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Molecular detection and macroelements changes in cattle infected with bovine ephemeral fever virus in Assiut Governorate, Egypt

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ABSTRACT

Bovine ephemeral fever (BEF) is a dangerous febrile viral disease that affects cattle and causes large financial losses. The aims of this study were to study clinical findings of BEF virus (BEFV) infection, identify certain risk factors and evaluate infected cattle's mineral changes. One hundred and seven cattle were used in this investigation. Whole blood and serum samples were collected for laboratory analysis. Reverse transcriptase polymerase chain reaction (RT-PCR) had been employed for BEFV diagnosis. The observed clinical findings of BEFV infection in cattle were fever, respiratory manifestation (serous nasal discharge & cough), lacrimation, lameness, enlarged of superficial lymph nodes, drop of milk production, ruminal stasis, and recumbency. Of the studied diseased cattle, 43 (43%) of 100 whole blood samples showed molecularly positive results for G1 gene. The infection rate of BEFV had no significant variation by locality, age, sex, breed and season in the studied diseased cattle. Serum calcium level of confirmed BEFV positive samples was significantly lower ($p<0.001$) than serum calcium level in clinically healthy ones. Serum phosphorus and magnesium levels in BEFV infected cattle did not differ significantly ($P<0.05$) from that of clinically healthy ones. The importance of establishing efficient prevention and control strategies in Egypt must be emphasized in order to reduce the prevalence of BEFV infection in cattle.

Introduction

BEF, commonly known as Three-day sickness, is a non-contagious viral disease that affects cattle and water buffaloes (El-Allawy *et al*., 2021; Rezatofighi *et al*., 2022). In tropical and subtropical areas of Africa, Asia, Australia, and the Middle East, BEF is a serious febrile disease (Lavon *et al*., 2023). It is caused by BEFV, a rhabdovirus belonging to genus *Ephemerovirus* and family *Rhabdoviridae* (Lavon *et al*., 2023). BEFV is a single-stranded, negative-sense RNA virus that resembles a bullet (Elgendy *et al*., 2022). A nonstructural glycoprotein and five structural proteins of the matrix, nucleoprotein, phosphoprotein, surface glycoprotein, and RNA-dependent RNA polymerase, are encoded by the 14,900 nucleotides viral genome (Rezatofighi *et al*., 2022; Lavon *et al*., 2023). Glycoprotein (G) has four different antigenic sites (G1-G4) identified on its surface and is essential for both the induction of the host body's immune response and the process of virus replication (Rezatofighi *et al*., 2022). Direct contact or fomites do not transmit BEFV; but it spread by mosquitoes and biting midges like *Culicoides* (Zahid *et al*., 2022; Lavon *et al*., 2023). BEF is recognized by high morbidity, which may exceed up to 80% and extremely low mortality (1–2%) but if treatment is delayed, it can worsen (Benevenia *et al*., 2024; Puspitadesy *et al*., 2024). Even though it only lasts for a short period, the disease can result in significant financial losses because it reduces the quantity and quality of milk produced, keeps beef herds in worse condition, abortion, lowers bull fertility, and in severe cases, can even be fatal (El-Allawy *et al*., 2021; Lavon *et al*., 2023). Biphasic fever, salivation, muscle stiffness, difficulties with swallowing, serous nasal and ocular discharge, dyspnea, lameness, general lethargy, limb paralysis, sternal and lateral recumbency with pulmonary and subcutaneous emphysema are clinical characteristics of BEF (Bazargani *et al*., 2013; Elgendy *et al*., 2022; Benevenia *et al*., 2024). Given the need to have practical and reliable BEF diagnostic methods that enable the establishment of strategies to manage the disease and lower viral transmission (Benevenia *et al*., 2024). RT-PCR is the most effective molecular method employed for diagnosis of this disease (El-Allawy *et al*., 2021). The G gene is important for the molecular identification and categorization of viruses (Tokgoz *et al*., 2024). Early paralysis is reversible due to the biochemical alterations caused by BEFV infection, such as an increase in blood fibrinogen and a drop in total calcium levels (Chandra *et al*., 2004). Considering the financial impact of BEFV infection in cattle, the aims of the present study were to investigate the clinical findings of BEF, determine distinctive risk factors, and evaluate serum macroelemetns changes in cattle associated with BEFV infection.

