



Preparation and Characterization of Dog Erythrocytic Membrane Antigen

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Authors' contributions

All authors are equally contributed for sample collection, providing research idea, collection of articles, typing editing and correction of articles. All authors read and approved the final manuscript.

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ABSTRACT

Dog erythrocytic membrane antigen plays a major role for determining blood group. Structural and molecular characterization of erythrocytic membrane antigen improves the production of blood typing antisera, to study the auto antibody production in canine autoimmune haemolytic anemia. The proteins in the lipid domain arranged from the inside of the erythrocyte to the outside. The integral membrane proteins include membrane protein 3 visible in coomassie Brilliant blue-stained polyacrylamide gels. The erythrocyte cytoskeleton consists of spectrin, ankyrin, actin, and protein 4.1 form a filamentous network under the lipid bilayer of erythrocytic membrane. The cytoskeletal proteins interact with integral proteins and lipids of the bilayer forms network for maintaining membrane integrity. The external clustering of membrane protein 3 creates a recognition site for auto antibodies. The auto antibodies from the erythrocytes of dogs with acute immune haemolytic anemia have similar electrophoretic mobility to the human erythrocyte Rh related proteins and glycoproteins and able to immunoprecipitate membrane proteins that on SDS-PAGE and western blotting. The DEA 1 blood group system consists of antigens 1.1, 1.2, 1.3 which are most significant in terms of transfusion reactions and alloantibodies produced against DEA 3, 5 and 7 are not much clinical significance. Blood samples collected from registered donor dogs and RBC membrane were isolated by hypotonic freeze thaw method. The ghost membrane and cytoplasmic marker were identified in western blot.

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1. INTRODUCTION

Mammalian whole blood consists of two major basic parts. The non-cellular liquid part is called plasma which makes up approximately 55% of total blood volume and consists of 90% water, 7% protein and 3% other organic and inorganic molecules and ions. The cellular components of the blood make up approximately 45% of the total volume. In the cellular parts, 99% of the cells are red blood cells and the remaining cells are white blood cells and platelets [1]. The normal mammalian red blood cell is a flexible biconcave disc like structure approximately 7 - 8 micrometers in diameter. Erythrocytes are unique component in all mammalian cells and travel thousands of miles in body through tubes of various sizes for delivering oxygen to the tissues. The approximate life span of erythrocytes in circulation is 120 days and depends on the ability of the membrane to remain intact and flexible. Storage of erythrocyte for long term causes damage of membrane leads to release of proteases causes destruction of RBC. The erythrocyte membrane consists of two parts, a lipid bilayer and the cytoskeleton domain. In most of the mammalian cells, the lipid bilayer has structurally similar [2]. The cytoskeleton domain of erythrocyte membrane differs from one cell to other cells because it does not contain the structural protein tubulin and not involved in cell motility or phagocytosis [3]. The lipid domain contains cholesterol and phospholipids and composed of nearly equal parts of lipid and protein. The cholesterol equally distributed between the two halves of the lipid bilayer and the other lipids are asymmetrically distributed as glycolipids, phosphatidyl choline, and sphingomyelin which are located in the outer portion of the bilayer [4]. The phosphatidyl inositols, phosphatidyl ethanolamine and phosphatidyl serine occur in the inner layer of phospholipid bilayer. Sometimes the internal amino phospholipids become exposed to the plasma, trigger the clotting mechanism leads to thrombus formation in side the blood vessels [5]. The lipid domain fluidity is determined by the molar ratio of cholesterol to phospholipid, degree of unsaturation of phospholipid acyl chains and phosphatidyl choline sphingomyelin ratio. The sphingomyelin induces rigidity to the membrane and phosphatidyl choline forms highly fluid lipid regions. Proteins in the lipid domain of erythrocytic membrane located from the inside of

