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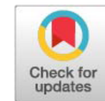
Use of Physiological and Clinical Biomarkers as Indicators in Field Study of Bovine Ephemeral Fever



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ABSTRACT

In this study therapeutic trials of two different regimes for BEFV were evaluated under field conditions on the basis of clinical biomarkers. A comparative analysis of physiological biomarkers was also made. For this experimental study 30 BEF positive cattle at their first lactation were selected and equally divided into three groups A, B, and C. A fourth group "D" comprising 10 healthy cattle was made as positive control while group C was negative control. The animals in group A were treated with antibiotic (fluoreconical @ 0.08 mg Se/kg BW) and NSAID (phenylbutazone @ 8mg/kg BW) after every 8 h and immune booster (Selevit® @ 20ml) daily by IV route. The members in groups B were treated similarly except that they were not given immune booster. Both groups were treated for three consecutive days. The effectiveness of a particular treatment regime was evaluated on the basis of disappearance of clinical biomarkers, while values of BEF positive animals' hematological and serum biochemical parameters were analyzed and compared with altered values in different conditions. These physiological biomarkers were evaluated at short intervals and processed for total MCV, MCH, MCHC, and lymphocytic and leukocyte count, most importantly. It was recorded that there was significant increase in number of WBCs and neutrophils, but a significant decrease was noticed in number of lymphocytes. While the serum tests showed significant decrease in iCa, P, Na, K, BUN, ALP, PTH. While significant increase was observed in Glu, Cl, Isuline and cortisol but no significant changes in Creatinine concentration. In group A clinical biomarkers fever, respiratory problem, lameness, anorexia, and milk production became almost normal after 72 h starting from 24 h, while in group B they went down, but not as significantly as in group A. These results confirmed the use of immune booster along with other therapy to successfully treat the BEF in field conditions.

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Authors' Contribution
MZ conducted the research. AZD supervised the research. MI and KM co-supervised the research. MH and NK helped in lab work and compilation of results. MU wrote the manuscript.

Key words
BEF, Clinical biomarkers, Physiological biomarkers, Serum biochemical parameters, Cattle.

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INTRODUCTION

Bovine ephemeral fever (BEF) is an economically important disease in cattle. It has its impacts in the form of abortion, decreased milk production, temporary infertility in bulls and prolonged recovery in some animals as well as trade restrictions. In outbreaks of BEF, the morbidity rate may be as high as 80%. The average mortality rate is 1-2% but can be higher in healthy animals. Although mortality is usually low, cattle in good condition are usually affected more severely and mortality rates can be higher up to 30% in very fat cattle. In tropical areas where BEF is endemic, subclinical infections are common. Outbreaks of BEF often follow periods of rainfall. In more temperate regions, epidemics occur during summer months and tend to decline with the onset of winter. Transmission does not occur by direct contact or by fomites. Virus persistence of BEF occurs in arthropod (Quinn *et al*., 2001).

Geographical distribution of BEF revealed that it is sporadic in many provinces of Iran, near south and hot areas. The temporal parts of Asian countries on south line like Bangladesh, Pakistan, Iran, and Iraq are the endemic areas of this disease of cattle and buffalo (Roya, 2008). In 1999, three cases of BEF were reported in Pakistan. Generally, the disease occurs in sporadic form in Pakistan. But in summer 2014 disease was found in exotic and indigenous cattle as well as in water buffaloes. It severely damaged the dairy industry. Usually, the disease was diagnosed on the basis of clinical signs in Pakistan.

Animals affected with BEF should be rested. Anti-inflammatory drugs such as phenylbutazone, flunixin meglumine and ketoprofen have proved useful for treatment. In another experimental study comparative

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efficacy of two therapeutic regimes, i.e., diclofenac sodium (NSAID) @ 1mg/Kg B/W along with rumenotoric 2 bolus BID and NSAID along with Oxytetracycline LA @ 20mg/ Kg B/W IM and supportive rumenotoric 2 bolus BID, were compared and later combination was found more successful in treating BEF positive animals. There is no evidence that the virus persists in animals following recovery from acute illness (Quinn *et al*. 2001).

The present study is aimed at comparing two different therapeutic regimes to successfully treat BEF in field conditions of Pakistan. For efficacy evaluation, clinical biomarkers like change in milk production, fever, lameness, respiratory problems and anorexia were used. Asi and his fellow scientists described that these sign are according to the BEF (Asi *et al*., 1999). Further, physiological biomarkers including haematological and serum chemistry parameters of BEF positive animals were also analysed and compared with different conditions. The study helped in devising successful treatment protocol for BEF positive animals in field conditions of Pakistan. Moreover, it also helped to understand physiological parameters in BEF positive animals.

MATERIALS AND METHODS

Study area

Study was conducted in 2017 in Rajanpur, Punjab, Pakistan. District Rajanpur is located in the southern part of the Punjab; topography of the land is mixed comprising of riverine, plateau, mountainous and semi-hilly areas. The temperature during summer months may rise up to 52°C and fall below 4°C during winter in the study area. The average annual rainfall is 7.08 mm.

