# Cloning and expression of Equine herpesvirus 1 glycoprotein D as a fusion protein in E. coli from a local isolate from El Zahraa Stud for Arabian Horses to be used as a diagnostic antigen for detecting the antibodies either in vaccinated or infected horses

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## ABSTRACT

The current study focuses on 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 .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 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 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, then the sequenced product of the local isolate was cloned Into PET 151 D topo plasmid with an N-terminal tag containing the V5 epitope and a 6xHis tag and transformed into Top 10 Ecoli cells just to maintain the stability and propagation of the cloned gene then the cloned plasmid isolated to be transformed again in Ecoli BL21 cell which include the T7 promotor for expressing the glycoprotein D, western blotting carried out on the induced culture at different expression times using the alkaline phosphatase labeled N-terminal anti histidine monoclonal antibodies revealed single bands at the expected size, the same samples have been reacted with serum collected from aborted and infected mares revealed band at the expected site using anti equine IgG labeled horse radish peroxidase, concluding that the expressed envelope glycoprotein D of EHV-1 (EHV-1gD) could be used as a good candidate for manufacturing of diagnostic Antigen for detection of the circulating antibody either in vaccinated or latent infected horses.

## INTRODUCTION

Twelve portal proteins form a ring in the nucleocapsid, which is used by viral DNA to enter into the capsid.(Newcomb et al., 1989) (Baker et al., 1990)The tegument corresponds to the space between the nucleocapsid and the envelope. This is composed of 12 viral proteins and enzymes involved in the initiation of viral replication.

Nucleocapsids and tegument are surrounded by an envelope presenting 11 viral glycoproteins on its surface the eleven glycoproteins of EHV-1 (i.e. gB-gp14, gC-gp13, gD-gp18, gE, gG, gH, gI, gK, gL, gM and gN) are conserved in other alpha herpesvirus and therefore

named according to the nomenclature established for HSV-1. Glycoproteins are essential in infection processes including virus adsorption, penetration, and cell-to-cell spread. Compared to HSV-1 and the majority of other alpha herpesviruses, EHV-1 encodes an additional glycoprotein, gp2, with homologues present only in EHV-4 and AHV-3. As for other alpha herpesviruses, EHV-1 can infect a large range of cell types in the respiratory tract, lymphoid organs and the nervous system. Cells infected by direct contact with EHV-1 or by cell-to-cell contact with infected cells. Envelope glycoproteins of EHV-1 play key roles in the entry of the virus into host cells. EHV-1 uses the same glycoproteins as other alpha herpesviruses (e.g. HSV, bovine herpes virus (BHV) and pseudorabies virus (PRV)) to bind to permissive cells. EHV-1 glycoprotein C (gC) binds to heparan sulphate-containing glycosaminoglycans on the cell surface (Frampton et al., 2005; Osterrieder, 1999; Osterrieder et al., 1996). Glycoproteins D and M (gD and gM) are required for virus entry (Csellner et al., 2000) (5), but another unique receptor, still unknown and distinct from virus-receptors previously described for alpha herpesviruses is also involved (Frampton et al., 2005). Once attached, the virus penetrates the cell by either fusion of the virus envelope and cell membrane or by non-classical endocytosis/phagocytosis (Frampton et al., 2007), which release the nucleocapsid and tegument proteins of EHV-1 into the cell The envelope glycoprotein D of EHV-1 (EHV-1gD) is essential for virus infectivity due to its function in entry of virus into cells (Csellner et al., 2000; Whittaker et al., 1992), and it is one of the most potent inducers of virus-neutralizing antibody among the spectrum of EHV-1 proteins (Stokes et al., 1997)., The envelope glycoprotein D residues from 4 to 22 near the N terminal makes continuous epitope of the gD and does not require entire three dimensional molecule processing (Flowers and O'Callaghan, 1992), glycoprotein D has multiple forms when expressed on the surface of insect cell through baculovirus expression system as (56, 52, and 48 kDa) (Love et al., 1993).

## MATERIAL AND METHOD

1-The glycoprotein D of Equine herpes virus 1 of the Kentucky D reference strain has been synthesized by gene art synthesis technology to be used as control positive by life Technologies Company The fragment was cloned into pMAT-T .

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 upon to their own responsibility and these samples have been delivered by 18th 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 kit Catalog no. 69504 (Qiagen, Germany) and according to the manufacturer's protocol.

