Isolation of Field Isolate of Equine herpes virus-1 Based on Molecular Detection and Characterization of Glycoprotein D

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ABSTRACT

Equine herpesvirus (EHV)-1 is a pathogen of horses, well known for its ability to induce abortion and nervous system disorders neonatal foal pneumonitis, highly contagious among susceptible horses, with viral Transmission to cohort animals occurring by inhalation of aerosols of virus-laden respiratory secretions. Extensive use of vaccines has not eliminated EHV infections, and the worldwide annual financial burden from these equine pathogens is immense. Clinical signs may occur despite the presence of a virus-specific immune response in the horse. The current study focus on outbreak recorded in El Zahraa Stud for Arabian horses during 2014 and recorded officially for the first time by OIE in Egypt by the notification presented by Chairman, General Organization for Veterinary Services (GOVS), Ministry of Agriculture and Land Reclamation, the samples collected from the stud were either aborted fetal tissues or nasal swaps from apparently healthy animals and previously aborted mares the current study focuses on isolation and molecular characterization of EHV-1 of the local isolate Egypt/VSVRI/Zahraa/2014 using the glycoprotein D of EVH-1 due to its role in virus infectivity and its function in entry of virus into cells and is considered as one of the most potent inducers of virus-neutralizing antibody among the spectrum of EHV-1 proteins based on sequence analysis and multiple alignment revealed single nucleotide substitution at the base pair number 121 from the start codon which give lead to single Amino Acid Substitution from CAG (Glutamine(Gln/Q)) which is considered as a polar Amino Acid to AAG (Lysine(Lys/K)) which is a basic Amino Acid which is very characteristic for the local isolate than the rest of isolate and strains.

INTRODUCTION

EHV-1 is a viral pathogen with a high prevalence in domestic horses worldwide O'Neill et al., 1999; Bryans & Allen, 1989). EHV-1 infection has enormous economic impact on the breeding, competitive and recreational horse industries, as losses through abortion, respiratory disease, neurological disorders and death of full-term newborns occur perennially (Allen et al., 1999; Gilkerson et al., 1998). The high prevalence of the virus results from difficulty in preventing primary infections and the subsequent establishment of latent infection in the lymphocytes and neuronal tissues (Baxi et al., 1995; Chesters et al., 1997; Edington et al., 1994), The neurological signs of EHM reflect a diffuse, multifocal myeloencephalopathy secondary to vasculitis, hemorrhage, thrombosis and ischemic neuronal injury (Edington et al., 1986; Wilson, 1997). The sudden onset and early manifestation of ataxia, paresis and urinary incontinence, the involvement of multiple horses on the premises, and a recent history of fever, abortion or respiratory Disease in affected horse

populations are typical features, although there is considerable variation between outbreaks with respect to epidemiological and clinical findings (Wilson, 1997). Prevention is difficult because many horses are latently infected, allowing the virus to circulate silently in horse populations, and currently available vaccines do not confer protection against neurological manifestations of infection (Patel and Heldens, 2005; Slater, 2006). In view of Glycoprotein D (gD) role in virus entry and as targets for immune responses in other herpesviruses, the envelope glycoproteins of EHV-1 have been investigated for immunogenicity using murine models of EHV-1 respiratory disease (Tewari et al., 1994; Osterrieder et al., 1995; Stokes et al., 1997; Kukreja et al., 1998; Packiarajah et al., 1998; Zhang et al., 1998). Inoculation with EHV-1 glycoprotein D (gD) expressed by a recombinant baculovirus (Love et al., 1993) or as plasmid DNA led to protective responses in mice (Tewari et al., 1994; Ruitenberg et al., 1999). Inoculation of mice with EHV-1 gD DNA followed by recombinant protein (prime-boost) was more effective than inoculation with either protein or DNA alone (Ruitenberg et al., 2000b). However, the efficacy of such approaches in horses is not yet proven, and there have been few reports of testing recombinant glycoproteins in the horse (Audonnet et al., 1999; Ruitenberg et al., 2000a) the aim of this study is to monitor the circulating strains of Equine herpes virus-1 in the Egyptian field based on sequence and phylogenetic analysis to build up accurate data for updating the vaccine strains used against the disease.

