

Molecular and biochemical studies on bovine ephemeral fever

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Abstract

Bovine ephemeral fever (BEF) in cattle has been reported to be associated with a range of biochemical changes which are similar to those seen in milk fever. This study aimed to clarify the biochemical alterations that associate infection of cattle with BEF with special references to the mechanisms involved in the development of hypocalcemia. The study was conducted on 30 cases of cattle infected with BEF based on the characteristic clinical signs which were confirmed by isolation of virus and RT-PCR. Another 6 healthy cows were used in the study as control. The evaluated parameters included biochemical variables such as serum values of total protein (TP), albumin (Alb), glucose (Glu), total calcium (tCa), ionized calcium (iCa), inorganic phosphorus (P), magnesium (Mg), sodium (Na), potassium (K), chloride (Cl), creatinine (Cr), blood urea nitrogen (BUN) and serum activity of alkaline phosphatase (ALP). Hormonal profile included parathyroid hormone (PTH), insulin (Ins), and cortisol (Cor). The results showed that BEF-infected animals demonstrated a significant decrease ($P<0.05$) in serum concentrations of TP, Glu, iCa, P, Na, K, BUN and ALP while the mean values of serum levels of Glu and Cl were significantly increased ($P<0.05$). The mean values of serum levels of PTH were significantly decreased ($P<0.05$) while serum concentrations of Ins and Cor showed a significant increase. It was concluded that the clinical signs of bovine ephemeral fever are related to the hypocalcemia resulting from suppression of parathyroid hormone which seems to be mediated by respiratory alkalosis caused by the disease. This explanation needs future studies to provide a direct link between measurement of blood indicators of acid-base status, blood biochemical parameters and urine analysis. However, this work can provide a good knowledge about the pathogenesis of the disease that can lead to better management and proper treatment.

Keywords: Bovine Ephemeral Fever, Molecular, Biochemical, Hypocalcemia

Introduction

Bovine ephemeral fever (BEF) also known as Three Day Sickness is an arthropod vector-borne disease of cattle and occasionally buffaloes which is caused by the *Ephemerovirus* virus of the *Rhabdoviridae* family of virus (Uren, 1989; Nandi and Negi, 1999). Bovine ephemeral fever is endemic in most tropical and subtropical areas of Africa, Australia, the Middle East and Asia and spread of the disease depends on the season and weather conditions (Kirland, 1995; Walker, 2005). The disease is characterized by very obvious clinical signs which can be quite severe. These include sudden onset of fever, stiffness, disinclination to movement accompanied by lameness (St. George et al., 1984). These clinical signs can be exacerbated by severe environmental stress or forced exercise (St. George, 1985; St. George et al., 1985). Recovery usually occurs within three to four days of the onset of clinical signs, hence the term ephemeral.

Lactating cows, bulls in good condition, and fat steers are the worst affected, and their recovery may take up to a week (Uren et al., 1992).

The mortality of the disease is low; however, it can cause significant economic impact through reduced milk production in dairy herds, loss of condition in beef cattle and loss of draught animals at the time of harvest, temporary infertility in bulls as well as trade restrictions (St. George et al., 1984).

Although the pathogenesis of the disease is complex, the study of ephemeral fever in cattle has defined a range of biochemical changes in blood which seemed to be similar to those of milk fever (St. George et al., 1995). However, the biochemical dyscrasias are far more extensive in BEF. Milk fever, or post-parturient hypocalcemia is a metabolic disease of dairy cows close to calving caused by a reduced blood calcium levels when demand for calcium for milk production exceeds the body's ability to mobilize calcium reserves in bone (Goff and Horst, 2003).

Whereas the cause of hypocalcemia in milk fever is well documented to be the result of blood calcium depletion because of milk production, the reason for the decreased calcium levels in BEF is not well established. Therefore, the aim of the present study was to define the blood biochemical alterations associated with BEF with particular emphasis on the mechanisms involved in the change in blood calcium levels based on isolation of virus and confirmation by RT-PCR.

