



Verification of Molecular and Conventional Techniques used in The Diagnosis of Equine Herpes Virus in Some Egyptian Governorates

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ABSTRACT

Equid herpesviruses (EHVs) affect equine health and can cause significant economic losses to the equine industry worldwide. In the current study, the circulation of two infectious equid herpesviruses (EHV-1 and EHV-4) among different horse populations in some farms was monitored. In the present study, 50 samples of nasal secretions and tissue homogenates from neurological disease cases, abortion, neonatal foal deaths, and 36 serum samples. Samples of swabs and organs inoculated in embryonated chicken egg and Madin darby bovine kidney cell line. 29 samples were positive in egg injection but no detected CPE in cell line for three passages. DNA was extracted and subjected to conventional PCR to detect the two herpesviruses' presence using specific primers. Three isolates of EHV-1 and four were detected. One EHV-1 and two EHV-4 were subjected to phylogenetic analysis. Phylogenetic analysis confirmed the existence of the isolated EHV-1 and EHV-4. They were more closely related to other previously isolated EHV-1 and EHV-4 from Egypt and other countries. Antibodies against EHV-1 and EHV-4 were tested using ELISA. The results showed that EHV-1 and EHV-4 are endemic and can be a continuous threat for horses in the absence of vaccination programs and frequent virus reactivation.

Keywords: DNA, ELISA, EHV-1, Equine, herpesvirus.

Original Article:

DOI:<https://dx.doi.org/10.21608/javs.2021.140052>

Received : 07 October, 2020.

Accepted : 27 December, 2020.

Published in January, 2021 .

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J. Appl. Vet. Sci., 6(1) : 1 – 8 .

INTRODUCTION

Horses are constantly exposed to the world's widespread equid herpesviruses (EHVs) in horse populations. The viruses belong to the Alphaherpesvirinae subfamily [six viruses: equid herpesviruses type 1 (EHV-1), EHV-3, EHV-4, EHV-6, EHV-8, and EHV-9] or the Gammaherpesvirinae subfamily [three viruses; EHV-2, EHV-5, and EHV-7 (Davison *et al.*, 2009)]. Horses are the natural host to EHV-1, EHV-2, EHV-3, EHV-4, and EHV-5, while donkeys are the primary host of EHV-6, EHV-7 (AsHV-2), and EHV-8 (AsHV-3). EHV-9 (Abdelgawad *et al.*, 2016). In Egypt, monitoring is sporadic but EHV-1 or EHV-4 has been reported to circulate in the horse population (Walid Azab *et al.*, 2019). EHV-1 and its close relative, EHV-4, cause significant economic losses to the equine industry due to clinical illnesses associated with lost time for training and performance (Patel and Heldens 2005).

Although both viruses cause respiratory disease, the only infection with EHV-1 may result in abortion, perinatal mortality and neurological disorders with clinical signs that vary in severity but can result in complete paralysis (Patel and Heldens 2005). The infection of horses with either of the two viruses is clinically and serologically difficult to distinguish because of their high genetic and antigenic similarity (Pusterla 2005). The viruses' source(s) could be from apparently healthy and/or nonhealthy in-contact carriers. These carriers could have transmitted the viruses to the horses during transportation, training periods, race competitions, or breeding (Ma *et al.*, 2013 and Sarani *et al.*, 2013). It is also possible that the horse keepers, jockeys, or animal health workers transmitted the viruses from infected horses to the horses by direct contact during grooming, riding, or medical examinations /treatment (Ma *et al.*, 2013).

Active and / or latent infection by EHV-1 and EHV-4 occurs among the equine population. These horses harbor the viruses and serve as their disseminators following the stress and reactivation of latent infections. EHV-4 infection seems to be more prevalent than EHV-1 infection among the equine population (Taghi *et al.*, 2015). In Egypt, the virus was isolated from the aborted fetus on the chorioallantoic membrane of embryonated chicken eggs as a first record by (Hassanain *et al.*, 2002). Other succeeding ones followed this trial by some authors, who isolated and identified the local EHV-1 strain from aborted Arabian mares and internal organ of their foeti from a private stud with a history of recurrent abortion during 2005 and 2006 (Warda *et al.*, 2013).