Selection of animals

BEF positive cattle (n=30) at their first lactation were selected based on the basis of clinical biomarkers like fever, respiratory problem, lameness, anorexia, and decreased milk production. The animals were divided into three groups A, B, and C each comprising 10 animals. A fourth group D comprising 10 healthy cattle at their first lactation was made as negative control while group C was positive control.

For treatment trials, thirty cattle suffering from clinical BEF were randomly selected and divided into 3 groups of 10 as A, B and C. A fourth group "D" comprising 10 healthy cattle (not suffering from clinical BEF) was also made. The animals in group A were treated with Vitamin E plus selenium as immune booster. This group was given phenylbutazone at 8mg/kg BW 8 hourly interval by IV route and fluoreconical at 0.08 mg Se/kg BW. This treatment was given for 3 consecutive days. The members in group B were treated with phenylbutazone at 8mg/kg BW 8 hourly interval by IV route and fluoreconical at 0.08 mg Se/kg BW. This treatment was also given for 3 consecutive days. The animals in groups C served as positive control (infected untreated) and that of groups D as negative control (non-infected, non-treated). The effectiveness of a particular treatment regime was evaluated on the basis of reversal of clinical signs, haematological and serum biochemical parameters. Blood samples (5 ml) were collected at days 0, 3, 7 and 14, from each cattle in each group and were transported to University Diagnostic Laboratory, University of Veterinary and animal Sciences, Lahore.

Hematological studies

Blood samples (5 ml) were collected at days 0, 3, 7 and 14, from each cattle in each group from the jugular vein using hypodermic 1.5"x18G needle attaché to disposable syringe. These samples were poured into EDTA coated vacutainers (purple toped). After collection the samples were transferred to ice cooled container for transport to the laboratory.

The blood samples were processed for total leukocyte count, differential leukocyte count, haemoglobin, red blood cell count, mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and platelet count. The blood samples were analysed by Automated Hematology Analyzer (Sysmex).

Serum biochemical studies

Blood samples (5 ml) were collected from each animal, through the jugular vein using hypodermic 1.5"x18G needle attaché to disposable syringe. The blood samples were poured into plain vacutainers (red toped) and were allowed to clot. After clotting, the samples were centrifuged at 3500 rpm for 5 min and clear straw coloured supernatant (serum) was collected into appendorf tube by pasture pipette. The serum samples were stored at -20°C till further analysis.

Serum samples were transferred to Minerals and Metals Analysis Laboratory, Department of Environmental Sciences, University of Veterinary and Animal Sciences, Lahore. All serum samples were tested for inorganic phosphorus (P), magnesium (Mg), sodium (Na), potassium (K) and chloride (Cl). One ml of each sample was digested and distal water was added to make volume up to 25 ml. Analysis of magnesium and calcium was done with atomic absorption spectrophotometer, inorganic phosphorous with spectrophotometer while analysis of sodium and potassium was accomplished using flame photometer. All the results were recorded.

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Serum samples were transferred to Quality Operation Laboratory, University of Veterinary and Animal Sciences, Lahore for estimation of alanine transaminase (ALT), aspartate transaninase (AST), alkaline phosphatase (ALP), lectate dehydrogenase (LDH), selenium Se, blood urea nitrogen (BUN), total protein (TP), albumin (Alb), glucose (Glu), total calcium (tCa), ionized calcium (iCa), creatinine phosphokinase (CK), and serum activity of alkaline phosphatase (ALP). Hormonal profile included parathyroid hormone (PTH), insulin (Ins), and cortisol (Cor). Enzymatic and calorimetric test procedures were followed according to the guidelines of manufactures of kits used for these estimations. Results were obtained from laboratory and recorded.

Statistical analysis

Data from therapeutic trials were analyzed through two way ANOVA technique under Completely Randomized Block Design by using Statistical Analysis System 9.1 (SAS Software). Data regarding risk factors and different treatment groups was analyzed by chi-square test. P < 0.05 value was considered significant.

RESULTS

Figure 1 shows the effect of vitamin E+ selenium, phenylbutazone and fluoreconical (A) phenylbutazone and fluoreconical (B) on various hemtological and bio-chemical components of cattle infected with BEF. It was observed that animals affected from BEF in Group A recovered more fast than that of animals in Group B as shown in Figure 1. The blood samples were processed for total leukocyte count, differential leukocyte count, haemoglobin, red blood cell count, mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and platelet count. It was recorded that there was significant increase in number of WBCs and neutrophils. But a significant decrease was noticed in number of lymphocytes. No significant difference in number of red blood cells (RBCs), haemoglobin (Hb), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), hematocrit (HCT%), and platelets were observed as shown in Table I. Serum was also obtained from blood samples. The serum samples were tested for alanine transaminase (ALT), aspartate transaninase (AST), lectate dehydrogenase (LDH), (CK) selenium Se, creatinine, blood urea nitrogen (BUN), total protein (TP), albumin (Alb), glucose (Glu), total calcium (tCa), ionized calcium (iCa), inorganic phosphorus (P), magnesium (Mg), sodium (Na), potassium (K), chloride (Cl), and serum activity of alkaline phosphatase (ALP). It was recorded that significant decrease occurred in iCa, P, Na, K, BUN, ALP, PTH, while significant increase was observed in Glu, Cl, Isuline and cortisol but no significant change in creatinine concentration as shown in Table II.

