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Research Article

Isolation and Characterization of Antichloramphenicol Antibodies using SDS Page

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Background: Antichloramphenicol antibodies can be produced in small or large animals depending on the requirement of the researcher. Previously most researchers have raised antibodies in small animals such as rabbits due to their easy availability and handling. In the present study antichloramphenicol antibodies were produced in large animals because large volumes of serum was needed for various studies.

Objective: The objective of the present study was to isolate and characterize antichloramphenicol antibodies produced in camels, donkeys and goats for development of a CAP Enzyme Linked Immunosorbent Assay.

Methods: The methods employed were SDS-PAGE electrophoresis which involved the analysis of crude and purified goat, camel and donkey antichloramphenicol antibodies. Purification of the antichloramphenicol antibodies was carried out by precipitation using ammonium sulphate. Immunization of experimental animals was carried out using standard immunological methods.

Results: The results indicated that the crude anti-CAP antibody produced in camels, goats and donkeys showed 7 protein bands of molecular sizes 11.7, 40, 61.6, 134.3, 145, 169.5 and 182 kda. However the protein band of molecular weight 11.7 kda was not observed in the purified antibody from the 3 animal species. The protein bands of the camel appeared smaller and were more distinct as compared to those of donkeys and goats.

Conclusion: From this study it was concluded that purified camel antibodies are smaller and more specific followed closely by goat antibodies and donkey antibodies.

Keywords: anti-chloramphenicol (CAP) antibodies, camels, goats and donkeys

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1. Introduction

Antibodies have been produced in various animal species for use in research. Camel antibodies have been used in the development of assays such as prostate specific assay (Zubritsky 2005) caffeine assays (Landenson et al, 2006) and chloramphenicol Enzyme Linked Immunosorbent Assay (Wesongah et al, 2007). Antibodies produced in rabbits are popular because they are easy to produce and also these animals are easily available worldwide. These animals are also easy to breed and handle. However large animals are preferred source of antibodies in case of large scale production. Camel antibodies have been reported to be small and quite stable (Zubritsky 2005, Ladenson 2006,). In the present study we isolated and characterized antichloramphenicol antibodies produced in goats, donkeys and camels using SDS- PAGE.

2. Materials and Methods

2.1 Reagents and Chemicals

Ammonium sulphate (BDH 10033), Sodium dodecyl sulphate, Acrylamide, Ammonium persulphate, SigmaMarker, G50 Sephadex medium were obtained from Sigma (Poole, Dorset, UK).

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2.2 Experimental Animals

Two animals from each of three selected species, camels, goats and donkeys all males were purchased and housed in a barn at the Kenya Agriculture Research Institute – Trypanosomiasis Research Centre (KARI-TRC) permission was obtained from Institutional Animal Care and Use Committee (IACUC) of Trypanosomiasis Research Centre. On arrival at the KARI-TRC all the animals were eartagged. They were sprayed with a solution containing 12.5% w/v amitraz (Triatix ®Coopers (K) Ltd.) for tick control. The animals were orally given albendazole ®(Norbrook Ltd, Newry, United Kingdom) for deworming. They were left to acclimatize for three weeks before the actual treatment commenced in accordance with the Guide for the care and Use of Laboratory Animals (Institute of Laboratory Animal Resources 1996). The animals were maintained on hay, lucerne and had water *ad libitum*.

2.3 Preparation of Reagents used for antibody purification and characterization

Ammonium sulphate solution

Saturated ammonium sulphate $(NH_4)_2SO_4$ was prepared by dissolving 550g of Ammonium sulphate (BDH 10033) in 950 ml of distilled water and filtered and the pH adjusted to 7.0 with ammonium hydroxide making up to 1 litre and stored at +4°C.

G50 Sephadex medium

10g of G50 medium sephadex was dissolved in 100ml PBSEx1+NaN3 and kept at +4 °C overnight the following day the sephadex was washed three times before packing the column.

Gel for electrophoresis

Preparation of gel for electrophoresis was carried out as shown in **Table 1**.