The circulation of four infectious equid herpesviruses (EHV-1, EHV-2, EHV-4, and EHV-5) among different Arabian horse populations and donkeys in Egypt were monitored through measuring antibody titers against EHV-1 and EHV-4 using virus neutralization test and type-specific ELISA; the result revealed that EHV-1, EHV-2, EHV-4, and EHV-5 are endemic and can be a continuous threat for horses in the absence of vaccination programs and frequent virus reactivation (Walid Azab *et al.*, 2019). Standard diagnostic methods for EHV-1 and related viruses are well-established, including virus isolation and serological assays, particularly virus neutralization tests (VNT) and type-specific ELISA assays (OIE, 2018). Quantitative PCR (qPCR) methods offer alternatives to virus isolation and have been proven sensitive and time-effective (Pusterla *et al.*, 2005).

The current study's objective was to determine the prevalence of infection with EHV1 and EHV4 among horses and donkeys in Egypt and molecular comparison between the local isolate of each of them and the previously sequenced Egyptian and universal strains.

MATERIALS AND METHODS

1. CLC's Enzyme-linked immunosorbent assay (ELISA) :

Using the ELISA kit (Ingezim rinoneumonitis 14.HEV.K1 lot A69377 Ref10-3100-02) . Samples of 36 equine sera were tested by ELISA for the detection of EHV-1 and EHV-4 specific antibodies as described by the manufacturer. Thirty-six Serum samples taken from symptomatic cases were investigated for the presence of EHV-1 and EHV-4 specific antibodies.

2. Virus isolation

2.1. On tissue culture

Fifty clinical samples, which had been stored at -70°C , included nasal secretions and tissue homogenates from cases of neurological disease, abortion, and neonatal foal deaths. The samples were collected from different horse farms. They were prepared and injected in 50 tissue culture flask (25ml.) containing MDBK confluent sheet and Leighton, flying tube-cover slip- having MDBK for one hour then suspended in RBMI maintenance media containing antibiotics. The flasks were incubated at 37°C in a humidified 5% CO_2 atmosphere for seven days and checked every day for the presence of a viral cytopathic effect (CPE). The coverslips were collected after 24 hours for use in the fluorescent antibody technique. The cultures were passaged at weekly intervals for a total of three passages. In the absence of any specific CPE, the cell cultures were recorded as negative. (Stasiak *et al.*, (2018)).

2.2. On embryonated chicken eggs (ECE)

The prepared laboratory samples were inoculated in ECE 9-11 days through the chorioallantoic route. The inoculated eggs were examined daily until nine days for monitoring pock lesion.

3. Fluorescent antibody technique

Fluorescent antibody technique was applied on frozen chorioallantoic membrane showing pock lesion for identification of the isolated virus using EHV-1 antiserum manufactured from animal and plant health inspection service .national veterinary services laboratories. Ames, IA 50010 lot#340EDV1001

4. Molecular studies

4.1. Nucleic acid extraction

EH1, EH4

Whole nucleic acid extraction from selected seven positive isolated samples was performed using the QIAamp mini elute virus spin kit (Qiagen, Germany, GmbH). Briefly, 200 μl of the sample suspension was incubated with 25 μl of Qiagen protease and 200 μl of A.L. lysis buffer at 56°C for 15 min. After incubation, 250 μl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 50 μl of elution buffer.

4.2. Oligonucleotide Primers

Supplied from (Metabion Germany) are listed in table (1).

Table 1: Primers sequences, target genes, amplicon sizes

| Target agent | Target gene | Primer sequence (5'-3') | Length of amplified product (bp) | Reference |
|--------------------------|----------------|-----------------------------|----------------------------------|-------------------------|
| EH1 | Glycoprotein B | F/GCAAACAACAGAGGGTTCGATAGAA | 342 bp | Hatshejani et al., 2015 |
| | | G | | |
| R/GTCGATGTCGTAAAACTGAGAG | | | | |
| F/TATTGTTCCGCCACTCTTGACG | | 508 bp | | |
| R/GTAGAATCGGAGGGCGTGAAGC | | | | |
| EH4 | | | | |

Table 2: Table illustrating the results of different tests performed on samples used at this study

| Kind of samples | Nasal and tissue homogenates | | | | Sera samples |
|----------------------|------------------------------|----|-----------|------------|--------------|
| | Isolation | | Molecular | | |
| Type of test | ECE(confirmed by FAT) | TC | PCR | Sequencing | ELISA |
| +ve | 29 | 0 | 7 | 3 | 30 |
| -ve | 21 | 0 | 0 | 0 | 6 |
| Total tested samples | 50 | 50 | 7 | 3 | 36 |

4.3. PCR amplification

The Primers of EH1, EH4 PCR were utilized in a 25 µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler. The primary denaturation step was done at 95 °C. for 5 min, followed by 35 cycles of 94°C for 30 sec., 55°C for 40 sec. and 72°C for 40 sec. A final extension step was done at 72°C for 10 min.

