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Effects of albumin depletion by TCA/Acetone method on plasma electrophoretic protein profile of diarrhoeic buffalo calves

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Abstract
For identification of diagnostic/prognostic indicators for infectious diarrhoea in buffalo calves, blood and faecal samples from 30 diarrhoeic calves were collected. Six healthy buffalo calves' constituted goup I as control. Out of 30 diarrhoeic calves from which blood and faecal samples were collected, 3 animals were found positive for *Balantidium coli* (Group II), 16 animals for *Strongyle/Strongyloid spp.* (Group III), 3 animals for *Eimeria spp*. (GroupIV), and rest 8 animals were not having any parasitic burden and grouped as non- parasitic one (GroupV). After removal of excess of albumin, separation of plasma proteins by SDS-PAGE revealed clearly disease related changes in protein/peptide bands. In group- I, total number of protein/peptide bands in TCA/Acetone treated precipitate were 17, while 17, 15, 14 and 19 number of major protein bands were present in groups-II to V respectively. Similarly, 5 major protein bands were present in supernatant of control group whereas 8, 9, 9, 9 protein bands were present in diarrhoeic groups II to V respectively. Proteins having molecular wt. of approx. 43.3 kD corresponding to alpha-1 acid glycoprotein was found to be down regulated in groups III and IV indicating lowered ability of the animals to neutralize toxins whereas upregulation of proteins having molecular wt. of approx. 62.2 and 44.6 kD corresponding to α-2 macroglobulin and α-1 protease inhibitor respectively. The main functions of these proteins to mainly inhibit proteases and is found to be increased in chronic liver diseases pointing towards the damaging effects of infection on liver.

Keywords: Albumin depletion, TCA/Acetone, SDS-PAGE, protein profiling, buffalo calves

1. Introduction
Livestock is the backbone of the dairy industry and agricultural production system in Indian economy as well as in socio-economic development of millions of rural household. Calf diarrhea is a commonly reported disease and a major source of economic loss to farmers and cattle industry. Diarrhoea being the leading cause of death in calves aged less than six months is caused by a variety of pathogens including bacteria, viruses, protozoa and intestinal parasites [1].

Animal body shows two types of immune reaction to any type of injury. One is specific immune reaction mediated by antibodies and the other is innate nonspecific immune reaction like fever, cytological reactions etc. This innate nonspecific immune reaction of the body is otherwise known as acute phase response. The main aim of acute phase response is to maintain homeostasis and tissue healing. In the acute phase response serum/ plasma level of some kind of proteins are found to decrease while the levels of some other proteins increase many folds. Canines with coccidiosis infection showed significantly lower concentrations of α1-globulins, while ancylostomosis infection had higher values of α-2 globulins by serum protein electrophoresis [2].

The evaluation of serum proteins and their electrophoretic pattern is a well-established laboratory method in the diagnosis of many diseases in humans, which has replaced the biochemical determination of the concentrations of albumin and the ratio of albumin to globulins. The results of serum protein electrophoresis can be one of the most useful diagnostic aids in a wide spectrum of diseases, including infectious and inflammatory diseases, renal or gastrointestinal disorders, immunodeficiency states, as well as paraproteinaemias caused by lymphoid or plasma cell neoplasia [3-4]. Plasma has a huge amount of albumin which hampers the visibility of other proteins in electrophoretic separation. So excess of albumin was removed by using 10%TCA/Acetone. When excess of albumin was removed from plasma, there were two portions, one was

supernatant in which excess of albumin was present and other was precipitate in which rest of the proteins were present [5]. Although many studies have been carried out to determine the usefulness of serum proteins and their electrophoretic pattern in various disease conditions and disorders in animals. Serum protein evaluation is still a relatively little-used diagnostic tool in veterinary medicine.

2. Material and Methods

2.1 Materials

i. Animals

Under this study, thirty buffalo calves (1–6 months of age, male and female) suffering from diarrhoea either referred to the TVCC, or in the nearby villages of Hisar district were used for sampling. Samples were collected after 3-5 days of illness. In addition to these, six healthy calves from LUVAS farm were kept as control and constituted Group-I.

ii. Clinical examination

The most prominent clinical signs among the buffalo with diarrhoea were mild to severe diarrhoea, depression, dullness and depraved appetite. The animals were weak and reluctant to move.