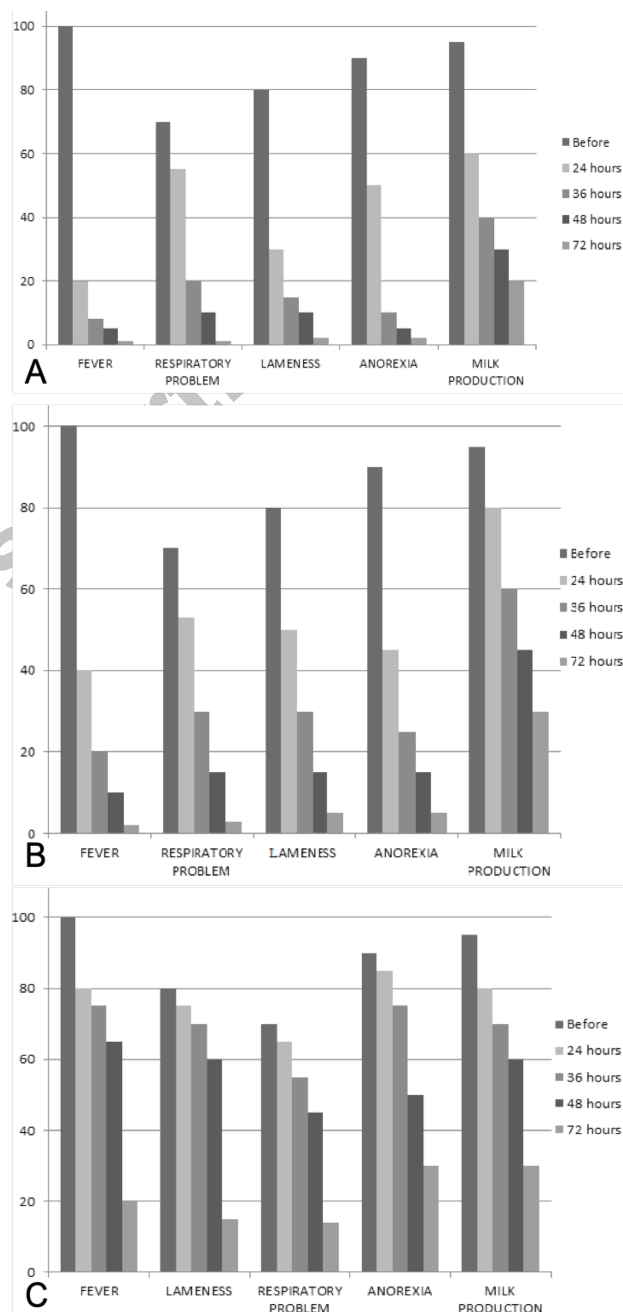

Fig. 1. Reversal of clinical biomarkers in groups a (A), b (B) and c (C).

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Table I.- Hematological parameters (Mean ± SD) in animals affected with BEF and treated with immune booster, antibiotic and antipyretic. Control positive (sick but without treatment) and control negative healthy animals.