4.4. Analysis of the PCR Products

The PCR products were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products were loaded in each gel slot. A gel pilot 100 bp DNA ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

4.5. Nucleotide sequencing

The sequencing of products of 3positive samples (1EHV-1 and2 EHV-4) was done by genetic analyzer 3500(life technology)using a big dye terminator V3.1sequencing kit. Bioedit and main workbench six software do an analysis.

RESULTS

1- Isolation

Twenty-nine out of 50 samples show pock lesion on the CAM of ECE from the 1st passage. MDBK cell line fails to show any CPE for three passages



Fig.1: Pock lesions at the CAM of inoculated ECE

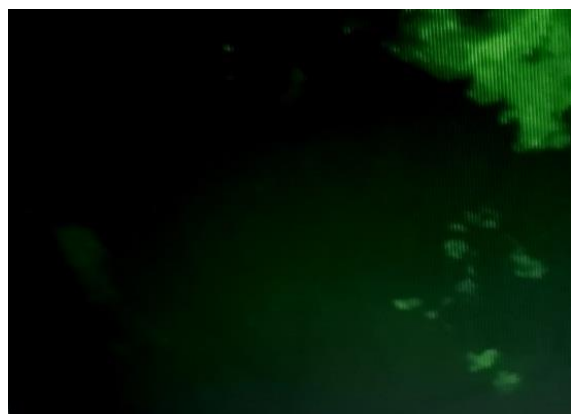


Fig.2: Indirect fluorescent antibody technique

2. Enzyme-linked immunosorbent assay (ELISA)

Thirty out of 36 examined serum samples were positive for the presence of EHV-1 and EHV-4 when tested with ELISA. Samples with S/P ratio ≥ 0.3 were considered positive.

3. PCR results

Table 3: The result of 7 tested samples by PCR:

| Sample | EH1 Result | EH4 Result |
|--------|------------|------------|
| 1 | - | + |
| 2 | + | - |
| 3 | - | + |
| 4 | + | - |
| 5 | - | + |
| 6 | + | - |
| 7 | - | + |

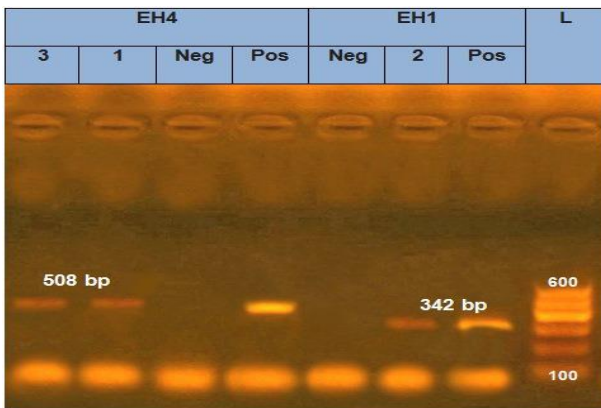


Fig.3: Showing PCR results on agarose gel

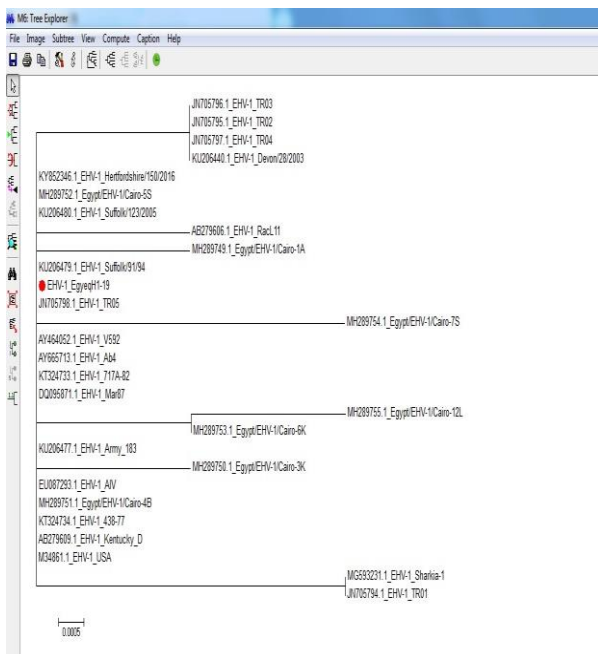


Fig.4: Phylogenetic tree of the sequenced Equine herpes1 sample isolated from a horse in 2019.

