



A First Molecular Phylogeny of an Egyptian Equine Herpesvirus-4 Strain Derived from a Fetal Arabian horse

Al-Shammari ZS¹, Ahmed BM², Haroun M^{*1,4}, Affy AF³, Elsanousi AA² and Shalaby MA²

¹Virology Unit, Veterinary Laboratory, Department of Animal Resources, Qatar, P.O. Box 23211

²Department of Virology, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt, P.O. Box 12211

³Department of Virology, Animal Health Research Institute, Dokki, Egypt

⁴Department of Microbiology and Parasitology, Faculty of Veterinary Science, University of Nyala, P.O. Box 155, Nyala, Sudan

*Corresponding author: M Haroun, Virology Unit, Veterinary Laboratory, Department of Animal Resources, P.O. Box 23211, Doha, Qatar, Tel: +97444560545; Fax: +97444653086; E-mail: mismail_99@yahoo.com; mohamedharounismail@gmail.com

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Abstract

An Egyptian equine herpes virus-4 isolate derived from aborted-fetal liver specimens of Arabian breed horses collected between March 2014 and February 2015 was molecularly detected and characterized. Viral deoxyribonucleic acid fragment equivalent to 580bp specific glycoprotein B primer sets was retrieved from a liver specimen assigning the identification of EHV-4 VRLCU 412-2015 strain.

The investigation affirms the previous study of prevalence of EHV-4 in Egypt, and that EHV-4 subtype is one of the main viral causes of early upper respiratory tract infections among equine populations with seldom-perinatal foal mortalities and mare abortions. Sequence analysis of 80% of the amplified fragment followed by compound multiple nucleotides-amino acids sequence alignments of the virus glycoprotein B gene to that of the Gen Bank-derived EHV-4 reference strains revealed 100% homology. Being the first phylogenetic analysis of the Egyptian EHV-4 subtype, the study related the isolate to 6 Turkish EHV-4 strains of the year 2011.

Keywords: Equine herpesvirus-4; Sequence analysis; Phylogeny, Arabian horses; Egypt

Introduction

Equine herpes virus-4 (EHV-4) is a global infectious DNA viral disease primarily affecting horses leading to respiratory and seldom abortive squeals [1-3]. The virus belongs to the genus *Varicello virus*, Subfamily *Alphaherpesvirinae* within the family *Herpesviridae* [4-5].

Of the nine up to date worldwide known equine herpes viruses, the *Alphaherpesvirinae* viruses, EHV-1, EHV-3 and EHV-4 are known to pose significant public health hazards among domesticated equine species and significant economic impact on horse industry [6]. The reality that EHV-4 can infect most horses by two years of age rendering

foals the main sources of early infection [7], and that there are serious clinical implications of EHV-1 infection [8] signify the request to properly implement quarantine measures aiming at controlling the diseases.

With respect to the industrial value of the Arabian horses in their native countries, there are few studies concerning the impact of the epidemiology of EHV in the area. In Egypt, the early detection of anti-EHV-2 antibodies conducted by Matumoto and others [9] studying equine rhinopneumonitis infection was the first. A long time later the existence of EHV-1 subtype in the country was recorded [10,11].

However, it was only in 2011 could patently EHV-4 subtype beside EHV-1 and EHV-2 were incriminated as viral agents responsible for respiratory and/or abortive cases among Arabian mare breeds collected between 2005 and 2006 [12]. Yet the total horse population is estimated by 67000, a figure that represents about 0.02% of the total equine population in Egypt [13], their health situation necessitates continuous following.

While recurrent clinical abortive cases among the Egyptian pregnant mare communities, coupled with suspected silent and overt respiratory EHV-4 infections among male and female horses are continuously observed, the limited national preventive efforts done to combat the disease are shown too ineffective.