the erythrocyte to the outside. Structurally these membrane proteins can be divided into an internal hydrophilic portion with membrane-spanning hydrophobic portion and an external hydrophilic portion with attached carbohydrate. The blood groups of mammals are determined by the structure of external carbohydrates compounds of membrane and the membrane-spanning proteins are classified as integral proteins [6]. The integral proteins include membrane protein 3 which is visible in Coomassie blue-stained polyacrylamide gels and several glycoporphins present in membrane visualized by periodic acid-Schiff-stained gels [7]. The membrane protein 3 is an anion exchange protein which is transporting carbon dioxide from the tissues to the heart for purification but the exact function of most glycoporphins is unknown. Most of the RBC membrane proteins are insoluble in water so it is necessary to solubilize the phospholipid bilayer by using detergents [8]. The red blood cell membranes were solubilized by sodium dodecyl sulfate (SDS) and then the extracted membrane proteins were separated by acrylamide gel electrophoresis (SDS-PAGE). The sialooligosaccharide rich glycoproteins detectable by staining with the periodic acid-Schiff's reagent [9]. The sialoglycoproteins visible in PAS staining are called glycoporphins. The glycoporphin is heavily glycosylated so electrophoretic migration of glycoporphin is relatively low when compared to other membrane components [10]. The red blood cell membrane proteins can be classified into transmembrane proteins and peripheral membrane proteins that associate with the cytoplasmic side of the lipid bilayer [11]. The band 3 and glycoporphins are transmembrane proteins and transmembrane proteins associate with the peripheral proteins forms protein meshwork. The main component of the cytoskeleton is a spectrin tetramer which bind with the actin filament and band 4.1 and forms a junctional complex. These junctions are linked to the end of some spectrin tetramers and forms a net like meshwork [12]. The cytoskeletal proteins glycoporphins C, D and band 3 are importance for maintenance of shape and mechanical properties of the red blood cell in circulation and The glycoporphins C and D are anchored to the membrane by the cytoskeleton [11].

Isolation of canine erythrocytic ghost membranes and analysis of protein component by gel

electrophoresis and mass spectrometry, advances in imaging technologies followed by biochemical, structural, and functional characterization of the various protein components of the erythrocytic membrane is very important to study the blood group antigen, transfusion reaction, allo antibodies and auto immune haemolytic anemia and other hereditary related disorders [13].

In this preliminary studies includes molecular characterization of dog erythrocytic membrane antigen, western blot analysis of membrane antigen and characterization of membrane and cytoplasmic marker was carried out with canine DEA1.1 blood group antigens and which is needed for the development of membrane antigen and production of monoclonal antibody and polyclonal antibodies for blood grouping. Hence with is introduction the research study carries following broad objectives

1. Isolation of dog erythrocytic membrane antigen
2. Characterization erythrocytic membrane and cytoplasmic marker

2. MATERIALS AND METHODS

2.1 Sample Collection

The 5 ml of Canine of DEA1.1 positive and negative blood samples collected in EDTA vial from registered healthy donor dogs of Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, India with owner's permission.

2.2 Blood Typing

Blood typing was performed by immunochromatography methods using methods using blood typing kit.(Alvedia, Limonest, France).The canine RBC was washed three times in phosphate buffered saline and 2% cell suspension was tested along with positive negative controls according to the recommended protocol.

2.3 Preparation of Canine Erythrocytic Membrane Antigen

The DEA1.1 ghost membrane antigen was prepared as per the method described by Francesco Di Girolamo (2010) with slight modification. 5 ml of DEA1.1 canine blood was taken in a tube and initially the blood sample was centrifuged at 1500 rpm for 15 min at 4°C The supernatant plasma and Buffy coats are removed