MATERIAL AND METHODS

1-The glycoprotein D of Equine herpes virus 1 of the Kentucky D reference strain synthesized by gene art synthesis technology by life Technologies Company and The synthesized gene Glycoprotein D CDS was assembled from synthetic oligonucleotides and /or PCR products. The fragment was cloned into pMAT-T using Sfil and Sfil cloning sites where the plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The final construct verified by sequencing. The sequence congruence within the used restriction sites was 100%.

2-The local isolate in the current study has been isolated form a wave of Abortions has been recorded in El Zahraa Stud for Arabian Horses, collected samples were either aborted fetal tissues either Lung and livers as well as Aborted placenta or 8 nasal swaps from mares suffering from repeated Abortions and frequent respiratory manifestation These samples have been collected by the authorized personnel in El Zahraa Stud for Arabian horses and according to their own responsibility and these samples have been delivered by 18t February 2014 to the Genetic Engineering Research Department, Veterinary Serum and Vaccine Research Institute for confirming the cases.

3-DNA extraction and purification carried out by using Qiagen DNeasy Blood & Tissue extraction (Qiagen, Germany) and according to the manufacturer's protocol. Purified DNA recovered in 100-µl elution buffer and stored at -20°C for further testing.

4- PCR primer pairs: upstream primer F1-30A was 21 mer after the CACC

overhang including the first ATG of the Glycoprotein D of EHV1 5` CAC CAT GTC TAC CTT CAA GCT TAT 3` While The downstream primer R1-30A was 20 mer from the nucleotide number 1189 till 1209 which is the end of the glycoprotein D of EHV1 5`ACA CTG TTT ACG GAA GCT GG 3`,with the following conditions (25 µl of Dream Taq Green PCR Master Mix (2X), which includes DreamTaq DNA Polymerase,2X Dream Taq Green buffer, dNTPs, and 4mM MgCl2 by Thermo Fisher Scientific Inc., MA, USA) and 1 µl of 0.5 µM of both F1-30A and R1-30A primers and 5 µl of 0.5 µg template DNA with the following cycling conditions 95°C for 5 min followed by 35 cycle s of 95°C for 30 s, 52°C for 30 s and 7 2°C for 1 min and 30 seconds and a final extension step at 72°C for 7 min, The second primer pairs were designed to flank the area from the beginning of the glycoprotein D of EHV1 including the first ATG of the gene was 21 mer F2 5`ATG TCT ACCTTC AAG CTT ATG 3` to flank an area of about 1040 bp from the start ATG of the gene where the selected primer length were 20 mer from the nucleotide number 1021 bp till 1040 bp R2 5`GAG TTG CTC TTA GAC GTT TT 3`. With the following cycling conditions 95°C for 5 min followed by 40 cycle s of 95°C for 30 s, 41°C for 30 s and 7 2°C for 1 min and 20 seconds and a final extension step at 72°C for 7 min Primers are synthesized by synthesized by (BIOSEARCH TECHNOLOGIES South McDowell Boulevard Petaluma, CA, USA)

PCR carried out using Use a thermostable, proofreading DNA polymerase • 4 x 1.25 mL DreamTaq Green PCR Master Mix (2X), which includes DreamTaq DNA Polymerase, 2X DreamTaq Green buffer, dNTPs, and 4 mM MgCl2 cat No #K1081, and the PCR primers above to produce your blunt-end PCR product .on the control plasmid pMAT using the both primer pairs revealed PCR products at the expected size one was about 1209 bp corresponding to the expected size of the whole length glycoprotein D gene of EHV1 and the other product was about 1040 bp corresponding to the expected size flanked by the second primer pairs, these results assures that the designed primers was working efficiently as they have given products at the expected size exactly, also these results truly assures that the samples delivered by African horse sickness Department, VSVRI had no EHV1 viral DNA in their samples, the same PCR conditions carried out on the samples collected from El Zahraa Stud for Arabian horses using the primers flanking the 1040 bp primers where 6 samples of the nasal swaps was strong positive while the intensity of the PCR band varies may be according the viral DNA load on the isolated samples but all the positive samples give the band at the exact expected size and the remaining two samples were negative, the remaining sample of the aborted fetal tissue give the product at the expected size but gave some non-specific bands below the expected band size.