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`

Using 25 µl of DreamTaq Green PCR Master Mix (2X), according to Thermo Fisher Scientific Inc., MA,USA with 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)

5- PCR carried out using Use a thermostable, proofreading DNA polymerase • 4 x 1.25 mL DreamTaq Green PCR Master Mix (2X), 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 effiently 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.

64-PCR 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.

7- after sequence, verification the sequenced blunt-end PCR product has been cloned into pET151/D-TOPO cloning vector allows expression of recombinant protein with an N-terminal tag containing the V5 epitope and a 6xHis tag. According to the manufacturer instruction cat No#K151-01 ,the cloned gene used to transform electrocompetent E. coli Top 10 Ecoli cells And Incubated at 37C for 1 hour with shaking and plated on selective plate containing100 µg/ml ampicillin.

8- PCR carried out on the selected colonies using the primer pairs flanking the whole length Glycoprotein D (1209) and the primers flanking the 1040 bp then to get sure of the correct orientation of the insert inside the pET 151 plasmid PCR carried out using the T7 forward primer of the plasmid 5´-TAATACGACTCACTATAGGG-3´supplied with the pET151/D-TOPO cloning kit and the R2 primer inside the insert 5`GAGTTGCTCTTAGACGTTTT 3`. Yielding a PCR product of about 1200bp which is the expected size to be flanked by these 2 primers with the following cycling conditions 95°C for 5 min followed by 40 cycle s of 95°C for 30 s, 47.5°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 then another PCR carried out with the F1 primer of the insert 5` CACCATGTCTACCTTCAAGCTTAT 3` with the T7 reverse of the vector 5´-TAGTTATTGCTCAGCGGTGG-3´ Yielding a product of about 1285 bp which is the expected size at the following cycling conditions 95°C for 5 min followed by 40 cycle s of 95°C for 30 s, 52°C for 30 s and 7 2°C for 1 min and 20 seconds and a final extension at 72°C for 7 min assures that the insert exist in the correct orientation, then to get sure of that the insert is in frame inside the insert the PCR product harboring the T7 forward primers and the R2 primer has sent for sequencing by GATC Company, Germany by using ABI 3730xl DNA sequencer which combines the traditional Sanger technology with the new 454 technology,

9- The cloned plasmid extracted again using the alkaline lysis protocol according to Sambrook and russel and the extracted plasmid electrophoresed to get sure of the correct size of the cloned plasmid in comparison to the original plasmid size and pET 151/D/LacZ as a control plasmid and pMAT plasmid purchased from life technologies that harboring the synthesized Glycoprotein D of the Kentucky D reference strain compared with super coiled DNA ladder life technologies cat.No.15622-012.

10-By confirming accurate plasmid size pET151 harboring the gene of interest, the extracted plasmid used in transforming the BL21 Ecoli for expressing the cloned glycoprotein D gene of EHV1 of the local isolate following the manufacturer instructions and the recombinant plasmids were grown in 10 ml L.B Media supplemented with Ampicillin at 37 8C with shaking until A550 was(0.5-0.8) The cultures were then induced with 1 mM IPTG (Boehringer Ingelheim) for up to 18 h at 24 at 37 C with vigorous shaking. Aliquots of the induced culture were harvested by centrifugation at various time points for every hour for 4-6 hours and tested for expression and stored at -80 C to be tested by 9-

9-Western blot analysis (Wellington et al., 1996) by using Bio-Rad's 0.45 µm pore–size nitrocellulose membrane and reacted with Anti-HisG-HRP Antibody monoclonal antibodies (Novex) and the pooled serum samples from the mares were the local isolate has been isolated and characterized from El Zahraa Stud for Arabian horses.