Acrylamide Solution

28.38 g acrylamide and Bis- acrylamide 1.62 g was weighed and dissolved in 100ml distilled water, filtered and degassed.

10% Sodium dodecyl Sulphate (SDS)

Ten grams of SDS was added to 90 ml of distilled water and stirred gently to dissolve and made up to 100ml with distilled water.

Stacking gel

Stacking gel was prepared by mixing 1.5 ml of acrylamide, with 2.5 ml of 0.5m Tris buffer pH 6.8 plus 6 ml of distilled water plus 100 µl of 10% SDS then 150 µl of 10 %ammonium persulfate was added and vortexed before adding 70 µl of TEMED.

Electrophoresis buffer

Electrophoresis buffer was prepared by dissolving 3g of (0.025M) Tris, 14.4g of (0.192M) glycine, 1.0 g of (0.1 % SDS) in 1000 ml distilled water, pH 8.3.

SigmaMarker

SigmaMarker proteins were reconstituted with 100µl of deionised water and resulted in a solution containing approximately 2-3.5 mg of protein per ml of 62Mm Tris base, pH 8.0 1Mm EDTA, 4% sucrose, 0.5% dithiothreitol, 2% SDS and 0.005% bromophenol blue.

1.5 M Tris-HCL PH 8.8

1.5 M Tris-HCL PH 8.8 was prepared by weighing 27.23g of Tris base and add 80 ml of distilled water. The pH was then adjusted to pH 8.8 with 6 N HCL and the solution made upto 150 ml with distilled water then 0.4% of SDS (0.6g) is added and stored at 4°C.

0.5 M Tris-HCL PH 8.8

0.5 M Tris-HCL pH 6.8 was prepared by weighing 6.1g of Tris base and adding 80 ml of distilled water. The pH was then adjusted to pH 6.8 with 6 N HCL and the solution made upto 100 ml with distilled water then 0.4% of SDS is added and stored at 4°C.

Silver staining solution

This solution consisted of two solutions A and B. Solution A was prepared by adding 0.8g of $AgNO_3$ in 2.5 ml of distilled water. Solution B was prepared by adding 1ml of 2 m NaOH in 20 ml H_2O and then 1.5 ml concentrated ammonia was added. While shaking solution A was added slowly to solution B and made to 100 ml by adding distilled water.

Gel fixing solution

This solution was prepared by mixing 50 % of methanol (Analar) with 10 % acetic acid and made up to 100 ml.

Colour developer

7.5g of sodium carbonate was weighed and dissolved in 200ml of water while stirring, to this solution 800µl of 37% formaldehyde and 10mg of sodium thiosulfate (Na_2So_3) was added and the solution made upto 250 ml with distilled water.

2.4 Purification and characterization of anti-chloramphenicol antibody

Anti-chloramphenicol antibody collected during the final booster (797 days post immunisation) raised in goat 1, donkey 2 and camel 1 gave high optical densities and were therefore selected for purification by precipitation using ammonium sulphate and gel filtration.

Purification was carried out as follows; In a 50 ml beaker 1ml hyperimmuneserum was added to 1 ml cold saline (0.85%, pH 7.0) then 2ml of cold saturated ammonium sulphate was added dropwise while stirring on high-speed magnetic stirrer. The mixture was stirred for 30 minutes on ice while a white precipitate (globulin) formed. This mixture was then centrifuged for 30 minutes at 3000 rpm in refrigerated bench top centrifuge. The supernatant was then discarded and the precipitate taken up in 2 ml of saline per ml of the original serum. This procedure was repeated twice. The final supernantant was colourless.

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The final precipitate was taken up in 1ml or less of saline and dialysed extensively in PBS overnight to remove all the ammonium sulphate.

In order to improve on resolution the amount of ammonium sulphate in the globulin phase was reduced by gel filtration on sephadex ® G50 (Pharmacia Upsalla Sweden). The gel was swollen in excess buffer (0.1m PBSE pH 7.2, 0.02% NaN_3) and then degassed. The column was packed with the gel and then equilibrated with the buffer. The sample was then applied on the bed surface in a volume of about 5% of gel volume. The first eluted sample (about 50 ml) was concentrated in polyethylene glycol 8000 (PEG) P-2159 1kg lot 092K0156. (Sigma, UK) before dialysis (12 KDS cut off) against PBS pH 7.2 at +4 °C overnight. The crude antibody, the antibody purified by precipitation and the antibody purified further by passing through sephadex column were tested using CAP ELISA and optical densities obtained compared using Statview statistical package (Wesongah and Guantai, 2012).