and the RBC pellet was washed three times with PBS with 10 mM phenylmethylsulfonyl fluoride protease inhibitor. The samples were centrifuged at 800 rpm for 20 min at 4°C and the remaining leukocytes and platelets were completely removed and the number of RBCs were counted in haematology analyzer. The 2×10^{10} RBC concentration was used for ghost membrane preparation. The pellet was diluted 10 times with 150 mM of NaCl which contain 10mM of phenylmethylsulfonyl fluoride protease inhibitor. The samples were continuously freeze thawed with LN2 for 4 times and centrifuged at 13000 rpm for 30 min at 4°C. The supernatant was removed and the pellet was again resuspended with 2 ml of 0.15% NaCl and shaken vigorously then centrifuged at 13000 rpm for 15 min and the supernatant was discarded. The lysed cell pellet was treated with 10 volumes of ice cold PBS containing 10 mM of PMSF of 20 mM EDTA. The lysed cells were washed three times with the same buffer containing 10 mM of NaOH until the pellet become white ghost. The centrifuged membranes were white, showing no traces of attached hemoglobin. The EDTA-lysed membrane preparations were then resuspended in the isotonic phosphate buffer with 10 mM of PMSF, 0.2% OPG and 0.1% triton X 100with 10 mM phenylmethylsulfonyl fluoride. The protein concentration were estimated by using Bradford assay along with known concentration of BSA and stored at -80°C.

2.4 SDS Page Analysis of Canine Erythrocytic Membrane Antigen

The SDS PAGE was performed in 12% polyacrylamide gel according to the method described by Laemmli [14]. The freshly prepared ghost membrane proteins mixed with sample loading buffer and boiled for 10 min after cooling the protein samples loaded in gel and SDS-PAGE was performed with tricine running buffer. The molecular weight of canine ghost membrane antigen was calculated from migration relative to standard pre stained protein markers (Page Ruler, Thermo fisher Scientific). There were seven bands with known molecular weights of canine ghost membrane antigen in the gel.

2.5 Western Blot Analysis of Canine Erythrocytic Membrane Antigen Marker

The DEA1.1 canine ghost membrane antigen separated by SDS PAGE and transferred in to methanol treated Poly vinylidene fluoride

membrane [15] by using dry blotting apparatus (Bio Rad). After complete transfer of protein into membrane, the membrane was washed with PBS for removing gel attached with membrane and the membrane was treated with 5% BSA for overnight blocking unbound area. The Poly vinylidene fluoride membrane was washed three times with PBST and treated with 1:5000 dilution of anti glycoporin A monoclonal antibody (anti glycoporin CD235a, Abecomics, USA) for 2 hrs and the membrane was washed three times with PBST. The membrane was treated with antimouse IgG conjugates (Thermo fisher Scientific 1:8000 dilution) for 1 hr. The Poly vinylidene fluoride membrane was washed three times with PBST and color developing substrate Diamino benzidine was added and the western blot patterns of erythrocytic membrane glycoporin a were analyzed.

2.6 Preparation of Canine Cytoplasmic Proteins

5 ml of DEA1.1 canine blood was taken in a centrifuge tube and initially the blood sample was centrifuged at 1500 rpm for 15 min at 4°C. The supernatant plasma and buffy coats were removed and the cell pellet was washed three times with PBS containing protease inhibitor 10 mM PMSF. The samples were centrifuged at 800 rpm for 20 min at 4°C and the remaining leukocytes were completely removed and the number of RBC was counted. The 2×10^{10} RBC concentrations was used for cytoplasmic organelles preparation. The pellet was diluted 10 times with 150 mM of hypotonic NaCl and incubated for 1 hr with gentle mixing. After complete lysis of RBC the suspension was centrifuged at 12000 rpm and the bright red supernatant was collected in a separate tube. The supernatant was subjected ultracentrifugation at 50000 rpm for 5 hrs. The supernatant was discarded and the pellet was resuspended in PBS. The concentration of protein was estimated by Bradford assay along with known concentration of BSA standard.

2.7 SDS Page Analysis of Canine Cytoplasmic Proteins

The SDS PAGE was performed in 10% polyacrylamide gel according to the method described by Laemmli (1970). The molecular weight of canine ghost membrane antigen was calculated from migration relative to standard pre stained protein markers (Page Ruler, Thermo fisher scientific). The molecular weights of canine

ghost cytoplasmic proteins obtained in the gels were documented.