The animals were screened for presence of parasitic ova by floatation technique. The animals which were found positive for the presence of *Balantidium coli, Strongyle and Strongyloid spp., Eimeria spp.,* constituted as Groups II, III and IV respectively as tabulated in Table-1. Animals having profuse diarrhoea but faecal samples were negative for parasitic ova constituted Group-V.

Table 1: Various groups under study and number of animals in each group.

Groups	Number of animals in each group	Faecal samples positive for
Group I (Control)	6	None
Group II	3	*Balantidium coli*
Group III	16	*Strongyle/strongyloid spp.*
Group IV	3	*Eimeria spp.*
Group V	8	Non parasitic diarrhoea

iii. Collection of blood samples

Approximately 5 ml blood was collected from jugular vein using 10 ml disposable syringe aseptically. Blood was poured in centrifuge tube containing heparin for separation of plasma. Faecal samples were also collected in sterile containers to avoid contamination and were subjected to parasitological examination. For separation of plasma the blood samples collected in centrifuge tube containing heparin were centrifuged at 3000 rpm for 10 minutes; the plasma was separated in aliquots and buffy coat layer was removed to harvest the red blood cells.

2.2 Methods

i. Albumin depletion from plasma

Excess of albumin was depleted from plasma by a modified protein precipitation procedure for efficient removal of albumin from serum (Chen *et al*., 2005) [5]. 20 µL sample of plasma was precipitated by rapid addition of four volumes of ice-cold acetone containing 10% w/v TCA and was immediately mixed by gentle vortexing. The mixture was incubated at -20 ºC for 90 min and centrifuged at 8000 × g, at 4 ºC for 20 min. The supernatant was removed and collected. Then 1 mL of ice-cold acetone was added to wash the precipitate. The sample was incubated on ice for 15 min and centrifuged as above. The acetone-containing supernatant was removed and the precipitate was collected. One ml ice-cold acetone was added to the 10% TCA/Acetone containing supernatant to completely precipitate the proteins in the supernatant. These two portions were resuspended in the buffer of pH 6.8 and 0.5 M molarity.

ii. Plasma protein electrophoretic profile

12% resolving and 4% stacking gels were polymerized between two glass plates in a gel caster, with a comb inserted at the top of the stacking gel to create the sample wells. After the gel was polymerized, the inner chamber was filled with running buffer and the comb was removed. The samples to be analyzed and the Prestained Protein Molecular Weight Marker (Thermo Scientific Prestained protein molecular weight marker #26612, 120kD-20kD) were mixed with sample buffer in 1:1 ratio. The mixtures were heated at 95°C for 5 minutes and then cooled before loading. A constant electric field (90 Volt) was applied across the gel, causing the negatively-charged proteins to migrate across the gel towards the positive (+) electrode (anode). Depending on their size, each protein was moved differently through the gel matrix. Then the gel was stained with 0.25 % Coomassie Brilliant Blue R-250 for 1hr with continuous shaking. Then it was destained by overnight incubation in destaining solution. The molecular weights of the peptides were estimated by EZ Gel documentation system using Image lab software.

3. Results

Pooled plasma samples from each diarrhoeic group as well as healthy control group were subjected to precipitation and precipitation electrophoretic separation of various proteins.

Table 2: Approximate molecular weight of protein bands in precipitate portion

S. No.	Marker	Group I	Group IV	Group III	Group II	Group V
1.	120kD	120	120	120	120	120
2.	-	107.4, 94.7	112.2, 96.8	111.4, 96.8	106.6, 93.3	116.5, 104.3, 91.3
3.	90kD	-	90	90	-	-
4.	-	87.5, 80.1, 65.2, 59.3, 53.5	85.2, 70.3, 63.9, 56.9	85.2, 68.9, 63, 56.1, 51.7	85.2, 76.9, 62.2, 52	83.4, 75.3, 63, 56.9, 51.7
5.	50kD	-	-	-	-	-
6.	-	49.1, 41.3, 37.4, 34.2	49.6, 44.1, 37,	45.9, 40.3, 36.6	48.3, 44.6, 40.2, 36.8	48.1, 44.4, 40.3, 36.7
7.	34kD	-	-	-	-	-
8.	-	31.4, 27.4	31.4	31.4	33.9, 31.1, 27.1	33.8, 30.9, 26.9
9.	-	43.3	-	-	43.3	43.3
10.	-	37.4	-	-	37.4	37.4
11.	26kD	-	-	-	-	-
12.	-	-	23.3	22.8	-	-
13.	20kD	20	20	20	20	20

