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Isolation, purification, and immuno biochemical characterization of IgG from dog (*Canis lupus familiaris*)

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Abstract
Background: Immunoglobulin G (IgG) plays a pivotal role for secondary immune response in animals which trigger passive immunization against harmful pathogens in dogs.
Purpose: The purpose of the present study was to isolate, purify, and immuno biochemically characterize IgG isolated from serum of dog (*Canis lupus familiaris*).
Methods: The serum was isolated from dog which had undergone ammonium sulphate precipitation, which was dialysed overnight in a refrigerator followed by Lowry Method from where the protein concentration was estimated to be 122.22 mg/ml and the Total Protein Concentration was estimated to be 6.147 g/dl that is 61.47 mg/ml. This study continued through Column Chromatography estimation by obtaining a bell shaped graph (Absorbance vs Number of Fractions) followed by SDS-PAGE from where Rf values of different bands were obtained. Marker (Known) and samples P1, P2, P3, P4 (Unknown) graphs were obtained. The marker graph was estimated by Log of Molecular Weight of Protein Ladder Marker and the unknown graphs were estimated by Anti-Log of Molecular Weight of Protein Ladder Marker. After that DID Test was done where a single precipitin line of purified IgG of Canine dog was observed against antiserum developed in rabbit.
Results: A single precipitin line of purified IgG of Canine dog observed against antiserum developed in rabbit and was found to be immune-reactive by DID Test and was fully confirmed by SDS-PAGE.
Discussion: The dog serum was isolated by Ammonium Sulphate, purified by Column Chromatography, followed by Immunobiochemical Characterization which was confirmed by SDS-PAGE by obtaining a single precipitin line of IgG by DID Test.

Keywords: DID test, immunoglobulin G, SDS-page, column chromatography, Lowry method

Introduction
Immunoglobulin G (IgG) is mainly responsible for secondary immune response in animals. Rodney Porter and Gerald M Edelman first elucidated the characteristic Y-shaped structure of antibodies. In 1972, they were awarded the Nobel Prize for Medicine and Physiology for their discovery. Rodney Porter (1966) [15] described that there are two methods for the formation of subunits of immunoglobulins, the first one is by enzymatic hydrolysis with papain or pepsin leading to a combined site that contains fragment (Fab) of 45,000 molecular weight and formation of light chain and also N-terminal. Rodney Porter (1972) [16] elucidated in his Nobel lecture about production of antibodies by dissociation of precipitates along with salt solution These Y-shaped molecules were eventually identified as immunoglobulin G (IgG), the structure of which is composed of four polypeptide chains- two heavy (50KDa) and two light chains (25kDa)- linked by non-covalent bonds and disulphide bridges. R J Heddle and D Rowley during 1975 measured the level of IgG in dog serum, colostrum, milk, parotid saliva and small bowel fluid using the single radial immune-diffusion method.
Abdel-Rahman, *et al.* (2017) [1] obtained the molecular weights of camel IgG of four bands were 63, 52, 40, and 33 kDa, this study was conducted by SDS-PAGE. Dogs (*Canis lupus familiaris*) are used in biomedical research because they have certain similarities with humans. Santos F.N *et al.* (2014) [18] enunciated that production and characterization of IgY antibody

against canine IgG is very essential for serological markers in infection and also used for elaboration of diagnostic kits. Dogs are especially suitable for cardiovascular studies due to the resemblance in heart connectivity and size to the human heart. Experiments on dogs led to the discovery of insulin to treat diabetic patients, the development of blood transfusion procedures and the creation of the electrical defibrillator to restore normal heart rhythm. The efficiency of some new cancer drugs are tested in dogs with the same cancers as humans, as they can have a benefit for both humans and dogs. So dog is used for experimentation of this study and they should be taken care of, also well maintained so that crude IgG can be isolated from them properly for immunobiochemical analysis and characterization. In this study, focus has been given on IgG because they are the most common antibody present in blood and other body fluids (75% to 80%) of all the antibodies present in the body. This antibody is capable of protecting against infection by remembering which germ is been exposed before in the body and it is the one and only antibody that can cross placenta of pregnant woman to protect the baby (foetus). So the present study is to isolate, purify and immuno biochemically characterize IgG extracted from dog serum.