2.8 Western Blot Analysis of Canine RBC Cytoplasmic Marker

The DEA1.1 canine ghost membrane antigen separated by SDS PAGE and transferred to methanol treated PVDF membrane [15] by using dry blotting apparatus (Bio Rad, USA). After complete transfer of protein to membrane, the membrane was washed with PBS for removing gel attached with membrane and the membrane was treated with 5% BSA for overnight blocking un bounded area. The PVDF membrane was washed three times with PBST and treated with 1:5000 dilution of mice alpha anti tubulin monoclonal antibody (Sigma, USA) for 2 hrs and the membrane was washed three times with PBST. The membrane was treated with antimouse IgG conjugates (1:8000 dilution) for 1 hr. The PVDF membrane was washed three times with PBST and color developing substrate TAB was added and patterns of alpha tubulin cytoplasmic marker were analyzed in western blotting.

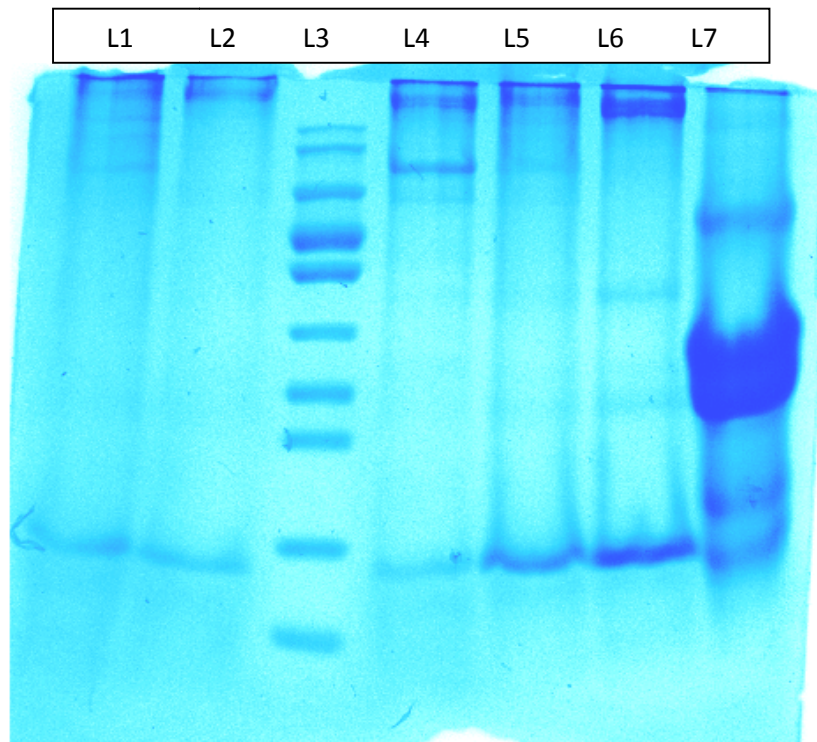
3. RESULTS AND DISCUSSION

3.1 SDS Page of DEA1.1 Canine Erthrocytic Membrane Antigen

The SDS PAGE profile of dog erythrocytic membrane antigen showed 7 distinct bands of spectrin, ankyrin, actin, protein 4.1. glycoproin A, glycoporin B glycoporin C and some of the cytoskeletal proteins also present which are interact with integral proteins and phospholipid bilayer to maintain membrane integrity of erythrocytic membrane. The erythrocyte cytoskeleton consists of several proteins that form a filamentous network under the lipid bilayer and some of the transmembrane and integral membrane glycoprotein and sialoglycoprotein which is embedded in phospholipid bilayer not visible in SDS PAGE (Fig. 1).

