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PRODUCTION OF AN IN-HOUSE RABBIT ANTI-CAMEL IMMUNOGLOBULIN G (IgG) CONJUGATED WITH HORSERADISH PEROXIDASE (HRP) FOR USE IN IMMUNOBLOTS

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ABSTRACT

The present study was aimed to develop anti-camel immunoglobulin G (IgG) conjugate. Camel serum was obtained from healthy camels and the IgG was extracted from this serum using the commercially available IgG purification kits. The electrophoretic profile of camel IgG showed four bands of molecular weight 66, 50, 44 and 33 kDa. Two rabbits were immunised with the purified camel IgG for production of rabbit anti-camel IgG. IgG fraction from serum collected from the immunised rabbits at the end of the immunisation protocol was purified using the commercially available IgG purification kits. The purified IgG tested for reactivity using ELISA against camel serum and then coupled to horseradish peroxidase. The produced conjugate then tested for reactivity using western immunoblotting against serum from camels infected with *Haemonchus longistipes* and normal camel serum. The conjugate was able to react with camel serum and was able to detect three components of *H. longistipes* of molecular weight 126, 76 and 18 kDa.

Key words: Conjugation, dromedary camels, *H. longistipes,* HRP, IgG, immunoblots

Immunoassays are the most popular techniques for diagnosis of infectious diseases and are dependent on the availability of anti-species conjugates, particularly those used for antibody detection (Aregawi and Feyissa, 2016). Different types of labeling systems are available depending on the proposed assay. Fluorochromes, such as fluorescin isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC) are generally suitable for immunohistochemistry and flow cytometry (Johnstone and Thorpe, 1982; Poojan and Kumar, 2011; Avens and Bowman, 2010). Radioisotopes such as iodine (125I) are used in radioimmunoassay (Liddell and Cryer, 1991; Zahi *et al,* 2010), while enzymes are used for increasingly popular enzyme-immunoassays (Ivey *et al,* 2011).

Enzymes have achieved a high degree of popularity in immunoassays mainly because of their high specificity for the substrate, which can be, converted to coloured products that can be either soluble or insoluble. Enzymes also have the advantage over other labels in that they are readily available, inexpensive and have high specific activity (Tijssen, 1985). The most commonly used enzymes include horseradish peroxidase (HRP), alkaline phosphatase (AP) and β-galactosidase (β-G) (Greenfield, 2014). Overall, HRP conjugates are considered to be superior to AP and ß-G conjugates due to the higher specific enzyme activity and immunological reactivity of HRP conjugates (Porstmann *et al,* 1985). The reaction products of HRP are easily detectable, while that of AP and β-G are less strongly coloured (Liddell and Cryer, 1991).

There are a number of methods used to couple HRP to immunoglobulin, however, the periodate method (Wilson and Nakane, 1978) is the most commonly used. This method is popular because it couples the antibody to HRP through the carbohydrate portion of each molecule and since this is not usually involved in the active site of the antibody and the enzyme, the method is less likely to reduce the immuno-reactivity. Immunoassays such as western immunoblotting depends mainly on the availability of anti-species conjugates. Commercial anti-species conjugates can be purchased from the market, however, anti-camel conjugates are too

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expensive in these markets. The present study was aimed to develop anti-camel immunoglobulin G (IgG) conjugate.

Materials and Methods

Parasites

Adult *Haemonchus longistipes* worms were obtained from the abomasa of naturally infected slaughtered camels at Al-Ahsa Central Abattoir, KSA following the method of Smith and Smith (1996). The examined abomasum contained only *H. longistipes* parasites identified by their barber pole appearance and long spicules (Soulsby, 1986).

Serum samples

Blood samples were collected from the jugular vein of *Haemonchus longistipes* infected and uninfected camels and from the ear vein of the immunised rabbits into glass bottles without anticoagulant for serum preparation.

Production of polyclonal antibodies to camel immunoglobulin

Two rabbits were immunised with the purified camel IgG using an immunisation regime adapted from Greenfield (2014). Each rabbit was injected intramuscularly with 100µg antigen emulsified in FCA on day 0 and on day 14. Two booster doses of 50µg antigen in FIA were administered to each rabbit subcutaneously on day 49 and 77 and a final boost of 25 µg of antigen in FIA on day 196. Serum was collected from these rabbits on days -1, 24, 56, 84, 203 and 210.