Reliable preventive measures aiming at controlling the perpetuation of the disease among these animals would expect to embark on availability of preliminary data, the basic of which understands the molecular epidemiology of EHV-4 in the country. As a first trial to deal with this investigation, the authors initiation were to establish a basic analysis aiming at revelation of the phylogenetic identity of the virus; a necessity that would expect to meet viral diseases prevention requirements and further, aid sustaining future controlling EHV-4 in Egypt.

Materials and Methods

Animals and samples

Twelve aborted-fetal horse liver specimens collected from a single equine stable between February 2014 and March 2015 were used in the investigation. All specimens were treated aseptically, allocated in sterile specimen containers, chilled, transferred immediately to the laboratory and stored in pieces in liquid nitrogen for further processing.

Viral DNA extraction

Wizard[®] SV Genomic DNA Purification System (Promega, USA) kit was used for EHV DNA purification following the manufacturer instructions with minor modification of an overnight incubation of each 20 mg-liquid nitrogen frozen liver sample with 275 µL digestion mix at 55°C. Control viral DNA extractions followed the manufacturer's cell culture protocol without the modification. The final clear lysate was processed according to the manufacturer instructions and stored each in about 70 µL aliquot at -86°C till used.

Identification of EHV-4 by nested-PCR:

The OIE reference nested-polymerase chain reaction (n-PCR) EHV-4 580 bp specific glycoprotein B (gB) primers synthesized in METABION, GmbH, USA were used for amplifying the extracted PCR

products. Following Emerald Amp, Takara-Bio, Japan instructions, each 5 µL viral DNA extract was initially amplified by the primer sets BS-4-P1 (5'-TCT ATT GAG TTT GCT ATG CT-3', nucleotide positions 1705-1724) and BS-4-P2 (5'-TCC TGG TTG TTA TTG GGT AT-3', nucleotide positions 2656-2637) [14].

Generation of the PCR products was performed by GenAmp PCR System 9700 thermocycler following one hold step at 94°C for 10 mins for primary denaturation; 40 cycles of 94°C for 60 sec for denaturation, 60°C for 60 sec for primer annealing and 72°C for 3 mins for extension; one hold at 72°C for 10 mins for final extension and 4°C for cooling. Using 10% 10 µL first PCR product each, the primer sets BS-4-P3 (5'-TGT TTC CGC CAC TCT TGA CG-3', nucleotide positions 1857-1876) and BS-4-P4 (5'-ACT GCC TCT CCC ACC TTA CC-3', nucleotide positions 2456-2437) were used for the secondary amplification run following the initial thermo-profile amplification program. Agarose gel genomic DNA product identification succeeded the standard procedures.

DNA sequence and analysis

Sequencing of the viral genomic DNA template of the isolate was performed according to Sanger dideoxynucleotide-sequencing technology following Telford and coworkers [15,16]. 25 µL 5ng/µL second n-PCR product was used in presence of 10pmol n-PCR primer sets for amplifying and sequencing. Following the Laboratory Information Management System (LIMS), data were obtained 48h later and analyzed according to NCBI/BLAST [17].

Based on BLAST result, the highly similar sequences were downloaded as FASTA sequences. Multiple sequences alignment editing, correcting, frame and amino acid adjusting were performed using Windows XP/7/vista/8 and 8.1BioEdit-supported software [18]. The adjusted sequence file was exported to FASTA files, imported into MEGA 6.06 DNA alignment tool and converted into MEGA format 6 [17] for phylogenetic analysis using the neighbor joining method. Finally, utilizing a statistical support of one thousand bootstrap replicates, the phylogenetic trees topology was obtained and saved as PDF. All sequence data were submitted to the Gen Bank [19] to obtain a Gene Bank accession number.