The insoluble fraction was prepared by resuspending cells after Thawing the samples (from Pilot Expression and resuspend each cell pellet in 80 µl of 1X SDS-PAGE sample buffer (0.5 M Tris-HCl, pH 6.8 1.25 ml Glycerol (100%) 1.0 ml β mercaptoethanol 0.2 ml Bromophenol Blue 0.01 g SDS 0.2 g

then bring the volume to 10 ml with sterile water) then Boiled 5 minutes and centrifuged briefly then electrophoresed.
11-The soluble fraction was prepared by resuspending cells after in 500 µl of Lysis Buffer (50 mM potassium phosphate, pH 7.8,400 mM NaCl,100 mM KCl,10% glycerol,0.5% Triton X-100,10 mM imidazole) then repeated Freezing and thawing cycles for three times at 42C°, sonication and addition of lysozyme solution carried out for further efficiency of lysis process ,then Centrifuge samples at maximum speed in a microcentrifuge for 1 minute at +4°C to pellet insoluble proteins. then the supernatant Transferred to a fresh tube and stored on ice, then Mix together equivalent amounts of supernatant and 2X SDS-PAGE sample buffer (0.5 M Tris-HCl, pH 6.8 2.5 ml, Glycerol (100%) 2.0 mlβ-mercaptoethanol 0.4 ml, Bromophenol Blue 0.02 g, SDS 0.4 g) and boil for 5 minutes then blotted on Bio-Rad's 0.45 µm pore–size nitrocellulose membrane.

## RESULTS

Different trials have been carried out on samples delivered by African horse sickness department Veterinary serum and vaccine research institute to assure the existence of the viral genomic DNA in these samples or not, either these samples were infected cell monolayers or pock lesion samples of Chorioallantoic membrane of the inoculated Embryonated chicken Eggs according to the data supplied by the African horse sickness department ,2 primer sets have been used to detect the viral DNA the primers were designed either to flank the whole coding sequence of the glycoprotein D of EHV1 spanning about 1209 bp while the other pairs were used to flank an area of about 1000 bp fragment starting from the initiation codon ATG, but the results were negative for both primers, so appositive control should be used to verify that these primers are working effiently or not to assure either the tested samples have the EHV1 DNA or not, so a control plasmid harboring the glycoprotein D of EHV1 of the Kentucky Strain as reference strain to use it as appositive control for the envelope glycoprotein D, The synthesized Glycoprotein D CDS was assembled from synthetic oligonucleotides and /or PCR products. The fragment was cloned into pMAT-T which is Ecoli adapted plasmid using Sfil and Sfil cloning sites and The final construct was verified by sequencing.

1-synthesized Glycoprotein D of EHV1 (3589bp) from the transformed Top 10 Ecoli while Lane 2 is the negative non-transformed Top10 Ecoli assuring the correct size of the control plasmid harboring the gene of interest as shown in Figure (1).


**Fig (1)** First lane is100bp DNA ladder, Lane 1 is the extracted control pMAT plasmid carrying the whole length coding sequence.

2-PCR carried out on the control plasmid to assure the efficacy of the primes flanking the whole coding sequence of

the glycoprotein D cloned in the pMAT control plasmid, giving bands at the expected size 1209 bp which is the size of the coding sequence of the glycoprotein D of Equine herpes virus-1 as shown in figure (2).

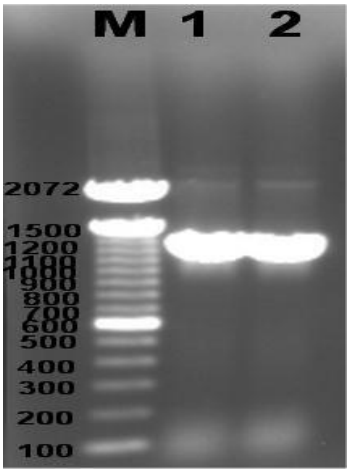


**Fig (2)** PCR products carried on the control pMAT plasmid flanking the whole length glycoprotein D of EVH1 where the first Lane Is 100 bp Ladder and the lanes 1&2 the (1209) glycoprotein D of EHV1 at the expected size.

3-PCR carried out on the field samples isolated from El Zahraa Stud for the Arabian horses using another set of primers flanking only 1040 bp starting from the first ATG Methionine codon of the gene till base number 1040 of the coding sequence, the collected samples either were nasal swaps from animals with previous history of repeated abortions, animal showing signs of respiratory manifestation or aborted fetal tissues giving bands at the expected size when compared against the PCR product of the control plasmid using the same set of primers.

4- Sequence analysis of the field isolate using the primers in both directions flanking the whole coding region of the Glycoprotein D of EHV-1 1209 bp showing great conservation along the whole coding region except unique change at the nucleotide 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 unique characteristic change in Glycoprotein D of Equid herpesvirus1isolateEgypt/VSVRI/Zahraa 2014 Accession no.# KM406326. 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 EHV.

