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Methods of preparation of dog erythrocytic membrane antigen

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Abstract
Erythrocyte membrane consists of lipid bilayer, cytoskeleton domains and the lipid domain. The structural protein tubulin was not involved in cell motility or phagocytosis and the lipid domain contain nearly equal parts of lipid and protein. Preparation and characterization of dog erythrocytic membrane are important for identification of canine blood groups. Physical method of preparation of ghost membrane showed less purity and time consuming. The chemical hypotonic osmolysis method showed high yield of membrane protein as well as less contamination with cytoplamic protein. The combination of chemical osmolysis, detergent solubilisation and sonication gave good ghost erythrocytic membrane profile in SDS - PAGE. The normal erythrocytic membrane protein pattern depends on the method of preparation and the degree of washing. Thoroughly washed membranes lack of visible band 8 and whereas globin and partially washed membranes have relatively higher quantities of bands. Small amounts of spectrin may also be lost during *in vitro* lysis and washing procedure.

Keywords: Dog erythrocytic membrane, lipid bilayer, canine blood groups, chemical osmolysis

Introduction
Mammalian whole blood consists of two basic parts. Plasma, the non-cellular liquid part, makes up approximately 55% of total blood volume. It is approximately 90% water, 7% protein, 3% other organic and inorganic molecules and ions. The cellular components of the blood make up approximately 45% of the total cell volume. Approximately 99% of the cells are red blood cells [13, 14]. The intracellular compartment of the mature mammalian red cell consists entirely of a cytoplasm containing aqueous solution of simple inorganic, organic molecules and macromolecules, with a very high concentration of the protein haemoglobin. This lack of cellular structure enables mammalian red blood cells to efficiently squeeze through the smallest capillaries and mammalian red blood cells produce ATP by glycolysis and lactic acid fermentation. Erythrocyte membrane consists of lipid bilayer. The cytoskeleton domains and the lipid domain which are structurally similar in most mammalian cells and the structural protein tubulin was not involved in cell motility or phagocytosis and the lipid domain contain nearly equal parts of lipid and protein called as sialoglyco protein. The cholesterol equally distributed between the two halves or leaflets of the lipid bilayer and the other lipids were asymmetrically distributed as glycolipids, phosphatidylcholine and sphingomyelin which were located in the outer half of the bilayer and phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine occur in the interior layer facing the cytoplasm. The lipid domain fluidity of erythrocyte was determined by the molar ratio of cholesterol to phospholipid, degree of unsaturation of phospholipid acyl chains and phosphatidylcholine sphingomyelin ratio [14, 16].
The canine erythrocytic membrane glycophorin was the major auto antigen causing autoimmune haemolytic anaemia and was identified by using specific polyclonal antiserum in western blotting. They used polyclonal antisera specific for DEA 1.I, 1.2, 3, 4, 5 and 7 in western blotting against dog erythrocytic membrane protein [1]. They found no reactivity with any antiserum and antisera can agglutinate intact RBC with conformational blood group antigens. The epitopes recognized by the reagents may be denatured during SDS-PAGE [13]. The membrane molecular weight of glycophorins ranges from 42 and 29 kD and are the major blood group specificity auto-antigens in canine erythrocytes. They concluded that canine erythrocyte glycophorins with the molecular weight 42 and 29 kD of canine represent that the rhesus antigen of human being.

The isolation and characterization of dog erythrocytic membrane are important for identification of canine blood groups and immune mediated hemolytic diseases [1].

Materials and Methods

Blood sample collection

The dog blood samples were collected from Veterinary Peripheral Hospital, Tamil Nadu Veterinary and Animal Sciences University, India for research purpose. The blood samples were collected in EDTA vial and the packed RBC was prepared and counting was done in haematology analyser. 2X 10^{10} cells/ml were taken for erythrocytic membrane preparation.

Physical method of preparation of dog erythrocytic membrane antigen

Centrifugation

5 ml of canine blood sample was centrifuged at 3000 rpm for 10 min. The supernatant, plasma and buffy coats were removed. The RBC pellet was washed for three times with PBS and again centrifuged at 12000 rpm for 15 min. The upper platelet layer was removed with pipette and the RBC pellet was washed three times with PBS. The cell pellet was vigorously shaken for 1 min and again the RBC pellet was centrifuged at 20000 rpm for 30 min. The supernatant was removed and the pellet was re-suspended in PBS and stored at -80 °C.