Materials and methods

Animals and Ethical approval

Throughout the study period from May 2023 to June 2024, a total of 107 (100 diseased and 7 clinically healthy) cattle of different ages, sexes, and breeds from various villages in Assiut Governorate were admitted to Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Assiut University. The 100 investigated diseased cattle were divided into three groups: group (1) were 29 cases that suffered from enlarged of superficial lymph nodes, respiratory manifestation (serous nasal discharge & cough) and ruminal stasis, group (2) were 30 cases which suffered from fever, enlarged of superficial lymph nodes and ruminal stasis and group (3) were 41 cases that suffered from fever, drop of milk production, lameness, recumbency, respiratory manifestation (serous nasal discharge & cough), lacrimation and ruminal stasis. Each cattle used in this study was cared for in compliance with ethical standards. The investigation was given approval number 06/2025/0308 by the Research Ethical Committee of the Faculty of Veterinary Medicine at Assiut University in Assiut, Egypt.

Clinical evaluation

Clinical examinations of the studied cattle were conducted in compliance with Jackson and Cockcroft (2002).

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Sampling

Five milliliters of whole blood were drawn from the jugular vein of the investigated cattle and separated into two parts after they had been properly restrained. One part (2 ml) was put into sterile ethylene diamine tetraacetic acid (EDTA) vacutainer tubes and kept at -20°C for subsequent extraction of RNA. The second portion (3 ml) was put into sterile plain vacutainer tubes free from anticoagulant and left to clot at room temperature. After clotting, it was centrifuged at 3000 r.p.m. for 20 minutes, and serum was carefully obtained and stored in separate tubes at -20°C for further biochemical analysis (Saied, 2017; Abd El baset *et al*., 2020).

Molecular diagnosis

Viral RNA extraction

The viral RNA was extracted from 100 whole blood samples of examined diseased cattle using the EasyPure® Simple Viral DNA/RNA extraction kit (TransGen Biotech, China), following the manufacturer's directions.

Primers

The particularity of the primers selected for the G1 gene of BEFV in the present investigation (Metabion International AG, Germany) was previously assessed (Zheng *et al*., 2007; El-Allawy *et al*., 2021). Table 1 illustrates the primer sequences and their positions within the viral genome.

Table 1. Nucleotide sequence of the chosen primers of G1 gene of BEFV and the size of the RT-PCR products.

Primer		Sequences of nucleotides	Size of product (bp)
G_1 gene	380F	5´- AGA GCT TGG TGT GAA TAC -3´	380
	380R	5´-CCA ACC TAC AAC AGC AGA TA-3´	

Identification of G1 gene of BEFV using RT-PCR

Specifically, an RT-PCR was constructed to amplify the BEFV G1 gene. The extracted RNA was reverse transcribed to cDNA using the ABT 2X RT mix kit (Applied Biotechnology, Egypt) following the manufacturer's instructions and kept at -20°C until it was used. Using the ABT 2X RT mix kit (Applied Biotechnology, Egypt), the extracted RNA was reverse transcribed to cDNA in accordance with the manufacturer's instructions and stored at -20°C until it was needed. cDNA fragments with a length of 380 bp were amplified using primer sets 380 forward and 380 reverse. The ABT red master mix (2X) (Applied Biotechnology, Egypt) was utilized in this investigation as a source of polymerase enzymes and DNTP. PCR was performed using a PCR thermocycler (Techine, UK) using the following reagents: 8 µl of ABT red master mix (2X), 0.5 µl of each primer 380F and 380R (5 pmol), 4 µl of cDNA material, and 3 µl of PCR molecular grade water make up the final 16 µl volume. One initial denaturation at 94°C for five minutes (40 cycles of denaturation at 94°C for one minute, 46°C for one minute for the annealing step, and 72°C for one minute for extension) and a final extension at 72°C for ten minutes were the thermal cycling conditions.

Analysis and detection of PCR products

To determine the reaction, seven microliters of the amplified DNA product were injected. Using a gel UV transilluminator (Syngene, UK), the amplicons were become visible after 75 minutes of 1.5% agarose gel electrophoresis stained with ethidium bromide (10 mg/ml) at 90 V and 155 mA. A 100-bp DNA ladder was employed to measure the amplicon size.

Samples selection for biochemical parameters

Biochemical parameters studies used 25 samples positive for BEFV (6 samples of group (1), 9 samples of group (2), 10 samples of group (3)) and 7 samples as a control group (clinically healthy).