Monitoring antibody production

An antibody-ELISA was used to measure the amount of antibody in the sera collected from the immunised rabbits on days 24, 56, 84 and 203, post primary injection of the immunogen and the titre of the final serum collected on day 210. Each serum was tested against the purified camel IgG at a dilution of 1/100 as antigen. Sera were evaluated over a 2-fold dilution range from 1/250 – 1/8000 for the sera collected 24-203 days and over a 2-fold dilution range from 1/500-1/1024000 for the final serum collected at day 210. In each case, pre-immunisation serum at a similar dilution range was included as a negative control. All serum samples were tested in duplicates and the assay performed using a peroxidase labelled anti-rabbit IgG conjugate. Serum samples showing an absorbance value greater than two standard deviations (2xSDEV) above the mean of the negative control were considered positive. The antibody titre of the final serum was taken as the last dilution that continued to show an absorbance of more than 2xSDEV above the mean of the negative control.

Purification of IgG antibodies from serum

The IgG from camel serum and serum raised in rabbits against camel IgG was purified using the commercially available ImmunoPureR IgG kit (Pierce Chemical Company, USA). The method utilising 1ml columns of immobilised protein A covalently coupled to 6%, crosslinked, beaded agarose (Protein A affinityPakTM columns). Fractions eluted from these columns were dialysed against PBS. The concentration of the purified camel IgG is measured using the commercially available BCA kit. Antibody reactivity of dialysed rabbits IgG fractions was tested against the purified camel IgG by antibody-ELISA. The purified camel IgG was used at 1/100 dilution. Rabbit IgG preparations were tested at an eight 2-fold dilutions from 1/100 to 1/12800. Normal rabbit serum at similar dilution series was also included in the test as a negative control. IgG preparations dilution showing an absorbance value greater then 2xSDEV above the mean of the similar dilution of the negative control was considered positive.

Detection of purified camel IgG by SDS gel electrophoresis (SDS-PAGE)

Purified IgG samples were electrophoresed on 7-20% gradient polyacrylamide gels as described by Laemmli (1970). Protein banding pattern on the gels was visualised by Coomassie Blue stain and their molecular weight were determined and compared to pre-stained protein ladder of molecular weights ranged from 10-200 kDa (Cell Signaling Danvers, MA, USA).

Labeling IgG antibodies with horseradish peroxidase

Before labeling, the selected IgG fractions were dialysed with slow stirring overnight against several changes of distilled water at 4°C and freeze-dried overnight using a freeze-dryer (LABCONCO, USA). These lyophilised IgG fractions were then conjugated to HRP (EC 1.11.1.7; CAS RN 9003-99-0; Sigma-Aldrich, Inc. Missouri, USA) using the modified periodate method (Wilson and Nakane, 1978). The resulting IgG-HRP conjugate was dialysed overnight against PBS at 4°C. Prior to storage an equal volume of glycerol was added to the conjugate which was then stored at -20°C until needed.

Reactivity of labeled IgG antibodies in immunoblotting

H. longistipes crude soluble extract was stored frozen in SDS-sample buffer and heated

immediately before use to 100°C for 3 minutes to re-dissolve any crystallised SDS. Cooled samples were then electrophoresed on 7-20% gradient polyacrylamide gels as described by Laemmli (1970). After electrophoresis, Western blots were prepared by electro-blotting separated antigens in unstained gels onto nitrocellulose paper according to the method described by Towbin *et al* (1979) and Burnette (1981) using a Trans-Blot SD semi-dry transfer cell (Bio-Rad, USA). Antigens were detected by western immunoblotting against serum from camels infected with *H. longistipes* and normal camel serum as a negative control. The unbound sites on the nitrocellulose membranes were blocked overnight with 5% dried milk in blocking buffer (50mM Tris, 150mM sodium chloride, 1mM EDTA, 0.05% NP40, 0.25% gelatin, 0.02% thimerosal, pH 7.4) prior to each incubation. The blocked membrane was rinsed with PBS, cut into strips and incubated overnight with the above sera diluted 1/50 in blocking buffer. The incubation was carried out at room temperature. The unbound antibodies were then removed by washing the membranes with 7 changes of PBS over 2 hours, before the produced peroxidase-labelled, rabbit anti-camel IgG diluted to 1/200 in blocking buffer was added and incubated for 2 hrs. After washing the membranes in PBS as above to remove excess conjugates, the labeled antigen/antibody complexes were visualised by incubation in substrate solution (4-chloro-1-α-naphthol 0.5 mg/ml in 20 mM Tris, 500 mM sodium chloride, pH 7.5, Sigma Ltd., UK) with hydrogen peroxide (0.06%) as the substrate. Colour development was allowed to occur for 30 minutes and the reaction was stopped by washing in distilled water and permanent records made by photography.

Results

SDS gel electrophoresis (SDS-PAGE) profile of purified camel IgG

The electrophoretic profile of camel IgG showed four bands of molecular weight 66, 50, 44 and 33 kDa (Fig 1).