Homogenization or physical disruption

5 ml of canine blood sample was centrifuged at 3000 rpm for 10 min. The plasma and buffy coats were removed The RBC pellet was washed three times with PBS and again centrifuged at 12000 rpm for 15 min. The upper platelet layer was removed with pipette and the RBC pellet was washed three times with PBS. The cell pellet was agitated by using glass rod and triturated vigorously for 30 min and centrifuged at 10000 rpm for 10 min. The supernatant was removed and the cell pellet was washed three times and stored at -80 °C.

Sonication

5 ml of blood sample centrifuged at 3000 rpm for 10 min. The supernatant plasma and buffy coats are removed. The RBC pellet was washed three times and again centrifuged at 12000 rpm for 15 min. The upper platelet layer was removed with pipette and the RBC pellet was washed three times with PBS. Again the pellet was centrifuged at 12000 rpm for 30min. The supernatant was removed and the pellet was washed three times and the pellet was sonicated with ultrasonic probe for 30 second burst with 30 second intervals for 10 min.

Chemical methods

Hypotonic osmolysis method

5 ml of blood sample was centrifuged at 3000 rpm for 10 min. The plasma and buffy coats were removed and the cell pellet was vigorously shaken for 1 min and vortexed for 2 min. The RBC pellet was washed three times with PBS and again centrifuged at 12000 rpm for 15 min and the supernatant was removed. 5 ml of 0.15M NaCl was added to the RBC pellet, shaken vigorously and then centrifuged at 12000 rpm for 15 min. The supernatant was removed and pellet again re-suspended with 5 ml of 0.15% NaCl and shaken vigorously then centrifuged at 12000 rpm for 15 min and the supernatant was discarded. Then the pellet was washed three times with PBS and pellet was re-suspended in isotonic NaCl solution with 10mM of PMSF and stored at -80 ˚C.

Hypotonic osmolysis method by using hypotonic phosphate buffer

The DEA1.1 ghost membrane antigen was prepared as per the method described by Di Girolamo *et al.* (2010) [7] with slight modification. 5ml of DEA1.1 canine blood sample was centrifuged at 1500 rpm for 15 min at 4 °C. The supernatant plasma and buffy coats were removed and the RBC pellet was washed three times with PBS containing 10 mM PMSF protease inhibitor. The samples were centrifuged at 1800 rpm for 20 min at 4 °C and remaining leukocytes and the platelets were completely removed. The 2 $X10^{10}$ RBC concentration was used for ghost membrane preparation. The pellet was diluted 10 times with 150 mM of NaCl which contain 10 mM of PMSF and centrifuged at 13000 rpm for 30 min at 4 °C. The supernatant was removed and the pellet was again re-suspended in 2 ml of 0.15% NaCl and shaken vigorously, centrifuged at 13000 rpm for 15 min and the supernatant was discarded. The lysed cell pellet was treated with ice cold 10 volumes of 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 haemoglobin. The EDTA-lysed membrane preparations were then re-suspended in the isotonic phosphate buffer containing 10 mM of PMSF and 0.1% tritonX 100 with 10 mM PMSF. The protein concentration were estimated by Bradford assay and stored at -80 ˚C.

Tris EDTA lysis method

5 ml of blood sample was centrifuged at 3000 rpm for 10 min. The supernatant, plasma and buffy coats were removed and the cell pellet was vigorously shaken for 1 min and vortexed for 2 min. The RBC pellet was washed three times with PBS and again centrifuged at 12000 rpm for 15 min. The supernatant was removed and washed red cells were lysed by mixing with 10 volumes of 1 mm EDTA, 10 mm Tris-HCI, pH 7.4, for 10 min in the cold. The lysed cells were washed three times with the same EDTA buffer. The centrifuged membranes were white, showing no traces of attached haemoglobin. The EDTA-lysed membrane preparations were sonicated and re-suspended in the isotonic buffer with protease inhibitor and stored at -80 ˚C.