Then the virus has been isolated on chorioallantoic membrane of Embryonated Chicken Egg according to (Hassanien MM, Maysa H, El-bagoury F, Magda AK, El-Kabbany MMA, Daoud MA (2002). Trials for isolation and identification of equine herpesvirus abortion in Egypt. Vet. Med. J. Giza 50(4): 977-986. 2002) and Vero cell monolayer to propagate the virus, then pock lesions and the infected monolayer cell lysate collected (this step carried out

by African Horse sickness Department, VSVRI) then PCR carried out on these samples to verify the presence of the EHV1 viral DNA in either the infected monolayer cell lysate or the pock lesion.
5-PCR for sequence analysis carried out on the sample Number 7 which was the most intense Band at the expected size which may reflect the higher viral DNA load among these samples using the primers flanking the whole length of the glycoprotein D of EHV1, then the PCR product excised and purified from the gel using Quiaquick purification kit (Qiagen) according to the manufacturer instruction then sent for sequencing.

RESULTS

1- Plasmid profiling to assure the size of the control transfected plasmid into Top 10 Ecoli cells in comparison to the non-transformed cells as shown in Figure (1)

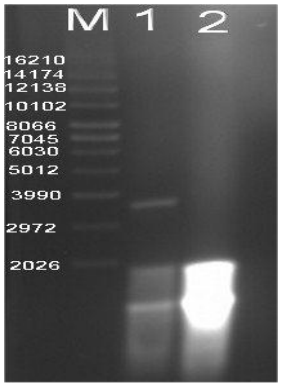


Fig (1) First lane is100bp DNA ladder, Lane 1 is the extracted control pMAT plasmid carrying the whole length synthesized Glycoprotein D of EHV1 (3589bp) from the transformed Top 10 Ecoli while Lane 2 is the negative non-transformed Top10 Ecoli

2- PCR using the primer set that flanks the coding sequence of the glycoprotein D (1209) bp of Equine herpes virus inside the control pMAT plasmid harboring the control gene of the Kentucky D reference strain as show in figure (2)

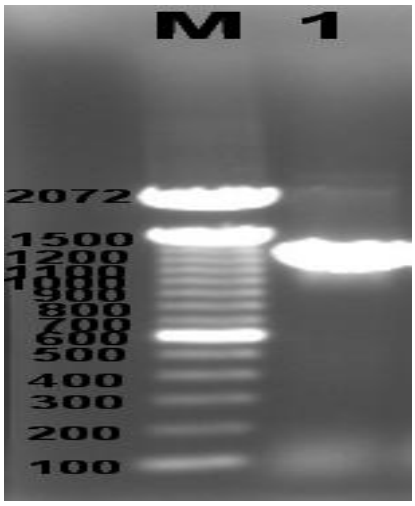
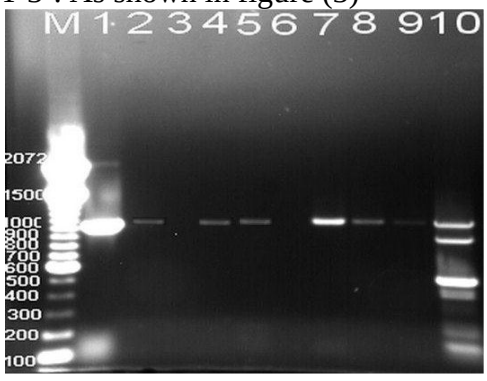


Fig (2) PCR products carried on the control pMAT plasmid flanking the whole length glycoprotein D of EVH1 where the first Lane Is 100 bp Ladder and the lanes 1 the (1209) glycoprotein D of EHV1 at the expected size.