2.5 SDS-PAGE electrophoresis

Crude and purified goat 1, camel 1 and donkey 2 anti-chloramphenicol antibody were analyzed with SDS-PAGE as described by Laemmli 1970. Slab gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was performed as follows;

The gel mould was assembled using glass plates and plastic spacers. The separating gel solution was prepared as shown in **Table 1**. The high and low gel concentrations was mixed and poured into the gel mould. This was allowed to polymerize for 15-30 minutes. A stacking gel was then polymerized on top of the separating gel. A plastic comb was then pushed into the solution, ensuring that no air bubbles are trapped and the gel was allowed to polymerize for 20 to 30 minutes.

Table 1: Sodium dodecyl Sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Modification of Laemmli 1970)

Ingredients	Final acrylamide	
	Low	High
30% Acrylamide	6.4 ml	11ml
1.5 M Tris-HCL pH 8.8	3.75ml	3.75 ml
Distilled water	4.65ml	5 ml
10% SDS	150 µl	150 µl
10% Ammonium persulfate	300 µl	300 µl
TEMED	60 µl	60 µl
Total Ingredients	30ml	30ml

When the gel had polymerized, the gel mould was clapped to the electrophoresis tank using bull-dog clips and the electrode reservoirs filled with the electrophoresis buffer. The samples were diluted with the sample buffer (1:50). The samples were then loaded into the slots in the stacking gel (15 microlitre/slot), the electrodes connected to the power pack and electophoresed at 200 volts until the blue dye incorporated in the sample buffer had reached the bottom. The power was finally turned off, the gel was then removed from the mould and stained for 30 minutes with silver nitrate. All the runs were done at room temperature.

2.6 Immunization of experimental animals and collection of hyperimmune sera

The CAP immunogen was adminisitered at an initial dose of 3mg for camels and donkeys and 2 mg for goats. Subsequent inoculations were dosed at 1mg and 0.5mg respectively. The emulsions were injected subcutaneously into four sites of each animal (left and right front quarters and left and right hind quarters). Blood samples were collected from the jugular vein for all immunized animals immediately before each booster and anti-CAP antisera prepared and stored at -20 °C for analysis. Immunization of experimental animals and collection of hyperimmune sera from the animals was carried out in two phases. In phase I all the experimental animals were immunized and then given boosters monthly and test bled every two weeks for 5 months.

Phase II of the immunization was initiated 114 days after the 4th booster of phase I. The experimental animals were given boosters starting from the 5th booster, 6th, 7th, 8th, 9th and 10th booster at two monthly interval followed by monthly test bleeding for a period of 16 months. The final booster (11th) was carried out 797 days following the 10th booster and test bled after 2 weeks. This was done to allow the antibody to mature,

Serum samples were prepared from the test bleeds and stored at -20 °C for analysis later.

2.7 Determination of Optical densities

The anti-CAP antibody raised in the 3 different animal species was assessed using checkerboard titration. 96 well microtitre plates (immulon 4, Dynatech Labs, Chantily, USA) were coated horizontally (12 columns) with doubling serial dilutions of antibody starting from either 1/100, 1/200. 1/400 or 1/800 depending on the strength of the antibody. After an overnight incubation at 4°C, the plates were kept at -20 °C . When required for use the plates were thawed and washed five times and a doubling serial dilution of conjugate added vertically (8 rows) starting from either 1/100, 1/400. 1/500, 1/800, 1/1k, 1/2k, or 1/16k depending on the strength of the antibody. The plates were developed and optical densities (ODs) determined using CAP ELISA (Wesongah et al, 2007). The ODs obtained from antisera collected on different days after booster immunizations and between and within the different animal species were compared. The anti-CAP antibody from the final booster (797 days post-immunization) from each of the three animal species that gave high ODs were purified and characterized.