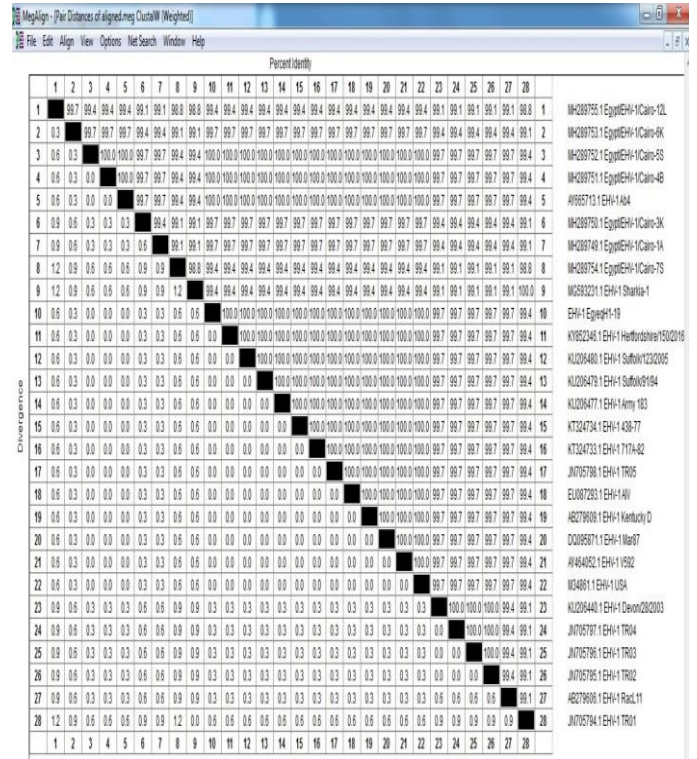


Fig.5: Sequence distance of the sequenced equine herpes1 samples in comparison with Egyptian isolates and universal isolates.

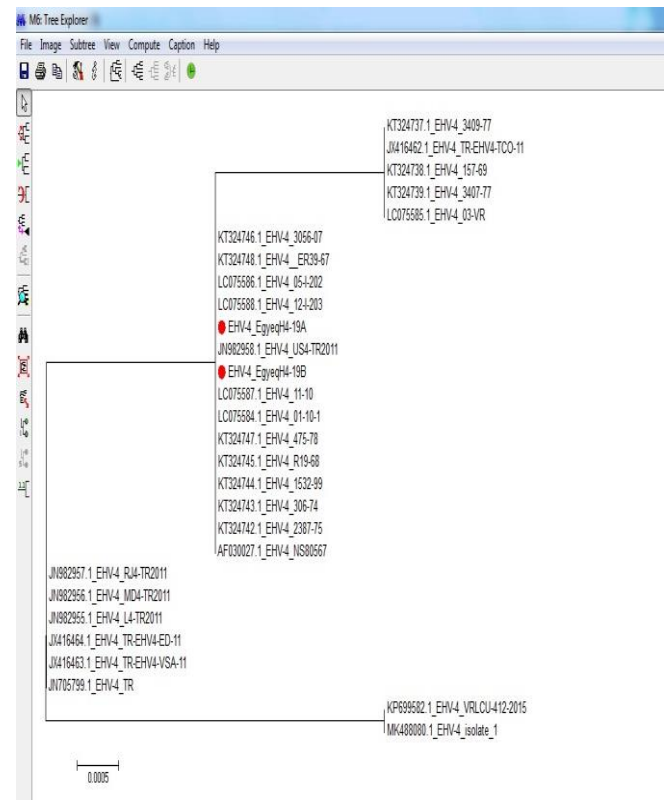


Fig.6: Phylogenetic tree of 2 sequenced Equine herpes4 samples isolated from 2 horse in 2019

MegAlign - [Pair Distances of aligned.meg ClustaW (Slow/Accurate, IUB)]