Materials and Methods

Isolation of IgG

Crude IgG was extracted from dog serum. Then dog serum (3 ml) and 4.1M concentration of ammonium sulphate (3 ml) was taken in a centrifuge tube and gently mixed. Then the tube was kept in ice at about 1 hour at 4 ^{0}C. After 1 hour, the tube was taken out from ice and centrifuged at 5000 rpm for 15 min at 37 ^{0}C. After that the supernatant was discarded out and clear precipitate was obtained which was diluted with PBS (3 ml). After dilution, the sample was mixed by Vortexing and then the entire mixed sample was poured carefully into a Dialysis bag which was kept overnight at 4^{0}C. On the next day, the sample was taken out from the refrigerator and again centrifuged for 5000 rpm for 15 min to obtain the supernatant. Here the freshly obtained supernatant was considered as the Lowry Sample. After that in a separate tube 10 ml of 2% Na_2CO_3 (Sodium Carbonate) was taken as well as 200 µl of Na-K Tartarate (Sodium Potassium Tartarate) was also taken in the same tube and mixed properly and then the tube was wrapped carefully by an aluminium foil and kept aside. This mixture of 2% Na_2CO_3 and Na-K Tartarate was called Lowry Reagent. Then the three test tubes were taken Blank (B), Standard (S), and Test (T). In each of the B, S, and T tubes 3 ml of 2% Na_2CO_3 and Na-K Tartarate mixture was given. After that separately, in B 100 µl of distilled water (DW) was given, in S 100 µl of Bovine Serum Albumin (BSA) (BSA standard was approximately formulated at 2mg/ml in an ultrapure 0.9% sodium chloride solution) at was given and in T, 100 µl of Lowry sample was given and all the tubes were mixed properly and then kept for incubation for 15 min. While the incubation was going on, in a fresh separate tube, 600 µl of Folin reagent and 600 µl of DW is taken and mixed properly with the help of micropipette. After that, the B, S, T tubes were taken from the incubator and 300 µl of Folin Reagent (FR) and DW mixture was given to each tube separately. The colour change was immediately noticed in three separate tubes and then the tubes were again kept inside the incubator for 30min. After that the tubes were taken out and spectrophotometric readings of the three tubes were taken at 750 nm and Protein Concentration was calculated to be 122.22 mg/ml Fig (1). While on the other hand, the rest of the dog serum after completion of the ammonium sulphate precipitation experiment was taken which was later used for the calculation of Total Protein Concentration. From the rest of the dog serum, in B, 3 ml of 2% Na2CO3 was taken only. While in S, 3 ml of 2% Na_2CO_3 was taken and 30µl of Na-K Tartarate was taken. In T, 3 ml of 2% Na2CO3 was taken and 30µl of dog serum (remaining) was taken. All the tubes were properly mixed and set in the incubator for 5 min at 37 ^{o}C. The colour change was noticed in the tubes after incubation and then spectrophotometric readings of only S and T were taken at 578 nm from where the Total Protein Concentration of the sample was calculated to be 61.47 mg/ml (Fig 2 & 3).

