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Lipid and acute phase proteins' profiling in diarrhoeic buffalo calves

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Abstract
In dairy industry diarrhoea being the main cause of death in calves during early stages of life. It is caused by a variety of organisms. Irrespective of etiological agents, the physiological and biochemical conditions of the host always get affected due to stress and other causative agents. Out of 30 diarrhoeic calves from which blood and faecal samples were collected, 3 animals were found positive for *Balantidium coli*, 16 animals for *Strongyle/ Strongyloid spp.*, 3 animals for *Eimeria spp.* and rest 8 animals were not having any parasitic burden and grouped as non- parasitic one. The blood collected was estimated for haematological parameters whereas plasma was estimated for triglycerides, acute phase protein profile of diarrhoeic calves. Plasma acute phase protein Ceruloplasmin was also found to be increased significantly (p<0.05) indicating inflammatory reactions due to systemic infections.

Keywords: caeruloplasmin, fibrinogen, triglyceride, diarrhoea, buffalo calf

Introduction
Livestock sector is mainly responsible for socio-economic development and upliftment in developing countries like India. Diarrhoea, being the leading cause of death in calves is a major source of economic loss to the cattle industry. Diarrhoea has multifactorial causes including bacteria, viruses, protozoa and intestinal parasites. Several parasites like *Balantidium coli*, *Strongyle/Strongyloid spp.*, *Eimeria spp*. are associated with calf diarrhoea. Along with these, entero-pathogens like *Escherichia coli*, *Salmonella typhimurium*, *Clostridium perfringens*, bovine rotavirus, coronavirus and the protozoan parasite *Cryptosporidium parvum* are also responsible for causing diarrhoea (Izzo *et al.*, 2011, Gastroenteritis, dehydration and loss of body fluid leads to alterations in various serum metabolites (Edwards and William, 1972) [8, 7].

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. These proteins are known as acute phase proteins (APP). These proteins include protease inhibitors, coagulation proteins (e.g. fibrinogen, prothrombin), complement proteins, transport proteins (e.g., Haptoglobin (Hp), Ceruloplasmin (Cp), hemopexin) and some other kind of proteins, like C reactive protein, serum amyloid A (SAA), serum amyloid P (SAP), acid glycoprotein (AGP) etc. It is well known that acute phase proteins (APPs) are either positive (up-regulated; Hp, Fb and SAA) or negative (down-regulated; albumin, transferrin and α-fetoproteins) regulated depending on the response to the challenge (Kaneko, 1989) [8]. Among the positive acute phase proteins, the serum level of some APP increase 10 to 100 or even1000 folds within a few hours after injury. They are called as major APPs. Proteins whose levels increase 2-10 times and their value decline to normal after longer period are known as moderate APPs and those with slight increase in serum level are known as minor APPs. Moderate and minor APPs are more pronounced in chronic inflammation (Ceron *et al.*, 2005) [3].

Use of acute phase proteins as biomarkers for animal disease diagnosis and health status assessment has got high potential in modern veterinary practice. Research on serum acute phase proteins (APP) provides a lime light in the area of non specific biomarkers. This acute phase response can be accompanied by alterations in lipid metabolism in the form of higher serum triglycerides and lower high-density lipoprotein levels (Cabana *et al.*, 1989) [2].

Materials and Methods

Materials

Animals

In this study, thirty buffalo calves (1–6 months of age, male and female) suffering from diarrhoea either referred to the Veterinary Clinical Complex, 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.

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. Based on clinical signs, faecal consistency and appearance, the type of diarrhoea is presented in Table-1.

Table 1: Various types of diarrhoea and clinical observations associated with it.

Group No.	Appetite	Diarrhoea (Faecal consistency)	Microscopic Faecal examination		
			Balantidium coli	***Strongyle/ strongyloid spp***	***Eimeria spp.***
1	Normal	Semi solid and greenish coloured	-ve	-ve	-ve
2	Slight anorectic	Loose and whitish yellow	+ve	-ve	-ve
3	Slight anorectic	Loose with some undigested ingesta	-ve	+ve	-ve
4	Anorectic	Bloody diarrhoea	-ve	-ve	+ve
5	Mild to severe anorexia	Fluidy with greenish discolouration	-ve	-ve	-ve

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-2. Animals having profuse diarrhoea but faecal samples were negative for parasitic ova constituted Group-V.