Materials and Methods

This study was carried out on 30 cases during an outbreak of bovine ephemeral fever in Kafr El-Sheik governorate. Diagnosis was made through physical examination based on the characteristic clinical signs which were confirmed by isolation of virus and RT-PCR. Majority of cases were pregnant heifers or heifers. Another 6 healthy cows were used in the study as control.

Heparinized blood samples were collected from infected cattle for separation of buffy coat for virus isolation. Another blood samples were collected from the animals of both groups and serum samples were separated and stored at -20°C until assayed for other biochemical and hormonal assays.

Baby hamster kidney cell line (BHK21) and African green monkey kidney cell line (VERO) (kindly supplied by Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt) were used for isolation, propagation and titration of BEF virus. For isolation a confluent monolayer of BHK 21 and VERO cell cultures 50 ml prescriptions were inoculated with 0.2ml/prescription of 10% leukocytic fraction in normal saline and left for one hour to allow virus adsorption. Subsequently infected cells were washed with Hank's Balanced Salt Solution (HBSS) then supplemented with maintenance media, incubated at 37°C and subjected to daily microscopical examination to detect the induced cytopathic effects (CPE). Minimum Essential Medium (MEM) with Hank's salts and L-glutamine without sodium bicarbonate purchased from Sigma was used for the growth and maintenance of cell cultures.

The growth and maintenance media were supplemented with 10% and 2% newborn calf serum respectively. The final pH of the growth and maintenance media was approximately adjusted to 7-7.3 by sodium bicarbonate solution 4.4%. Preparation of the media was done according to the instruction manual.

RNA was extracted using one-step RNA reagent (Bio Basic Inc.) according to the manufacturer's protocol. Reverse transcription of RNA was carried out with 18 nucleotide primer FG 5' TACAACAGCAGATAAAAC 3' as a forward primer

and 18 nucleotide primer RG 5' CATTATGGGATAGGATCC 3' as a reverse primer. The forward and the reverse primers were derived from the glycoprotein gene sequence (10263-10700 nt) of the Australian isolate BB7721. Reverse transcription and PCR were carried out according to the Slomka et al., (2009) using ONE STEP RT-PCR kit (QIAGEN). The RT-PCR conditions were 30 min at 50°C, 2 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 45°C, 1 min at 72°C and 10 at 72°C. The amplified product was resolved by electrophoresis in 0.9% agarose gel.

Serum values of total protein (TP), albumin (Alb), glucose (Glu), total calcium (tCa), inorganic phosphorus (P), magnesium (Mg), creatinine (Cr), blood urea nitrogen (BUN) and serum activity of alkaline phosphatase (ALP) were measured with calorimetric procedures using diagnostic kits of Spinreact (Spain) and following the manufacturer's instructions. Serum ionized calcium (iCa), sodium (Na), potassium (K) and chloride (Cl) were determined by using OPTI LION Automated Cassette-Based Electrolyte Analyzer (OPTIMEDICAL, USA). Total globulin (Glo) was determined by subtracting albumin from total proteins.

Parathyroid hormone (PTH), insulin (Ins), cortisol (Cor) were detected by enzyme-linked immunosorbent assay (ELISA) using commercially available test kits of Cobasintegra (Roche, Germany) and following the manufacturer's instructions.

Statistical analysis

All the values were presented as mean± standard deviation (SD). Mean values of BEF infected and control animals were compared by Student's t-test at 0.05 level of probability.

Results

There was a sudden onset of fever- as high as 41°C compared with the normal temperature of about 38°C in the healthy animals. The temperature returned to normal within 36 hours. Affected animals were anorectic, depressed and losing weight. There was severe drop in milk production in milking cows. Most animals demonstrated shivering, stiffness, lameness, an incoordinated gait and were difficult to move. Some animals were in lateral recumbency and showed no interest in the surroundings. Recumbent animals were bloated, had ruminal stasis, and some lost their swallowing reflex. The respiratory manifestations included a rapid, shallow respiration and stringy nasal discharges. Other animals had aspiration pneumonia and emphysema with subcutaneous accumulation of air along the back. No abortion was recorded among pregnant animals.