File Edit Align View Options Net Search Window Help

Percent Identity

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | | | | |
|----|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|------|------|---------------------------------|---------------------------------|-----------------------------|
| 1 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.4 | 97.6 | 1 | JN982958.1 EHV-4 USA-TR2011 | |
| 2 | 0.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.4 | 97.6 | 2 | EHV-4 EgeqH4-19A |
| 3 | 0.0 | 0.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.4 | 97.6 | 3 | EHV-4 EgeqH4-19B |
| 4 | 0.0 | 0.0 | 0.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.4 | 97.6 | 4 | LC075588.1 EHV-4 12-I-203 |
| 5 | 0.0 | 0.0 | 0.0 | 0.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.4 | 97.6 | 5 | LC075587.1 EHV-4 11-10 |
| 6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.4 | 97.6 | 6 | LC075586.1 EHV-4 05-I-202 |
| 7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.4 | 97.6 | 7 | LC075584.1 EHV-4 01-I-10-1 |
| 8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.4 | 97.6 | 8 | KT324748.1 EHV-4 ER39-67 |
| 9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.4 | 97.6 | 9 | KT324747.1 EHV-4 475-78 |
| 10 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.4 | 97.6 | 10 | KT324746.1 EHV-4 3056-07 |
| 11 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.4 | 97.6 | 11 | KT324745.1 EHV-4 R19-68 |
| 12 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.4 | 97.6 | 12 | KT324744.1 EHV-4 1632-99 |
| 13 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 100.0 | 100.0 | 100.0 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.4 | 97.6 | 13 | KT324743.1 EHV-4 306-74 |
| 14 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 100.0 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.4 | 97.6 | 14 | KT324742.1 EHV-4 2387-75 |
| 15 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.8 | 99.4 | 97.6 | 15 | AF030027.1 EHV-4 NS80567 |
| 16 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 100.0 | 100.0 | 100.0 | 99.6 | 99.6 | 99.6 | 99.6 | 99.6 | 99.6 | 99.6 | 100.0 | 99.6 | 99.2 | 97.4 | 16 | LC075585.1 EHV-4 03-VR |
| 17 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.0 | 100.0 | 100.0 | 99.6 | 99.6 | 99.6 | 99.6 | 99.6 | 99.6 | 99.6 | 100.0 | 99.6 | 99.2 | 97.4 | 17 | KT324739.1 EHV-4 3407-77 |
| 18 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.0 | 0.0 | 100.0 | 99.6 | 99.6 | 99.6 | 99.6 | 99.6 | 99.6 | 99.6 | 100.0 | 99.6 | 99.2 | 97.4 | 18 | KT324738.1 EHV-4 167-69 |
| 19 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.0 | 0.0 | 0.0 | 100.0 | 99.6 | 99.6 | 99.6 | 99.6 | 99.6 | 99.6 | 100.0 | 99.6 | 99.2 | 97.4 | 19 | KT324737.1 EHV-4 3409-77 |
| 20 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.4 | 0.4 | 0.4 | 0.4 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 99.6 | 99.6 | 97.8 | 20 | JN982957.1 EHV-4 RJ4-TR2011 |
| 21 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.0 | 100.0 | 100.0 | 100.0 | 99.6 | 100.0 | 99.6 | 97.8 | 21 | JN982956.1 EHV-4 MD4-TR2011 | | |
| 22 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.0 | 0.0 | 100.0 | 100.0 | 99.6 | 100.0 | 99.6 | 97.8 | 22 | JN982955.1 EHV-4 L4-TR2011 | | |
| 23 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.0 | 0.0 | 0.0 | 100.0 | 99.6 | 100.0 | 99.6 | 97.8 | 23 | JX416464.1 EHV-4 TR-EHV4-ED-11 | | |
| 24 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 99.6 | 100.0 | 99.6 | 97.8 | 24 | JX416463.1 EHV-4 TR-EHV4-VSA-11 | | |
| 25 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 99.6 | 99.2 | 97.4 | 25 | JX416462.1 EHV-4 TR-EHV4-TCO-11 | |
| 26 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.4 | 0.4 | 0.4 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.4 | 99.6 | 97.8 | 26 | JN705799.1 EHV-4 TR | | |
| 27 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.8 | 0.8 | 0.8 | 0.8 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.8 | 0.4 | 98.2 | 27 | KP699582.1 EHV-4 VRLCU-412-2015 | |
| 28 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.8 | 0.8 | 0.8 | 0.8 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.8 | 0.4 | 0.0 | 98.2 | 28 | MK488080.1 EHV-4 isolate 1 | |

Fig.7: Sequence distance of the 2 sequenced equine herpes4 samples in comparison with Egyptian isolates and universal isolates