Table 2: 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

Collection of blood samples

Approximately 5 ml blood was collected from jugular vein aseptically. For separation of plasma, the blood samples collected in centrifuge tube containing heparin were centrifuged at 3000 rpm for 10 minutes and the plasma was separated in aliquots. The plasma was stored in liquid nitrogen in aliquots till further analysis for estimation of biochemical parameters.

Methods

Estimation of Acute Phase Proteins in plasma

Caeruloplasmin

Its concentration in plasma was estimated by the method of Ravin (1961) [11]. Ceruloplasmin Catalyzes the oxidation of some polyamines and its action on p-phenylenediamine was used by Ravin as a measure of the amount present. 0.1 ml of plasma was taken into three test tubes, one for control and two for the test. One ml sodium azide (0.5%) was used in the control. Then 8 ml of the acetate buffer (440 mmol/l, pH 5.5) was added to each tube followed by one ml p-phenylene-diamine (0.5%). This was shaken to mix and placed in water bath at 37 ºC for one hour, removed and 1ml of sodium azide was added to each test tube. Again the mixture was shaken well and the tubes were cooled at 4-10 ºC for 30 minutes. Then reading was taken at 550 nm with the control tube as blank.

Fibrinogen

Fibrinogen in plasma samples was measured by method of Martinek and Berry (1965) [10]. Its estimation from plasma is based on precipitation by 1.20 M phosphate buffer. Two test tubes were taken one as test and other as blank and s0.23 ml of plasma was pipetted into each of them. 3 ml of fibrinogen reagent was added to the sample test tube and 3.0 ml. of 0.9% (w/v) sodium chloride to blank and mixed by gentle lateral shaking. Readings were taken in Spectrophotometer at 450 nm against the blank adjusted to 100% transmittance. Standard curve was prepared using 0.25 ml of standard fibrinogen (20mg/dl to 80mg/dl) in place of 0.25 ml plasma in routine assays.

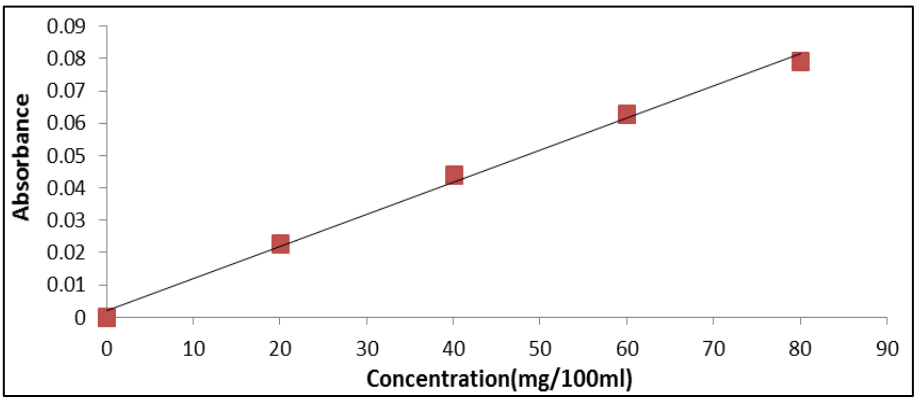

Fig 1: Standard curve of fibrinogen

Estimation of Plasma triglycerides

The levels of plasma triglycerides were measured by Glycerol Phosphate Oxidase (GPO), a kit based method. This procedure involves enzymatic hydrolysis of the triglycerides by lipase to glycerol and free fatty acids. The glycerol produced is then measured by coupled enzyme reactions. Triglycerides are first hydrolyzed by lipoprotein lipase to glycerol and free fatty acids.

Glycerol is then phosphorylated by adenosine-5-triphosphate (ATP) forming glycerol-1-phosphate (G-1-P) and adenosine-5-diphosphate (ADP) in the reaction catalyzed by glycerol kinase (GK). G-1-P is then oxidized by GPO to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). Peroxidase catalyzes the coupling of H_2O_2 with 4-aminoantipyrine (4-AAP) and 3, 5-Dichloro-2-hydroxybenzene sulfonate (DHBS) to produce a quinone-imine dye that shows an absorbance maximum at 540 nm. The increase in absorbance at 540 nm is directly proportional to triglyceride concentration of the sample.

