*Original Article*

# Bovine ephemeral fever epidemics in Kingdom Saudi Arabia: clinical, epidemiological and molecular investigation

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## Abstract

Introduction: Bovine ephemeral fever virus (BEFV) is an arthropod borne Rhabdovirus affects cattle and water buffalo causes acute febrile disease.
Methodology: The clinical picture and epidemiological pattern of BEF were described among cattle in epidemics of 2007, 2009 and 2011 in four geographical regions of Kingdom Saudi Arabia (Eastern, Jizan, Qasim, and Riyadh). Serum samples were tested using VNT. Virus isolation and molecular characterization were carried out for the first time in KSA.
Results: The main clinical symptoms were fever, stiffness, lameness, salivation and subcutaneous emphysema. The prevalence and the mortality rate of BEF have decreased from 70% and 4.6% in 2007 to 30% and 0.6% in 2011, respectively in the 4 studied areas. There was no region association with higher prevalence of BEF. The intracluster correlation (ICC) was estimated for the first time in KSA as 0.0034. BEFV had been isolated from 11 out of 20 samples (55%) and isolation was confirmed by VNT. The molecular detection of BEFV by RT-PCR and real-time RT-qPCR were found more sensitive for diagnosis of the disease than virus isolation; 80% and 90% for the former tests and 55% for the latter. Three isolates were sequenced, they showed 84.7% - 100% identities in between and shared 90.4%-96.5% sequence identity with a previously published sequence from Australia (KF679404). The generated sequences belonged to 3rd cluster of BEFV glycoprotein.
Conclusions: BEF occurrence has cyclic nature and the efficacy of vaccines prepared from local strains has to be evaluated and considered in diseases control.

## Introduction

Bovine ephemeral fever (BEF) is a viral disease of cattle and water buffaloes which causes economic losses such as: reduction in milk production in dairy herds; loss of condition in beef cattle; and loss of draught animals at the time of harvest [1]. The causative agent of the disease is bovine ephermeral fever virus (BEFV); which belongs to genus *Ephemerovirus* in the family *Rhabdoviridae*. BEFV is a bullet or cone-shaped virion possessing a single stranded, negative-sense RNA genome, and five structural proteins comprising a nucleoprotein (N), a polymerase-associated protein (P), a matrix protein (M), a large RNA-dependent RNA polymerase (L) and a glycoprotein (G) spanning the viral envelope. The G protein is a class I transmembrane glycoprotein that forms clear projections on the virion surface [2, 3]. Four distinct neutralizing epitopes (G1, G2, G3 and G4) have been determined [4].

Clinical signs of BEF include bi-phasic fever, anorexia, muscle stiffness, ocular and nasal discharge, ruminal stasis and recumbence [1]. The disease is characterized by high morbidity and low mortality with various complications [5]. Reported outbreaks show that, there is an increase in the morbidity and mortality rates such in Saudi Arabia during 1990 and 1996 [6] and in Egypt 2000 [7]. Serum samples of 1,480 cattle in Kingdom of Saudi Arabia (KSA) were examined for the presence of specific neutralizing antibodies to BEFV and the results indicated the transmission of the virus in non-vaccinated cattle [8].

The severe clinical manifestations and enormous economic losses recorded recently [1] have created

substantial awareness, among both individual and industrial owners, of the epidemiology and control of BEF. In this study, we describe the clinical picture and the epidemiology of BEF outbreaks that occurred in the summer of 2007, 2009 and 2011 among cattle belonging to farmers in four geographical regions of KSA (Eastern, Jizan, Qasim, and Riyadh). The methods used include: isolation of BEFV, molecular detection by RT-PCR and real time RT-qPCR followed by sequencing and phylogenic analysis of amplified segment of G glycoprotein gene of the isolated BEFV from these outbreaks was the other aim of this study.