Purification of IgG

The crude IgG was purified by Column Chromatography and 36 fractions (test tubes) were taken. At first in the column 1.5 ml of the Lowry sample was given and at that time the end of the column should remain closed. After washing step of the column was done by phosphate buffer saline (PBS), 36 fractions were arranged and marked accordingly and kept under the column. After all the tubes were filled up then the spectrophotometric readings of the 36 fractions were. Then a bell shaped graph was also made (Absorbance vs no of tubes) as shown in Fig (4 & 5). Then, among the 36 fractions, from test tube number (10-24), the values were gradually increasing and then decreasing, so these tubes (10-24) were considered which were kept overnight at 4 ^{o}C. On the next day, the tubes (10-24) were taken out and the samples present in these tubes were kept in 5 distinct tubes, the rest of the tubes were discarded. Then 2 gm of Sodium Hydroxide (NaOH) and 10gm of Sodium Carbonate (Na_2CO_3) were measured and taken and dissolved in 500 ml of DW in a tube and vortexed well. The 5 tubes were there and one extra tube (named B) was taken. In a fresh tube 15 ml of 2% Na_2CO_3 was taken and in another fresh tube, 10 ml of 2% Na_2CO_3 was taken. In the 15 ml tube, 300 µl of Na-K Tartarate was taken and in the 10 ml tube, 200 µl of Na-K Tartarate was taken. Here, basically there are 7 tubes, one fresh B tube, one fresh S tube and five fresh T tubes (the fractions '10-24' were compiled into 5 pools, called T_1-T_5). From the 15 ml and 10 ml tubes, 3ml each was given in B, S, T1-T5 tubes and mixed properly. Then in B, 100 µl of DW was given. In S, 100 µl of BSA was given and from T_1 to T_5, samples from the 5 tubes (kept aside earlier) was given. In a separate tube, 1300 µl of Folin reagent and 1300 µl of DW was taken and mixed properly. The B, S, T_1-T_5 tubes were kept inside the incubator for 15 min. After taking out the tubes from the incubator, 300 µl of Folin reagent and D W mixture was given in B, S, T_1-T_5 tubes and mixed properly and the colour change was immediately noticed. Then the tubes were again kept inside the incubator for 30 min. After that spectrophotometric readings were taken at 750 nm as shown in Fig (6). Then Protein Concentration of only T_1-T_5 were calculated to be 1 mg/ml, 2.069 mg/ml, 5.372 mg/ml, 3.093 mg/ml, 5.255 mg/ml.

Immunobiochemical Characterization of IgG

After completion of Column Chromatography experiment Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was successfully done. By taking samples P1-P5 (which were earlier named as T1-T5, obtained from Column Chromatography) and Crude sample (Sample made earlier for Lowry Method) SDS-PAGE was done and the bands in the gel were obtained. Five wells were

there in the gel. The first one was Marker (Known) well, the second one was P1 (Unknown) well, the third one was P2 (Unknown) well, the fourth one was P3 (Unknown) well and the fifth one was P4 (Unknown) well. The fifth well did not appear clearly so it was not considered. Then the Retardation Factor (Rf) value of Marker and P1-P4 was calculated and logarithmic excel graph (log of Molecular Weigt vs Rf) of Marker was plotted and (anti log of Molecular Weight vs Rf) of P1-P4 were plotted accordingly with respect to the Rf values obtained. Now, the logarithmic excel graphs of marker and P1-P5 was plotted. For marker (log of M.W vs Rf) graph was plotted and for P1-P4 (anti log of M.W vs Rf) were plotted considering Fig (7). The Log values of Marker (Known) graph were calculated to be Log (235) is 2.36, Log (170) is 2.23, Log (130) is 2.11, Log (93) is 1.96, Log (70) is 1.84, Log (53) is 1.72, Log (41) is 1.61, Log (30) is 1.47, Log (22) is 1.34, Log (18) is 1.25, Log (14) is 1.14, Log (9) is 0.95. On the basis of these log values the marker graph was obtained shown in Fig (8). The Log values of P1-P4 (Unknown) were calculated to be Anti-Log (2.36) is 229.08, Anti-Log (2.23) is 169.82, Anti-Log (2.11) is 128.82, Anti-Log (1.96) is 91.2, Anti-Log (1.84) is 69.18, Anti-Log (1.72) is 52.48, Anti-Log (1.61) is 40.73, Anti-Log (1.47) is 29.51, Anti-Log (1.34) is 21.87, Anti-Log (1.25) is 17.78, Anti-Log (1.14) is 13.8, Anti-Log (0.95) is 8.91. On the basis of these Anti-Log values the graphs of P1-P4 were obtained as shown in Fig (9). After that, Double Immunodiffusion (DID) Test was performed. At first in a small beaker D W was taken and heated in a microwave for some few minutes and then taken out. After that, 100 ml of PBS was poured into a conical flask and heated in a microwave. After heating the conical flask was taken out and 1.5 gm of agar was dissolved into it. Then two slides were taken out and washed thoroughly. The conical flask containing PBS and agar mixture is again heated inside the microwave for complete dissolve of the agar. Then the PBS and agar was poured carefully over the two slides with the help of a pipette and left for 2 hour for solidification. After solidification, in each of the two slides four wells were made, so total eight wells were made. In each of the well of the slides just one drop of PBS and agar mixture was given. After that in first slide, in the first well 20µl of Goat IgG was given. In the second well 20 µl of Rabbit anti goat IgG was given. In the third well, 20 µl of Rabbit anti goat IgG was given. In the fourth well, 20 µl of Goat IgG was given. Then, in the second slide, in the first well 20µl of Goat IgG was given. In the second well 20 µl of Rabbit anti goat IgG was given. In the third well, 20 µl of Rabbit anti goat IgG was given. In the fourth well, 20 µl of Goat IgG was given. Then the slides were kept for incubation overnight. On the next day, the slides were taken out from the incubator to observe the bands in the well as shown in Fig (10). Hyper-immune serum was raised in rabbit against crude IgG of dog. Single precipitin line was observed in DID Test when the partially purified IgG was reacted with hyper-immune serum. At first, the wells of the DID slides were raised with Goat IgG and Rabbit-Anti goat IgG. Then a single precipitin line of purified IgG of canine dog was observed against antiserum developed in rabbit.