Monitoring antibody response in immunised rabbits

Serum collected from the immunised rabbits on days 24, 56, 84 and 203 following primary injection of the antigen showed a progressive increase in absorbance values when tested by ELISA against purified camel IgG (Fig 1). At a serum dilution of 1/250 approximately 4-fold increase in the absorbance value from day 24 to day 203-post primary injection was observed. The anti-serum showed an absorbance value of 0.624 at day 24 which was increased to 2.626 at day 203 (Fig 2).

The antibody titre of the final serum collected on day 210 post immunisation that showed an absorbance value (0.423) greater than 2xSDEV (0.184) above the mean of negative control serum (0.229) was 1/8000 as defined by antibody ELISA against purified camel IgG (Fig 3).

Purification of IgG

Purified camel IgG antibody concentration eluted from protein A affinity column was 3.6 mg/ml when measured by QuantiProTM BCA Assay kit (Sigma Chemical Company, UK).

Reactivity of anti-camel IgG raised in rabbits

The ELISA absorbance values of the dialysed rabbit IgG decreased with increasing dilutions of the antibody when titrated against purified camel IgG. An absorbance of 3.189 at a dilution of 1/100 was seen in this IgG, which decreased to 0.708 at a dilution of 1/3200 (Fig 4) but still more than 2xSDEV above the mean of the normal rabbit serum.

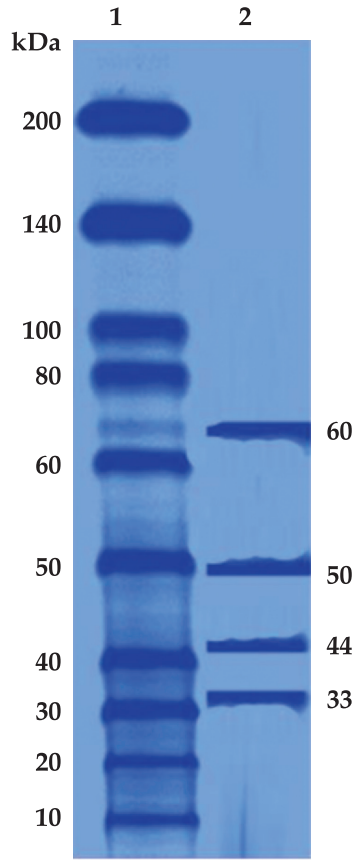

Fig 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis profile of camel immunoglobulin G. Lane 1: Molecular weight marker, Lane 2: Camel immunoglobulin G.

Reactivity of HRP-conjugated IgG by immunoblotting

The conjugated IgG was able to detect three antigenic components in *H. longistipes* soluble extract with molecular weight of 126kDa, 76kDa and 18kDa when tested against serum collected from the naturally infected camel (Fig 5, lane 2). Pre-stained protein ladder of molecular weights ranged from 10-200 kDa was used in the current study (Cell Signaling Danvers, MA, USA).

Discussion

Polyclonal antibodies in hyper-immune serum raised to a single antigen usually contains high proportion of high affinity antibodies with high ability to bind antigen (Greenfield, 2014). Such high affinity and high sensitivity of polyclonal antibodies makes them better reagents in immunoassays. However, the successful production of polyclonal antibodies requires the availability of a highly purified antigen so that the antibody produced will be able to detect the target antigen in complex mixtures. The present study was aimed at producing antiserum to camel IgG and eventually produced anti-camel IgG conjugate to detect *Haemonchus longistipes* antigenic components in infected camel sera by immunoblotting.

A number of IgG purification methods are available of which affinity chromatography with protein A sepharose is the most popular since it produces high yields of pure antibody (Greenfield, 2014). The method is simple, highly efficient (Chan, 1996) and a well-established mean for purification of IgG molecules (Tijssen, 1985; Greenfield, 2014). Although IgM antibodies from some species have been reported to bind to protein A (Tijssen, 1985), the conditions used in the present study, however, were likely to produce predominantly IgG antibodies particularly the high salt buffer conditions under which over 80% of IgM molecules are reported to be removed from the column with washing buffer (Tijssen, 1985). Electrophoretic profile of camel IgG carried out in the present study revealed four bands of molecular weight of 66, 50, 44 and 33 kDa. Similarly, previous workers using 12% homogenous gels under reducing conditions visualised four bands; two bands at 50 and 30 kDa in case of IgG1 while IgG2 and IgG3 produce only one band at 46 and 43