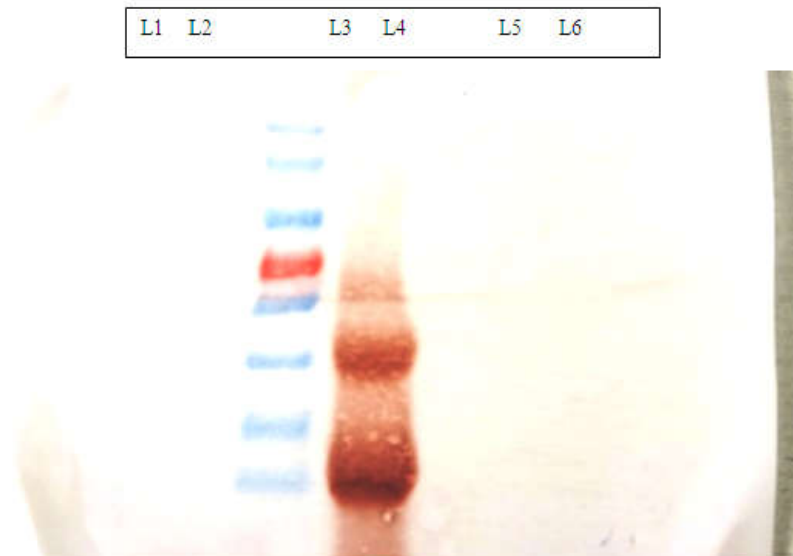
3.2 SDS Page of DEA1.1 Canine Cytoplasmic Proteins

The SDS PAGE profile of ultracentrifuged dog erythrocytic cytoplasmic fraction showed 5 distinct bands in 10% SDS PAGE. Some of the transmembrane proteins were integrated in cytoplasm also seen in SDS PAGE profile of cytoplasmic fraction (Fig. 3).



L1&L2 - Ghost membrane prepared by hypotonic saline solution with 0.2% OBG
L3 – PAGE ruler prestained protein marker (Page Ruler Thermo fisher scientific)
L4,L5,L6 - Ghost membrane prepared by 50 mM of phosphate buffer saline with 0.1% triton X
L7 – Skim milk powder as standard

Fig. 1. SDS PAGE – Erythrocytic membrane protein



L1 &L2 – Cytoplasmic protein (no bands)
L3– 1 kDa pre stained protein marker(Page Ruler, Thermo fisher scientific)
L4 – Canine RBC membrane antigen (46- 56 kDa, 48 - 56 kDa, 66-85 kDa)
L5&L6 - BSA standard (no bands)

Fig. 2. Western Blot analysis of ghost membrane antigen marker

3.3 Western Blot Analysis of Canine Ghost Membrane Antigen Marker

The antiserum raised in mice against DEA1.1 showed only diffused mass in western blot and did not shows any specific distinct band in western blot analysis of dog erythrocytic ghost membrane antigen due to epitope of membrane antigen damaged in denaturing gel electrophoresis. The ghost membrane antigen transferred PVDF membrane treated with glycoporin A monoclonal antibodies showed clear bands in molecular weight ranges from 28-117 kDa which is the similar to the finding of Barker et.al. (1991). The rabbit anti mouseglycoporin antiserum precipitated three large clear bands of molecular weight 33-40 kDa,48-56 kDa and 66 -85 kDa respectively (Fig. 2).

3.4 Western Blot Analysis of Canine Erythrocytic Cytoplasmic Marker

The western blot analysis of cytoplasmic marker along with alpha antitubulin monoclonal antibody showed clear bands in molecular weight ranges 50 kDa protein (Fig. 4). Monoclonal Anti- α -tubulin antibody recognizes an epitope located at the C-

terminal end of the α -tubulin isoform in a variety of organisms which were used various immunochemical techniques including immunoblotting, solid-phase RIAand immunocyto chemistry using tissues or cultured cell line preparations and immunoprecipitation