Groups	A: Immune booster + Phenylbutazone + Florfenicol (n=10)				B: Phenylbutazone + Florfenicol (n=10)				C: Infected untreated (Positive control) (n=10)				D: Non-infected, Non-treated (Negative control) (n=10)			
Parameters	1st Day	3rd Day	7th Day	14th Day	1st Day	3rd Day	7th Day	14th Day	1st Day	3rd Day	7th Day	14th Day	1st Day	3rd Day	7th Day	14th Day
WBCs ($\times 10^3/\mu l$)	13.15^{a}± 1.36	11.10^{a}± 0.77	9.01^{a}± 0.99	8.72^{a}± 1.43	12.80^{a}± 1.15	9.40^{a}± 0.96	8.82^{a}± 0.98	8.52^{a}± 1.14	13.25^{a}± 1.45	12.65^{a}± 0.73	11.15^{a}± 0.30	9.40^{a}± 0.96	9.05^{a}± 0.98	8.93^{a}± 0.96	8.55^{a}± 1.52	8.20^{a}± 1.71
Lymphocytes ($\times 10^3/\mu l$)	3.55^{a}± 2.36	4.70^{a}± 1.67	5.31^{a}± 1.55	5.38^{a}± 1.50	3.80^{a}± 1.45	4.77^{a}± 1.95	5.12^{a}± 1.45	5.00^{a}± 1.34	3.35^{a}± 0.85	5.15^{a}± 1.08	5.20^{a}± 1.30	5.30^{a}± 1.43	5.25^{a}± 0.98	5.33^{a}± 1.06	5.30^{a}± 1.98	5.20^{a}± 1.91
Neutrophils ($\times 10^3/\mu l$)	3.55^{a}± 1.36	11.10^{a}± 0.77	9.01^{a}± 0.99	8.72^{a}± 1.43	12.80^{a}± 1.15	9.40^{a}± 0.96	8.82^{a}± 0.98	8.52^{a}± 1.14	13.25^{a}± 1.45	12.65^{a}± 0.73	11.15^{a}± 0.30	9.40^{a}± 0.96	9.05^{a}± 0.98	8.93^{a}± 0.96	8.55^{a}± 1.52	8.20^{a}± 1.71
RBCs ($\times 10^6/\mu l$)	7.55^{a}± 1.036	7.40^{a}± 1.07	7.56^{a}± 1.02	7.59^{a}± 0.81	7.50^{a}± 1.05	7.47^{a}± 1.06	7.68^{a}± 0.48	7.51^{a}± 0.64	8.05^{a}± 1.15	7.96^{a}± 0.53	7.05^{a}± 0.68	7.40^{a}± 0.95	7.45^{a}± 0.58	7.39^{a}± 0.97	7.51^{a}± 1.05	7.41^{a}± 0.51
MCH (pg)	14.65^{a}± 2.14	14.13^{a}± 1.88	13.89^{a}± 1.17	13.95^{a}± 1.61	13.82^{a}± 1.91	13.31^{a}± 1.89	13.82^{a}± 2.08	13.99^{a}± 1.54	14.85^{a}± 1.95	13.65^{a}± 1.73	14.35^{a}± 0.88	14.65^{a}± 0.96	15.05^{a}± 1.64	14.03^{a}± 1.68	14.89^{a}± 1.27	14.95^{a}± 1.41
MCV (fl)	48.35^{a}± 3.94	48.13^{a}± 4.99	49.78^{a}± 5.30	49.45^{a}± 5.63	49.08^{a}± 4.51	50.80^{a}± 4.99	51.05^{a}± 5.60	49.81^{a}± 5.46	50.50^{a}± 5.25	49.55^{a}± 4.53	48.91^{a}± 3.98	50.85^{a}± 5.26	49.05^{a}± 4.64	51.02^{a}± 5.68	50.79^{a}± 5.17	49.75^{a}± 5.46
MCHC (g/dl)	31.58^{a}± 2.94	31.83^{a}± 3.29	32.88^{a}± 3.30	32.95^{a}± 3.31	32.82^{a}± 2.81	32.80^{a}± 3.31	32.78^{a}± 2.60	32.81^{a}± 2.65	34.05^{a}± 2.25	34.55^{a}± 2.53	34.21^{a}± 2.88	33.95^{a}± 2.96	33.15^{a}± 3.64	33.32^{a}± 2.68	33.09^{a}± 3.27	33.05^{a}± 3.65
Platelets ($\times 10^3/\mu l$)	642.11^{a} ±70.24	651.93^{a}± 54.9	662.88^{a}± 3.30	664.9^{a}± 75.92	654.42^{a}± 63.66	650.92^{a}± 54.94	660.78^{a}± 90.60	662.28^{a}± 90.65	659.59^{a}± 65.76	638.75^{a} ±96.93	654.23^{a}± 82.38	665.45^{a}± 70.37	635.85^{a}± 70.45	656.82^{a}± 76.68	651.09^{a}± 98.97	650.15^{a}± 99.95
HCT (%)	31.78^{a}± 3.94	33.18^{a}± 4.65	32.78^{a}± 3.41	33.95^{a}± 5.21	32.02^{a}± 3.71	31.95^{a}± 3.17	32.78^{a}± 2.61	33.81^{a}± 5.31	32.15^{a}± 3.35	31.75^{a}± 2.59	33.01^{a}± 3.78	32.95^{a}± 4.96	31.55^{a}± 3.94	32.42^{a}± 2.98	32.09^{a}± 3.67	31.95^{a}± 4.65
HGB (g/dl)	10.91^{a} ±1.724	11.23^{a}± 1.69	11.08^{a}± 1.69	11.11^{a}± 1.81	11.202^{a}± 1.921	11.165^{a}± 1.565	11.08^{a}± 1.67	11.02^{a}± 1.65	11.389^{a}± 1.326	10.75^{a}± 0.93	11.23^{a}± 1.38	11.15^{a}± 1.67	11.05^{a}± 0.745	11.082^{a}± 0.68	11.19^{a}± 1.897	11.18^{a}± 1.843

WBCs, white blood cells; RBCs, red blood cells; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration; HCT, hematocrit; HGB, haemoglobin. Means in the same rows showing different superscripts but significant difference from each other ($P<0.05$). The animals in group A were treated with immune booster Selevit® @ 20ml/day which contains Vitamin E plus selenium. This group was also given phenylbutazone at 8mg/kg BW 8 hrly interval by IV route and florfenicol at 0.08 mg /kg BW. This treatment was given for 3 consecutive days. The members in groups B were treated with phenylbutazone at 8mg/kg BW 8 hrly interval by IV route and florfenicol at 0.08 mg/kg BW. Immune booster (Vitamin E and selenium) was not given to the animals of this group to observe the results with immune booster and without immune booster. This treatment was given for 3 consecutive days. The animals in groups C were serving as positive control (infected untreated) and that of groups D as negative control (non-infected, non-treated).