5-comparison between the size of the control plasmid pMAT harboring the control synthesized glycoprotein D of the reference Kentucky D Strain at the exact size 3589 compared to the size of the cloned Glycoprotein D of the local strain Egypt_VSVRI_Zahraa_2014 accession number #KM406326 in the PET 151 D topo plasmid 6975 bp which the exact expected size of the cloned plasmid as shown in figure (3)

6- PCR carried out to assure the correct orientation site of the insert inside the cloned pET 151 D topo plasmid using the T7 reverse primer inside the plasmid and the Forward Primers including the CACC overhang and the ATG of coding region of the Glycoprotein D of the local isolate Egypt/VSVRI/Zahraa2014 as shown in figure(4).


**Fig (3)** first Lane showing supercoiled DNA ladder lane1 showing the control pMAT carrying the synthesized control glycoprotein D of EHV1 by gene art technology, Lane 2 showing the cloned pET151/D-TOPO vector harboring the characterized Glycoprotein D of Equid herpesvirus 1 isolate Egypt/VSVRI/ Zahraa2014.

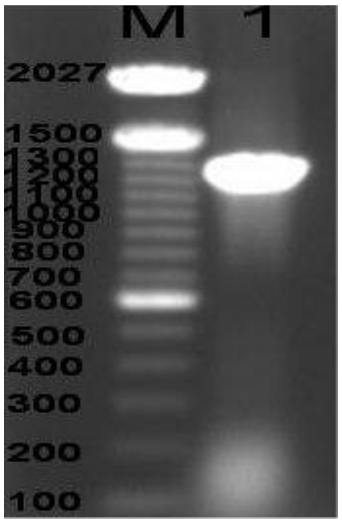


**Fig (4)** PCR product using the forward primer F1-30A of the insert and the T7 reverse primer showing a band at the expected size correct (1285 bp) which assures the correct orientation of the insert to be in frame to assure the expression of the insert downstream the T7 promotor.

7- Schematic figure showing the genetic Map of the insert inside the cloned plasmid showing the annotation of each land mark inside the cloned product as shown in figure (5).

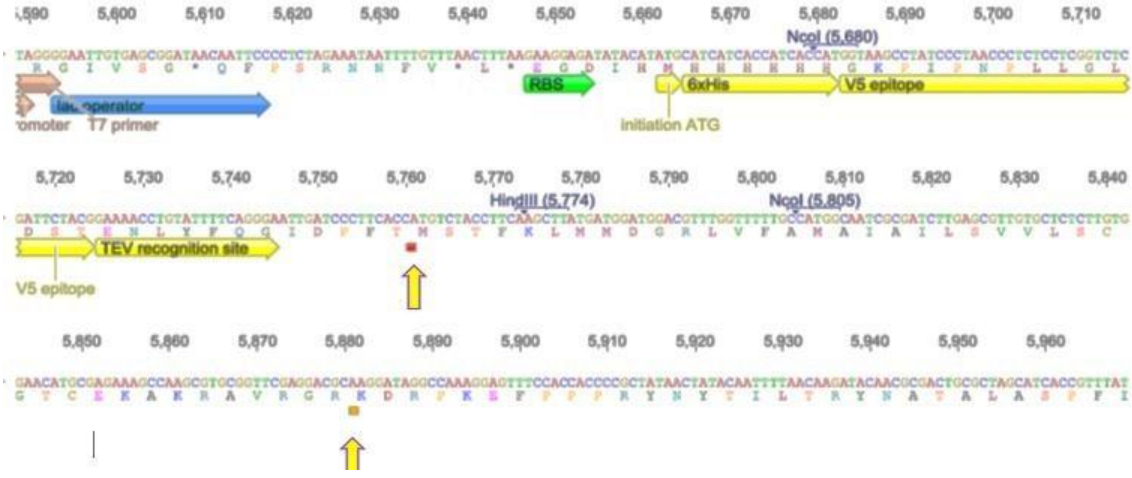


**Fig (5)** schematic representation showing the correct in frame orientation of the N-terminal cloned glycoprotein D of Equid herpesvirus 1 isolate Egypt/VSVRI/Zahraa2014 in pET151/D-TOPO showing the T7 primer, ribosome binding site (**RBS**), the initiation codon (ATG), 6x histidine, V5 epitope and the TEV recognition site, the vertical arrows

showing upstream CACC overhang followed by the beginning of the in frame cloned gene showing the same sequence of the amino acid residues of the PHA03265 conserved domain region of the glycoprotein D of equine herpes virus 1 of the local isolate and the change at the nucleotide 5881 (in the frame) from C to A leading to shift in the amino acid residue from **Q** (glutamine)to **K** (lysine) corresponding to residue number 121 of the glycoprotein D of Equid herpesvirus 1. By Geneious 4.8.3 software