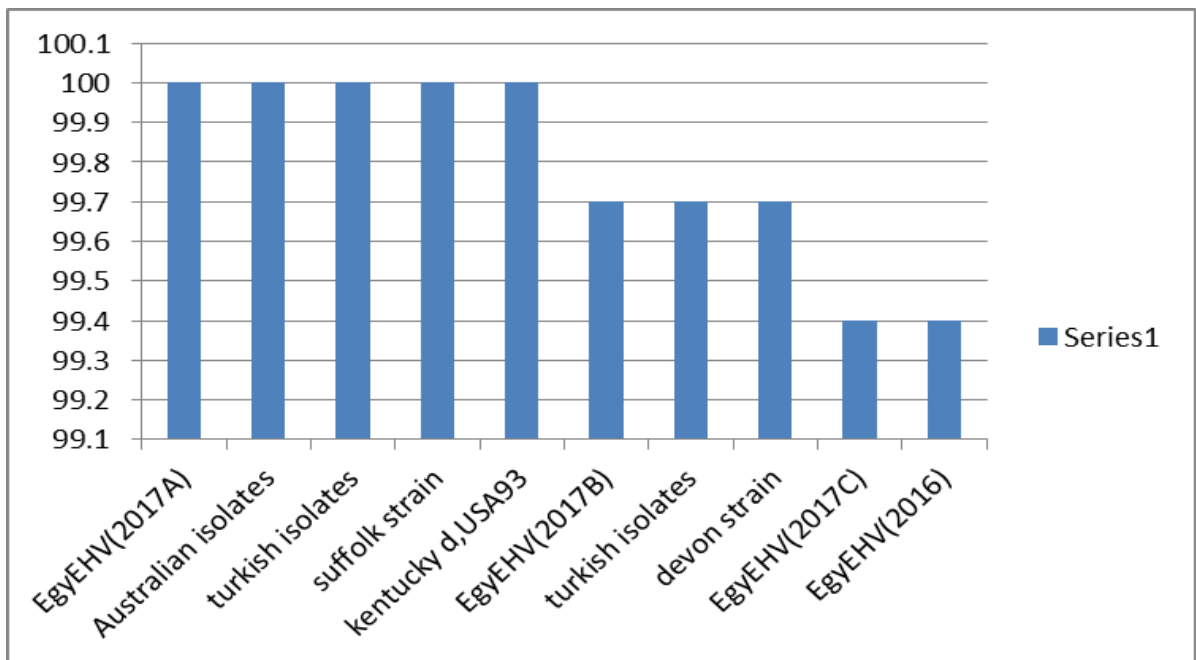


Fig 8: Chart illustrating the percentage of identity between the recently sequenced Egyptian EHV1 isolates in 2019 and the previously sequenced Egyptian and universal strains.

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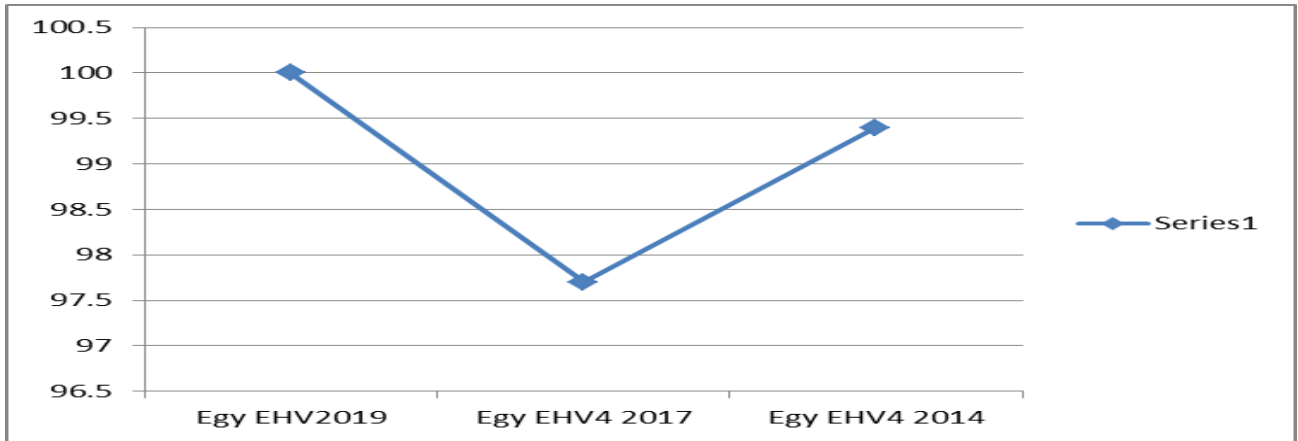


Fig. 9: Chart illustrating the percentage of identity between the recently sequenced Egyptian EHV1 isolate in 2019 and the previously sequenced Egyptian strains.