2.8 SDS-PAGE gel staining and colour development

After running the PAGE gel it was fixed with 50% methanol and 10% acetic acid on a rotator 5minutes/overnight and rinsed with distilled water. The

gel was washed in 50% methanol twice for 5minutes per wash rinsing with distilled water after each methanol wash. 10% of 25% aqueous glutaraldehyde was added to the PAGE gel and kept at room temperature for 30 minutes. The PAGE gel was then taken through a third cycle of rinsing with water and methanol alternately. Silverstaining of the PAGE gel was then carried out using solution A and B. Solution A was slowly added to Solution B (carefully to avoid formation of a precipitate). The gel was stained in the silver solution for 15 minutes and then rinsed with distilled water for 5 minutes. A colour developer was then added for 30 minutes for visualization under ultraviolet light and the colour reaction was stopped with 7% acetic acid. After visualization the gels were stored in the acetic acid at +4 ^{o}C.

2.9 Documentation of gels and estimation of the protein molecular sizes

After colour development the gels were visually inspected by illumination with ultraviolet light. The results were photographically documented using a digital still camera (Rollei fototechnic SN. 9U9ROLC2GA0004L, GmbH Salzdahlumer str.196 D-38126 Braunschweig, Germany) and downloaded onto a Toshiba laptop computer from which it was printed on a supercopier A4 paper. The migration distances of the protein bands of the antibody from the camel, donkey and goat serum and sigmaMarker were measured directly from photographs of the gels. The migration distances of protein bands were estimated by standard polynominal curves generated with the logarithm of relative migration of proteins on the X axis and the logarithm of the molecular size of the standard molecular size proteins on the Y axis with the Microsoft Excel program as previously described by Rochelle et al, (1985) and Wand et al, (2003). The protein profiles from the camels, donkeys and goats were compared for any protein band that was common in the 3 animal species and also in the crude and purified antibody.

3. Results

3.1 SDS-PAGE Analysis of anti-chloramphenicol antibody

A polynomial standard curve (**Figure 1**) was used to determine molecular sizes of protein bands obtained from a 10% SDS-PAGE analysis of anti-chloramphenicol antibody produced in camels, donkeys and goats.

Following purification of the anti-chloramphenicol antibody with Ammonium sulphate the crude and purified antibody from the camel, donkey and goat were analysed using SDS-PAGE electrophoresis. A total of nine and eight protein bands of molecular sizes ranging from 11.7 to 182 kda and 40 to 182 kda were observed in the crude and purified antibody respectively of all the three animal species as shown in **Figure 2** and **Figure 3**. Protein band of molecular size 11.7 kda was not observed in the purified antibody. The protein bands of the camel appeared smaller and were more distinct as compared to those of donkey and goats. Distinct differences in resolution between the purified and crude protein bands were only observed in the camel.There were no differences observed in the number of protein bands when the antibody from the three animal species was purified first by ammonium sulphate (AS) followed by passing through sephadex G50 column as shown in **Figure 3**.