8- Comparison to assure the correct inframe orientation inside the cloned plasmid using the heterologous primers between the glycoprotein D insert of the local strain Egypt/VSVRI/Zahraa2014. And the pET 151 D topo plasmid as shown in figure Giving products at the expected site assuring the correct inframe orientation of the insert inside the plasmid as shown in Figure (6).

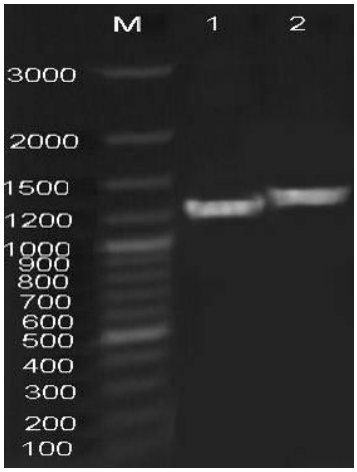


**Fig (6)** first lane is 100bp DNA ladder, Lane1 showing about 1200bp PCR product using the T7 forward primer inside pET151/D-TOPO vector harboring the characterized Glycoprotein D of Equid herpesvirus 1 isolate Egypt/VSVRI/Zahraa2014and the R2 primer inside the clone (1200 bp), Lane 2 showing PCR product about (1285 bp) using F1 primer of the insert with T7 reverse primer inside pET151/D-TOPO vector harboring the characterized Glycoprotein D of Equid herpesvirus 1 isolate Egypt/VSVRI/Zahraa2014.

**9**- Comparison between the control pMAT plasmid harboring the synthesized control glycoprotein D gene of the Kentucky D Strain 3589 bp, the control pET 151/D/LacZ 8832 bp ,and the cloned plasmid pET 151 D topo harboring the glycoprotein D of the local isolate Egypt/VSVRI/Zahraa2014 6975 bp as shown in figure(7).

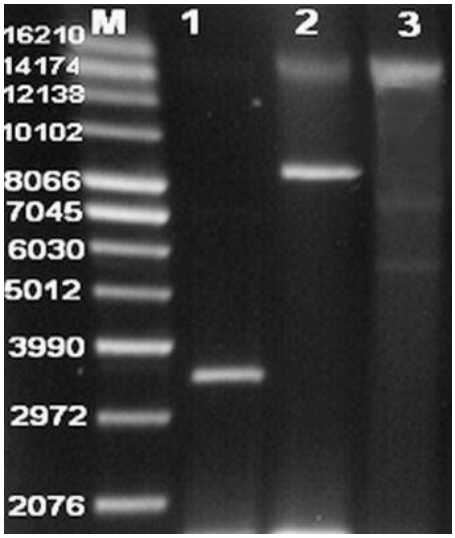


**Fig (7)** Lane 1 the control pMAT plasmid harboring the synthesized control glycoprotein D gene, lane 2 pET 151/D/LacZ, lane 3 cloned pET151/D-TOPO harboring Glycoprotein D of Equid herpesvirus 1 isolate Egypt/VSVRI/Zahraa2014, to show the comparison between the sizes of the control plasmids and the cloned pET151/D-TOPO harboring Glycoprotein D of Equid herpesvirus 1 isolate Egypt/VSVRI/Zahraa2014.

10- western Blot analysis of the produced Glycoprotein D of Equid herpesvirus 1 isolate Egypt/VSVRI/Zahraa2014 as a fusion protein using the N-terminal Anti His monoclonal antibodies detecting the expressed protein bands at the expected size about 48 KD on different expression times from Zero hour till 6 hours after the induction using the isopropyl $\beta$-D-thiogalactoside (IPTG) as shown in Figure (8).