3- PCR carried out on the field samples using the primer pairs that flanks about 1040 bp from the start of the coding sequence giving bands at the expected size using F2 5`ATG TCT ACCTTC AAG CTT ATG 3 and the selected primer length were 20 mer from the nucleotide number 1021 bp till 1040 bp R2 5`GAG TTG CTC TTA GAC GTT TT 3`. As shown in figure (3)



Fig(3) PCR products using the F2 R2 primers flanking about 1040 bp carried out on the nasal swaps samples and aborted fetal tissues collected from the El Zahraa Stud for Arabian horses where the First lane is 100bp DNA ladder

while Lane 1 PCR carried on the control pMAT plasmid, lane 2,4,5,7,8,9 from the collected nasal swaps and Lane 10 was from the aborted fetal tissues gave bands at the expected size and the intensity of bands may correlate to the initial viral DNA load on these samples, while samples of Lane 6 and Lane 3 were negative.

4- Multiple sequence alignment using neighbor joining method revealed that there is single nucleotide substitution at the base pair number 121 from the start codon which give lead to single Amino Acid Substitution from CAG (Glutamine(Gln/Q)) which is considered as a polar Amino Acid to AAG (Lysine(Lys/K)) which is a basic Amino Acid while the remaining part of the whole revealed no change which prove that the Glycoprotein D coding gene show great conservation among all strains which indicates that it is a good candidate to be used as a subunit diagnostic antigen to detect the level of the circulating antibodies against EHV1 as shown in Figure (4)

Fig (4) Sequence analysis of the Glycoprotein D of Equid herpesvirus 1 isolate Egypt/VSVRI/Zahraa2014 Accession No # KM406326.

	Ref	Seq	Ident	Sim	i	ord	cl	Isolate
2	VSVRI_Zahraa_2014	VSVRI_Zahraa_2014	100	100	1	1	1	complete cds VSVRI/Zahraa/2014
3	VSVRI_Zahraa_2014	AP012321_1__EVH1	98.25	99.43	2	2	1	Equid herpesvirus 1 isolate: 5586.
4	VSVRI_Zahraa_2014	AY665713_1__EVH1	99.75	100	3	3	1	Equine herpesvirus 1 strain Ab4
5	VSVRI_Zahraa_2014	M60946_1_HSEGP1718	99.5	100	4	4	1	Equine herpes virus isolate="Ab1"
6	VSVRI_Zahraa_2014	AB279607_1__EVH1	99.75	100	5	5	1	Equid herpesvirus 1 RacL11.
7	VSVRI_Zahraa_2014	AB279610_1_EVH1	99.75	100	6	6	1	Equid herpesvirus 1 Kentucky D.
8	VSVRI_Zahraa_2014	AY464052_1__131265_132473	99.75	100	7	7	1	Equine herpesvirus 1 strain V592
9	VSVRI_Zahraa_2014	M59773_1	99.75	100	8	8	1	Equine herpesvirus 1 M59773.1 GI:330890
10	VSVRI_Zahraa_2014	M62923_1_HSEGLYPD	99	100	9	9	1	Equine herpesvirus type-1 (EHV-1) gene, M62923_1_HSEGLYPD
11	VSVRI_Zahraa_2014	M87497_2_HSEE249GDX	99	100	10	10	1	M87497.2 GI:15617231
12	VSVRI_Zahraa_2014	KF644573_1__EVH1	99	100	11	11	1	Equid herpesvirus 1 strain T616 delta71
13	VSVRI_Zahraa_2014	KF644574_1__EVH1	99	100	12	12	1	Equid herpesvirus 1 strain T-616
14	VSVRI_Zahraa_2014	KF644580_1__EVH1	99	100	13	13	1	Equid herpesvirus 1 strain T-529 10/84
15	VSVRI_Zahraa_2014	KF644575_1__EVH1	99	100	14	14	1	Equid herpesvirus 1 strain 94-137
16	VSVRI_Zahraa_2014	AB453826_1__EVH9	96.26	99.64	15	15	1	Equid herpesvirus 9 ORF72,giraffe strain.
17	VSVRI_Zahraa_2014	AP010838_1__EVH	96.26	99.64	16	16	1	Equid herpesvirus 9 DNA, strain P19.
18	VSVRI_Zahraa_2014	JQ343919_1__130059_131267	90.79	95	19	17	1	Equid herpesvirus 8 strain wh
19	VSVRI_Zahraa_2014	AF030027_1_AF030027_Equine	76.36	83.15	17	18	2	Equine herpesvirus 4 strain NS80567
20	VSVRI_Zahraa_2014	S65633_1__glycoprotein	76.36	83.12	18	19	2	equine herpesvirus 4 S65633.1