3.5 Discussion

The erythrocytic cytoskeleton are plays important role in erythrocyte shape, flexibility and lipid organization [11]. The ghost membrane protein spectrin is the high copy number and bigger size dominant membrane protein and consists of two long flexible structures that are twisted together to form heterodimers proteins with different molecular weights ranges from 240,000 and 220,000 daltons [9]. Erythrocytic membrane protein actin binds to the tail of spectrin tetramers to generate a series of polygons with as the sides. The erythrocytes membrane protein 4.1 promotes and regulate the actin-spectrin association which forms net-like structure by the protein-protein interaction of spectrin, actin and protein 4.1 which lies in the lipid bilayer of RBC membrane. The erythrocytic cytoskeleton protein ankyrin, binds near the middle of the spectrin tetramers to form a link from the network to

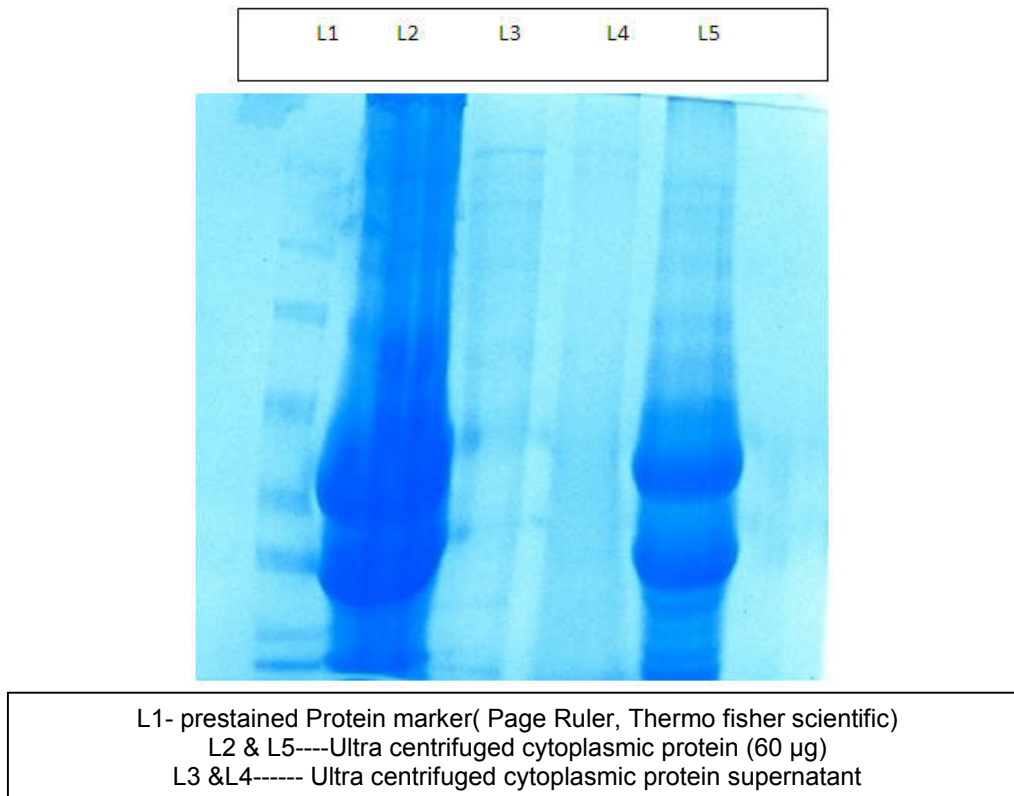
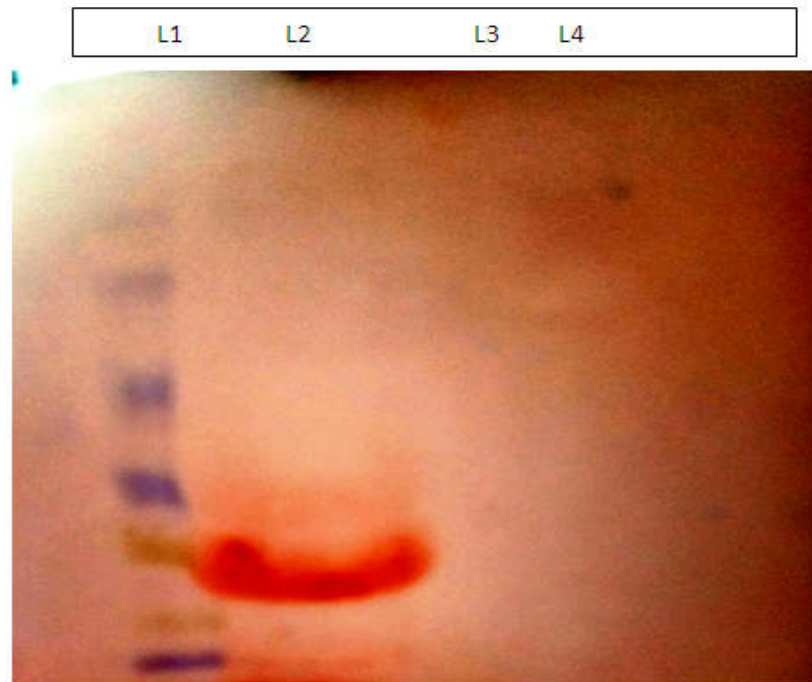