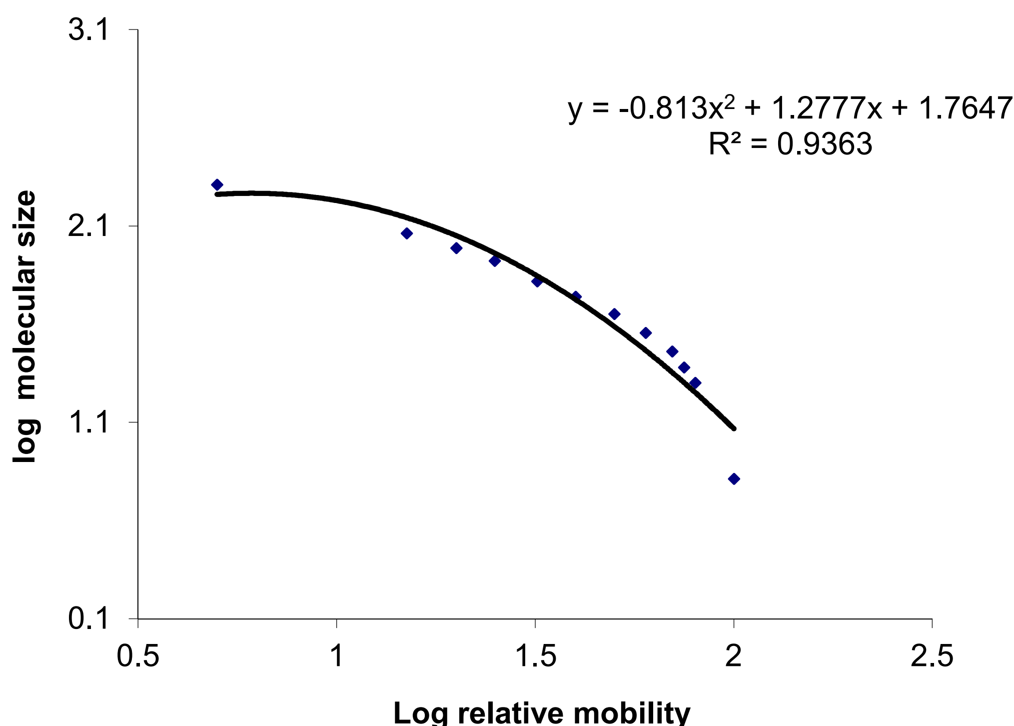

Figure 1: A polynomial curve of SigmaMarker in 10% SDS-PAGE with known molecular sizes verses relative mobility

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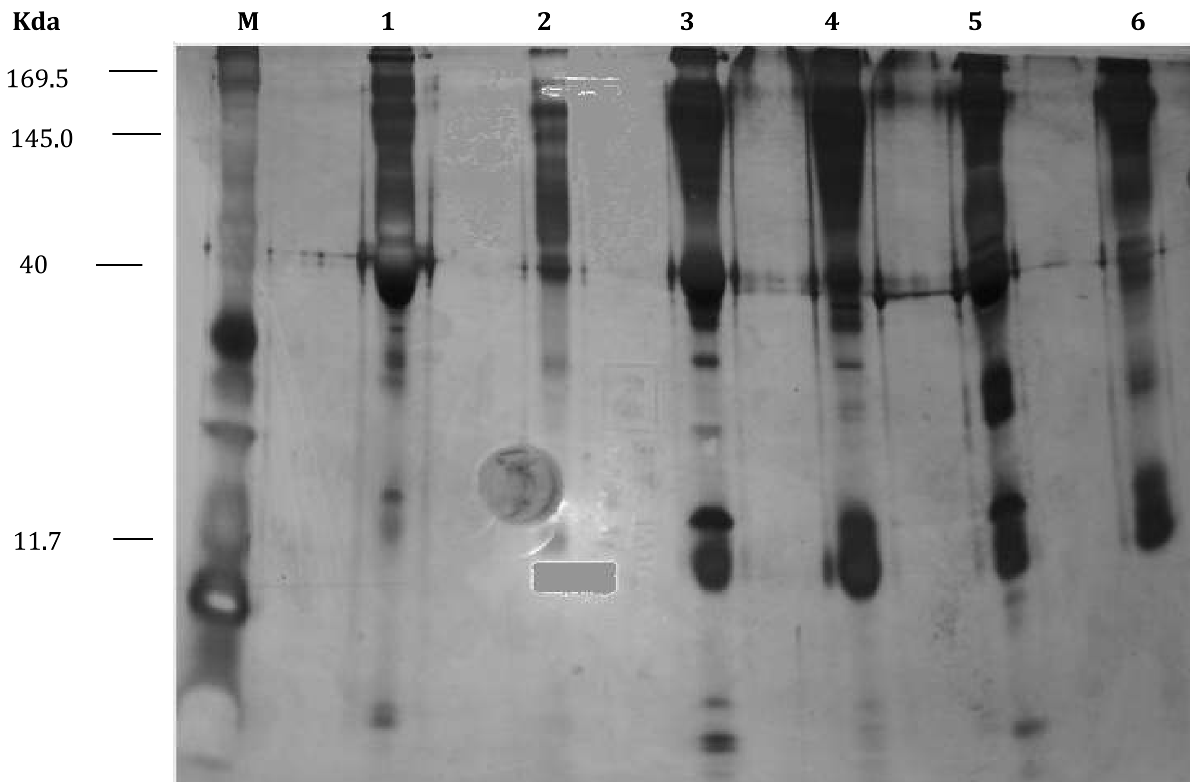