Table (1) showing The identity percent of 402 amino acid residue of the glycoprotein D of the local isolate VSVRI/Zahraa/2014 Accession

5- The identity percent revealed the highest identity 99.75% and 100% similarity with Equine herpes virus 1 strain Ab4, Equid herpes virus 1 RacL11, Equid herpes virus 1 Kentucky D, Equine herpes virus 1 strain V592, Equine herpes virus 1 M59773.1 GI:330890 Accession numbers. (AY665713_1, AB279607_1, AB279610_1, AY464052_1, M59773_1) respectively and 99.5% and 100% similarity with Equine herpes virus isolate="Ab1" Accession number # M60946_1 and showing 99 % identity and 100% similarity with Equine herpes virus type-1 (EHV-1) gene, M62923_1_HSEGLYPD,M87497.2 GI:15617231, Equid herpes virus 1 strain T616 delta71, Equid herpes virus 1 strain T-616, Equid herpes virus 1 strain T-529 10/84, Equid herpesvirus 1 strain 94-137 Accession numbers (M62923_1, M87497_2, KF644573_1, KF644574_1, KF644580_1, KF644575_1) respectively and showing 98.25% identity and 99.43% similarity with Equid herpesvirus 1 isolate: 5586 Accession number # AP012321_1 and revealed 96.26% identity and 99.64% similarity with Equid herpes virus 9 ORF72,giraffe strain and Equid herpes virus 9 DNA, strain P19 Accession numbers (AB453826_1 and AP010838_1) respectively and revealed 90.79% identity and 95% similarity with Equid herpes virus 8 strain wh Accession number # JQ343919_1 and revealed 76.36% identity and 83.15% similarity with Equine herpes virus 4 strain NS80567 Accession number #

AF030027_1 and revealed 76.36% identity and 83.12% similarity with Equine herpes virus 4 strain S65633.1 Accession number #S65633_1.

6-Phylogenetic analysis of the Egyptian isolate and other published EHV-1strains performed based on 402 amino acid residues of the glycoprotein D of Equine herpes virus1 Which covers 402 amino acid the showing the PHA03265 conserved domain region of the glycoprotein D of equine herpes virus 1 where it include two main neutralizing sites first one constitutes about 19 linear amino acid residues near by the N-terminus of the glycoprotein D spans from (residues 4 to 22) and another 20 residues spans from (residues 267 to 285) give rise to bands about 55 kDa polypeptides and 47 kDa polypeptides in infected cell extracts (Flowers and O'Callaghan, 1992) . The phylogenetic analysis revealed that The Phylogenetic relationship was performed using N-J method (MEGA 6) software. The distance of estimation based on method done by Saitou N & Nei M (1987) method by using MEGA 6 software. The bootstrap probabilities, as determined for 1000 resampling, showing the clusters of Equine herpes viruses and the precise location of the Egyptian isolate among other strains and isolates. Rooted Phylogenetic tree constructed to calculate and examine the evolutionary relationship of the sequences, in which the length of the horizontal line connecting one sequence to another is proportional to the estimated genetic distance between the sequences. The rooted tree showed that there are two main distinct clusters

- The first cluster includes two strains of EHV4 :

1- The Equine herpes virus 4 strain NS80567. 1 Accession number # AF030027_1

2- The equine herpes virus 4 S65633. Accession number #S65633_1

- The second cluster includes two main branches.

1- The first main branch includes with Equid herpes virus 8 strain wh Accession number # JQ343919_1.

2- The second branch includes two main sub branches

a- First one includes two strains of EHV-9 Equid herpes virus 9 ORF72,giraffe strain and Equid herpes virus 9 DNA, strain P19 Accession numbers (AB453826_1 and AP010838_1) respectively

b- Second one includes 4 clusters of EVH-1

- First cluster includes Equid herpes virus 1 strain T616 delta71, Equid herpes virus 1 strain T-616, Equid herpes virus 1 strain T-529 10 /84, Equid herpes virus 1 strain 94-137 Accession numbers (KF644573_1, KF644574_1, KF644580_1, and KF644575_1).
- Second cluster includes Equid herpesvirus 1 isolate: 5586 Accession number # AP012321_1.