Results and Discussion

The successful molecular retrieval of a viral DNA fragment equivalent to 580 bp EHV-4 gB specific gene from a fetal liver specimen out of the 12 examined samples is identifying the detection of the Egyptian strain EHV-4 VLRCU-412-2015. Besides, it confirms the preliminary tentative clinical diagnosis of the investigation. The result sustains the previous antigenic and molecular evidences that, the virus beside others, are playing a role in the recurrent abortive cases among Egyptian mares [12]. Although the study did not detect EHV-1 genomes in any of the 12-tested liver specimens (data not shown), the finding re-enforced the common notion of significant prevalence of EHV-4 subtype among the investigated mares, an epidemiological situation that has been demonstrated in 2011 comparing EHV-4 to EHV-1 and 2 subtypes [12]. The parallel study conducted by Al-Shammari and others [22] had already detected total anti-EHV-1 and 4 antibodies (Ab) among the Egyptian horses. Although their study did not include a discriminative investigation between ratio of the positive EHV-1 and EHV-4 Ab sera, both findings cohort with the descriptive epidemiology of EHV-4; that the virus possesses high reactive infectious potentiality compared to other latent EHV, especially EHV-1

[21]. The previous studies of Patel and Heldens [23] and Lunn et al. [24] concluded that exposure to EHV-4 is very common in young foals at early postnatal live than EHV-1, a reality that could explain the high incidence of EHV-4 infection and consequently, the high prevalence of their Ab over other EHV subtypes. The fact that this study has involved the molecular detection, characterization and finally phylogenetic analysis of the so-called VLRCU 412-2015 strain as an EHV-4 subtype among the subject Egyptian horses would expect to further sustain the understanding of EHV situation in the country. Obtaining the Gen Bank accession number KP699582.1 (Table 1), sequence analysis of 566 bp of the identified EHV-4 VLRCU-412-2015 gB viral specific gene fragment was performed [25].

| Strain | Date/Location | Accession No. | Identity % |
|---|--------------------------|---------------|------------|
| EHV-4 VLRCU-412-2015, glycoprotein B gene partial cds | 2015/Egypt current study | KP699582.1 | 100 |
| EHV-4 strain NS80567, complete genome | Before 1988/Ireland | AF030027.1 | 99 |
| EHV-4 glycoprotein gB gene, complete cds | Before 1988/Ireland | M26171.1 | 99 |
| EHV-4 MD4-TR2011 glycoprotein B gene, partial cds | 2011/Turkey | JN982956.1 | 99 |
| EHV-4 TR-EHV4-ED-11glycoprotein B gene, partial cds | 2011/Turkey | JX416464.1 | 99 |
| EHV-4 TR-EHV4-VSA-11glycoprotein B gene, partial cds | 2011/Turkey | JX416463.1 | 99 |
| EHV-4 L4-TR2011 glycoprotein B gene, partial cds | 2011/Turkey | JN982955.1 | 100 |
| EHV-4 strain nonfunctional glycoprotein B (glyB) gene, partial sequence | 2011/Turkey | JN705799.1 | 100 |
| EHV-4 TR-EHV4-TCO-11 glycoprotein B gene, partial cds | 2011/Turkey | JX416462.1 | 99 |
| EHV-4 AQHY12LT2014/1/HM gB gene for glycoprotein B gene, partial cds | 2014/Japan/USA* | AB986064.1 | 99 |
| EHV-4 US4 TR2011 glycoprotein B gene, partial cds | 2011/Turkey | JN982958.1 | 99 |
| EHV-4 RJ4-TR2011 glycoprotein B gene, partial cds | 2011/Turkey | JN982957.1 | 100 |

Table 1: Gen Bank accession data and identity of the reference EHV strains aligned to EHV-4 VLRCU-412-2015 strain (NCBI Blast) *Isolated in Japan from infected horse imported from USA.

This besides multiple alignment of nucleotides and amino acid sequences of 80% (520 bp) of the sequenced gene to that of eleven reference EHV-4 Gen Bank database isolates; the genetic identity of the strain was demonstrated (Table 1, Figure 1).