Statistical analysis

The data was subjected to standard error of means (SE) and analysis of Variance (ANOVA) for statistical significance (Snedecor and Cochran, 1967) [12].

Results

Acute phase proteins

Ceruloplasmin

The mean value of Ceruloplasmin level was 22.95±1.42 mg% in healthy control group whereas it was significantly increased in diarrhoeic groups as compared to healthy control group. Its values in all diarrhoeal groups were 27.19±3.35, 29.94±1.08, 25.28±2.8, 30.95±2.07 mg%.

Fibrinogen

The mean value of fibrinogen was 3.81±0.46 in healthy control group whereas in other diarrhoeic groups its values were 4.10±0.79, 4.63±0.27, 3.99±0.34, 5.12±0.35 in groups II to V respectively.

Table 3: Changes in Ceruloplasmin and fibrinogen values (Mean±SE) in calves.

Parameters	Group I (n=6)	Diarrhoeic animals (n=30)			
		Group II (n=8)	Group III (n=16)	Group IV (n=3)	Group V (n=3)
Caeruloplasmin (mg %)	22.95±1.42^{a}	27.19±3.35^{ab}	29.94±1.08^{ab}	25.28±2.81^{ab}	30.95±2.07^{b}
Fibrinogen (g/l)	3.81±0.46	4.10±0.79	4.63±0.27	3.99±0.34	5.12±0.35

Values with common superscripts do not differ significantly between groups at $p<0.05$. 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.

Triglyceride

The mean values of triglyceride in healthy calves were 16.39±2.79 mg/dl. The levels were found to be increased to 20.87±3.66, 19.48±3.96, 21.76±0.80, 22.47±4.19 mg/dl in all diarrhoeal groups II to V respectively, as compared to healthy control group I.

Table 4: Changes in triglyceride values (Mean±SE) due to diarrhoea in buffalo calves**.**

Parameters	Group I (n=6)	Diarrhoeic animals (n=30)			
		Group II (n=3)	Group III (n=16)	Group IV (n=3)	Group V (n=8)
Triglycerides(mg/dl)	16.39±2.79	20.87±3.66	19.48±3.96	21.76±0.80	22.47±4.19

Values with common superscripts do not differ significantly between groups at $p<0.05$.

Values are Means and S.E.is denoted by vertical bars.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.

Discussion

Two acute phase proteins (APP_S) namely Ceruloplasmin and fibrinogen were estimated in the present study to see the effects of diarrhoea caused by parasitic and non-parasitic infections. Parasitic infections had effects on Ceruloplasmin (Cp) concentrations and the levels were found to be increased significantly. Monitoring the levels of the APP_S can provide a mean to assess the innate immune systems response to diseases (Kaneko, 1989) [8]. In general Ceruloplasmin is a moderate APP and normally present in the blood of healthy animals, but on stimulation, the concentrations increase 5-10 fold, reaching a peak concentration 2 to 3 days after stimulation and decrease more slowly. So increased Cp level in parasitic and non-parasitic infections causing diarrhoea can

be used as a “molecular thermometer” of the innate immune systems response in buffalo calves. Parasitic and non-parasitic infections did not change the fibrinogen concentration significantly. It is a large protein of 340 kD that constitutes nearly 5% of the total plasma protein. It is composed of three domains linked by disulphide bridges and contains 3% to 5% carbohydrate (Doolittle *et al.*, 1988) [4] and is a moderate APP. Its synthesis has been reported to be increased during an acute phase response consistently during inflammation in horses and cattle (Eckersall, 2008) [5] whereas in buffalo calves effects were non-significant. Acute phase response can be accompanied by alterations in lipid metabolism in the form of higher serum triglycerides and lower high-density lipoprotein levels (Cabana *et al.*, 1989) [2].

Plasma triglyceride levels were found to be increased slightly in diarrhoeic calves suggesting the breakdown of stored fats to supply energy to the anorectic animals. Similarly, Kumar *et al.* (2010) [9] reported the increase in plasma triglycerides in buffalo calves infected with Ascariosis.

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