Third cluster includes two main sub Equine herpesvirus 1 strain Ab4, Equine herpes virus isolate="Ab1", Equid herpesvirus 1 RacL11, Equid herpesvirus 1 Kentucky D and Equine herpesvirus 1 strain V592.

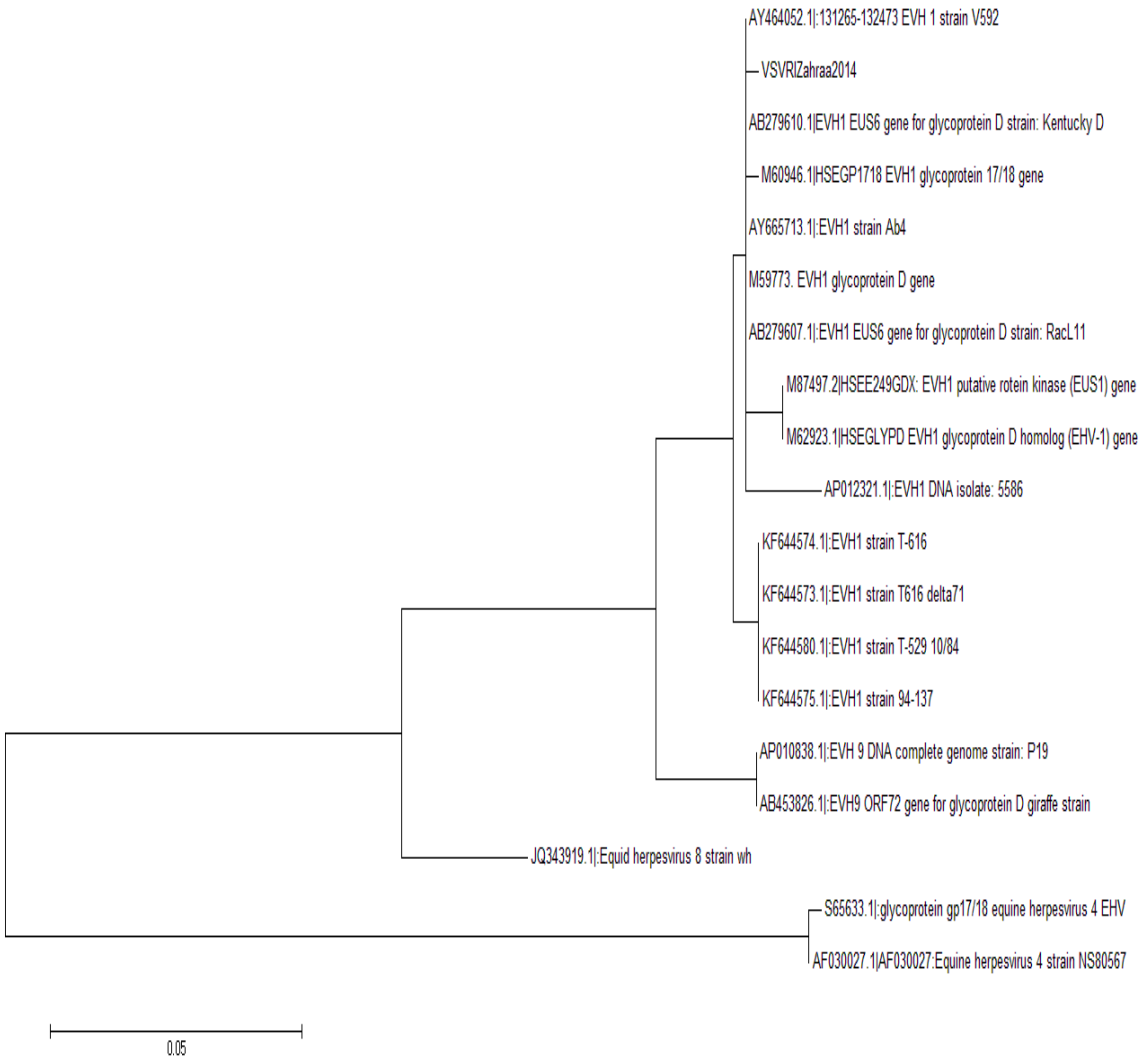
- branches.

a- The first branch includes EU putative rotein kinases of herpes simplex viruses homologue to glycoprotein D of EHV-1 HSEGLYPD_661_1867 and HSEE249GDX_4694_5900 Accession numbers M62923_1 and M87497_2 respectively

b- The second branch includes.