## Methodology

### Study Regions

The Kingdom of Saudi Arabia has one of the largest sheep, goat and camel populations in the Middle East area. The cattle population receives little attention in this area due to customer preferences and are mainly kept by either large industrial one species farms or individual farmers who keep few animals (usually > 10 animals) sometimes mixed with sheep, goats and camels [9]. The target population in this study was the cattle owned by individual farmers because the animals receive less veterinary supervision than the large industrial herds [8]**.** The highest densities of cattle are found in the Eastern, Jizan, Qasim, and Riyadh regions of KSA [9] – the study area. Summer in the study area is very hot which facilitates the wide spread of mosquitoes; the most important vector of BEF virus. Animals within the study areas have suffered BEF epidemics in different years of the last decade and so the epidemiology of the disease was studied as explained below.

### Animals and samples

A sample size of 385 was used and this was calculated as follows and described previously [8].

$$n = t^2 \times p\,(1\text{-}p)/m^2$$

where n = required sample size, t = 95% confidence interval, p = assumed prevalence of BEF in the study area (50%), m = margin of error (5%).

Four hundred cattle were examined clinically from each of the 4 studied regions in different epidemics. These cattle were inspected and physically examined twice daily [10].

Blood samples were collected via jugular venipuncture into clot activator tubes (Vacuette Greiner Bio-One, Frickenhausen, Germany). Blood samples were allowed to clot at ambient temperature, followed by centrifugation at 4,000 × g for 15 minutes,

and serum was harvested and stored at –20 ºC until serological analysis. All serum samples were subjected to a Micro-neutralization test which is known as the Virus neutralization test (VNT) [8].

Approximately 5 ml of whole-blood in 2011 epidemic was collected for virus isolation from 20 selected animals with fever above 40°C (typical of BEF) into vacutaner tubes containing EDTA, followed by separation of leucocytes fraction and preservation at – 80°C till use.

### Epidemiological investigation
Prevalence estimation

Apparent Seroprevalence (AP) was defined as the proportion of serum samples in the studied population that had antibodies against BEFV. Sensitivity (Se) and specificity (Sp) [8] and the true overall seroprevalence of BEFV antibodies among examined animals (TP) were calculated after adjusting for the sensitivity (Se) and specificity (Sp) of the serological tests as follows.

$$TP = (AP + Sp - 1) / (Se + Sp - 1).$$

The 95% confidence interval (CI) for TP was estimated using the Wald method [11]

$$CI = p \pm Z * \sqrt{\frac{p*(1-p)}{n}}$$

Where p is the seroprevalence, Z = 1.96 and n is the number of samples.

Mortality rate and relative risk of mortality rate estimation:

The mortality rate in different years of epidemics and in different regions was estimated as the proportion of animals studied that died after showing BEFV infection signs.

The relative risk of mortality among different regions and different years of epidemics was calculated using the mortality in the Eastern region and in 2011 as the baseline groups, by the following equation according to [12]:

$$(D1/N1)*(N2/D2)$$

Where D1 is the number of dead animals in a particular region or year. N1 is the total number tested in this region or year. D2 is the number of dead animals in Eastern region or in 2013. N2 is the total number tested in Eastern region or in year 2011.

Intracluster correlation coefficient

Calculation of intra-area correlation coefficients for seropositive status of individual cattle against BEFV

infection was obtained using the following equation [13].

$$ICC = (MSC-MSE)/(MSC+(Ma-1)*MSE)$$

$$MSC = \sum m * \frac{(p1-p)^2}{n-2}$$

$$MSE = \sum y * \left(\frac{1-p1}{M-n}\right)$$

$$Ma = \frac{\left(M - \sum\left(\frac{\sum m^2}{\sum M}\right)\right)}{(n-2)}$$

$$p = \sum y / \sum m$$

Where MSC is the mean square between areas (clusters), MSE is the mean square within areas, n is the number of areas, m is the number of cattle per village, y is the number of seropositive cattle per village, M is the sum of m (total number of cattle), n is the number of clusters, p1 is the proportion of seropositive cattle per area and p is the overall proportion of seropositive cattle or among all villages.