Figure 2: SDS-PAGE gel electrophoresis of crude and purified antichloramphenicol antibody raised in camel, donkey and goats; Lane **M**-Sigma Marker comprised of wide range molecular proteins (6.5 to 205kda) Lane **1** camel crude. **3** crude donkey, **5** crude goat with proteins 11.7 to 169 kda , Lane **2** camel purified, **4** purified donkey and Lane **6** purified goat with proteins 40 to 169,5 kda.

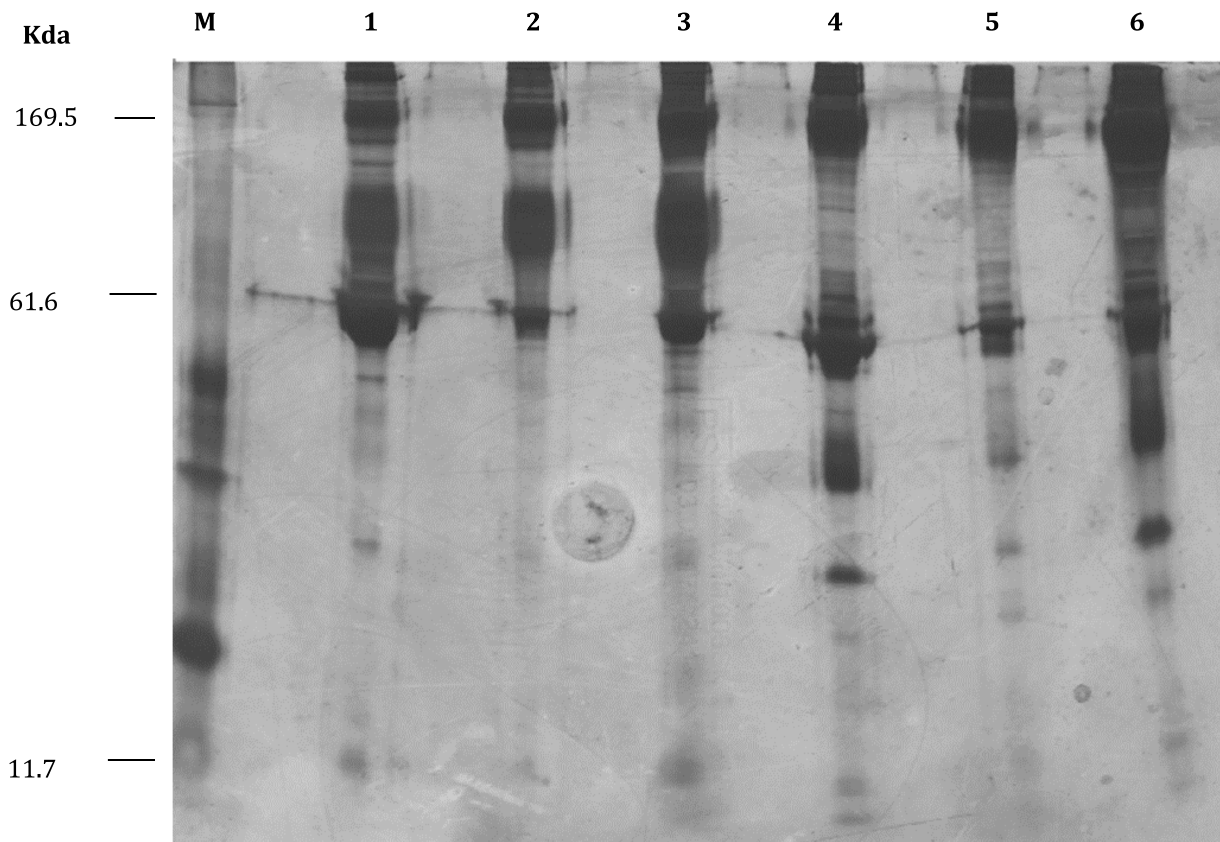

Figure 3: SDS-PAGE gel electrophoresis of crude and purified antichloramphenicol antibody, Lane **M**-SigmaMarker, Lane **1**, camel crude, **2** camel purified by ammonium sulphate, **4** Goat crude, **5** Goat purified by Ammonium sulphate, Lane **3** and **6** is camel antibody and goat purified by ammonium sulphate followed with sephadex 50 respectively.

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4. Discussion

Purification of the antibody with ammonium sulphate precipitation followed by dialysis from the three different animal species was found to enhance the specificity and sensitivity of the antibody as indicated by the high optical densities obtained from ELISA checkerboard titration (Wesongah and Guantai, 2012). This shows that removal of the other proteins of molecular size 11.7 kda reduced nonspecific binding and this consequently increased the specificity of the anti-chloramphenicol antibody produced in the different animal species. However further purification of the antibody using sephadex G50 column was not observed to improve the specificity of the antibody. This observation was supported by the SDS–PAGE analysis which showed no differences in the number of protein bands between the ammonium sulphate purified and the ammonium sulphate purified followed by sephadex G50 purification. This shows that filtration by sephadex G50 had no effect on the activity of anti-chloramphenicol antibody produced in the three animal species. Mohanty and Elazhary (1989) also made similar observation during purification of IgG from bovine serum. These authors carried out studies on purification of IgG from bovine serum with Caprylic Acid treatment followed by Ammonium Sulphate precipitation (CAAS) and Ammonium Sulphate Precipitation (ASP) alone and their results suggested that IgG purified by ASP was better than that obtained by CAAS method in terms of the yield of the IgG monomers and the recovery of the antibody activity. Murilla et al, 1996 also reported that affinity purification of antiserum might be one possible means of further increasing the specificity of the ELISAs.