Freeze thaw chemical osmolysis method

The DEA1.1 ghost membrane antigens were prepared as per the method described by Di Girolamo *et al.* (2010) [7] with slight modification. 5 ml of DEA1.1canine blood centrifuged at 1500 rpm for 15 min at 4 °C. The supernatant plasma and buffy coats were removed and the RBC pellet was washed three times by PBS with 10 mM PMSF protease inhibitor. The samples were centrifuged at 800 rpm for 20 min at 4 °C and remaining leukocytes and platelets were completely and 2 $X10^{6}$ RBC concentrations was used for ghost membrane preparation. The pellet was diluted 10 times with 150 mM of NaCl which contain 10mM of PMSF. 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 re-suspended with 2 ml of 0.15% NaCl and shaken vigorously, centrifuged at 13000 rpm for 15 min and the supernatant was discarded. The lysed cell pellet was treated with ice cold 10 volumes of 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 was white ghost. The lysed ghost membrane preparations were re-suspended in the isotonic phosphate buffer with 10 mM of PMSF, 0.2% OPG and another isotonic phosphate buffer with 0.1% triton X 100 and 10 mM PMSF. The protein concentration of ghost membrane protein was estimated and samples were and stored at -80 °C.

SDS - PAGE was performed as described by Sheng-Xiang *et al.* (1997) with slight modification. The reduction and alkylation of membrane protein samples were carried out by adding 1 µg DTT to 50 µg of RBC membrane proteins and the samples were incubated for 30 min at room temperature. The membrane protein were separated by SDS-PAGE by using 10 µl of loading buffer containing β-mercaptoethanol, SDS, glycerol, TRIS-HCl and bromophenol blue and 30 µl of RBC membrane proteins and the mixture was heated for 10 min at 70 ˚C. The samples were subjected in electrophoresis with 4-12% polyacrylamide gel using tris glycine running buffer containing 4 µl of reducing agent. Finally the gel was fixed in 40% methanol, 10% acetic acid solution and stained with Coomassie blue dye and after destaining SDS profile was analyzed.

Results and Discussion

In this study dog erythrocytic membrane antigen was prepared by various methods and the membrane proteins were separated by SDS PAGE. The high speed centrifugation removed other components of blood, disruption of the cell membrane and the internal content of RBC was removed. In this method lysis of RBC is incomplete and the purity was less with moderate amount of contamination of cytoplasmic protein and less yield of ghost membrane [6, 7]. Homogenization or physical disruption was an easy and simple method and this process is very sensitive and care must be taken to avoid complete destruction of the ghost membrane which leads to low yield of membrane protein. In sonication method, the high frequency sound waves were used to lyse the cells. Mechanical energy from the probe initiates the formation of microscopic vapour bubbles that form momentarily and causing shock waves which radiated through blood samples and lyse the cells [18-20]. Chemical osmolysis method was widely used method for preparation of erythrocytic membrane. Osmotic lysis can be used to disrupt some cells such as mammalian red blood cells and WBC. In this method, a buffered hypotonic solution followed by simple mechanical agitation can effectively lyse the cells by osmotic swelling, the cells to the point of lysis and mechanical agitation was employed to break open swollen cells which were not lysed. Most of the good quality ghost RBC membrane prepared by using phosphate buffer method and additionally 0.5 mM of the protease inhibitor, phenylmethylsulfonyl fluoride (PMSF) was added to the lysis buffer. EDTA-lysis ghost RBC membrane method used for estimating different components of ghost RBC membrane

The anionic, cationic and non-ionic detergent was commonly used for disrupting cells. All detergents damage the cell wall or membrane and these will lead to release of intracellular content. One of the most commonly used anionic detergent was sodium dodecyl sulfate (SDS) which reorganizes the cell membrane by disturbing protein-protein interactions and another commonly used compound for cell lysis was triton X100, which was non-ionic detergent which solubilized membrane proteins and showed good separation of membrane protein in SDS PAGE (Harrison 2011) [10].

The erythrocytic membrane protein consists of integral membrane protein, trans-membrane protein and extra cellular surface membrane proteins. The integral membrane protein consists of Anion transport protein (band 3), glycophorins: A, B, C, Na--K ATPase, blood group antigens Rh lipoproteins. The trans-membrane protein consists of spectrin (bands 1 & 2), spectrin binding proteins, ankyrin (band 2.1), syndeins (bands 2.1-2.6), bands 4.1-4.2, Bands 4.1-4.2, actin (band 5), glyceraldehyde-3-phosphate dehyndrogenase (band 6), band 7. The extra cellular surface membrane protein consists of acetylcholine esterase and blood group antigen. The spectrin consists of a complex of two polypeptides (bands 1 and 2) which have molecular weights of about 240,000 and 220,000 dalton respectively. The 20 to 25% of the total membrane proteins are spectrin polypeptide. Spectrin is water soluble, and its two subunits have the capacity to form dimers or tetramers depending upon the conditions of isolation and methods of purification**.** The structural components of the erythrocytic cytoskeleton were the heterodimers of double stranded spectrin which form tetramers by head-to-head associations. These tetramers may be connected into microfibrillar oligomeric complexes of actin**.** Receptor proteins b and d tightly twisted in surface membranes which was directly inserted into hydrophobic peptitide portions into the lipid bilayer [12]. The glycophorin A, is a sialic acid-rich glycoprotein and the dimeric form of glycophorin A migrates with an apparent molecular weight of 83,000 daltons. Glycophorin A monomer (PAS 2) had approximately 45.000 and glycophorin B (PAS 3) was about 25.000 daltons [2, 19, 20].