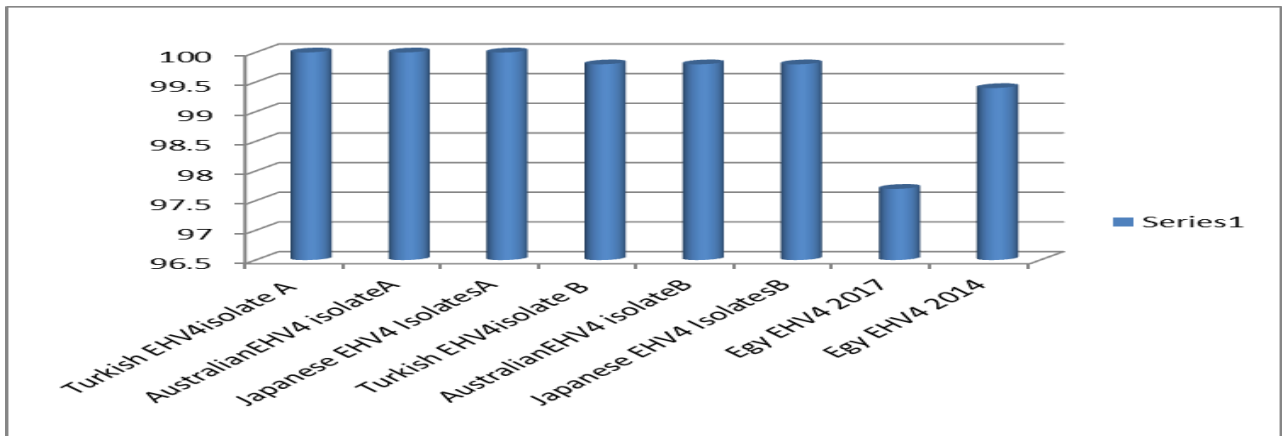


Fig.10: Chart illustrating the percentage of identity between the recently sequenced Egyptian EHV4 isolates in 2019 and the previously sequenced Egyptian and universal strains.

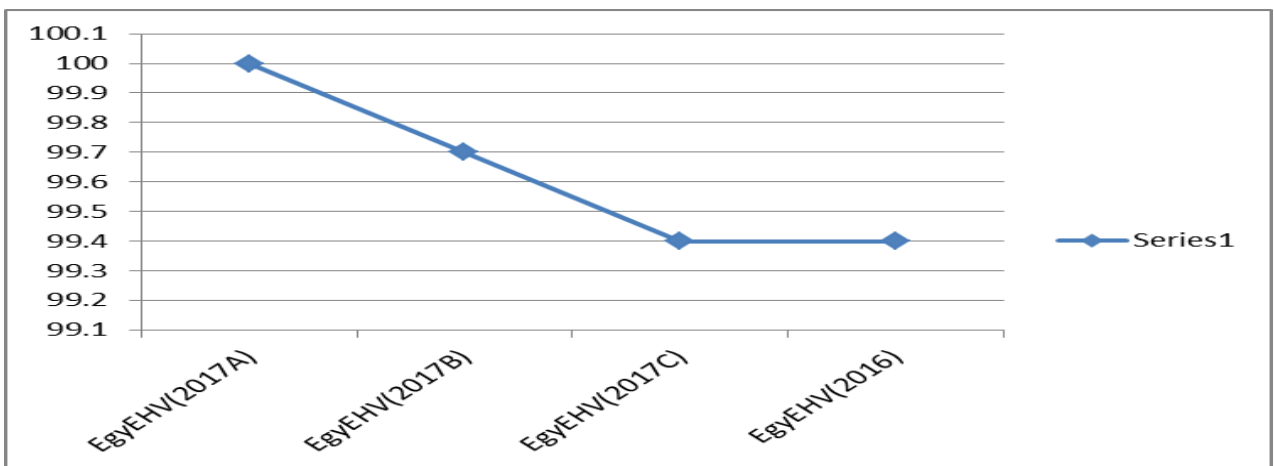


Fig.11: Chart illustrating the percentage of identity in between the recently sequenced Egyptian EHV4 isolates in 2019 and the previously sequenced Egyptian strains.

DISCUSSION

Equine herpesviruses (EHVs) are widespread in equine populations worldwide. While the infection with equine α -herpesviruses (EHV-1 and EHV-4) has been linked to several clinical outcomes. Equine herpesvirus 1 (EHV-1) causes respiratory disease, abortion, neonatal death, and neurological disease in equines and is endemic in most countries. Coinfections with these viruses were detected in both clinically infected and healthy horses and donkeys. This dual infection was reported before in horses and wild equids (**Amer et al., 2011; Back et al., 2015; Abdelgawad et al., 2016**). Furthermore, donkeys in Egypt are always stressed due to the assigned hard work, which might increase the probability of EHV shedding after reactivation (**Walid Azab et al., 2019**).