The revelation of 100% nucleotides identity and amino acid homology of EHV-4 VLRCU-412-2015 with three of the Gen Bank aligned strains and 99% with the rest (Table 1) would positively influence the understanding of the molecular epidemiology of the identified Egyptian strain.

Given that the isolate showed 100% sequence homology with six out of eight of year 11 Turkish EHV-4 Gen Bank reference strains, the nucleotides and amino acids group phylogenetic root percentages were 64% and 65%, respectively (Figures 1 and 2).

These findings might give clues that there was trans-boundary infection owing to geographical vicinity and/or both horse groups had contracted the disease from the same source.

However, since the total available number of the highly similar sequence Gen Bank-derived EHV-4 reference strains aligned to EHV-4 VLRCU-412-2015 at the time conducting this investigation was eleven, a wide-based future comparative study with more reference strains is recommended to retrieve more informative phylogenetic data.

As well, while the sources of the samples used for revealing the first EHV-4 Egyptian strain were collected during the year 2005-2006 [12], retrospective studies are required to show whether the identified strains were phylogenically similar or unique to EHV-4 VLRCU-412-2015.

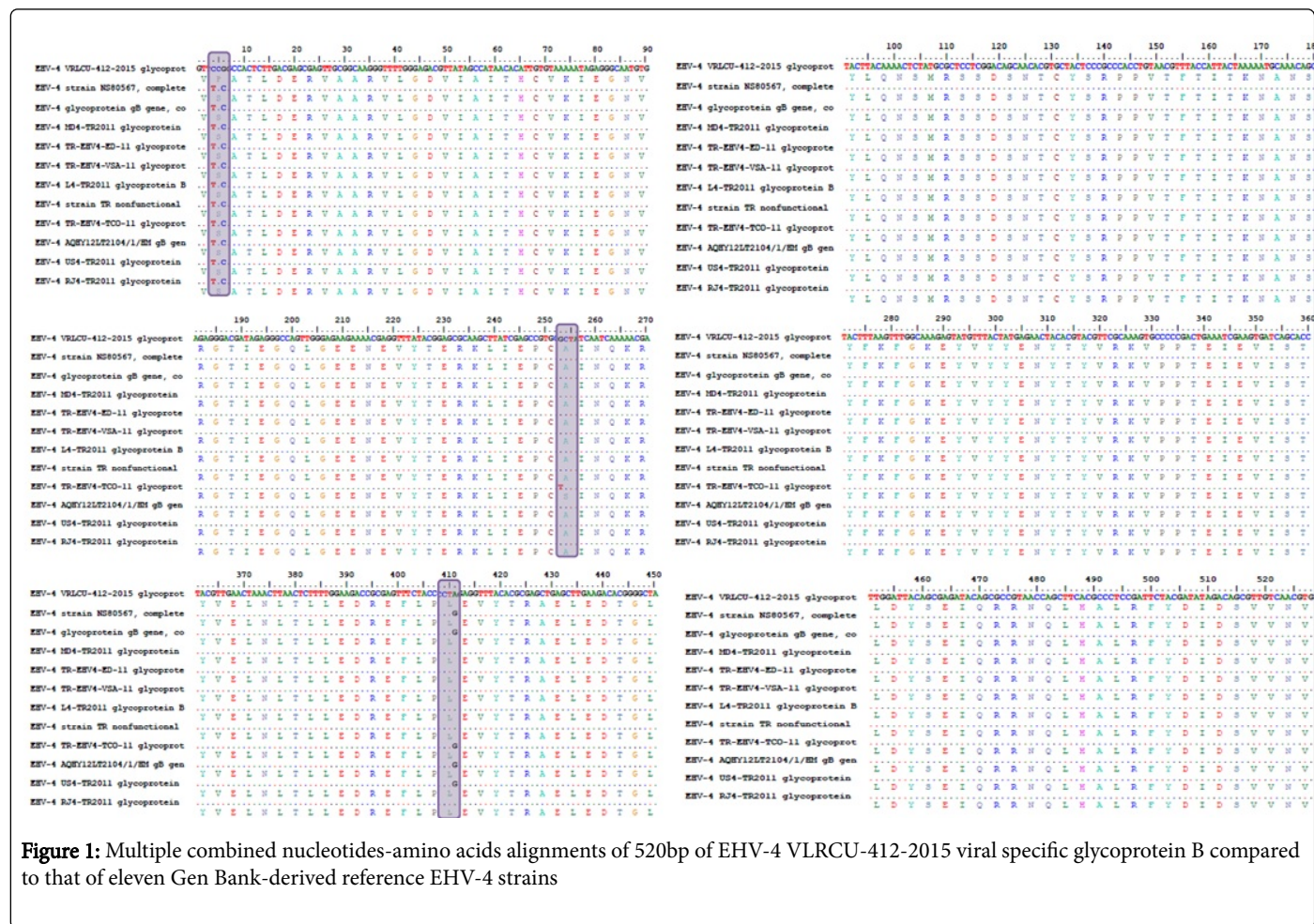


Figure 1: Multiple combined nucleotides-amino acids alignments of 520bp of EHV-4 VLRCU-412-2015 viral specific glycoprotein B compared to that of eleven Gen Bank-derived reference EHV-4 strains

Considering that horses have a socio-economical role in the life of the populations of the study area ranging from drafting to sporting obligates attention.

The repeated crossing of the borders to participate in the equestrian events could raise the susceptibility threshold to infection with EHV-4 and other EHV subtypes with a possible development of clinical latent cases [21].

Consequently, elaborated epidemiological future investigations recommended to pile data could aid augmenting the basis for application of reliable preventive measures to control the disease in the country.

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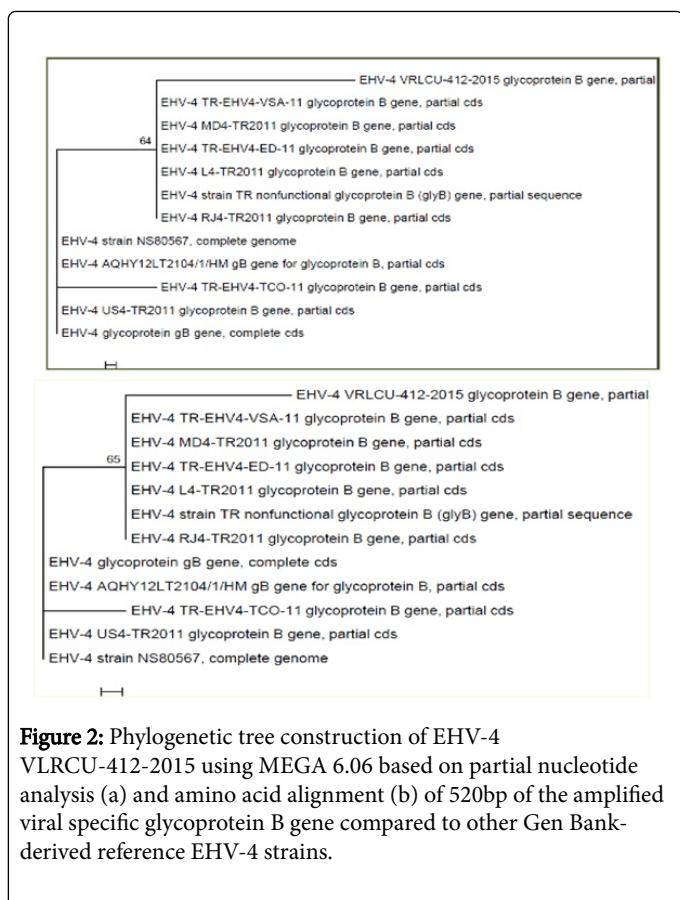


Figure 2: Phylogenetic tree construction of EHV-4 VLRCU-412-2015 using MEGA 6.06 based on partial nucleotide analysis (a) and amino acid alignment (b) of 520bp of the amplified viral specific glycoprotein B gene compared to other Gen Bank-derived reference EHV-4 strains.

