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# High-resolution melting (HRM) for genotyping bovine ephemeral fever virus (BEFV)

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ABSTRACT

In recent years there have been several major outbreaks of bovine ephemeral disease in the Middle East, including Israel. Such occurrences raise the need for quick identification of the viruses responsible for the outbreaks, in order to rapidly identify the entry of viruses that do not belong to the Middle-East BEFV lineage. This challenge was met by the development of a high-resolution melt (HRM) assay. The assay is based on the viral G gene sequence and generation of an algorithm that calculates and evaluates the GC content of various fragments. The algorithm was designed to scan 50- to 200-base-long segments in a sliding-window manner, compare and rank them using an Order of Technique of Preference by Similarity to Ideal Solution (TOPSIS) the technique for order preference by similarity to ideal solution technique, according to the differences in GC content of homologous fragments. Two fragments were selected, based on a match to the analysis criteria, in terms of size and GC content. These fragments were successfully used in the analysis to differentiate between different virus lineages, thus facilitating assignment of the viruses' geographical origins. Moreover, the assay could be used for differentiating infected from vaccinated animales (DIVA). The new algorithm may therefore be useful for development of improved genotyping studies for other viruses and possibly other microorganisms.



## 1. Introduction

Bovine ephemeral fever virus (BEFV) is a noncontagious arthropod-borne virus belonging to the genus *Ephemerovirus* in the family *Rhabdoviridae*. Similarly to other members of this family, BEFV exhibits a bullet-shaped morphology (Delte-Porta and Brown, 1979). Its genome is 14,900 bases long: a single-strand (ss) RNA in the negative polarity encoding five structural proteins – N, a nucleoprotein; P, a polymerase-associate protein; M, a matrix protein; L, a viral RNA polymerase-associate protein; and G, a surface glycoprotein – together with $G_{NS}$, a non-structural glycoprotein, and four open reading frames (ORFs) – α1, α2, β, and γ (Walker et al., 1991; McWilliam et al., 1997; Dhillon et al., 2000; Joubert et al., 2014).

BEF is an important viral disease of cattle and water buffalo in tropical, subtropical, and temperate climatic zones; it was reported in Africa, Asia including the Middle East, and Australia, but has never been documented in Europe or North and South America (Wang et al., 2001; Kirkland, 2002; Yeruham et al., 2002; Venter et al., 2003). The cattle disease, which is also known as three-day sickness, is characterized by stiffness, acute febrile reactions, lameness, and spontaneous recovery within 3 days (Nandi and Negi, 1999; Kirkland, 2002). In spite of its short duration, the disease can cause heavy economic losses, due to decreased milk production and lowered fertility in bulls, as well as fatality in severe cases (Nandi and Negi, 1999; Yeruham et al., 2003)

The virus is suspected to be transmitted by insects, and it has been isolated from a variety of insect vectors, including midges and mosquitoes (Davies and Walker, 1974). However, thus far, no insect vectors have been identified in controlled experiments.

Recently, four major outbreaks occurred in Israel, the first starting in 1999 among dairy-cattle herds in the Jordan Valley, from where it then spread to the Mediterranean coastal plain (Yeruham et al., 2002). The second outbreak started in 2004 and was much more widespread, covering most of Israel's Mediterranean Coastal Plain; the third, in 2010, covered the interior plain, and recently, in 2014–15, disease outbreaks were also recorded in the interior valley east of Haifa (unpublished data).

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HRM is a post-PCR analysis that enables direct characterization of DNA amplicons by producing DNA melt-curve profiles that can discriminate among nucleic acids according to sequence differences such as SNPs and small deletions. The technique enables mutation scanning, methylation, and genotyping (Garritano et al., 2009; Sarker et al., 2014; Amornpisutt et al., 2015; Naze et al., 2015; Sady et al., 2015). It is precise and accurate, and it is a nondestructive method that allows performance of downstream post-amplification procedures, e.g., gel electrophoresis or sequencing, following melt analysis (Druml and Cichna-Markl, 2014).

Melt-curve evaluation of qPCR products is usually performed to ensure primer specificity; typically it covers a temperature range of 65–95 °C in 0.5 °C increments. In HRM experiments, data is generally collected at narrower temperature increments than under standard melt-curve protocols, commonly 0.2–0.1 °C. Use of a highly concentrated saturation dye, e.g., LC Green PLUS, EvaGreen, SYTO9 or ResoLight, makes it possible to saturate every single nucleotide of the double-stranded DNA by intercalating the dye so as to label the PCR product along its entire length, thereby ensuring that all melting domains are detected (Reed et al., 2007; Erali et al., 2008; Druml and Cichna-Markl 2014). HRM analysis software is used to identify areas of stable pre- and post-melt fluorescence intensity from the HRM curve.