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Table II.- Serum biochemical parameters (Mean ± SD) in cattle suffering from BEF and treated with immune booster, antibiotic and antipyretic. Control positive (sick but without treatment) and control negative healthy animals.

Groups	A: Immune booster + Phenylbutazone + Florfenicol (n=10)				B: Phenylbutazone + Florfenicol (n=10)				C: Infected untreated (Positive control) (n=10)				D: Non-infected, Non-treated (Negative control) (n=10)			
Parameters	**1st Day**	**3rd Day**	**7th Day**	**14th Day**	**1st Day**	**3rd Day**	**7th Day**	**14th Day**	**1st Day**	**3rd Day**	**7th Day**	**14th Day**	**1st Day**	**3rd Day**	**7th Day**	**14th Day**
Calcium level (mg/dl)	7.50^{b}± 0.75	9.50^{b}± 0.77	10.01^{b}± 1.01	10.19^{b}± 1.03	6.80^{b}± 0.55	9.40^{b}± 0.96	9.90^{b}± 1.22	10.10^{b}± 1.02	7.80^{b}± 0.96	7.10^{b}± 0.73	6.40^{b}± 0.30	7.80^{b}± 0.96	9.80^{b}± 0.77	10.10^{b}± 0.96	10.15^{b}± 1.02	10.20^{b} ±0.71
Sodium (mmol/L)	139.9^{a}± 4.05	140.5^{a}± 2.77	139.91^{b} ± 4.11	140.19^{b}± 4.23	138.9^{a}± 3.45	139.9^{a}± 4.25	140.4^{a}± 4.93	141.1^{a}± 4.04	138.10^{a}± 2.77	140.0^{a}± 8.34	139.8^{b}± 4.24	140.6^{b}± 2.88	139.40^{a}± 3.86	140.6^{a}± 2.88	138.9^{b}± 3.45	140.30^{a}± 2.79
Chloride (mmol/L)	103.1^{a}± 4.04	103.9^{a}± 2.42	103.5^{b}± 2.46	103.89^{b}± 2.43	104.9^{a}± 1.55	104.1^{a}± 3.78	102.4^{a}± 2.79	103.10^{a}± 3.28	102.3^{a}± 3.30	102.1^{a}± 2.34	102.8^{b}± 4.40	102.6^{b}± 3.70	103.0^{a}± 2.91	103.10^{a}± 3.38	102.2^{b}± 2.45	103.30^{a}± 2.79
Potassium (mmol/L)	3.90^{a}± 0.34	4.15^{a}± 0.77	4.16^{b}± 0.85	4.19^{b}± 0.90	4.06^{a}± 0.48	3.70^{a}± 1.19	4.04^{a}± 0.48	4.07^{a}± 0.42	3.87^{a}± 0.44	4.10^{a}± 0.49	4.08^{b}± 0.48	4.17^{b}± 0.49	4.12^{a}± 0.50	4.16^{a}± 0.58	4.15^{b}± 0.52	4.2^{a}± 0.48
Total protein (g/dl)	6.81± 0.34	6.15± 0.77	5.80 ± 0.85	5.41± 0.90	6.82± 0.35	6.70± 0.19	6.10± 0.48	5.38± 0.12	6.87± 0.40	6.92± 0.49	6.88± 0.48	5.37± 0.49	5.38± 0.12	5.40± 0.13	5.36± 0.11	5.35± 0.14
Albumin (g/dl)	3.28± 0.13	3.29± 0.12	3.30± 0.15	3.31± 0.17	3.27± 0.15	3.29± 0.18	3.31± 0.19	3.30± 0.15	3.33± 0.16	3.32± 0.17	3.31± 0.15	3.30± 0.11	3.31± 0.12	3.28± 0.13	3.32± 0.11	3.30± 0.14