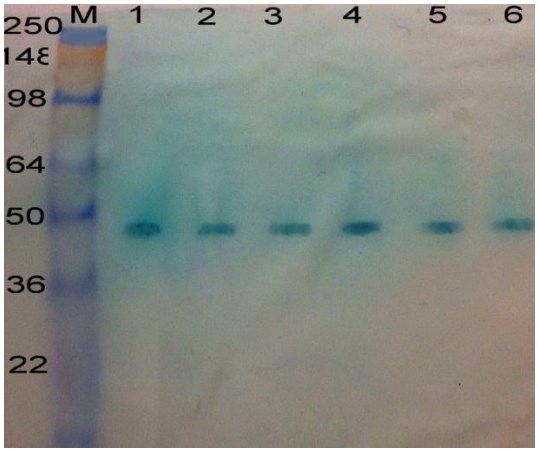


**Fig (8)** Western blot detection of EHV-l gD fusion polypeptides expressed by cloned pET151/D-TOPO vector harboring the characterized Glycoprotein D of Equid herpes virus 1 isolate Egypt/VSVRI/Zahraa2014 Accession No # KM406326 in BL21 Ecoli cells Electrophoresed on an SDS-8% polyacrylamide gel using N-terminal Anti His monoclonal antibodies Samples were taken as a time course study over a 6-h period every hour from first Lane Lanes: 1 to 6 1, 2, 3, 4, 5, and 6$^{th}$ hour. Showing bands at the expected size of the EHV-l gD fusion polypeptides about 48 KDa.

11- showing western blot analysis of the expressed glycoprotein D EHV-l gD fusion polypeptides expressed by cloned pET151/D-TOPO vector harboring the characterized Glycoprotein D of Equid herpes virus 1 isolate Egypt/VSVRI/Zahraa2014 using the rabbit anti-equine labelled horse radish peroxidase against the pooled serum sample collected from the infected cases with either respiratory signs or previous history of abortion using the rabbit anti-equine labeled horse radish peroxidase against the pooled serum sample collected from the infected cases with either respiratory signs or previous history of abortion giving bands at the expected size 48 KD as shown in Figure (18) which assures the biological reactivity of the expressed fusion protein with the serum of the infected horses as shown in figure (9).

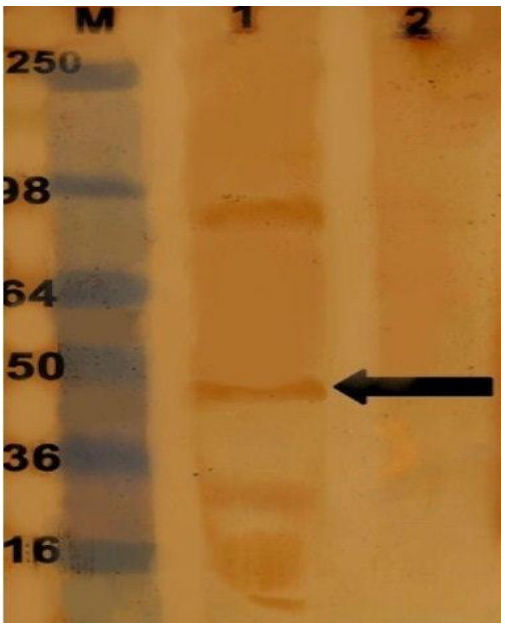


**Fig (9)** showing western blot analysis of the expressed glycoprotein D EHV-l gD fusion polypeptides expressed by cloned pET151/D-TOPO vector harboring the characterized Glycoprotein D of Equid herpes virus 1 isolate Egypt/VSVRI/Zahraa2014 Accession No # KM406326 in BL21 Ecoli cells Electrophoresed on an SDS-8% polyacrylamide gel using the rabbit anti-

equine labelled horse radish peroxidase against the pooled serum sample collected from the infected cases with either respiratory signs or previous history of abortion where the virus has been isolated showing at the expected size of the EHV-l gD fusion polypeptides about 48 KDa lane 1and lane 2 is negative control of non-induced Bl-21 cell .

