
Isolation and Phylogenetic Grouping of Equine Encephalosis Virus in Israel

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During 2008–2009 in Israel, equine encephalosis virus (EEV) caused febrile outbreaks in horses. Phylogenetic analysis of segment 10 of the virus strains showed that they form a new cluster; analysis of segment 2 showed ≈92% sequence identity to EEV-3, the reference isolate. Thus, the source of this emerging EEV remains uncertain.

Equine encephalosis is an arthropod-borne, noncontagious, febrile disease of horses. It was first described >100 years ago by A. Theiler (1) under the name equine ephemeral fever. The disease is caused by *Equine encephalosis virus* (EEV; genus *Orbivirus*: subfamily *Sedoreovirinae*: family *Reoviridae*) (2,3), which is transmitted by *Culicoides* spp. biting midges (4). Before 2008, EEV had been isolated only in South Africa, where 7 antigenically distinct serotypes, EEV-1–7, have been identified and characterized (3).

Orbiviruses encode at least 7 structural and 4 nonstructural (NS) proteins from 10 linear dsRNA genome segments (5). The smallest genome segment, segment 10 (Seg-10), encodes NS3, which mediates the release of virus particles from infected cells, and NS3A. The second largest of the EEV genome segments, Seg-2, encodes virus protein (VP) 2, the larger of the 2 outer-capsid proteins. By analogy with bluetongue virus (BTV), the *Orbivirus* type species, the virus serotype is determined by the specificity of interactions between VP2 and neutralizing antibodies generated during infection of the mammalian host. Consequently, VP2 and Seg-2 show sequence variations

that correlate with serotype and, thus, can be used to determine the virus serotype (6).

From October 2008 through January 2009, a febrile horse disease that was diagnosed as equine encephalosis was observed in dozens of stables across Israel (7). The recent emergence of novel orbivirus strains (including BTV and epizootic hemorrhagic disease virus) in Europe, North America, Asia, and Australia (8) is of major concern to the worldwide livestock industry. Furthermore, the similarity of EEV to African horse sickness virus, one of the most devastating pathogens of equids, warranted further investigation of the outbreaks and molecular characterization of the virus. The molecular and sequence analyses reported here confirm the existence of EEV in Israel and identify the virus and its serotype, as well as its phylogenetic roots.

The Study

During October–November 2009, samples of whole blood from 8 febrile horses (H1–8; temperatures 39.5°C–42°C) in Israel were collected into EDTA tubes and analyzed at the Koret School of Veterinary Medicine (Hebrew University, Rehovot, Israel). Vero cell (American Type Culture Collection, Manassas, VA, USA) culture results of blood from H3, H5, and H8 were positive for EEV (Table 1; Figure 1).

Total RNA was extracted from the fifth and sixth passages of all 3 samples by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) to obtain sufficient viral load for the subsequent analyses and replications. RNA was reverse transcribed into cDNA by using the Verso cDNA Kit (Thermo Fisher Scientific, Epsom, UK). PCR amplification of the gene encoding NS3 (Seg-10) was performed on the 3 isolates by using GoTaq Green Master Mix (Promega, Madison, WI, USA) with the following primers: 5'-GTT AAG TTT CTG CGC CAT GT²³-3', 5'-⁷⁴¹GTA ACA CGT TTC CGC CAC G⁷⁶⁰-3'. Thermal cycling conditions for the PCR were as previously described (9); the primer annealing temperature was modified to 53.5°C. PCR products were purified by using a cDNA purification kit (ExoSAP-IT; USB, Cleveland, OH, USA), and sequencing was conducted by BigDye terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA, USA) in an ABI 3700 DNA Analyzer (Applied Biosystems) by using ABI data collection and sequence analysis software. Further analysis of the NS3 sequence was performed with Sequencer software, version 4.8 (Gene Codes Corp., Ann Arbor, MI, USA). Sequences were deposited in GenBank under accession nos. HQ441245 for H5, HQ441246 for H3, and HQ441247 for H8. The NS3 genes (Seg-10) were compared with those of different EEVs (9) and other related orbiviruses (Table 2). Phylogenetic trees were generated by using the neighbor-joining and maximum-likelihood

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Table 1. Clinical signs for horses whose blood was tested to determine the cause of a febrile disease, Israel, October–November 2009