Table 1: Protein Concentration of sample (mg/ ml) of Lowry Method

Sl. No.	Cell No/ Name	Absorbance (750 nm)
1 (Standard)	1-	0.018
2 (Test)	2-	2.200

The Protein Concentration of sample was calculated on the basis of the following formula:

$$\text{Protein Concentration in Sample} = \frac{\text{OpticalDensityofSample+ConcofStandard}}{\text{OpticalDensityofStandard}}$$

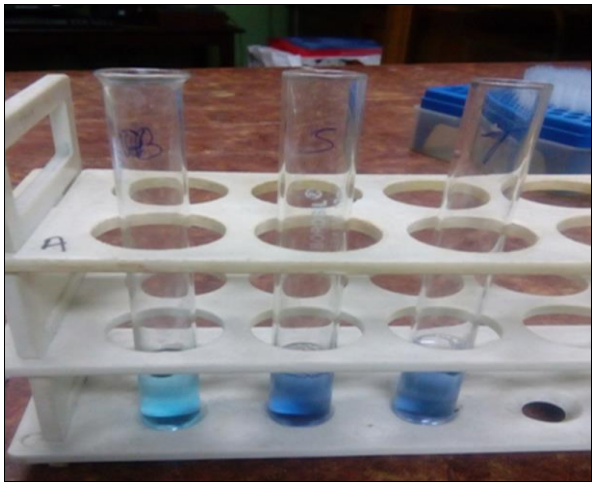

Fig 1: Colour change notified in B, S, T tubes after incubation for 5 min at 37 ºC. In B, S, T 3 ml of Reagent A was given while in S 30µl of Reagent B was given and in T, 30µl of dog serum was given. All the tubes were mixed and kept in incubator for 5 min at 37 ºC. After Incubation colour slightly turned bluish in the three tubes

Table 2: Total Protein Concentration Method

Sl. No.	Cell No/ Name	Absorbance (578 nm)
1 (Standard)	1-	0.166
2 (Test)	2-	0.157

The Total Protein Concentration was calculated on the basis of the following formula:

$$\text{Total Protein Concentration} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} * 6.5$$

Table 3: Spectrophotometric readings of 36 fractions of Column Chromatography

Sl. No.	Absorbance (at 280 nm)
1.	0.190
2.	0.284
3.	0.158
4.	0.250
5.	0.147
6.	0.249
7.	0.070
8.	0.195
9.	0.274
10.	1.914
11.	2.344