Glucose (mg/dl)	90.45± 0.90	88.29± 1.75	85.30± 2.15	80.31± 3.17	90.48± 0.95	88.90± 1.85	86.20± 1.35	82.30± 2.15	90.33± 0.85	91.32± 1.25	88.31± 1.15	86.30± 2.11	80.31± 3.26	80.45± 3.20	80.35± 3.11	80.30± 3.14
Total calcium (mg/dl)	7.65± 0.22	7.85± 0.23	7.99± 0.11	8.11± 0.10	7.68± 0.21	7.90± 0.20	8.20± 0.11	8.10± 0.12	7.65± 0.20	7.99± 0.18	8.11± 0.15	8.12± 0.11	8.11± 0.11	8.45± 0.12	8.35± 0.13	8.10± 0.14
Ionized calcium (mg/dl)	0.80± 0.10	0.82± 0.11	0.99± 0.12	1.12± 0.15	0.81± 0.10	0.85± 0.11	0.88± 0.13	1.11± 0.16	0.83± 0.12	0.81± 0.11	0.80± 0.10	0.88± 0.13	1.10± 0.14	1.15± 0.13	1.17± 0.17	1.16± 0.14
Inorganic phosphorus (mg/dl)	5.45± 0.78	6.73± 0.62	7.30± 0.50	8.61± 0.32	5.65± 0.73	6.50± 0.71	7.10± 0.45	8.30± 0.34	5.33± 0.88	5.99± 0.76	6.18± 0.56	6.20± 0.45	8.41± 0.26	8.35± 0.29	8.32± 0.27	8.30± 0.32
Magnesium (mg/dl)	1.54± 0.19	1.73± 0.15	1.53± 0.10	1.52± 0.09	1.55± 0.18	1.50± 0.17	1.52± 0.08	1.52± 0.07	1.51± 0.06	1.50± 0.05	1.49± 0.04	1.51± 0.05	1.52± 0.06	1.53± 0.09	1.51± 0.07	1.53± 0.06
Creatinine (mg/dl)	1.04± 0.07	1.01± 0.04	0.99± 0.03	0.96± 0.01	1.05± 0.05	1.04± 0.04	1.00± 0.03	0.98± 0.02	1.03± 0.03	1.04± 0.03	1.03± 0.05	1.02± 0.02	0.97± 0.02	0.98± 0.01	0.96± 0.01	0.96± 0.01
BUN (mg/dl)	10.25± 0.37	11.88± 0.35	12.76± 0.32	14.21± 0.34	10.56± 0.28	11.15± 0.29	11.99± 0.32	13.88± 0.35	10.33± 0.31	10.85± 0.31	11.18± 0.32	11.88± 0.33	14.23± 0.34	14.21± 0.33	14.23± 0.32	14.20± 0.34
ALP (U/l)	173.85± 5.15	186.23± 4.99	195.22± 4.08	203.62± 3.96	174.23± 5.10	180.15± 4.22	193.30± 3.95	202.65± 3.65	173.43± 5.23	174.95± 5.03	177.18± 4.59	199.86± 3.45	203.43± 3.5	201.95± 3.70	202.18± 3.50	203.86± 3.76
PTH (U/l)	17.89± 0.86	20.23± 1.99	25.37± 2.28	28.92± 3.32	18.23± 0.83	19.15± 1.34	23.30± 2.36	27.65± 3.30	18.43± 0.87	19.95± 1.30	18.80± 0.95	20.86± 1.45	29.03± 0.95	28.95± 0.99	29.18± 0.59	18.86± 0.87
Ins (mIU/ml)	5.75± 0.45	5.11± 0.46	4.22± 0.58	3.62± 0.66	5.80± 0.43	5.18± 0.48	4.10± 0.55	3.65± 0.65	5.83± 0.43	5.95± 0.49	5.18± 0.50	4.86± 0.59	3.48± 0.63	3.52± 0.65	3.46± 0.62	3.56± 0.64
Cor (mg/ml)	10.35± 0.71	8.34± 0.58	6.11± 0.42	4.89± 0.30	10.29± 0.68	9.11± 0.60	7.31± 0.45	5.01± 0.31	10.31± 0.70	9.95± 0.59	9.18± 0.51	9.86± 0.58	4.85± 0.29	4.58± 0.28	5.66± 0.35	4.86± 0.30