Horse no.	Clinical signs	Date of first clinical sign	Duration of clinical signs, d	Date of blood collection	Virus isolated*
1	Temperature 39.5°C, lack of appetite	Oct 25	5	Nov 2	No
2	Temperature 39.5°C, lack of appetite	Oct 29	3	Nov 2	No
3	Temperature 40°C, lack of appetite	Oct 30	3	Nov 3	Yes
4	Temperature 39.5°C, lack of appetite	Nov 8	Unknown	Nov 8	No
5	Fever, colic, lethargy, congested mucous membranes, rapid pulse, lack of appetite	Nov 5	5	Nov 9	Yes
6	Temperature 39.5°C, lack of appetite	Nov 17	4	Nov 22	No
7	Temperature 42°C, apathy	Nov 25	2	Nov 26	No
8	Temperature 39.7°C, lack of appetite, colic	Nov 26	3	Nov 27	Yes

*Positive cases were confirmed by reverse transcription PCR of dsRNA genome segment 10.

methods (Phylip Inference Package version 3.68, Seqboot Program; J. Felsenstein, University of Washington, Seattle, WA, USA) to create 100 datasets (bootstrapping) and the

DNA Maximum Likelihood Program version 3.5 (<http://cmgm.stanford.edu/phylip/dnaml.html>) to construct the trees. Finally, the Consense program version 3.5c (<http://cmgm.stanford.edu/phylip/consense.html>) was used to create a final consensus tree for our dataset. Broadhaven virus, a tick-borne orbivirus, was used as the outgroup in the phylogram for the gene encoding NS3.

The phylogenetic analyses of EEV Seg-10 grouped the Israeli isolates with other EEV isolates but as a distinct group with no close relation to African horse sickness virus, BTV, or epizootic hemorrhagic disease virus. Within the EEV group, 3 discrete clusters (A, B, C) were recognized; the Israeli isolates formed one of these clusters (C; Figure 2). The Israeli isolates have 85%–86% nt identity to cluster A and 75%–76% nt identity to cluster B.

In addition, full-length cDNA copies of individual EEV (from H3 and H8) genome segments were synthesized and amplified by reverse transcription PCR by using the anchor spacer–ligation method as described (10,11). Partial sequences (for the upstream 450 bp) of Seg-2 from the different Israeli isolates were identical, showing 92.3% nt and 95.7% aa sequence identity with Seg-2 and VP2 of the Kaalplaas isolate, the reference isolate of EEV-3 (GenBank accession numbers are listed in Table 2). Previous phylogenetic comparisons of Seg-2/VP2 from different BTV types showed a maximum of 71% nt and 78% aa acid identity between serotypes (6), indicating that the isolates from Israel also belong to EEV type 3.

Conclusions

Equine encephalosis virus has long been enzootic to southern Africa, but it has not been isolated in other parts of the world. We report the characterization of an EEV strain isolated outside of Africa. Phylogenetic analysis of Seg-2 showed 92% sequence identity to EEV-3 (Kaalplaas).

Analysis of Seg-10 (the gene encoding NS3) of different orbiviruses showed 2 clusters of South African EEV strains (A and B), in agreement with previously published studies (9). These 2 clusters appear to correlate with the geographic origins of the viruses in South Africa,

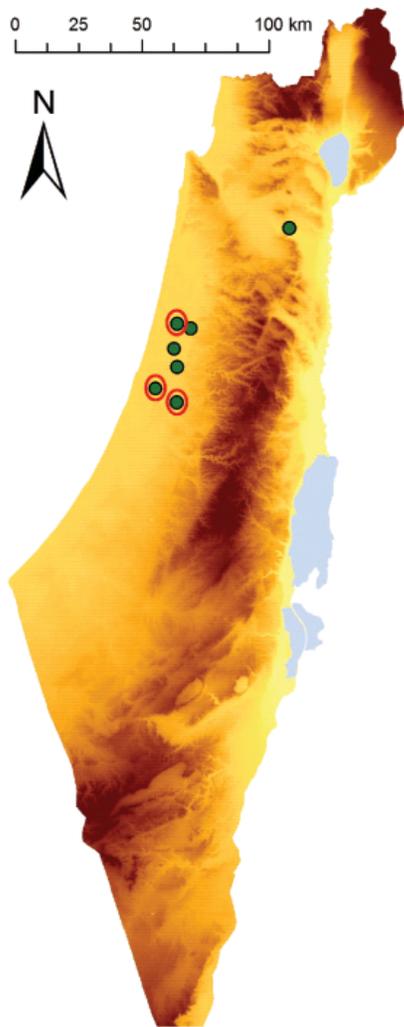


Figure 1. Geographic location of farms in Israel with horses showing signs of equine encephalosis virus (EEV) infection. Eight horses with suspected EEV infection lived on 7 farms. Red circles indicate farms with EEV-positive cases.

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