Gp - I, Healthy control; Gp -II, *Balantidium coli* positive; Gp- III, *Strongyle/Strongyloid* spp. positive; Gp- IV, *Eimeria spp.* positive; Gp –V, Non parasitic diarrhoeal group.

Major protein bands in the electrophoretic protein profile of precipitate in all groups were visualized. In group- I, total number of protein bands were 17 while 17, 15, 14 and 19 number of major protein bands were present in groups-II to V, respectively (Table 2 and fig 1). Protein bands of molecular weight nearly 43.3 kD were less intense in groups III and IV as compared to other groups. 62.2kD molecular weight protein band was present in lane 4 which represents group II. Protein bands of molecular weight nearly 44.6 and 44.4 kD were present in group II and V respectively.

Similarly, 5 major protein bands were present in supernatant of control group whereas 8, 9, 9, 9 protein bands were present in diarrhoeic groups II to V respectively as shown in table 3.

Table 3: Approximate molecular weight of protein bands of supernatant portion

S. No.	Marker	Group I	Group IV	Group III	Group II	Group V
1.	120kD	120	120	120	120	120
2.	-	118.1	119.3, 102.3	104.4	104.4	106.6
3.	90kD	-	-	-	-	-
4.	-	72.6	71.3, 62.2	75.5, 66.7, 59.9, 54.1	75.9, 66.3, 50.2, 39.7	78.1, 68.2, 39.9, 31.74
5.	50kD	-	-	-	-	-
6.	-	-	39.2	39.5	39.7	39.9
7.	34kD	-	-	-	-	-
8.	-	30.9, 21.7	31.5, 23.1	31.4, 22.6	31.4, 22.6	31.7, 22.6
9.	20kD	-	-	-	-	-

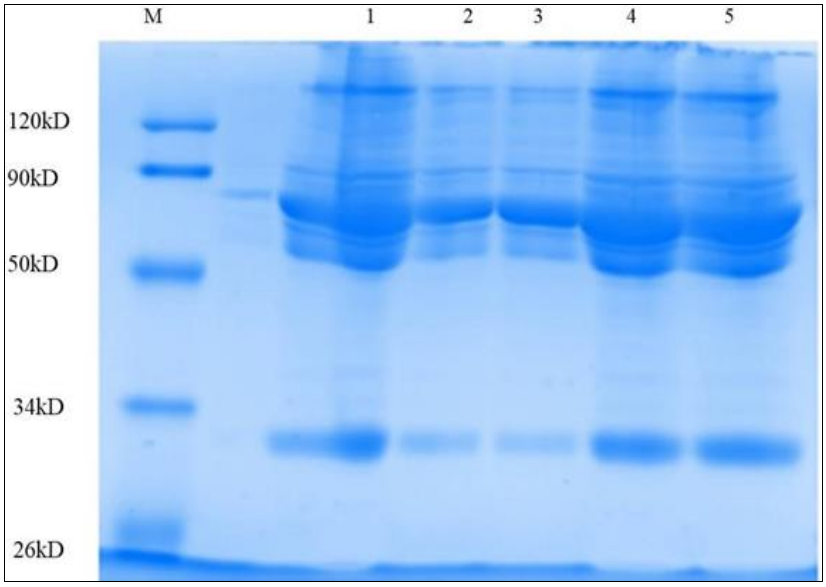

Fig 1: SDS-PAGE electrophoretic profile of plasma proteins in precipitate after albumin depletion from pooled plasma samples in various groups.