Risk factor identification

A binary logistic regression model was used to estimate the effect of the year and region on the seropositive status of cattle against BEFV. This model was built using SAS 9.2 software (SAS Institute Inc. 2008) which also calculates the crude odds ratios (OR) with 95% confidence interval (CI).

Evaluation of diagnostic tests

The sensitivity of different diagnostic tests; virus isolation, RT-PCR and RT-qPCR were estimated using the results of the VNT as the gold standard. [14].

*BEFV isolation*

The isolation of BEFV was carried out as described in brief below [15]. Suckling mice were inoculated with the leucocyte fraction of infected animals (100 µl) and subjected to 5 blind passages in suckling mice. Thereafter Vero cells were inoculated with BEFV extracted from the brain of the 5th passage infected suckling mice. The virus, kindly supplied by the Animal Health Institute (Riyadh Ministry of Agriculture, KSA), was passaged 5-10 times in Vero cells until a cytopathogenic effect (CPE) was evident.

*BEFV identification*

Isolates of the BEFV were identified in cell culture by VNT, according to Burgess 1974 [16], using the reference bovine ephemeral fever virus antiserum provided kindly from the National Institute of Animal Health, Kannondai, Tsukuba, Ibaraki, Japan.

*Molecular detection of BEFV*
RNA extraction

Isolation of RNA was carried out from Vero cell culture by using MagMax-96 viral RNA isolation kit provided by Applied Biosystems (Foster City, USA) according to the manufacturer´s instructions.

*RT-PCR for detection of BEFV*

The RT-PCR targeting the G protein gene by using primers previously described by [15] was carried out in a thermocycler (Applied Biosystems, Foster City, USA). RT-PCR was carried out using one step RT-PCR kits (Invitrogen, Carlsbad, USA). The RT-PCR buffer had a final concentration of 50 mM Tris-HCL, pH 8.3, 75 mM KCL, 3 mM $MgCL_2$, Superscript RNase H-Reverse Transcriptase, 1 mM dNTP, 0.5 µm of each primer, 2.5 U Taq DNA polymerase and 5 µl of viral RNA. The volume of the final mixture was 50 µl which was subjected for 5 minutes to 60°C (to denature RNA) and for 45 min to 42°C (for cDNA synthesis). This was then amplified by PCR using 40 cycles (94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min). This was followed by a final elongation step terminated the reaction at 72 °C for 10 minutes. Analysis of the amplified product was by electrophoresis on a 1.5 % agarose gel and visualization using a UV illuminator after staining with ethidium bromide.

*Real Time RT-PCR for detection of BEFV*

Published primers and probe sequences were used [16]. Briefly the RNA was reverse transcribed to cDNA as follows: 4µl of high capacity RNA-to cDNA Master Mix (Applied Biosystems, Foster City, USA), 2µl RNA and 14µl deionized water were transferred to the tube (total volume 20 µl), followed by centrifugation to spin down the content and to eliminate any air bubbles. The reaction tubes were loaded into a thermocycler (5 minutes at 25 °C, 30 minutes at °42 C, 5 minutes at °85 C and final hold at 4 °C). The real time PCR mix composed of 2 µl of RT-PCR product, 12.5 µl of TaqMan Universal Master Mix (Applied Biosystems, Warrington, UK), 100 ng of each of the primers, 1 µl of the probe and water to final volume of 25 µl. The reaction carried out into the real time PCR system (Applied Biosystems 7900HT, Foster City,

USA). The reaction conditions were 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 15 second at 95 °C, 1 minute at 60 °C. Analysis of the final results were conducted using the software supplied by the manufacturer of the real time PCR system.