Daily observation of inoculated VERO cell culture revealed appearance of CPE as cell rounding, granulated cytoplasm, cell aggregation and cell lysis that end with detachment of cells from the culture surface 4 days in the first and second passages, after 2 days post inoculation in the third passage and 24 hours post inoculation in the fourth & fifth passages (Fig. 1).

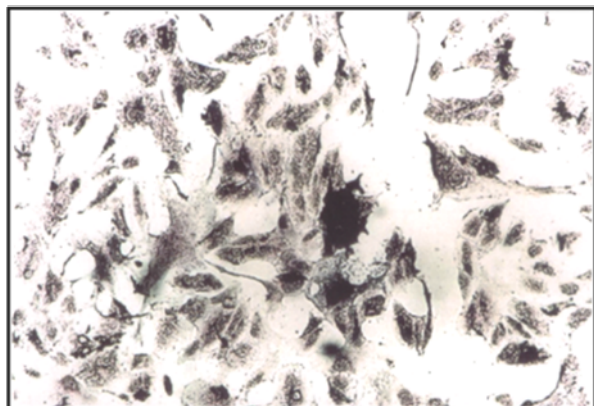


Fig. 1: CPE in inoculated VERO cells with BEF virus isolate (H&E stain, magnification: X40)

The expected amplified DNA band of 438 bp corresponds to a region in G glycoprotein was obtained using primers derived from the sequence of BEFV-Australian isolate BB7721. RT-PCR was used to detect BEFV from cell culture (BHK21 and Vero). Agarose gel electrophoresis analysis of the PCR products is demonstrated in Fig. 2.

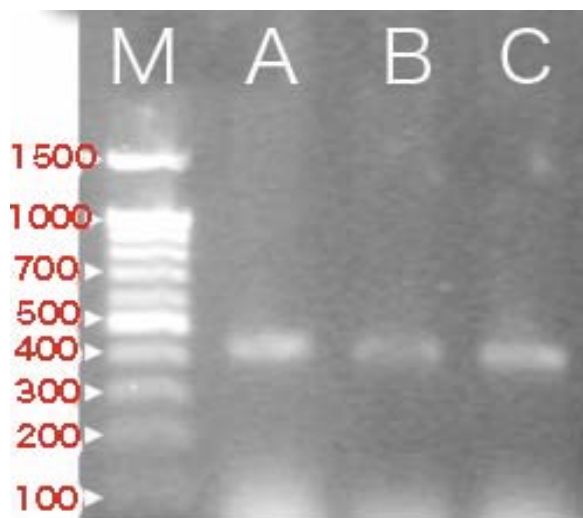


Fig. 2: PCR amplification of BEFV 438bp from BHK21 and Vero. Lane M: 100 bp ladder, lane A control, B BEFV from BHK21 and C BEFV from VERO

Results of serum biochemical results as shown in (Table 1) showed that compared to the control healthy animals, BEF-infected cattle demonstrated a significant decrease ($P<0.05$) in serum concentrations of TP, Glo, iCa, P, Na, K, BUN and ALP while the mean values of serum levels of Glu and Cl were significantly increased (Table 1). Serum concentrations of Cr did not show significant changes (Table 1).

Table 1: Serum biochemical variables in BEF-infected cattle compared to the healthy animals. (Values are means \pm SD)

Variable	Control group	OSD- group
TP (g/dl)	6.86 \pm 0.30	5.36 \pm 0.11*
Alb (g/dl)	3.36 \pm 0.11	3.30 \pm 0.17
Glo (g/dl)	3.50 \pm 0.20	2.60 \pm 0.05*
Glu (mg/dl)	80.36 \pm 3.27	90.33 \pm 0.85*
tCa (mg/dl)	8.10 \pm 0.10	7.86 \pm 0.23
iCa (mg/dl)	1.13 \pm 0.12	0.82 \pm 0.11*
P (mg/dl)	8.63 \pm 0.32	5.70 \pm 0.75*
Mg (mg/dl)	1.52 \pm 0.07	1.53 \pm 0.10
Na (mEq/l)	138.07 \pm 1.00	127.57 \pm 5.50*
K (mmol/l)	8.23 \pm 0.46	6.10 \pm 0.36*
Cl (mmol/l)	88.23 \pm 0.68	103.18 \pm 1.73*
Cr (mg/dl)	0.96 \pm 0.01	1.03 \pm 0.05
BUN (mg/dl)	14.10 \pm 0.34	10.66 \pm 0.28*
ALP (U/l)	203.72 \pm 3.97	174.97 \pm 5.11*

Significant differences in the values between the BEF and control animals are indicated by * $P<0.05$.