Red cell membrane proteins are usually separated and classified by sodium dodecyl sulfate polyacrylamide gel electrophoresis. There were seven major polypeptide bands determined by this technique. The normal erythrocytic membrane protein pattern in SDS PAGE depends on the method of preparation and the degree of washing of the membranes. Thoroughly washed membranes lack of visible band 8 and globin protein and partially washed membranes showed relatively higher number of bands and good resolution in SDS PAGE. Small amounts of spectrin may also be lost during *in vitro* lysis and washing procedure. The fluid-mosaic model postulates that membrane proteins are globular to account for the high content of alpha helix and are variably and asymmetrically embedded within the lipid bilayer [1, 2]. Membrane proteins, were classified as integral and peripheral. Bands 1, 2 (spectrin) and 5 (actin) join together to form a submembranous cytoskeleton of the red cell. Bands 2.1 (ankyrin), 2.2 to 2.6 (syndeins) and 4.1 to 4.2 have been implicated in linking spectrin to the membrane. The visco elastic spectrin-actin microfibrillar meshwork influences cell shape and provides anchoring sites at the cytoplasmic membrane surface for transmembraneous proteins [6].

Rasmussen and Rasmussen (2000) [17] developed a new method of protein isolation by using short proteinase treatment and homogenization in ionic buffers followed by two-stage centrifugation. They found that this method showed high mitochondrial integrity and purity but the contamination of genetic materials and protein degradation remains higher in proteinase treatment. Helenius and Simons (1975) [11] analyzed different buffer system used for tissue homogenization solublization and preservation of targeted proteins. They identified 0.1-1% triton X 100 increase the solubility of non-polar protein and 6-8 M of urea causes

disruption of protein hydrogen bonds and increases solubility of protein. They found that 1-100 mM of sodium chloride causes membrane disruption and protein solubility, 1-2% SDS causes membrane disruption, 1mM of EDTA inhibits the metalloproteases and prevention of changes in protein phosphorylation, 1-10 mM of β mercaptoethanol and dithiothritol causes cleavage of disulphide bond and protein denaturation.

Thermolysis method had potential and more common in large scale production. The periplasmic proteins in G (-) bacteria are released when the cells are heated up to 50 ºC. Cytoplasmic proteins can be released from cells within 10 min at 90 °C. The high protein release had been noticed after short high temperature shocks, than longer low temperature exposures but the results were highly unreliable due the protein solubility changes with temperature fluctuations [7, 15]. Freezing and thawing of a cell can cause the cells to burst due to frequent formation and melting of ice crystals. Gradual freezing causes formation of larger crystals which causes an extensive damage to the cell but freezing and thawing causes loss of enzyme activity [8-10].

The chemical methods used for preparation of ghost membrane were risky for the disruption of sensitive and fragile cells because the solvents and detergents used for membrane preparation can cause protein denaturation, damaging the final product. The problem related to chemical methods was the removal and recovery of the chemical disrupter and also the chemical methods also have low efficacy and more expensive and less useful as compared to physical disruption methods. The chemical solvents like alcohols, dimethyl sulfoxide, methyl ethyl ketone or toluene can be used for cell lysis. These solvents can extract cell wall lipid components which leads to release of intracellular components and these method can be used with wide range but some of the fine proteins were denatured [6] In addition to solvents, cell lysis can be achieved by hydrolysing the cell wall by alkali compound (pH 10.5-12-5) but the chemical costs for neutralization of alkali were high and the product may not be stable in alkali conditions. The enzymes were utilized for cell lysis process [10]. The chemical methods were not generally applied in large scale processes because the cost of enzymes was high and limited availability was limits their utilization in large scale processes but the added enzyme may complicate purification. Immobilization of enzymes rectify the problems and digestive enzymes will decompose the cell membrane. Different cell types and strains have different kind of cell walls and membranes and the uses of enzyme depends on types of cells [5].