References

- Slater DJ (2007) Equine herpesvirus. Equine infectious disease Section II. Saunders-Elsevier Publisher, Philadelphia 134-153.
- Association of Quaternary Horse Journal (2015) Equine herpesvirus-1.
- American Association of Equine Practice (2013) Equine herpesvirus-1 and 4 related diseases.
- Davison A, Eberle R, Hayward GS, Roizman B (2009) The order Herpesvirales. *Arch Virol* 154 (1): 171-177.
- International Committee of Virus Taxonomy (2014).
- Crabb BS, MacPherson CM, Reubel GH, Browning GF, Suddert MJ, et al. (1995) A type-specific serological test to distinguish antibodies to equine herpesviruses 4 and 1. *Arch Virol* 140 (2): 245-258.
- Allen GP (2002) Respiratory Infections by Equine Herpesvirus Types 1 and 4. In: Lekeux P. (Ed.).
- International Veterinary Information Services. Ithaca, New York, USA.
- Studdert MJ, Hartley CA, Dynon K, Sandy, JR, Slocombe RF, et al. (2003) Outbreak of equine herpesvirus type 1 myeloencephalitis: new insights from virus identification by PCR and the application of an EHV-1-specific antibody detection ELISA. *Vet Rec* 153: 417-423.
- Matumoto M, Ishiraki R, Shimizu T (1965) Serological survey of equine rhinopneumonitis infection among horses in various countries. *Archives-Virusforsch* 15: 609-624.
- Hassanien MM, Maysa H, El-Bagoury F, Magda, AK, El-Kabbany MMA, et al. (2002) Trials for isolation and identification of equine herpesvirus abortion in Egypt. *Vet Med J Giza* 50: 977-986.
- Warda SM (2003) Studies on equine abortion problem in Egypt. Zagazig University, Benha Branch, Egypt.
- Amer HM, Shaltout AK, El-Sabag IM, El-Sanousi AA, Shalaby MA (2011) Prevalence of equine herpes viruses 1, 2 and 4 in Arabian horse population in Egypt. *Afri J Microbiol Res* 5: 4805-4811.
- Food and Agriculture Organization (2011) Egypt: Country pasture/forage resources profile.
- Borchers K, Slater J (1993) A nested PCR for the detection and differentiation of EHV-1 and EHV-4. *J Virol Meth* 45: 331-336.
- Telford EAR, Watson MS, McBride K, Davison AJ (1992) The DNA sequence of equine herpesvirus-1. *Virol* 189: 34-316.
- Telford EAR, Watson MS, Perry J, Cullinane AA, Davison AJ (1998) The DNA sequence of equine herpesvirus-4. *J Gen Virol* 79: 1197-1203.
- NCBI/BLAST.
- BioEdit.
- NCBI/BankIt.
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol Biol Evol* 30: 2725-2729.
- Allen GP, Kydd JH, Slater JD, Smith KC (2004) Equid Herpesvirus 1 and Equid Herpes virus Infections, *Infectious Diseases of Livestock*. Oxford Press 76: 929-859.
- Al-Shammari ZS, Haroun M, Elsanousi AA, Shalaby MA (2015) Equine herpesvirus-1 and 4: investigating the disease humoral immunity profile among some Arabian and Foreign horses. *Egypt J Virol* Vol 12: 29-35.
- Patel JR, Heldens J (2005) Equine herpesvirus 1 (EHV-1) and 4 (EHV-4): epidemiology, disease and immunoprophylaxis: a brief review. *Vet J* 170 (1): 14-23.
- Lunn DP, Davis-Poynter N, Flaminio, MJ, Horohov DW, Osterrieder K, et al. (2009) Equine herpesvirus-1 consensus statement. *J Intern Vet Med* 23: 450-461.