Fig. 3. SDS PAGE – Cytoplasmic protein



L1 – Pre stained protein marker(Page Ruler, Thermo fisher scientific)
L2 – Cytoplasmic protein (50 kDa protein)
L3 and L4 – Canine ghost RBC membrane protein

Fig. 4. Western blot analysis of cytoplasmic protein

membrane protein 3.3. The erythrocytic membrane protein 4.1 links the cytoskeleton to another glycoprotein transmembrane protein to form polyphospholipids [16]. The inherited deficiencies of any of its proteins cause membrane instability, loss of membrane lipid and increased tendency of affected erythrocytes to fuse with each other causes intravascular thrombosis. The membrane strength of erythrocytes were largely dependent on the erythrocyte membrane cytoskeleton. In this experimental study all the seven membrane proteins were separated in SDS PAGE showed similar to the finding of Fukuda et al., [17] who is done similar type of study in human RBC.

The important cytoplasmic marker tubulin is an intracellular, cylindrical, filamentous structure and also the major building block of microtubules and present in almost all eukaryotic cells. Microtubules function as structural and mobile elements in mitosis, cell division, intracellular transport, flagellar movement and the building of cytoskeleton part of erythrocytic membrane. Tubulin consists of α -tubulin and β -tubulin and share considerable homology heterodimer shows the molecular weight of 50 kDa. Totally 7 distinct types of tubulin are identified and have distinct

subcellular localization. Micro tubular systems contain at least three α -tubulin isoforms and two isoforms are coded by two α -tubulin genes which are both transcribed and code for extremely similar proteins. The acetylated form of α -tubulin distributed intracellularly and play the static and dynamic role in cytoskeleton structure of erythrocytic membrane [6]. Identification and molecular confirmation of cytoplasmic marker tubulin importance for diagnosis of certain hereditary diseases, identification of tumour markers, drug targeting and blood typing [18]

The international canine blood grouping committee workshops classified six canine blood group antigens which are named as dog erythrocyte antigen 1.1, 1.2, 3, 4, 5, and 7 by using polyclonal sera [19,20] and monoclonal serum [16]. The DEA 1.1, DEA1.2, DEA1.3 antigens are strong antigenic potential and causing transfusion reaction and the alloantibodies against DEA 1.1 or 1.2 do not occur naturally but dogs can be sensitized following an incompatible first blood transfusion and pregnancy causes alloantibodies against DEA1.1 and DEA1.1. The alloantibodies against DEA 3, 5 and 7 are also present in some dogs but not clinical significance [20]. The structural