1- VSVRI/Zahraa/2014Accession number#KM406326
2- Equid herpesvirus 1 isolate: 5586. Accession number# AP012321_1
3- Equine herpesvirus 1 strain Ab4. Accession number# AY665713_1
4- Equine herpes virus isolate="Ab1". Accession number# M60946_1
5- Equid herpesvirus 1 RacL11. Accession number# AB279607_1
6- Equid herpesvirus 1 Kentucky D.Accession number# AB279610_1
7- Equine herpesvirus 1 strain V592 Accession number# AY464052_1.

Therefore, the VSVRI/Zahraa/2014 closely related to Equid herpesvirus 1 isolate: 5586 as shown in figure (6).



Fig(6) Phylogenetic analysis of the deduced amino acid sequence of the glycoprotein D of Equid herpesvirus 1 isolate Egypt/VSVRI/Zahraa2014 using MEGA5 software this rooted tree was generated by neighbor-joining method with 1000 bootstrap. The bar represents a sequence divergence of 0.01 residue per site, the neighbor-joining method done by. Saitou N & Nei M (1987).

DISCUSSION

The current study Based on molecular characterization of one of the circulating isolates of EHV-1 causing outbreak in El Zahraa Stud for Arabian horses based on sequence analysis of the envelope glycoprotein D of EHV-1 (EHV-1gD) due to its essential role in virus infectivity and its function in entry of virus into cells and is considered as one of the most potent inducers of virus-neutralizing antibody among the spectrum of EHV-1 proteins ,collected samples were either aborted fetal tissues either Lung and livers as well as Aborted placenta and nasal swaps from mares suffering from repeated Abortions, The current study used the gene synthesis technology introduced by Invitrogen to synthesis the glycoprotein D of the Kentucky Strain Accession number# AB279610.1 as reference strain to use it as appositive control for the envelope glycoprotein D of EHV-1 (EHV-1gD) cloned in pMA-T plasmid for optimizing the detection of the virus from field samples by PCR using 2 sets of primers one of them includes the whole length of the gene about 1209 bp while the nested one from the start ATG is 1000 bp ,seven out of nine samples have been reported positive by the 2 sets of primers, then the specific band at the expected size of the whole length of EHV-1 (EHV-1gD) gene of the local isolate was sent for sequence analysis, multiple alignment revealed single nucleotide substitution at the base pair number 121 from the start codon which give lead to single Amino Acid Substitution from CAG (Glutamine(Gln/Q)) which is considered as a polar Amino Acid to AAG (Lysine(Lys/K)) which is a basic Amino Acid ,and detect the differences and similarity between the Egyptian isolate and the rest isolates and strains around the world based on the phylogenetic data obtained from the sequenced gene of the local isolate due to the importance of that glycoprotein which is related to its role in antigenicity and infectivity of EHV-1 as the glycoprotein D of EHV-1 located in the conserved protein domain family PHA03265 of herpes viruses

CONCLUSION

It very recommended that to make sequence analysis of the circulating Equine herpes virus-1 in the Egypt and to monitor the changes in genome of the virus to determine their antigenic state, similarity and dissimilarity with other strains in the region and all over the world. To have asserted data about the situation of Equine herpes virus-1 in Egypt to be taken in consideration during the vaccine Formulation and to make continuous update of the local vaccines using the locally circulating strains which will reflect on the potency of the prepared vaccines to protect animals against the disease it is also recommended to use cells of equine origin or RK-13 for propagation of EHV-1 as Vero cells proved to be resistant for EHV-1 but may be permissive for EHV-4 (Frampton et al., 2005).

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