Gp - I, Healthy control; Gp -II, *Balantidium coli* positive; Gp- III, *Strongyle/Strongyloid spp.* positive; Gp- IV, *Eimeria spp.* positive; Gp –V, Non parasitic diarrhoeal group.

Lane M: Marker	Lane1: Group I	Lane2: Group IV
	Lane3: Group III	Lane4: Group II
	Lane5: Group V	

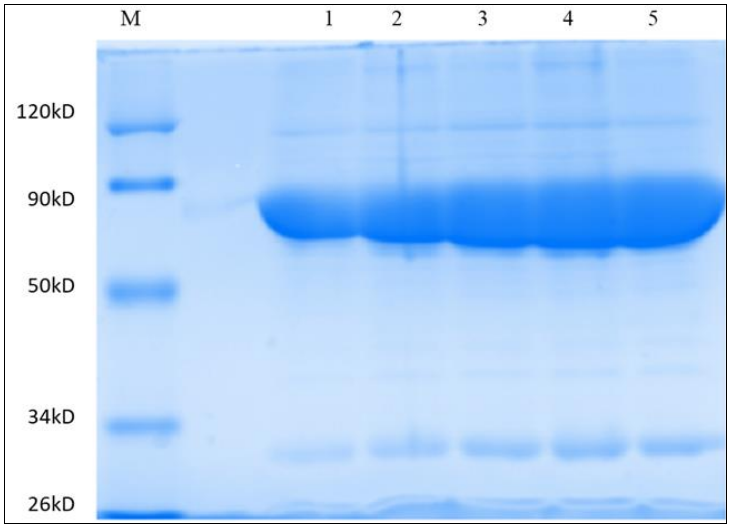

Fig 2: SDS-PAGE electrophoretic profile of plasma protein in supernatant after albumin depletion in pooled plasma samples of various groups

Gp - I, Healthy control; Gp -II, *Balantidium coli* positive; Gp- III, *Strongyle/Strongyloid spp.* positive; Gp- IV, *Eimeria spp.* positive; Gp –V, Non parasitic diarrhoeal group.

Lane M: Marker	Lane1: Group I	Lane2: Group IV
	Lane3: Group III	Lane4: Group II
	Lane5: Group V	

4. Discussion

SDS-PAGE system introduced by Laemmli (1970) was used for separation of plasma proteins. Separation on SDS-PAGE increased the complexity for interpretation of the electrophoretogram. The proteins were no longer grouped in the familiar regions, but were in a series of bands defined by relative molecular mass. The treatment and breakdown of complex proteins into their component subunits complicated interpretations. The high abundance of just a few of the proteins, such as albumin caused further difficulty in interpretation. After removal of excess of albumin, separation of plasma proteins by SDS-PAGE revealed clearly disease related changes in protein/peptide bands (Figure 1 and 2). Similarly, Fagliari *et al.*, 1998 [6] and Kiral *et al.*, 2004 [8] reported the variations in protein bands after removal of highly abundant serum proteins.

Protein bands having molecular weight of 43.3 kD were less intense in groups III and IV as compared to other groups indicating down regulation of these proteins in these groups and 62.2kD molecular weight protein band was present in group II. The protein having molecular weight nearly 43kD corresponds to alpha-1 acid glycoprotein (AGP) as revealed from literature. The ability of AGP to bind low molecular weight ligands may also have a general protective role as it binds to toxic lectins and endotoxins (Israili and Dayton, 2001). Protein bands of molecular weight nearly 44.6 kD were present in group II and V respectively.

The unique bands of molecular weight 62.2 kD and 44.6 kD in *B. coli* infected animals showed up regulation of these proteins which corresponds to alpha-2 macroglobulin and alpha-1 protease inhibitor respectively. The main function of this protein is to mainly inhibit proteases and is found to be increased in chronic liver diseases pointing towards the damaging effects of infection on liver.

Animals suffering from non-parasitic infection also showed expression of new protein having the molecular weight of approx. 44.4 kD similar to as observed in group II.

In conclusion, APPs concentrations increase continuously during some chronic and/or subclinical disease conditions whereas concentrations also decrease under certain disease conditions. So up regulation and down regulation of APPs are of equal importance under various parasitic and non-parasitic disease conditions.

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