Biochemical parameters analysis

Utilizing a spectrophotometer (Mecasys, Korea) and commercially available kits, the biochemical analysis was conducted to determine the serum levels of total calcium, phosphorus and magnesium (Biomed diagnostic, Egypt) in accordance with the manufacturer's instructions.

Statistical analysis

The statistical package for the social sciences (SPSS) version 16 software was used to conduct statistical analysis (2007). For analyzing the clinical and epidemiological findings, the Chi-square of independence was used. Biochemical parameters were compared between clinically healthy and molecularly positive cattle using a one-way analysis of variance (ANOVA).

Results

Clinical findings of investigated cattle associated with BEF

The observed clinical features of BEF in the 9 examined diseased cattle in group (1) were enlarged of superficial lymph nodes, respiratory manifestation (serous nasal discharge & cough) and ruminal stasis, while in 15 investigated diseased cattle in group (2) were fever (40-42oC), enlarged of superficial lymph nodes and ruminal stasis but in 19 studied diseased cattle in group (3) were fever (40-42oC), drop of milk production, lameness, recumbency, respiratory manifestation (serous nasal discharge & cough), lacrimation, and recumbency (Table 2).

Molecular detection of BEFV by RT-PCR

PCR was used to analyze cDNA samples in order to produce the required band at 380 bp as a result of the G1 gene of BEFV (Fig. 1). Molecularly positive results were found in 43 (43%) out of 100 whole blood samples.

Table 2. Clinical signs of BEF in studied diseased cattle.

Clinical findings		No. of investigated cattle	BEF		P-value
			Positive No. (%)	Negative No. (%)	
Group (1)	Enlarged of superficial lymph nodes, respiratory manifestation and ruminal stasis	29	9 (31.03%)	20 (68.97%)	0.29
Group (2)	Fever, enlarged of superficial lymph nodes and ruminal stasis	30	15 (50%)	15 (50%)	
Group (3)	Fever, drop of milk production, lameness, recumbency, respiratory manifestation, lacrimation and ruminal stasis	41	19 (46.34%)	22 (53.66%)	
Total		100	43 (43%)	57 (57%)	

No significant variation at $p<0.05$.

Fig. 1 Agarose gel electrophoresis of RT-PCR products following G1 gene amplification of BEFV in studied cattle. Line M: DNA ladder 100 bp, line C+ve: Control positive sample; lines 1, 2, 3, and 4: Positive samples and line C-ve: Control negative.

Potential risk factors

This study highlighted few risk factors, such as locality, age, sex, breed, and season that influence the occurrence of BEF in cattle. Age, sex, and breed of the affected cattle, as well as locality and season, did not significantly affect the BEF infection rate (Table 3).

Biochemical parameters analysis

Values of calcium, phosphorus and magnesium markers of BEFV infected and clinically healthy cattle are shown in Table 4. The serum calcium level of confirmed BEFV positive samples was significantly lower ($p<0.001$) than serum calcium level in clinically healthy ones. The serum phosphorus and magnesium levels in BEFV infected cattle did not differ significantly ($P<0.05$) from that of clinically healthy ones.

Discussion

BEF is an arthropod-borne disease that affects cattle and results in large economic losses (El-Allawy *et al.*, 2021). The noted clinical findings of BEF in the studies diseased cattle were fever (40-42oC), salivation, respiratory manifestation (serous nasal discharge & cough), lacrimation, lameness, enlarged of superficial lymph nodes, drop of milk production, ruminal stasis, and recumbency. These signs were similar to those found in earlier studies by Zaghawa *et al.* (2017); El-Allawy *et al.* (2021); Elgendy *et al.* (2022); Rezatofighi *et al.* (2022) and Puspitadesy *et al.* (2024). The clinical findings associated to BEFV infection which reported in our investigation could be caused by the disease-related inflammatory response led to a drop in calcium concentration, an increase in vascular permeability, the production of cytokines and interleukins (IL-2 and IL-6), and inflammatory biomarkers such cortisol and c-reactive protein while BEFV replication in reticuloendothelial tissues such as the lung, spleen, and lymph nodes, as well as nutritional selenium insufficiency, may be the cause of pulmonary and subcutaneous emphysema (Albehwar *et al.*, 2017; Abo-Sakaya and Bazan, 2020; El-Allawy *et al.*, 2021).

Table 3. Correlation between BEFV in examined cattle and possible risk factors according to RT-PCR result.