The mean values of serum levels of PTH showed a significant decrease ($P<0.05$) in BEF infected animals while significantly higher ($P<0.05$) levels of serum concentrations of insulin and cortisol were observed (Table 2).

Table 2: Hormonal profile in cattle infected with BEF compared to the control healthy animals. (Values are means \pm SD)

Hormone	Control	BEF-cattle
PTH (pg/ml)	29.65 \pm 3.38	18.46 \pm 0.88*
Ins (mIU/ml)	3.58 \pm 0.65	5.89 \pm 0.44*
Cor (ng/ml)	4.86 \pm 0.30	10.24 \pm 0.69*

Significant differences in the values between the BEF and control animals are indicated by * $P<0.05$.

Discussion

BEF is an acute febrile disease of cattle and water buffaloes caused by the bovine ephemeral fever virus a member of the genus *Ephemerovirus* in the family Rhabdoviridae (Nandi and Negi, 1999). Affected animals are only sick for a few days; hence the alternative name Three Day Sickness but the clinical severity of the disease is inconsistent with the

subsequent rapid recovery of most of the affected animals (Walker, 2005). Because the disease is most severe in the more valuable classes of cattle such as bulls, pregnant and lactating cows, and fat, well-conditioned cattle, significant economic losses can occur through loss of condition, decreased milk production, lowered fertility of bulls, mismothering of calves and delays in marketing and restrictions on the export of live cattle. (Young and Spradbrow, 1990). In this study, the diagnosis of BEF was based on the characteristic clinical signs which were typical for BEF and are in agreement with previous studies (Burgess and Spradbrow 1977; St. George et al., 1984; St. George et al., 1995). These clinical signs were confirmed by virus isolation and RT-PCR.

BEFV can be isolated in a number of common cell cultures including African green monkey kidney (Vero) and baby hamster kidney-21 (BHK-21). Cytopathogenic changes are visible on 2-5 days post inoculation including slight rounding of cell followed by destruction of the whole cell sheet within 24-48 hours. Reverse transcriptase polymerase chain reaction (RT-PCR) has been developed with many advantages as it is possible to detect as little as 2 fragments of viral RNA from infected tissue by ethidium bromide staining after 30 cycle of PCR (Wu et al., 1992). There is no need for virus replication, moreover, RT-PCR is not time consuming since all procedures involved take about 6 hours to be completed (Davis and Boyle, 1990). The application of RT-PCR on BEF virus infected leukocytes yielded a clear single band on agarose gel stained with ethidium bromide. The amplified DNA fragment corresponds to 438 bp. PCR confirms the diagnosis of BEF outbreak which is sensitive, specific and of value for rapid diagnosis (Khalil et al., 2001).

One of the most important problems in cattle infected with BEF is hypocalcemia which results in clinical signs have been proved to be similar to that seen in milk fever, a disease of multiparous dairy cows (St George et al., 1984; Uren et al., 1992; St George et al., 1995; Charbonneau et al., 2008). In milk fever initiation of lactation places one of the greatest stresses on Ca homeostasis and is associated with hypocalcemic parturient paresis among high producing dairy cows (Goff and Horst, 2003). In BEF, despite rigorous investigation of the clinical signs and the pathology of disease, the reason for the lowered serum calcium levels is not well known. Hypocalcemia can be produced under several conditions. The most important of them are malnutrition, hypoparathyroidism; hypoalbuminemia (about half of all calcium is bound to serum albumin, renal disease and magnesium depletion (Duncan et al., 1994; Kaneko et al., 1997) Acid-base imbalances also are known to affect serum ionized calcium levels (Duncan et al., 1994; Kaneko et al., 1997).