The physical methods are only viable at laboratory scale, due to their cost-effectiveness, scale-up difficulty and well suited for industrial scale High energy requirements and high pressure requirements were the main disadvantages of physical methods. The ultrasound method may offer significant energy savings when compared to solid shear mechanical methods. The difficulty of sterilization and cleaning procedures makes mechanical methods susceptible to contamination. Sonication method had severe health and safety issues, resulting from noise. The physical methods requires pressure and temperature. These conditions need to be strictly monitored as they may affect protein release, protein solubility and cause undesirable effects on the end products. Changing temperatures, used in thermolysis, may cause cells to burst or may damage cells with the formation of crystals. Temperature variation also affects the activity of enzymes and may alter three-dimensional structures and the major problem with physical methods is their high cost [8, 15]. Chemical methods used were risky to use for the disruption of sensitive cells, the used solvents and detergents can cause protein denaturation, damaging the final product and the chemical methods have low efficacy, making them more expensive and less useful as disruption methods [18]. A significant issue related to chemical methods is the removal and recovery of the chemical disrupter. The combination of physical methods like centrifugation and sonication, chemical method like hypotonic osmolysis and detergent solubilisation required for better SDS PAGE profile [20].

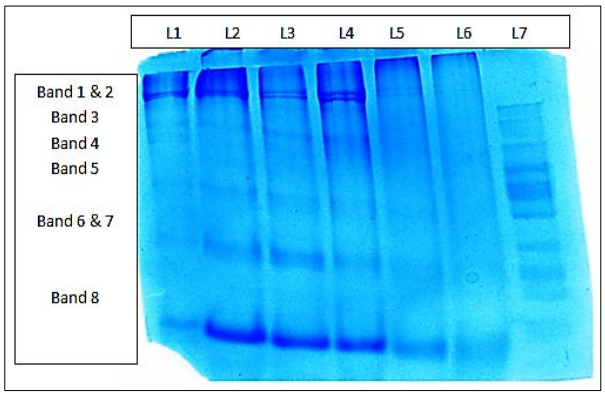

Fig 1: Dog erythrocytic membrane prepared by chemical osmolyis

L1 to L6: 6 numbers of different canine ghost membrane prepared by chemical osmolysis + sonication + detergent solubilization, L7: Protein marker
Band 1 & 1: α & β spectrin, band 3, 4 & 5: Anion transporter glycophorin A, B and 6 & 7: glycophorin C
Band 8: glycophorin B

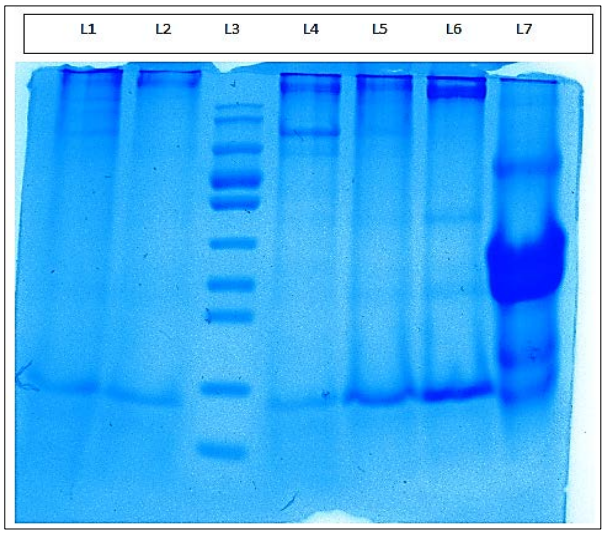

Fig 2: Dog erythrocytic membrane prepared by different methods

L1: Chemical osmolysis thermal shock + sonication,
L2: Chemical osmolysis enzyme digestion + sonication
L3: Protein marker,
L4: Chemical osmolysis + sonication
L5 & L6: chemical osmolysis freeze thaw method + sonication + detergent solubilization
L7: Physical method

Conclusion

Among these methods, the freeze thawing, chemical osmolysis with hypotonic phosphate buffer saline showed less contamination with cytoplasmic protein, minimum loss of

ghost membrane protein and SDS PAGE profile when compared to other physical and chemical methods of ghost membrane preparation, however further detailed studies are needed in future.

Declaration section
Ethical approval and consent to participate: Not applicable
Consent for publication: Yes I am agree
Availability of supporting data: Not applicable
Conflict of interest: Not interested

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