*Sequence analysis of purified PCR products*

Sequence analysis of the purified PCR product was performed using the Applied Biosystems 3500 Genetic Analyzer. The BigDye Xterminator purification kit provided by Applied Biosystems, Foster City (USA) was used for sequencing. Briefly, 45 µL SAM solution and 10 µL BigDye XTerminator were placed in each well using wide bore pipette tips (orifice of 1.0 mm or greater). The plates were sealed with adhesive cover and thoroughly mixed by vortexing at 1600 r.p.m. for 30 minutes and then centrifuged at 1000 r.p.m for 2 minutes. The plates were run in the Genetic analyzer 3500 using BigDye XTerminator run module. The nucleotide sequences determined in this study were initially analyzed using the basic local alignment tool (BLAST) (http://blast.ncbi.nlm.nih.gov/). The sequence identities among the Saudi sequences and between Saudi and homologous sequences from other countries were determined using the EMBOSS NEEDLE software program (http://emboss.bioinformatics.n1/cgibin/emboss/needle). Phylograms were constructed using the three nucleotide sequences generated in this study together with eight sequences obtained from GenBank the of BEFV reported from other countries. An online version of MAFFT software [17] was used to construct the phylogenetic trees, based on the neighbor-joining method [18] with a Jukes–Cantor substitution model [19].

**Figure 1.** Extensive subcutaneous emphysema in clinically affected cattle with BEF.



## Results

### Field investigation

BEF was observed as fever with anorexia in all diseased animals, abortion in 4% of cases, subcutaneous emphysema (Figure 1) in 2% of cases, respiratory distress (dyspnea) in 47% of cases, recumbence in 16% of cases, stiffness, lameness in 63% of cases, difficulty swallowing, salivation, nasal discharge in 50% of cases and decrease in milk yield in 25% of cases.

### Epidemiological findings

The AP and TP estimates of BEF by region and year are listed in Table 1. The TP ranged from 70.0% (95% CI: 65.5 – 74.5) in Riyadh region in 2007 to 28.7% (95% CI: 24.4 – 33.2) in the Eastern region in 2011. The logistic regression analysis showed that there was a significant decrease in the seroprevalence of BEFV

**Table 1.** Estimates of seroprevalence of bovine ephemeral fever virus in different regions of The Kingdom of Saudi Arabia during epidemics.

| Year | Region | | | | |
|------|--------|--------|--------|--------|--------|
| | Estimate | Eastern | Riyadh | Qasim | Jizan |
| **2007** | No. positive/ total tested | 207/400 | 210/400 | 209/400 | 200/400 |
| | AP | 51.8% | 52. 5% | 52.3% | 50.0% |
| | TP (95% CI) | 69.1% (95% CI: 63.7 – 74.5) | 70.0% (95% CI: 65.5 – 74.5) | 69.7% (95% CI: 65.5 – 74.5) | 66.7% (95% CI: 62.1 – 71.3) |
| **2009** | No. positive/ total tested | 97/400 | 120/400 | 90/400 | 95/400 |
| | AP | 24.3% | 30.0% | 22. 5% | 23.8% |
| | TP (95% CI) | 32.2% (95% CI: 27.6 – 34.8 ) | 40.0% (95% CI: 35.2 – 44.8) | 30.0% (95% CI: 25.5 – 34.5) | 31.7% (95% CI: 27.6 – 34.8) |
| **2011** | No. positive/ total tested | 86/400 | 97/400 | 99/400 | 91/400 |
| | AP | 21.5% | 24.3% | 24.8% | 22.8% |
| | TP (95% CI) | 28.7% (95% CI: 24.4 – 33.2) | 32.4% (95% CI: 27.6 – 34.8) | 33.1% (95% CI :28.5 - 37.7) | 30.4% (95% CI: 25.5 – 34.5) |

AP is apparent seroprevalence; TP is true prevalence.