In the present study significant changes in serum albumin and Mg concentrations were not observed excluding hypoalbuminemia and hypomagnesemia as causes for hypocalcemia. To rule out renal disease as a reason, serum levels of Cr and BUN were measured and the results revealed that serum Cr concentrations did not show significant changes excluding the possibility of presence of renal dysfunction. On the other hand, the mean values of BUN concentrations showed a significant decrease. In ruminants, urea excretion by the kidney is dependent on nitrogen balance and nitrogen content in diet (Duncan et al., 1994). Therefore, if the animal is a low nitrogen diet as in malnutrition or anorexia, almost all urea in the rumen is degraded to ammonia, which is then used to synthesize amino acids for protein synthesis (Duncan et al., 1994).

Signs of functional hypocalcemia usually develop when ionized calcium is significantly reduced for normal muscle function without necessarily a total Ca response fall in the total serum calcium levels which was seen in the present study. Serum ionized calcium concentration can be greatly affected by acid-base abnormalities. Acidosis causes displacement of calcium ions from their binding sites on albumin and an increase in ionized calcium concentration results. Conversely, alkalosis will cause a decrease in ionized calcium concentration and may lead to the symptoms of hypocalcemia (Duncan et al., 1994; Kaneko et al., 1997). Although, no direct measurements could be made to test the presence of alkalosis, the demonstration of high fever, accelerated respiration and emphysema in the infected animals together with the fact that growth of virus takes place mainly in the reticuloendothelial cells in the lungs (Burgess and Spradbrow, 1977) may be a plausible reason for the presence of respiratory alkalosis. Fever has been known for some time to affect the acid-base balance of the blood through the accompanying hyperventilation. Hyperventilation causes the body to lose carbon dioxide in expired air leading to decreased H^+ ions and elevated pH (Afzaal et al., 2004).

The expected response to alkalosis is the progressive decrease in the calcium ions efflux from the bone. This is achieved by stimulating the osteoblastic activity and suppressing the osteoclasts thus reduces bone Ca resorption (Bushinsky, 1996). Lower values of pCO_2 coincided with a rise in pH was reported in cows infected with BEF (Uren et al., 1992). Lopez et al. (2003) stated that metabolic and respiratory acidosis stimulate PTH secretion while, metabolic and respiratory alkalosis markedly decrease PTH values during normocalcemia and delay the PTH response to hypocalcemia. This explanation was provided very much in retrospect by testing of serum levels of parathyroid hormone which showed significant decrease in the BEF-infected animals suggesting that

the reduction in serum ionized calcium seen in the present study was due to decreased bone resorption caused by suppression of PTH secretion.

Reduction in serum ionized calcium in respiratory alkalosis also can be secondary to increased binding of calcium to serum albumin even though plasma total calcium does not change. This is because a portion of both hydrogen ions and calcium are bound to serum albumin. When blood becomes alkalotic, bound hydrogen ions dissociate from albumin, freeing up the albumin to bind with more calcium and thereby decreasing the freely ionized portion of total serum calcium (Afzaal et al., 2004). Ruminant stasis also may have contributed to the lower serum calcium levels, by reducing calcium absorption, but is not likely to be initiating cause (St. George et al., 1984).

Consistent with the report of St. George et al. (1984) and Uren et al. (1992), the present study reported a significant decrease in serum phosphorous concentrations which seemed to be secondary to phosphorus redistribution as a result of respiratory alkalosis (Amanzadeh and Reilly, 2006). During respiratory alkalosis, intracellular CO_2 decreases, causing intracellular pH to rise. This mechanism stimulates the glycolytic pathway, specifically phosphofructokinase, a key rate-limiting enzyme of glycolysis. Production of sugar phosphates is enhanced, which in turn induces intracellular phosphorus entry, thus decreasing serum phosphorus concentration (Hoppe et al., 1982; Amanzadeh and Reilly, 2006). Respiratory alkalosis also has been reported to enhance phosphorus uptake by muscle which largely accounts for the hypophosphatemia (Hoppe et al., 1982).

