Curtain *et al*., (1973).

Highly conserved mammalian proteins sometimes fail to elicit a humoral immune response in animals, such as rabbits, that are traditionally used for generating antibodies. Our data further confirmed that due to the phylogenetic distance between birds and mammals, there is a greater potential of producing a higher percentage of specific antibodies against mammalian antigens when using chickens (Carlander *et al.,* 1999). This makes production of antibodies against conserved mammalian proteins usually more successful in chicken than in other mammals and may be generally used for other highly conserved mammalian antigens (Gassmann *et al.,* 1990; Carroll *et al.,* 1983). In addition, anti-camel IgY antibodies tend to recognize the same protein in a number of mammalian species, making them more widely applicable.

To the best of our knowledge, this is the first report of using chicken for the production of anti-camel Igs. These antibodies might be used as a candidate for characterization of Igs from different mammalian species. For further researches, it is convenient to examine the ability of IgY to increase sensitivity and specificity of the immunoassay procedures such as techniques used for the assessment of passive immune transport and antibody response to the vaccines due to its advantages in contrast with mammalian Igs.

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ارزیابی آنتی بادی های IgY تولید شده در مرغ علیه ایمونوگلوبولین شتر

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چکیده

آنتی بادی زرده تخم مرغ (IgY) علیه ایمونوگلوبولین شتر تهیه و ویژگی آن با روش های ایمونودیفوزیون، ایمونوالکتروفورز و وسترن بلات نشان داده شد. در آزمون ایمونودیفوزیون و ایمونوالکتروفورز آنتی بادی های ضد شتر به صورت اختصاصی با آنتی بادی های شتر واکنش نشان دادند. وسترن بلات نشان داد که IgY تهیه شده، زنجیره سنگین ایمونوگلوبولین ها را در تعدادی از گونه های پستانداران تشخیص می دهد. آنتی بادی های ضد ایمونوگلوبولین شتر با تحت کلاس های IgG1,IgG2,IgG3 شتر و همین طور با تحت کلاس های گوسفند واکنش نشان دادند، اما هیچگونه واکنشی با تحت کلاس های ایمونوگلوبولین گاو و اسب مشاهده نشد. IgY پلی کلونال تهیه شده بر علیه ایمونوگلوبولین شتر به صورت اختصاصی با زنجیر سبک واکنش نشان داد. به طور کلی در این مطالعه، توانایی استفاده از ایمونوگلوبولین زرده تخم مرغ به عنوان یک منبع مناسب آنتی آنتی بادی برای ایمونوگلوبولین شتر نشان داده شده است.

واژه‌های کلیدی: هاپتوگلوبین، شیر، سرم، بیماریهای التهابی گاو.

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