12.	2.520
13.	2.399
14.	2.500
15.	2.500
16.	2.500
17.	2.500
18.	2.500
19.	2.500
20.	2.462
21.	1.922
22.	1.744
23.	1.449
24.	1.447
25.	1.237
26.	1.325
27.	1.114
28.	1.153
29.	0.968
30.	1.055
31.	0.913
32.	0.988
33.	0.800
34.	0.895
35.	0.761
36.	0.882

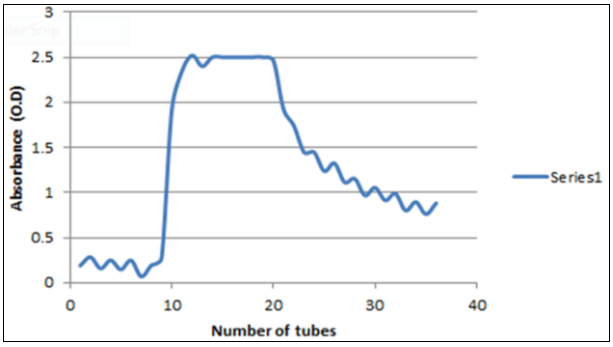

Fig 2: Bell shaped graph (Abs vs No of tubes) of Column Chromatography was plotted from the spectrophotometric readings of the 36 fractions. From the above graph, it was observed that starting from 10 up to 24 the graph line was increasing and then again decreasing as related to the spectrophotometric values obtained

Table 4: Spectrophotometric readings of T1-T5 at 750 nm

Sl. No.	Cell No/ Name	Absorbance (at 750 nm)
1 (Standard)	1-	0.043
2 (Test 1)	2-	0.043
3 (Test 2)	3-	0.089
4 (Test 3)	4-	0.231
5 (Test 4)	5-	0.133
6 (Test 5)	6-	0.226

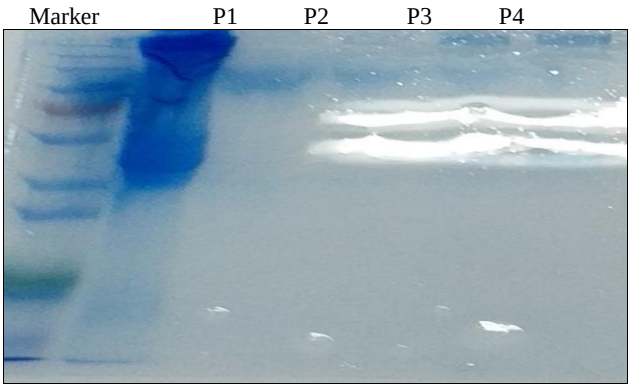

Fig 3: SDS-PAGE Gel Picture. Wells (1-5) are observed clearly but the fifth well was not observed clearly so it was not considered

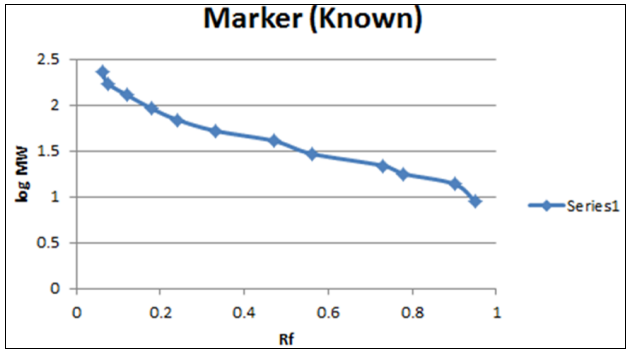

Fig 4: Marker (Known) graph where log M.W is at y axis and Rf values at x axis

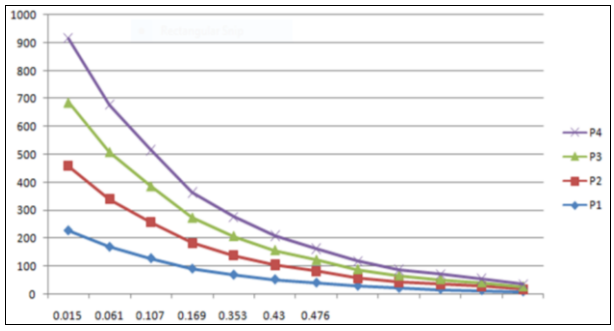

Fig 5: P1, P2, P3, P4 (Unknown) graphs where anti-log M.W at y axis and Rf values at x axis