Variable		No. of examined cattle	PCR		P-value
			No. of positive (%)	No. of negative (%)	
Locality	Manfalut city	19	8 (42.11%)	11 (57.89%)	0.97
	Assiut city	52	23 (44.23%)	29 (55.77%)	
	Abuteeg city	29	12 (41.38%)	17 (58.62%)	
	Total	100	43 (43%)	57 (57%)	
Age	8 months - 1.5 year	22	12 (54.55%)	10 (45.45%)	0.46
	< 1.5 - 3 years	45	18 (40%)	27 (60%)	
	< 3 - 5 years	33	13 (39.39%)	20 (60.61%)	
	Total	100	43 (43%)	57 (57%)	
Sex	Male	45	23 (51.11%)	22 (48.89%)	0.14
	Female	55	20 (36.36%)	35 (63.64%)	
	Total	100	43 (43%)	57 (57%)	
Breed	Native	58	27 (46.55%)	31 (53.45%)	0.40
	Mixed	42	16 (38.10%)	26 (61.90%)	
	Total	100	43 (43%)	57 (57%)	
Season	Spring	14	6 (42.86%)	8 (57.14%)	0.84
	Summer	71	32 (45.07%)	39 (54.93%)	
	Autumn	4	1 (25%)	3 (75%)	
	Winter	11	4 (36.36%)	7 (63.64%)	
	Total	100	43 (43%)	57 (57%)	

No significant variation at $p<0.05$.

Table 4. Serum biochemistry in BEFV infected cattle.

Variable	Clinically healthy group	Diseased group (1)	Diseased group (2)	Diseased group (3)	P-value
Calcium (mg/dl)	11.76±0.16[a]	7.95±0.65	8.08±0.54[b]	7.90±0.51[b]	0
Phosphorus (mg/dl)	5.31 ±0.41	5.23 ±0.44	6.03 ±0.36	5.77 ±0.34	0.44
Magnesium (mg/dl)	1.27 ±0.20	1.96 ±0.22	1.71 ±0.18	1.43 ±0.17	0.10

[a,b] Highly significant differences exist between the infected and healthy animals ($P<0.001$).

The current investigation used RT-PCR to confirm BEFV by using G1 gene. RT-PCR technique which was applied in this study had been established with numerous benefits including the ability to detect as little as two fragments of viral RNA from infected tissue (Wu *et al*., 1992), the lack of the need for virus replication, the fact that RT-PCR is time-efficient because all procedures take about 6 hours to complete (Davis and Boyle, 1990), the certainty that it is sensitive, specific, and useful for rapid diagnosis of the BEF outbreak in Egypt, and the significance that the optimized RT-PCR protocol can be used as a routine diagnostic tool for imported animals that may be BEFV carriers (Lapira *et al*., 2018). G1 epitope is a useful target gene for establishing sensitive and specific molecular diagnostic tool such as RT-PCR, for the recognition of BEFV infection because it has been genetically conserved among different isolates of BEFV (Pasandideh *et al*., 2018). G1 gene was found in 43 (43%) of the 100 whole blood samples of studied cattle by RT-PCR. Our molecular result (43%) was nearly similar to the findings of the previous research by Degheidy *et al*. (2011), who recorded that 45.45% (10/22) of the investigated cattle were molecularly BEFV positive. Our result was superior to those of a prior investigation by Chaisirirat *et al*. (2018), which found that 32% (24/75) of examined cattle had BEFV by RT-PCR. Our finding was lower than the earlier publication by El-Allawy *et al*. (2021) who concluded that the molecular positivity rate for the BEFV in cattle was 80% (40/50). This finding can result from Egypt's lack of a mandatory BEFV vaccination program.