## DISCUSSION

The current study assures what has been mentioned in The notification that has been received on 15/12/2014 from Prof. Dr Osama Mahmoud Ahmed Selim, Chairman, General Organization for Veterinary Services (GOVS), Ministry of Agriculture and Land Reclamation, Cairo, Egypt to the OIE recording an outbreak of Equine herpes virus1 in El Zahraa Stud for Arabian horses, Cairo, Ain Shams, AL QAHIRAH where the start date of the outbreak was 03/11/2014 and the disease confirmed by 06/11/2014 by Animal Health Research Center (AHRI) (National laboratory) using PCR Total number of animals at the farm: 480 Arabian Horses Total number of affected animals: 4, in contact animals: 60 Affected horses showed clinical signs Mild respiratory signs Fever Loss of appetite ,noting that Animals were vaccinated with autogenous vaccine locally manufactured at Veterinary Serum and Vaccine Research Institute. Note by the OIE World Animal Health Information and Analysis Department: Since this is the first time that infection with Equid herpesvirus-1 (EHV-1) is identified in the history of the country, the reason for notification as first occurrence applies to the country but the event is in fact limited to a zone, the results of this study assured by the OIE immediate notification by General Organization for Veterinary Services (GOVS) as the affected animals were vaccinated by the autogenous vaccine prepared by VSVRI (Veterinary Serum and Vaccine Research Institute and http://www.oie.int/wahis2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEventReport&reportid=16717 the initial trials in this study on the samples from the seed virus from which they prepare the autogenous vaccine and other samples including either infected cell monolayers (Vero cells) or infected chorioallantoic membrane pock lesion assured the absence of the Equine herpes virus 1 (EHV-1) DNA on these sample, so there is may be a problem in the antigen used to prepare autogenous vaccine for vaccinating the herd of El Zahraa Stud for Arabian horses as the outbreak occurs and there are recurrent cases of abortions in the vaccinated herd, all these claims assures that there is something related to the antigen from which the autogenous vaccine was prepared , so the current study designed to characterize one of the circulating isolates of Equid herpesvirus-1 (EHV-1) in the Egyptian field based on the molecular analysis carried out on the Glycoprotein D of Equid herpesvirus 1 isolate Egypt/VSVRI/Zahraa2014 that has been isolated from El Zahraa Stud for Arabian horses and to 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 (14),so the

initial trial to isolate the Equine herpes virus 1 (EHV-1) viral DNA using the primer flanking the whole gene and another set of primers that flank nearly about 1000 bp from the ATG of the gene on the samples delivered by the African Horse Sickness Department, Veterinary Serum and Vaccine Research Institute failed and assures the absence of Equine herpes virus 1 (EHV-1) viral DNA in these samples especially after using these two primers against the control plasmid carrying the whole length glycoprotein D of Equine herpes virus 1 of the Kentucky D reference strain Accession number# AB279610.1they gave two bands at the expected size for each one(1000bp and 1208 bp) assuring that the selected primers was efficient and competent to flank the products at the expected size when propped against the control plasmid harboring the synthesized gene, then these primers were tested against 9 samples purchased from El-Zahraa Stud for Arabian horses 8 of them were nasal swaps and one aborted fetal tissues 7 out of 9 samples was positive for EHV1 glycoprotein D including that one of the aborted fetal tissues , two nasal swaps samples were negative, then the virus has been isolated on chorioallantoic membrane of Embryonated Chicken Egg 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.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, where the infected BHK monolayer cell lysate (one sample) has no EHV1 viral DNA while the infected pock lesion (two samples) was positive using the two primer sets, assuring that Vero cell as monolayer cell culture may be not the relevant host for propagating the Ehv1 (Allen et al., 1983), then the viral DNA of the local isolate has been sent for sequence analysis by GATC Company, Germany by using ABI 3730xl DNA sequencer for characterizing the local isolate and to detect the similarity and differences between the local isolate and the reference strains and isolates all over the world, the glycoprotein D gene of the local isolate Egypt/VSVRI/Zahraa2014 Accession No # KM406326 revealed unique 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 this change is a unique change and considered very characteristic for the Egyptian isolate, the surface probability plot of the characterized the glycoprotein D gene of the local isolate Egypt/VSVRI/Zahraa2014 using protean analysis software revealed unique change in the shape of the peak especially at the site of the amino acid substitution at residue number 41 which is very characteristic for the local isolate than the rest of isolates. The antigenic index revealed no significant change in the shape of the plot between the local isolate and the rest of strains , while phylogenetic analysis based on the deduced amino acid sequence revealed