The Egyptian EHV1 isolate in 2019 was closely related to previously isolated Egyptian and universal isolates. The same results were found in the sequenced EHV4 Egyptian isolates. This data accords with the worldwide spread of equine herpesviruses (**Marie Garvey et al., 2019**). In this study, screening of horses acutely infected by EHV-1 and EHV-4 was investigated by isolating the causative viruses, PCR, and detection of viral antibody against BVH-1 and BVH-4.

Twenty-nine out of 50 samples show pock lesion on the CAM of ECE from the 1st passage. PCR investigated samples that show pock lesion on CAM for confirmation of the virus. EHV-1 was simultaneously detected in 3 samples while EHV-4 was detected in 4 samples. Two of the EHV-4 isolates and one EHV-1 were subjected to phylogenetic analysis. Sequencing was performed to confirm virus isolation and compare sequences retrieved directly from EHV isolated on pock lesions. Our sequences clustered together with other herpesviruses isolated worldwide.

Our isolated strain of equine herpes1 (EHV-1) has 100% identity to {The previously isolated Egyptian equine herpes1 in 2017A (MH289752.1 and MH289751.1); the Turkish strain JN705798.1(TR05); the Australian isolates (KT324734.1 and kt324733.1) and to the following universal strain KU206480(Suffolk/123/2005), KU206479.1 (Suffolk/91/94, DQ095871.1(mar87), ku206477.1 (Army_183), EU087293.1 berlin2008, AB279609.1 (Kentucky, D) and M34861.1(USA93))

Our isolated strain of equine herpes1 has 99.7% identity similar to {the previously isolated Egyptian equine herpes1 in 2017B (MH289753.1, MH289750. 1 and MH289749.1); the Turkish strain (JN705796.1, JN705795.1, and JN705797) and to the following universal strains KU206440.1

(devon/28/2003)}. Our isolated strain of equine herpes1 has 99.4% identity {the previously isolated Egyptian equine herpes1 in 2017C (MH289755.1 and MH289754.1) and that isolated in 2016 (MG593231.1)}.

Our two isolated strain of equine herpes4 are completely identical and has 100% identity with the Turkish strain(A) JN982958.1; to the Australian isolates(A) KT324748.1 ; KT324747.1; KT324746.1; KT324745.1; KT324744.1 ; KT324743.1; KT324742.1 and to the Japanese isolates(A) LC075588.1; LC075587.1; LC075586.1; LC075584.1. The analysis illustrate that our newly isolated strain were closely related to Australian isolates(B) KT324739.1; KT324738.1; KT324737.1 and to Japanese strains(B) LC075585.1, and to Turkish isolates(B) (JN982957.1 JN982956.1;JN982955.1; JX416464.1;JX416463.1; JX416462.1 ;JN705799.1) with a percentage of 99.8%. They were found to be similar to other previously isolated Egyptian equine herpes4 (isolated in 2017) with % of identity range from 99.4% as KP699582.1 in 2014 to 97.7% as Mk488080.1 in 2017.

In this study, we also examined the presence of specific antibodies against equine herpesvirus type 1 (EHV-1), and equine herpesvirus type 4 (EHV-4) in equine by ELISA for EHV-1 and EHV-4 specific antibodies. The level of antibodies to herpes virus from the examined symptomatic cases is an unprotective level.

CONCLUSION

It can be concluded that The present data indicate that virus isolation in conjunction with PCR assay would lead to a higher virus identification rate in multiple respiratory infections caused by EHV-1 and EHV-4 and subsequent phylogenetic analysis gave valuable information about the molecular epidemiology of EHV-1 and EHV-4 subtypes prevalent in Egypt.

Declaration of Competing interest

On behalf of all authors, I hereby declare that no conflict of interest may interfere with the publication of the manuscript.

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How to cite this article:

Morcos Ibrahim Yanni; Ebtsam, A. Abouelyazeed; Hala Abdelrehim Ali and Nadia Maher Hanna, 2021. Verification Of Molecular And Conventional Techniques Used In The Diagnosis Of Equine Herpes Virus In Some Egyptian Governorates. *Journal of Applied Veterinary Sciences*, 6(1): 1 – 8.

DOI: <https://dx.doi.org/10.21608/javs.2021.140052>