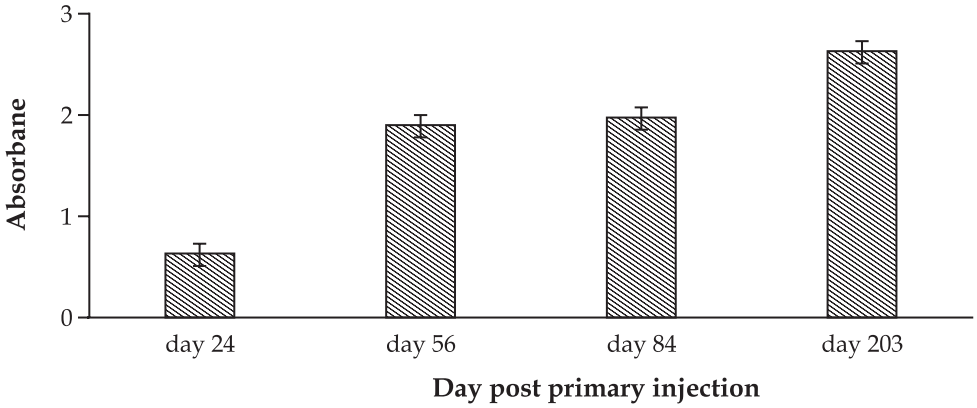

Fig 2. Monitoring antibody response in immunised rabbits

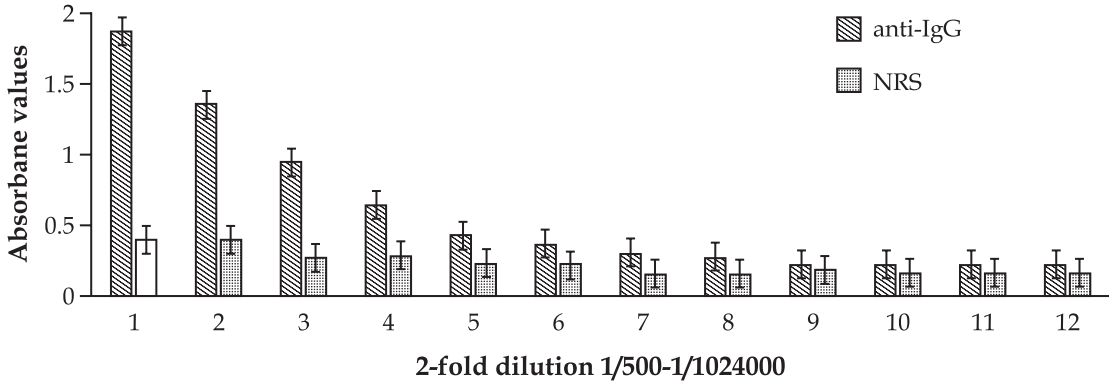

Fig 3. Antibody titre in the final serum collected on day 210 post-primary injection of camel IgG. 1-12 ≡ 2-fold dilution range from 1/500-1/1024000.