that the local isolate is closely related to the (Kentucky D reference strain,Ab4 strain,V592 strain and RacL11 strain) accession numbers (AB279610.1, AY665713.1, AY464052.1 and AB279607) respectively, then the characterized Glycoprotein D of Equid herpesvirus1 of the local isolate has cloned into Cloned into pET151/D-TOPO in frame with the N-terminal peptide containing the V5 epitope and the 6xHis tag and maintained in the Top10 Ecoli for maintenance and propagation of the clone, then the size cloned plasmid pET151/D-TOPO harboring Glycoprotein D of Equid herpesvirus 1 isolate Egypt/VSVRI/Zahraa2014 verified after cloning (6975 bp)in comparison to the synthetic control pMA-T plasmid harboring Glycoprotein D of Equid herpesvirus1of Kentucky D reference strain(3589 bp) and the expression control plasmid The pET151/D/lacZ (8832 bp) vector contains a lacZ gene that has been directionally TOPO Cloned into pET151/D-TOPO in frame with the N-terminal peptide containing the V5 epitope and the 6xHis tag each plasmid gave the correct size when electrophoresed in comparison to the supercoiled DNA ladder, then the correct orientation of the gene has been verified by either PCR using T7 forward primer inside the pET151/D-TOPO plasmid 5´-TAA TAC GAC TCA CTA TAG GG-3´ R2 primer inside my insert 5`GAG TTG CTC TTA GAC GTT TT 3`. Yielding a PCR product of about 1200bp and then another PCR carried out with the F1 primer of the insert 5` CAC CAT GTC TAC CTT CAA GCT TAT 3` with the T7 reverse of the vector 5´-TAG TTA TTG CTC AGC GGT GG-3´ Yielding a product of about 1285 bp which is the expected size to be flanked by these primer pairs which assures that the insert exist in the correct orientation and the assured by sequence analysis which revealed that the inserted glycoprotein D gene of the local isolate Egypt/VSVRI/Zahraa2014 was in the right Frame ,then transferred and to BL21 Ecoli for expressing the Glycoprotein D of Equid herpesvirus1 Egypt/VSVRI/Zahraa2014 to obtain a fusion protein candidate to be used as a diagnostic antigen to detect the circulating antibodies against Equine herpes virus 1 (EHV-1) using immunoblotting technique, The virus respiratory model shown elevated rates of viral clearance when using the GSTgD N-terminal fusion protein expressed in E. coli and associated with a good antibody and T-cell responses(Zhang et al., 2000), the produced antigen biological activity has been tested either using the anti-histidine (Anti-HisG) alkaline phosphatase labelled monoclonal Antibodies to detect Detects the N-terminal polyhistidine (6xHis) tag, and gave a band about 48 KD including the fusion protein tag at the expected size, and tested also against polyclonal antibodies raised in the serum collected from the previously infected animals and the specific reaction against the band of interest has been resolved using labelled anti equine IgG labeled horse radish peroxidase giving a band at the expected size (48 KD).it has been approved by western blotting that utilizes antisera against synthetic linear 19 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)

In 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 propagate the virus either on RK-13 cell or cells of equine origin like equine dermal cell will be more beneficial than using Vero cells for propagating the Equine herpes virus-1 as Vero cells is not a recommended cell line for propagating that virus as they do in VSVRI as Vero cell monolayer approved to be virtually resistant to EHV-1 but may permissive for EHV-4 (Frampton et al., 2005) which may have negative impact on the produced autogenous vaccine used for vaccinating the herd in El Zahraa Stud for Arabian horses, it is recorded that serial passage of the virus in hamsters or in cells of non-equine origin quickly gave rise to alterations in the viral DNA (GEORGE P. ALLEN et al) (Allen et al., 1983)., Equid (equine) herpesvirus (EHV) can be isolated by culture in susceptible cells of equine origin. Susceptible cell lines are primary equine foetal kidney (EFK) or equine fibroblasts ((equine dermal cells (EDC) or lung)). EHV can be isolated on RK-13, BHK-21, MDBK, PK-15 and SEK but the sensitivity is lower than for equine-derived cell lines (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.05.09_EQUINE_RHINO.pdf),at the same time it is very recommended to make very close in quality control on the vaccine produced in VSVRI to detect the viral DNA of Equine herpes virus-1 in each step of vaccine production to assure the presence of the antigen in sufficient titer before the process of inactivation, and to assess the titer of the produced antigen after inactivation process by serological techniques like ELISA and SNT using standardized either monoclonal or polyclonal antibodies supplied by reference laboratories and not to use the prepared hyper immune serum produced by their own in VSVRI as it may lead to false positive and misleading results that reflects negatively on the produced vaccine.

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