BUN, blood urea nitrogen; ALP, alkaline phosphatase; PTH, parathyroid hormone; Ins, insulin; Cor, cortisol. Means in the same rows showing different superscripts but significant difference from each other ($P<0.05$). For other experimental details, see Table I.

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DISCUSSION

The present study was carried out in district Rajanpur which is located in the southern part of the Punjab; topography of the land is mixed comprising of riverine, plateau, mountainous and semi-hilly areas. The temperature during summer months may rise up to 52°C and fall below 4°C during winter in the study area. The average annual rainfall is 7.08 mm.

The pathogenesis of BEF is linked with vascular inflammation reported by Young and Spradbrow (1980). So this point provided the rationale for its treatment with anti-inflammatory drugs and antibiotics to avoid secondary bacterial infection. Four Groups were made each of thirty animals including two treatment groups, one control negative and one control positive group. In treatment groups two types of treatment regimens were tested and compared. On comparison of these different treatments a significant difference was observed between the groups. It was recorded that group A showed better results in relieving fever, better feeding, decrease in lameness and reversal of haematological and serum biochemical parameters in 24-72 h while animals in group B took 72-96 h for the same results. The animals in group A were treated with Vitamin E plus selenium as immune booster. These animals were also given phenylbutazone at 8mg/kg BW 8 hourly interval by IV route and florfenicol at 0.08 mg Se/kg BW. While animals in group B were treated phenylbutazone at 8mg/kg BW 8 hourly interval by IV route and florfenical at 0.08 mg /kg BW. Uren *et al*. (1989) investigated the effect of two anti-inflammatory drugs on the development and persistence of clinical signs in cattle experimentally infected with BEF virus. In that study total of 16 cattle were given phenylbutazone sodium. The drug prevented fever and other clinical signs in six cattle when given daily during the incubation period at 8-h intervals for 5 days. When treatment with phenylbutazone was deferred until 2-4 h after the commencement of fever, the rectal temperature returned to normal within 4 h in four of six cattle and the development of other clinical signs also suppressed. Clinical signs of BEF developed in four untreated cattle infected at the same time. It was reported that the treatment with phenylbutazone blocked the host response which produces the clinical signs and did not have any anti-viral effect. In a similar experiment, a long-acting anti-inflammatory drug, flunixin meglumine, failed to prevent BEF or to modify the clinical signs once they had developed, except for the rectal temperature which returned to normal within 2-4 h of the administration of the drug. The efficacy of this drug was not improved by increasing the dosage to two to three times of the recommended level. These reports are result wise correlated with our study. In another study in Pakistan by Asi *et al*. (1999), three cases of cross breed cattle were reported on the basis of clinical signs showing that animals were suffering from BEF. They used the combination of antibiotic, non-steroidal anti-inflammatory (Metamizol and supportive treatment with vitamin B complex) and concluded that it is an effective treatment against BEF. This study is also consistent with our study. In both studies combination of NSAIDs, antibiotic and supportive therapies were used.

Cynthia and Scot (2010) reported that the use of anti-inflammatory drugs repeated after 3-4 h is effective remedy for BEF. Their results indicated the neutrophils number as 10.44 x 109/uL which significantly increased ($p<0.05$), while lymphocyte number was decreased to 2.90 x 109/L. They also found that there was no change in total white blood cells number (WBC), hemoglobin (Hb), packed cell volume (PCV), red blood cells (RBCs), mean corpuscle volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) values, and MID (it include monocytes basophils and eosinophil). The increased level of neutrophils during the peak of the temperature may be due to the bone marrow response against the virus, giving rise large number of band neutrophils. Our study also showed a significant decrease in lymphocytes. Previously it has been reported the increase in number of neutrophils is inversely related to the lymphocytes number (St. George *et al*., 1984). The increase in number of neutrophils, i.e., 9.6-12 x 109/L and 8.5 x 109/L as observed by St. George *et al*. (1984) and Uren and Morphy (1985) respectively, resulted in decrease of lymphocyte number which was recorded as 5.5-7 x 109/L and 3.8 x 109/L by the same scientists. This suggests that an increase in neutrophils causes the decrease in lymphocytes number.

Our results revealed that there was significant decrease, i.e., 7.84 mg/dl ($P>0.05$), in serum calcium level of infected cattle. The similar kind of serum calcium values, i.e., 2.04 mmol/l, were recorded by St. George *et al*. (1984) 2 mmol/L by Uren *et al*. (1992), 1.92 mmol/L by Uren and Murphy, 1985, and 7.86 mg/dl by Thabet *et al*. (2011). Uren *et al*. (1992) also reported hypocalcaemia which may occur due to activated neutrophils. When neutrophils are activated, the calcium in the plasma goes to the interior of the neutrophils then hypocalcaemia is observed (Forehand *et al*., 1989). Muscle fasciculation were also observed because of the low calcium level. The other possible reasons of hypocalcaemia may include ruminal stasis in which animal is unable to absorb calcium from the rumen or increased pH which may be up to 7.8 induced by high ammonia level. Some calcium may be taken up by increased amount of non-esterified fatty acid in the blood plasma (Murphy *et al*., 1990). The cause of

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decrease in serum calcium is yet unknown. There are many conditions: hypocalcaemia, hyperparathyroidism, acid base imbalance, decrease of serum albumin and malnutrition. These conditions play role in affecting the level of ionized calcium in serum (Duncan *et al*., 1994; Kaneko *et al*., 1997).

Signs of hypocalcaemia appeared due to significant reduction in ionized calcium that is required for muscular functions. Acid base imbalance has also effect on ionized calcium level in serum. When acidosis develops, it causes replacement of Ca ions from binding sites. It was also observed that fever affected the acid base imbalance in the blood accompanied with hyperventilation. The increase of pH and decrease of H+ are related to hyperventilation which causes the expiration of carbon dioxide from the body (Afzal *et al*., 2004). The efflux of calcium ions from the bones is related to increase in alkalosis in affected animals due to stimulation of osteoblastic activity and suppression of osteoclasts (Bushinsky, 1996). Higher level of alkalosis is responsible to reduce the ionized Ca concentration. The low level of ionized Ca concentration is conversely related to alkalosis which leads to development of signs of hypocalcaemia (Duncan *et al*., 1994; Kaneko *et al*., 1997). But there was no direct link of alkalosis with increase of body temperature, emphysema and increase of rate of respiration in BEF affected animal (Burgess and Spardbrow, 1977).