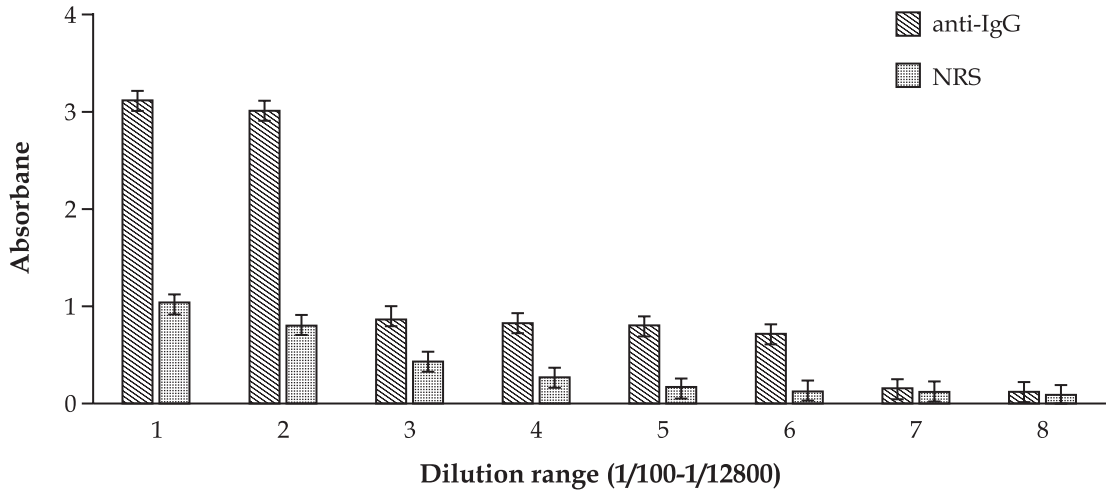

Fig 4. Reactivity of the dialysed rabbit IgG

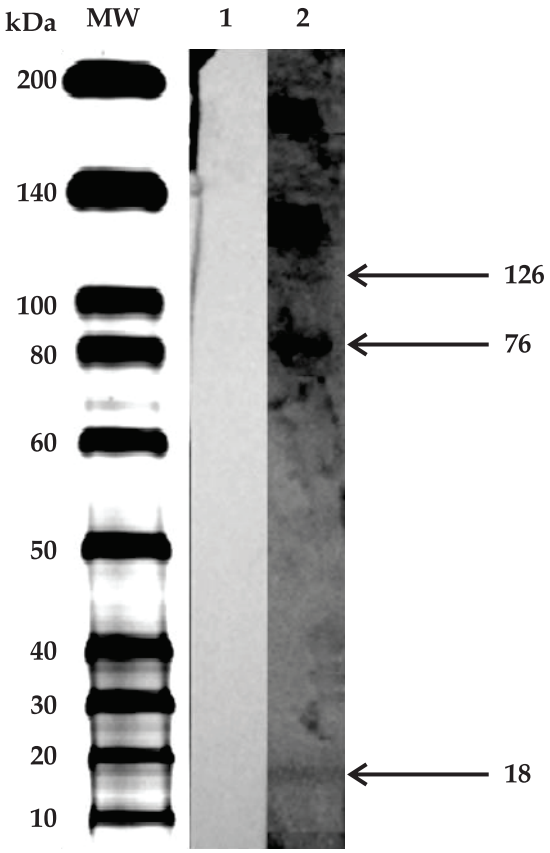

Fig 5. *H. longistipes* proteins identified by rabbit anti-camel IgG in immunoblotting (Lane 1 normal camel serum, lane 2 serum from camel infected with *H. longistipes*).

kDa, respectively (Blanc *et al*, 2008; Mohammadian *et al*, 2010; Shaker and Melake, 2012). Daley *et al* (2005) detected one light chain (22.7 kDa) and one heavy chain (49.4 kDa) for IgG1 and only one dominant heavy chain species of 42.1 kDa for IgG3 and IgG2. While, Abd El Hafez *et al* (2010) using 12% homogenous gels visualised three bands of 52, 46 and 30 kDa. Abdel-Rahman *et al* (2017) detected using 10% homogenous gels detected four bands of 63, 52, 46 and 33 kDa. The camel IgG as 2 bands with molecular masses of 150 and 75 kDa presented by (Khamehchian *et al*, 2014). These differences in bands numbers probably attributed to differences in sample preparation and the type of gels used.

Rabbits are widely used for production of specific antibodies and immunisation of these animals with camel IgG in the present study elicited strong antibody response when tested by antibody-ELISA. An increase in total globulin reaching 4.7 times the amount of globulin at day -1 (pre-immunisation serum) was observed in these animals confirming the usefulness of these animals in production of antibodies.

Immunoassays usually require purified antibodies as the efficiency of these assays is directly dependent on the purity of the antibody (Tijssen, 1985). Enrichment of antibodies is a necessary step achieved by purification and consequently the amount of enzyme needed to conjugate the antibody will be reduced. Anti-camel antibodies produced in the present study were successfully separated from rabbit serum using affinity chromatography. The column eluate in the present study contained the desired antibody as judged by its ability to recognise the camel IgG in ELISA.

The purified IgG antibodies were successfully, labelled with horseradish peroxidase using the modified periodate method (Wilson and Nakane, 1978). This is a well-established method for conjugation of the enzyme, which preserves antibody reactivity by coupling to the carbohydrate portion of the enzyme and antibody molecule, which is not

usually involved in the antigen binding (Liddell and Cryer, 1991). Conjugate produced by this method usually have higher activity than those produced by the two-step glutaraldehyde method with Nygren (1982) reporting five-fold higher detectability in spot-ELISA with periodate conjugates compared with those produced with the two-step glutaraldehyde method.

The IgG after conjugation to horseradish peroxidase retained its activity and was used in western immunoblotting to detect antigenic components of *H. longistipes* in serum of infected camels. The conjugate successfully detected three antigenic components of this parasite in camel sera. The low number of the parasite antigenic components detected in the present study could be attributed to the low number of antigens released during infection and representing excretory-secretory (ES) antigens. ES antigens of similar molecular weights were reported in *H. contortus* (Joshi and Singh, 1999; Bakker *et al,* 2004; Prasad *et al,* 2008) and in other helminth parasites (de Savigny, 1975; Ishiyama *et al,* 2009; Schnyder *et al,* 2011).

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