**Table 2.** Results of logistic regression for detection of the association between the region and year of bovine ephemeral fever epidemics and the seropositive status of cattle in The Kingdom of Saudi Arabia.

| Variables | | Tested | Positive | p-value | OR | 95% CI | SE |
|---|---|---|---|---|---|---|---|
| Location | Eastern | 1200 | 390 | 0.46 | 1. 0 | 0.9 – 1.2 | 0.55 |
| | Riyadh | 1200 | 427 | 0.05 | 1.2 | 1.0 1.4 | 0.55 |
| | Qasim | 1200 | 398 | 0.87 | 1.1 | 0.9 – 1.3 | 0.55 |
| | Jizan | 1200 | 386 | - | - | - | - |
| Year | 2007 | 1600 | 826 | 0.0001 | 3.5 | 3.0 -4.1 | 0.043 |
| | 2009 | 1600 | 402 | 0.0001 | 1.1 | 0.9 – 1.1 | 0.046 |
| | 2011 | 1600 | 373 | - | - | - | |

OR is odds ratio.

infection in 2011 compared with 2007 and 2009 (Table 2). Cattle in 2007 had 3.5 times the risk of getting BEFV infection than cattle in 2011 (96 % CI: 3.0 -4.1) (*p* < 0.0001). On the other hand, the effect of the region on the seroprevalence estimates was negligible (Table 2).

The mortality rate was higher in 2007 than in 2009 and 2011. The risk of death from BEFV infection in 2007 was 8.1 times more than in 2011. Also, the risk of death from BEFV infection in 2009 epidemic was two times more than it in 2011 (Table 3). On the other hand, there was a slight difference in mortality rates between different regions over the three years (Table 4).

The ICC estimates in this study was very low (0.0034).

*Virus isolation and identification*

The effect of intracerebral inoculation of BEF suspected samples on baby mice appear after the 3rd passage slightly and increased during the 4th and 5th passage. The inoculated mice showed convulsions, paralysis of hind limb, abnormal gait and death. The infected Vero cells showed specific CPE after the 3rd passage. The CPE appeared within 24-48 hrs. post infection of Vero cells and characterized by cell rounding, granulation of the cytoplasm, cellular degeneration that end with complete detachment of cells from the surface of cell culture flask. Isolates of the BEFV were identified in cell culture by virus neutralization test.

*Molecular detection and characterization*

BEFV was identified with RT-PCR using specific primers targeting the G glycoprotein gene. The cDNA were amplified producing a clear single band. The isolates were also confirmed as BEFV by using real time RT-PCR.

In this study for the first time in KSA partial sequencing of the G glycoprotein gene had been carried out. The amplified fragment was from 50bp to 470bp of the complete gene sequence. The amplified nucleotide sequence of the three isolates have GenBank accession numbers LC017738, LC017739 and LC017742.

The identity values among the nucleotide sequences of amplified part of Bovine ephemeral virus G Glycoprotein gene determined in this study ranged from 84.7% to 100%.; and shared 90.4%-96.5% sequence identity with a previously published sequence from Australia (KF679404).

**Discussion**

BEF is one of the economically important diseases affecting cattle in Saudi Arabia. The disease observed

**Table 3.** The relative risk (RR) of mortality during bovine ephemeral fever epidemics in The Kingdom of Saudi Arabia.

| Year | Mortality | | Total | RR |
|---|---|---|---|---|
| | Yes | No | | |
| 2007 | 73 (4.6%) | 1527 (95.4%) | 1600 | 8.1 |
| 2009 | 18 (1.1%) | 1582 (98.9%) | 1600 | 2.0 |
| 2011 | 9 (0.6%) | 1591 (99.4%) | 1600 | 1.0 |

**Table 4.** The relative risk of mortalities among different regions of BEFV epidemics in KSA.

| Region | Mortality | | Total | RR |
|---|---|---|---|---|
| | Yes | No | | |
| Eastern | 21 (1.8%) | 1179 (98.2%) | 1200 | 1.0 |
| Riyadh | 27 (2.3%) | 1173 (97.7%) | 1200 | 1.3 |
| Qasim | 25 (2.1%) | 1175 (97.9%) | 1200 | 1.2 |
| Jizan | 24 (2.0%) | 1176 (98.0%) | 1200 | 1.1 |