In light of the large number of recent BEF outbreaks in the Middle East, it was important to develop a technique that would rapidly identify origins of newly emerging viruses – for two reasons. First, to promptly determine whether a circulating virus is a local one or a new arrival, and then to determine its geographical origin. These data are obtainable because newly introduced viruses will have a greater chance to overcome the currently used vaccine than the local endemic virus (Hsieh et al., 2006). The second reason is to meet the need to distinguish between vaccine strains and field virus if the vaccine strain was not prepare from a local virus.

The analysis is based on testing the melting profile of homologous fragments from different samples that contain sufficient lineage-dependent GC content for HRM analysis. In order to identify regions that meet these requirements, an algorithm was developed and employed, that aimed to find fragments that are suitable for using in the assay, based on their GC content. In this study, by taking advantage of the algorithm, two suitable fragments were selected and used to identify virus lineage.

## 2. Materials and methods

### 2.1. Viruses

All Israeli viruses used were obtained from infected cattle. All other isolates – from Turkey, Australia, and Japan – were obtained as RNA or cDNA extractions.

### 2.2. RNA extraction

RNA was extracted from infected blood samples with the Viral Gene-spin kit (http://www.intronbio.com/Intro.asp) according to the manufacturer's instructions.

### 2.3. cDNA synthesis

The Verso cDNA kit (Fisher, https://www.thermofisher.com/order/catalog/product/AB1453A) was used for cDNA synthesis, using 500 ng of total RNA according to the manufacturer's instructions.

### 2.4. Generation of control protein G gene, RNA segment

The BEFV Protein G gene segment corresponding to region 750–1359 was amplified using the primers 5′-CTAATACGACTCACTATAGGGACCAAACAGAATCTGACT TCC-3′ (FWD control), and 5′-GATATTCCTCTATTCCCTCG-3′ (REV control). The underlined part corresponds to the T7 promoter sequence. The corresponding G gene regions from all studied isolates were cloned and sequenced. The PCR product of the control amplicon of isolate ISR2014 and the Australian vaccine were gel-purified and used as a template for synthesis of RNA control template, using the MEGAscript T7 Transcription Kit (Fisher, https://www.thermofisher.com/order/catalog/product/AM1334), according to the manufacturer's instructions. These products were then used as reference amplicons to evaluate the efficiency and sensitivity of the RT-quantitative PCR procedure. For the HRM calibration procedure, standard DNA amplicon concentrations of each isolate were used, so that the Cq difference between all samples will not exceed 4–5 cycles.

### 2.5. qPCR and HRM analysis

The RT-quantitative PCRs were performed with the SensiFast HRM mix (Bioline, www.bioline.com/us/sensifast −hrm-kit.html). The reaction mix preparation was as follows: 2× SensiFast HRM mix – 5 μL, template – 3 μL, primers – 0.4 μM final of each, dd$H_2O$ – to a final volume of 10 μL. Reaction conditions for the 1140–1351 amplicon were as follows: 95 °C for 0:30 min, 44× [95 °C for 0:05 min, 60 °C for 0:30 min + Plate Read], Melt Curve: 65 °C–95 °C, increment 0.5 °C,0:05 + Plate Read. Reaction conditions for the 1234–1340 amplicon were as follows: 95 °C for 2:00 min, 44× [95 °C for 0:05 min, 60 °C for 0:10 min, 72.0 °C for 0:15 min, Plate Read], Melt Curve: 70.0 °C–85.0 °C, Increment 0.1 °C for 0:01 min + Plate Read. Reaction conditions for the 79–140 amplicon were as follows: 95 °C for 2:00 min, 44× [95 °C for 0:05 min, 60 °C for 0:15 min, 72 °C for 0:15 min, Plate Read], Melt Curve: 70 °C–85 °C, Increment 0.1 °C for 0:01 min + Plate Read.

All reactions were carried out with the CFX 96 apparatus (Bio-Rad, Hercules, CA, USA) and analyzed with the Bio-Rad CFX Manager Precision Melt Program (Bio-Rad).

### 2.6. Bioinformatics

#### 2.6.1. Primer designand in silico PCR

Primers for conventional and quantitative PCR were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) and were tested *in silico* using the Geneious package (http://www.geneious.com/) with BEFV G gene sequences.

#### 2.6.2. GC analysis algorithm

The algorithm was implemented in the Java programming language (Oracle, CA, USA). It was compiled, tested, and run on the Eclipse Luna software (Eclipse Foundation, Ottawa, Canada) under a Windows 7 environment.

#### 2.6.3. Additional programs

For sequence analysis and reaction design, two main sets of programs were used: Geneious (Biomatters, Auckland, New Zealand) and the European Molecular Biology Open Software Suite (EMBOSS). In addition, the ClustalW2 Multiple Homology Analysis tools (http://www.ebi.ac.uk/Tools/msa/clustalw2/) were used.

Phylogenetic and molecular evolutionary analyses were conducted with the MEGA software, version 6.0 (Tamura et al., 2013). Design and analysis of the real-time PCR and HRM tests were per-