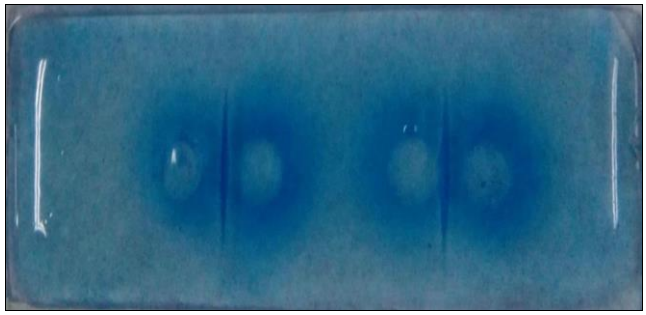

Fig 6: Single precipitin line of purified IgG of Canine dog observed against antiserum developed in Rabbit (DID)

Results

Hence, the present study was focused on how to isolate IgG from dog serum by Ammonium Sulphate Precipitation method followed by Lowry Method and Total protein Concentration, then to purify the IgG by Column Chromatography and for immunobiochemical characterization of this antibody, it was done by SDS-PAGE followed by DID test. In case of isolation protein concentration was calculated to be 122.22 mg/ml (Fig1) and total protein concentration was calculated to be 61.47 mg/ml (Fig 2 & 3). The purification of IgG was also estimated from (Fig 4 & 5), from where the spectrophotometric readings of 36 fractions were taken obtaining a bell shaped curve. For the Calculation of Rf values of Marker and P1-P4 (P5 not considered), at first the total length of the page was measured with the help of scale. The total length of the page was 6.5 cm. Then on the basis of Fig (7), in the first Marker well, 12 consecutive bands were observed and so the Rf values of those 12 bands were calculated. In well P1, Rf values of bands (1-7) were calculated. In P2 well, Rf values of (1-7) bands were calculated. In P3 well, Rf values of (1-7) were calculated. In P4 well, Rf values of (1-7) were calculated respectively. Now, the logarithmic excel graphs of marker and P1-P5 was plotted. For marker (log of M.W vs Rf) graph was plotted and for P1-P4 (anti log of M.W vs Rf) were plotted considering Fig (7). For the immunobiochemical characterization of IgG, in case of Marker (Known), the Rf values of 12 consecutive bands were calculated to be 0.061 cm, 0.076 cm, 0.12 cm, 0.18 cm, 0.24 cm, 0.33 cm, 0.47 cm, 0.56 cm, 0.73 cm, 0.78 cm, 0.90 cm, 0.95 cm. In case of P1 (Unknown), the Rf values of 6 consecutive bands (here the fifth band was not observed properly, so the band was calculated) were calculated to be 0.015 cm, 0.061 cm, 0.092 cm, 0.169 cm, 0.415 cm, 0.476 cm. In case of P2 (Unknown), the Rf values of 7 consecutive bands were calculated to be 0.015 cm, 0.061 cm, 0.123 cm, 0.169 cm, 0.353 cm, 0.430 cm, 0.492 cm. in case of P3 (Unknown), the Rf values of 7 consecutive bands were calculated to be 0.015 cm, 0.061 cm, 0.123 cm, 0.169 cm, 0.338 cm, 0.415 cm, 0.461 cm. In case of P4 (Unknown), the Rf values of 7 consecutive bands were calculated to be 0.015 cm, 0.061 cm, 0.107 cm, 0.169 cm, 0.353 cm, 0.430 cm, 0.476 cm. Now, the logarithmic excel graphs of marker and P1-P5 was plotted. For marker (log of M.W vs Rf) graph was plotted and for P1-P4 (anti log of M.W vs Rf) were plotted considering Fig (7). The Log values of Marker (Known) graph were calculated to be Log (235) is 2.36, Log (170) is 2.23, Log (130) is 2.11, Log (93) is 1.96, Log (70) is 1.84, Log (53) is 1.72, Log (41) is 1.61, Log (30) is 1.47, Log (22) is 1.34, Log (18) is 1.25, Log (14) is 1.14, Log (9) is 0.95. On the basis of these log values the marker graph was obtained shown in Fig (8). The Log values of P1-P4 (Unknown) were calculated to be Anti-Log (2.36) is 229.08, Anti-Log (2.23) is 169.82, Anti-Log (2.11) is 128.82, Anti-Log (1.96) is 91.2, Anti-Log (1.84) is 69.18, Anti-Log (1.72) is 52.48, Anti-Log (1.61) is 40.73, Anti-Log (1.47) is 29.51, Anti-Log (1.34) is 21.87, Anti-Log (1.25) is 17.78, Anti-Log (1.14) is 13.8, Anti-Log (0.95) is 8.91. On the basis of these Anti-Log values the graphs of P1-P4 were obtained as shown in Fig (9).