Ten percent SDS-PAGE analysis of crude anti-chloramphenicol antibody produced in camel, goats and donkey showed 9 protein bands of molecular sizes 11.7, 40, 61.6, 134.3, 145, 169.5 and 182 kda. Following purification of the antibody, protein band of molecular size 11.7 kda was eliminated. This could be due to its small size thus it diffused through the dialysis membrane. Of the nine protein bands isolated in the camel species two protein bands of molecular size 145 kda and 169.5 kda isolated in the present study are not markedly different from those of camel IgGs of 149.2 kda and 160 kda reported by Daley et al, 2005 and Herrera et al, 2005. In the present study the proteins isolated in the camel were smaller and more distinct visually compared to those of donkeys and goats. This could be due to the fact that camel IgG lack the light chain compared to other conventional mammalian antibodies (Omidfar et al, 2004). These heavy-chain antibodies (HCAbs) constitute approximately 50% of the IgG in Ilama serum and as much as 75% of the IgG in camel serum (Daley et al, 2005). Due to their small size camel antibodies are ideal tools for basic research and other applications such as tumor imaging and cancer therapy (Omidfar et al, 2004). However definite identification of the isolated protein bands was not possible because commercial antibodies were not readily available in the market. Therefore further research is recommended to identify the two protein bands of molecular weight 145kda and 169.5 kda isolated in the camel using commercial IgGs or other immunological tests. The protein content of the purified and crude antibody was not significantly different indicating that the crude antiserum had very low amounts of other proteins such as the low molecular weight protein of 11.7kda. This could be due to the eleven boosters administered to the animals thus selecting specific type of proteins (Wesongah and Guantai, 2012).

5. Conclusion

From this study it was concluded that purified camel antibodies are smaller and more specific followed closely with goats antibodies and finally donkey antibodies. This study recommends that further boosting of these animals may produce better antibodies that can be used in diagnosis of different types of cancer and other viral diseases using ELISA methods.

Conflict of Interest Declaration

The authors declare no conflict of interest.

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