The potential interactions of various risk factors, including locality, age, sex, breed, and season, with BEFV infection rate in examined cattle were investigated. Locality-wise, BEFV infection detection rate in the studied cattle was statistically non-significant in the three Assiut cities (Manfalut, Assiut, and Abuteeg). This result may be explained by the absence of any natural divisions or obstacles among Assiut Governorate's cities. Regarding age susceptibility, the rate of BEFV infection did not significantly differ among the age groups of cattle that were subjected to this study. This finding was similar to that of Mirazaie *et al*. (2017), who noted that BEFV affects all ages group of examined cattle. Our findings suggest that the study's cattle had an identical risk of contracting BEFV infection. In terms of sex vulnerability, the study found no statistically significant difference in the BEFV infection rate between male and female cattle. Our result was consistent with earlier studies by Momtaz *et al*. (2012) and Al-Sultany and Hassan (2013), who found no discernible difference in the prevalence of BEFV infection between males and females. Since both male and female cattle were equally susceptible to this non-sex-related disease, our outcomes may indicate that sex is not a significant contributing factor to bovine infection with BEFV. The rate of BEFV infection in native and mixed breeds of cattle did not significantly differ in the current investigation. This result was in contrast to previous investigations by Momtaz *et al*. (2012); Zaher and Ahmed (2011); Zaghawa *et al*. (2017) and El-Allawy *et al*. (2021), which showed that mixed breed, had a greater BEFV infection rate than native breed of cattle. It would appear from our findings that all cattle breeds had an equal chance of contracting BEFV infection. Studying seasonal variations and BEFV infection frequency revealed that the proportion of BEFV infection did not significantly vary throughout the year, however mathematically summer and spring seasons had a higher infection rate than autumn and winter seasons. This finding was in parallel with the findings of Kasem *et al*. (2014) and El-Allawy *et al*. (2021), who noted that the rate of BEFV infection was greater during hot months than during non-hot months. Our findings may be attributed to this disease can be seen throughout of year. Presence of BEFV infection in summer and spring more than other seasons may be due to the widespread of flying insect vectors (mosquito species), which are primarily responsible for the disease's transmission during summer. However, because of lack of rainfall during this time of year, the high temperatures and presence of standing water may provide an ideal environment for vector reproduction (Al-Sultany and Hassan, 2013; Kasem *et al*., 2014; Zaghawa *et al*., 2017; El-Allawy *et al*., 2021).

Serum biochemical markers, including calcium, phosphorus and magnesium were examined in cattle with and without BEFV infection. In our findings, the serum calcium level of confirmed BEFV positive samples was highly significantly lower ($p<0.001$) than serum calcium level in clinically healthy ones. These results were consistent with earlier studies by Ammar *et al*. (2005); Saied (2017); Abdullah *et al*. (2020); Zahid *et al*. (2022) and Dinçer *et al*. (2024), who reported that the serum calcium level of determined BEFV-positive samples was significantly lower than that of clinically healthy samples. Our outcome may be attributed to BEFV causes hypocalcaemia in infected cattle. One explanation for BEFV infection-induced hypocalcemia has been attributed to activated neutrophils, which cause calcium in the plasma to move within the neutrophils and return to normal near the end of the neutrophils' life (Abdullah *et al*., 2020). Other potential causes of hypocalcaemia include elevated pH, which can reach 7.8 due to a high ammonia level, or ruminal stasis, in which the animal is unable to absorb calcium from the rumen. Increased levels of non-esterified fatty acids in plasma may help the body absorb some calcium (Zahid *et al*., 2022). Hypoproteinemia could be the basis for the resulting hypocalcemia (Dinçer *et al*., 2024). In the current investigation, the serum phosphorus level in BEFV infected cattle did not differ substantially ($P<0.05$) from that of clinically healthy ones. Our findings were in accordance with earlier studies by Sayed *et al*. (2001); Jameel *et al*. (2012), who found no discernible difference in serum phosphorus levels between the BEFV-infected and clinically healthy groups. This result was different from prior investigations by Ammar *et al*. (2005); Saied (2017); Zahid *et al*. (2022) and Dinçer *et al*. (2024), which revealed that BEFV-infected cattle had a considerably lower serum phosphorus level than clinically healthy ones. According to our findings, phosphorus homeostasis is not substantially altered by BEFV or the inflammatory response it causes. The short-term and self-limiting nature of BEFV, which might not result in long-term systemic disruption, the efficient regulation by the kidneys, bones, and intestines, or the lack of cellular damage or organ failure that would cause notable phosphate shifts in BEFV infection are some possible explanations for this stability. The present study found no significant difference ($P<0.05$) between the serum magnesium level of BEFV-infected and clinically healthy cattle. Our results supported a previous study by Sayed *et al*. (2001), which reported no significant differences in serum magnesium levels between the clinically healthy and BEFV-infected groups. According to our findings, there is no direct correlation between BEFV and the animals' magnesium levels.

Conclusion

The current study identified BEFV infection in cattle. Calcium level decreases in serum of cattle infected with BEFV. According to the findings, improved disease control, screening programs, and greater awareness and preventive measures are all necessary to lessen the adverse effects of BEFV infection on animals' health.

Conflict of interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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