According to Uren rise of pH is related to decrease in values of pCO2 in BEF infected animals (Uren *et al*., 1992). In present study it was found that the secretion of PTH was stimulated due to metabolic and respiratory alkalosis with narmocalcaemia, and delayed PTH response may also lead to hypocalcaemia. Serum levels of PTH were significantly decreased in BEF infected animals. It showed that lower level of ionized Ca in serum was caused by PTH secretions (Lopez *et al*., 2003).

The reduced value of ionized Ca in the serum may be secondary to respiratory alkalosis and increased binding of Ca. But, total plasma Ca was not changed due to the attachment of calcium and hydrogen ions on the same binding site, i.e., serum albumin. As the blood became more alkaloid the H ions started dissociating from albumin. It freed the bound albumin to attach more Ca. Resultantly, ionized portion of total serum Ca also decreased (Afzal *et al*., 2004). In another study it was observed that for lowering the Ca in serum the ruminal stasis also played important role because it decreases calcium absorption (St George *et al*., 1984).

The studies presented by St. George *et al*. (1984) and Uren *et al*. (1992), showed that phosphorous concentration was significantly decreased, which indicated the secondary redistribution of phosphorous due to respiratory alkalosis (Amanzadeh and Reilly, 2006). Generally, intracellular CO2 is decreased due to respiratory alkalosis, which causes the increase in pH. The glycolytic pathway is initiated by this process, particularly phosphofructokinase which limits rate of enzymes of glycolysis. The intracellular phosphorous entry is initiated by the production of sugar phosphates therefore the concentration of phosphorous is decreased in serum (Hope *et al*., 1982; Amanzadeh and Reilly, 2006). There is a report which indicates that phosphorus uptake by the muscles is increased due to the respiratory alkalosis, which causes hypophosphatemia (Hope *et al*., 1982).

In this study hyponatremia was also observed in animals suffering from BEF. Afzal *et al*. (2004) described that in the distal and proximal tubules the hydrogen ions are secreted actively in exchange of sodium. The hyponatremia occurs when kidneys, in response to respiratory alkalosis, decrease secretion of hydrogen ions into the urine while increasing sodium secretion (Kaneko *et al*., 1997). To maintain the electrical neutrality, the excretion of sodium bicarbonate increases while chloride ions are retained by kidneys. It leads to hyper-chloremia which was recorded in this study. Similarly, hypokalemia was also recorded in BEF infected cattle in this study. In respiratory alkalosis, potassium ions (K+) are exchanged with hydrogen ions (H+) in the intracellular fluid. Then these potassium ions (K+) are pumped to extracellular fluid and their level in the serum decreases (Krapf *et al*., 1995). For the intracellular shifting of K+ there is also involvement of insulin (Duncan *et al*., 1994), that is why higher level of insulin in serum have relation with occurrence of hypokalemia observed in the animals infected with BEF (Mike, 2010).

Significant rise in the level of serum glucose was also recorded in BEF infected animals during the current study. Many scientists observed stress as the main reason behind hyperglycemia (Kaneko *et al*., 1997; Roussel, 1997; Moore, 1998). This was confirmed by recording the concentration of serum cortisol in the same animals during the current study. As adrenal glands produce cortisol in response of stress of different causes like systemic illness, febrile conduction and affections so the cortisol level was found significantly higher in these animals (Adcock *et al*., 2007; Torphy and Ho, 2007). In another study it was found that the promotion of hypoxic glycogenesis, inhibition of intracellular insulin and metabolism of glucose affect cortisol and blood glucose levels which lead to hyperglycemia (Roussel *et al*., 1997). The role of insulin in this postulate is confirmed by recorded results of serum concentration of insulin which was found significantly higher in BEF infected animals. There is reciprocal link between sensitivity of insulin and its secretion. So, the resistance of insulin resulted in higher level of its secretion

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to retain homeostasis of normal glucose level (Kaneko *et al*., 1997).

Lastly, it was also recorded in this study that there was a significant decrease of total protein (TP) in serum of the animals infected with BEF. This hypo-glubulinemia leads to inhibition of functions and number of antibody producing cells. Moreover, in BEF infected animals, the reported lymphopenia is a consistent property, which starts with the initiation of fever and returns to normal within 3 to 4 days (Burgess and Spradbrow, 1977; St. George *et al*., 1984; Uren *et al*., 1989). Similarly, in experimentally infected animals the level of neutralizing antibodies was low on 1st or 2nd days of development of clinical signs and this level became normal within 1 to 2 days after cessation of viremia (Young and Spradbrow, 1990).

CONCLUSION

The study proved that use of immune booster (Selevit®) along with antibiotic (fluoreconical) and NSAID (phenylbutazone) to successfully treat the BEF in field conditions using clinical biomarkers as indicators. Further it analyzed and compared physiological biomarkers parameters with different conditions.

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Statement of conflict of interest

The authors declare no conflict of interest.

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