In this work, hyponatremia was recorded in cattle infected with BEF. Hydrogen ions are actively secreted in the proximal and distal tubules in exchange with the sodium (Afzaal et al., 2004). As a renal response to respiratory alkalosis the kidney may reduce hydrogen ion secretion into the urine in exchange for increased sodium excretion which may result in hyponatremia (Kaneko et al., 1997). Renal correction of respiratory alkalosis may also include an increase in excretion of bicarbonate primarily as sodium bicarbonate instead of chloride to maintain electrical neutrality. This exchange would also lead to chloride retention and thus hyperchloremia which was reported in our study. Hypokalemia was observed in cattle infected with BEF. Low serum potassium concentrations can be seen in respiratory alkalosis as K^+ in the extracellular fluid is exchanging with intracellular H^+ ions that are pumped extracellularly (Krapf et al., 1995). Insulin also is known to enhance shift of potassium intracellularly (Duncan et al., 1994) therefore, the increase in serum insulin levels in this study can contribute in part in the development of hypokalemia seen in the BEF-infected cattle. The significant reduction in serum activity of

alkaline phosphatase could be associated with hypoparathyroidism and low levels of phosphorus (Mike, 2010).

The mean values of serum glucose concentrations were significantly higher in the BEF-infected cattle. Taking in consideration that cattle tend to produce marked stress hyperglycemia (Kaneko et al., 1997; Roussel, 1997 and Moore, 1998), this increase serum glucose levels could be attributed to stress response as indicated by the significant increase in serum cortisol concentrations reported in the infected cattle. Cortisol is a hormone, produced by the adrenal gland when the body is under stress for various reasons such as febrile conditions, general systemic illness and infections (Adcock et al., 2007; Torpy and Ho, 2007). The effect of cortisol on blood glucose levels can be achieved by promoting hepatic gluconeogenesis and inhibiting cellular insulin-receptor affinity exerting a postreceptor influence within cells producing insulin resistance that inhibits the action of insulin on glucose metabolism and thus hyperglycemia results (Roussel et al., 1997). This idea is further supported by observing the results of serum insulin concentrations which showed a significant increase. Insulin sensitivity and secretion are reciprocally related; thus, insulin resistance results in increased insulin secretion to maintain normal glucose homeostasis (Kaneko et al., 1997).

A significant decrease in serum TP was observed in animals infected with BEF as a result of hypoglobulinemia which may reflect an inhibition in the number and/or function of lymphocytes (antibody producing cells). Lymphopenia has been reported to be a consistent feature in cows infected with BEF starting with the onset of fever to return to normal levels after 3-4 days (Burgess and Spradbrow 1977; St. George et al., 1984; Uren, 1989; St. George et al., 1995). In cattle experimentally infected with BEF virus, low levels of neutralizing antibody were detected within the first or second day of clinical disease and lasted for one or two days after the cessation of viremia (Young et al., 1990).

Furthermore, cortisol is known to weaken or suppress the activity of the immune system by inhibiting lymphoid mitosis and reducing immune cell number and function (Ramaekers et al., 1975; Chase et al., 1995). Therefore hypoglobulinemia could be related in part to increased serum cortisol levels.

In conclusion, the information obtained from this study suggests that the clinical signs of bovine ephemeral fever appear to be related to hypocalcaemia. This hypocalcemia is the result of suppression of parathyroid hormone which is likely mediated by disturbances in acid-base status resulting from respiratory alkalosis caused by the disease. Although the fall in serum ionized calcium together with clinical presentation of the disease can be considered objective supporting evidence for respiratory alkalosis, this

explanation needs future studies to provide a direct link between measurement of blood indicators of acid-base status, blood biochemical parameters and urine analysis. However, this work can provide a good knowledge about the pathogenesis of the disease that can lead to better management and proper treatment. These findings further can recommend that the involvement of some drugs inducing a compensated metabolic acidosis can be considered a justifiable addition to the treatment regimen of BEF.

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