Discussion

Crude IgG was obtained after Ammonium Sulphate Precipitation which was dialysed against PBS which could be corresponded with Duong-Ly K (2014) [2] and Miller, S.A *et al.* (1988) [12]. The protein concentration of crude IgG from dog serum was determined by the method of Lowry *et al.* 1951 [9] and the result was 122.22 mg/ml. The total protein concentration of the serum sample was obtained 61.47 mg/ml. which almost corresponded to Kresge *et al.* (2005) [8]. Purified dog IgG was prepared by Column Chromatography and the respective graph was obtained according to the spectrophotometric values. This result was almost similar to that of Still *et al.* (1978) [20]. From the Column Chromatography, the total protein concentrations of the samples (T1-T5) were obtained as 1 mg/ml, 2.069 mg/ml, 5.372 mg/ml, 3.093 mg/ml, and 5.255 mg/ml respectively. The crude and purified samples were analysed by one-dimensional SDS-PAGE by Reynolds *et al.* (1970) [17]. Here the respective bands were obtained of marker and from P1-P4. The P5 well did not appear clearly, so it was not considered. The Rf values of the individual bands of the respective wells (Marker, P1-P4) were calculated to obtain the graphs. The graphs were obtained by calculating the logarithm of the molecular weight of protein-ladder markers. These results could be corresponded to the experiments conducted by Staikos *et al.* (2009) [19], Manning *et al.* (2004) [10], Hartinger *et al.* (1996) [6], Margolis *et al.* (1969) [11], Wiltfang *et al.* (1991) [21], Guan *et al.* (2015) [5], Pederson *et al.* (2007) [14]. After that a single precipitin line of purified IgG of Canine dog observed against antiserum developed in Rabbit (DID**)** which can be corresponded to Graham, S. (1996) [4]. Ouchterlony, O. (1948) [13] who showed precipitin line in purified IgG desorbed from diphtheria bacteria by *in vitro* method for testing the toxin producing capacity.

Conclusion

This work opened a new dimension regarding isolation, purification and immunobiochemical characterization of IgG from dog serum. The use of immobilized antigens for the isolation of antibodies seemed to be an optimal method in the field of Column Chromatography which was based on extremely specific interactions. The antibodies could either be used to purify natural proteins or be used for recombinant protein purification in conjunction with other methods. For proteomic and genomic projects, protein separation from plasma, serum, cerebrospinal fluid (CSF), urine, and other body fluids or cellular sources were considered to be an important step. Novel antibodies could be uncovered from autoimmune disease patients. Traditionally, IgG binding proteins, generally produced by gram positive bacteria was widely explored in antibody isolation. It was anticipated that the improvements of these methods would have great influence on large scale production of antibodies, and of course the generation of new affinity based methods for the increasing number of proteins and antibody derivatives available for the protein engineering and the proteomics revolution.

Conflict of interest

We certify that there is no Conflict of Interest with any financial organization regarding the material discussed in the manuscript.

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Author contributions
Conceptualization, Investigation, Resources, Original Draft Preparation, Review and Editing, Supervision: P. Chatterjee.

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