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clinically as epidemics in many regions of the KSA is associated with complications and difficulty in diagnosis. In this study infected cattle showed clinical signs similar to those reported in KSA [6,9,20], in Egypt [7], in Tanzania [21] and in Turkey [22]. The prevalence of BEF and the mortality rate was at its maximum in 2007. The severity of the 2007 epidemic in the KSA may be due to the absence of herd immunity - the last reported outbreak of BEF in the KSA was in1998. The prevalence estimates in 2009 and 2011 are similar to the BEF prevalence estimates in the same region by [8]. Another reason for the difference of seroprevalence estimates in different epidemics may be the strain of the causative virus. In 2011 there were three different strains of the virus isolated. In addition, the average number of seroconversions had a strong geographical component that reflects patterns of vector biology responsible for BEF virus transmission [23]. Furthermore, the lower neutralizing antibody titers in 2007 suggesting reduced immunity reflects the importance of population immunity for the control of BEFV [24].

On the other hand, there was no effect of region on the seroprevalence and mortality rate of the disease which disagrees with the findings of Zaghawa *et al.*, 2015 in the same regions. We expect that in Zaghawa *et al.*, 2015, the seroprevalence estimates were similar in 3 of the 4 examined regions and the seroprevalence was significantly high only in one region; Qasim region. Li *et al.* (2015) [25], reported that seroprevalence of BEF was similar in all the regions tested in China. Our findings confirm the even, wide spread nature of the disease in the KSA. The very low ICC obtained in this study confirms the homogeneity of cattle population in different localities of KSA and the absence of clusters of herd immunity.

BEFV was successfully isolated from diseased cattle. The clinical appearance of the inoculated mice and CPE in cell culture are typical of BEF [26,27]. Our RT-PCR results support others [28] in the belief that RT-PCR is a superior test for the provision of rapid and specific data for BEFV detection. The conventional RT-PCR is sensitive, specific and rapid test for detection of BEFV in clinical samples [29], moreover we found the RT-PCR assay to be useful for testing RNA samples extracted from peripheral blood mononuclear cells and so it could be an important tool for the screening of BEF infection [30].

Real time RT-PCR is an advanced form of polymerase chain reaction that maximizes the potential of the technique, in which the efficiency of the reaction can be precisely calculated, there is no need to run PCR products on agarose gels and importantly the data can be used to perform truly quantitative analysis of gene expression. In this study the isolated BEFV was confirmed also by application of real time PCR. The use of qRT-PCR for BEF diagnosis offers rapid confirmation of infection and provides real-time data about the presence of the disease in a district to veterinarians and cattle owners [28]. The sensitivity of the real time PCR in relation the RT-PCR for detection of BEF virus in clinical samples are in accordance with Hsieh *et al.*, (2005), as they recorded that real time PCR detected more positives in clinical samples than RT-PCR (36.36 or 18.18%, respectively).

The sequence data confirmed that these isolates were BEFV and that at least one of the bovine ephemeral fever virus glycoprotein G genes belonged to the 3rd cluster that includes the Australian strains. This close relation with the Australian sequence is difficult to interpret in this study, there is a need for further investigation of BEF in the KSA. However the comparison with sequences submitted to GenBank from Turkey [22] and Iran [27] allows a first look at the global geodynamics of BEF virus and shows that it is poorly understood [31]. The molecular diagnosis of BEF is established for the first time in KSA. The isolated virus can be effectively used for the sero-epidemiological studies in serum neutralization test and for preparing local vaccine to control the disease in KSA.

## Conclusions

It is the first scientific report on isolation and identification of BEFV in KSA with the establishment of tentative and molecular diagnosis. Moreover, the isolated BEFV can be effectively used for the sero-epidemiological studies in a serum neutralization test, and, hopefully, for preparing local vaccine to control the BEF in KSA.

## Authors' contributions
A.Z., F.H and A.N. contributed to the concept, design, sampling and laboratory analysis. A.Z., A.S and Y.H were

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responsible for data analysis and manuscript writing. All authors approved the final manuscript.

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