and biochemical character of dog erythrocytic antigen is important for the development and characterization of improved blood typing, production antisera and identifying the blood group antigens may act as autoantigens in canine autoimmune haemolytic anaemia [21]. Generally the human blood group antigens which are classified based on the glycolipids and glycoproteins on the surface of the red blood cell membrane [5]. The canine Rhesus (Rh) blood group antigens are also of major clinical importance similar to that of human because of their involvement in haemolytic disease of the new-born puppies, transfusion reactions and AIHA. The Rh -30 polypeptides and the Rh glycoproteins are the two major Rh-related integral membrane groups of glycoprotein determining the blood groups in mammals [22]. The amino acid changes in the Rh, polypeptides are responsible for antigenic polymorphism in the Rhesus system [23]. The autoantibodies derived from AIHA affected erythrocytes of dogs are able to immunoprecipitate membrane proteins and the dog Rh related proteins and glycoproteins in SDS-PAGE have similar electrophoretic mobility to the human erythrocytic membrane glycoprotein [24]. In our studies the dog erythrocytic membrane antigen characterized by SDS PAGE showed similar patterns of membrane protein of human erythrocytic membrane antigen.

Western blot analysis of erythrocytic membrane protein with polyclonal antisera specific for DEA 1.1, 1.2, 3, 4, 5 and 7 against electrophoretically separated and transferred erythrocyte membranes prepared from dog RBC showed no reactivity but same antisera could agglutinate intact RBC with conformational blood group antigens. In western blot analysis, epitopes recognised by the antisera against blood group antigen may be denatured during SDS-PAGE. In this experiment the polyclonal antisera raised against DEA1.1 positive membrane antigen showed no reactivity in western blotting and shows visible agglutination in microscopic and slide agglutination test with intact DEA1.1 positive and negative RBC due to denaturation of epitope in SDS PAGE.

The intracellular interactions of Rh glycoprotein, Rh30 polypeptide and glycoprotein B causes cell surface expression of Rh complex. The 30.5 kDa and Rh50A protein expressed in microsome of cell free translational system. Deglycosylation and inhibition of glycosylation causes reduction of 36 kDa protein without altering 30.5 kDa

protein motility which shows that the 30.5 kDa proteins are unglycosylated and Asn -37 cDNA sequence only glycosylated in the Rh glycoprotein isolated from erythrocytic membrane [23]

The erythrocytic membrane glycoprotein carries some blood group specific genes. Glycophorins with molecular weight of 42 kDa and 29 kDa, are the major autoantigens in canine autoimmune haemolytic anaemia [24]. The canine erythrocyte membrane glycoproteins were identified by using the specific antiserum in western blotting [24]. The canine erythrocyte membrane glycoproteins of MW 42 kDa and 29 kDa represent the rhesus antigen of human. The human Rhesus polypeptide has a complex structure with multiple transmembrane loops having both intracytoplasmic or extracellular regions [25]. The rabbit antisera raised against known peptide sequences of the Rhesus polypeptide and two of these antisera are specific for internal or transmembrane portions of the Rhesus molecule that are not accessible to the antibodies when incubated within intact erythrocytes and not causing agglutination. Same Rhesus antisera was used in western blotting with canine RBC membranes prepared from individual dogs of a range of different blood groups shows reactivity and the molecular weight of three of the canine blood group antigens DEA 1.2, 4 and 7 have a membrane protein have similar homology with human Rhesus blood group.

4. CONCLUSION

In this research study the dog erythrocytic membrane antigen prepared and characterized, analyzed and compared with human erythrocytic membrane antigen which gives preliminary idea about the Rhesus-like molecule importance for blood typing in mammals. The present study has demonstrated the membrane molecular marker glycoprotein A of canine erythrocytic membrane antigen and cytoplasmic marker alpha tubulin shows similarity to the human Rhesus blood group and the potential role for the development of transfusion reaction and study of acute immune haemolytic anemia and other hereditary defects associated with erythrocytic membrane. But in depth detailed further detailed studies are needed in future.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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