

Full Length Research Paper

# Cloning and phylogenetic analysis of *gD* gene of human herpes simplex virus type 2 in Iranian isolates

Hassan Momtaz<sup>1</sup>, Payam Ghasemi Dehkordi<sup>2\*</sup>, Abbas Ali Rezaeian<sup>2</sup>, Ramin Yaghobi<sup>3</sup>,  
Mohammad Kargar<sup>2</sup> and Manochehr Momeni<sup>4</sup>

<sup>1</sup>Department of Microbiology, Faculty of Veterinary Medicine, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran.

<sup>2</sup>Department of Microbiology, Islamic Azad University, Jahrom Branch, Jahrom, Iran.

<sup>3</sup>Shiraz Transplant Research Center, Namazee Hospital, University of Medical Science, Shiraz-Iran.

<sup>4</sup>Biotechnology Research Center, Islamic Azad University, Shahrekord Branch, Shahrekord-Iran.

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Herpes simplex virus type 2 (HSV-2) is a member of herpesviridae family and subfamily of alphaherpesvirinae. HSV-2 infection often causes genital herpes in women and men, abortion, infant herpes and non-infectious meningitides. Glycoprotein D and B, respectively, are coded by *gD* and *gB* gene. They are the adhesion of HSV-2 attached to the surface of the epithelial cells and are used for making vaccine. To determine the phylogenetic analysis of HSV-2 *gD* gene and cloning of it in *E. coli*, infected samples from Esfahan and Chaharmahal Va Bakhtiari provinces in Iran, which were multiplied by PCR and then the segments having 1013bp lengths related to 3 infected samples for *gD* gene were selected in cloning pCR 4-TOPO plasmid and sequencing. After determining the nucleotide sequences of HSV-2 *gD* gene, they were compared with samples reported in Iran and other countries. The results showed 2.8 - 10% genetic differentiation which enjoyed more affinity with nucleotide sequenced in USA (K02373) with 97.2% and the biggest difference is in Sweden sample (EU018093) with 90%. The current research showed high homology in the sequence of *gD* gene of HSV-2. Then, with presence of these affinities in the research samples and other species of Iran and other countries, a good vaccine can be made with high efficiency against all HSV-2 species in the world.

**Key words:** Herpes simplex virus type 2 (HSV-2), PCR, *gD* gene, phylogenetic analysis, Iran, Chaharmahal Va Bakhtiari province, Esfahan province.

## INTRODUCTION

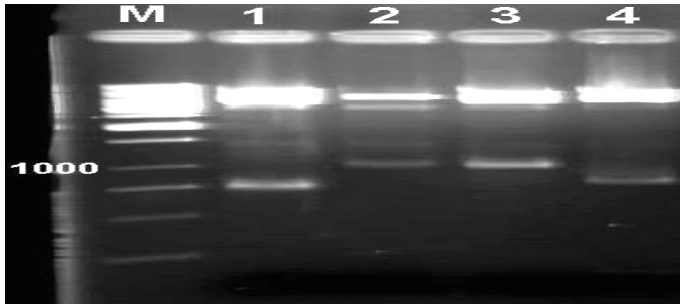
Genital herpes was first determined by John Astruc in 1736. Then, more information was achieved by the research step about these lesions, kinds of genital and labials herpes; until finally in 1925, Herpes simplex virus. Cause of these lesions was cultivated and separated in the laboratory. Herpes simplex virus type 2 (HSV-2) is a member of herpesviridae family and subfamily of alphaherpesvirinae. Herpes simplex virus often causes the genital herpes and nearly spreads via sexual

intercourse, while Herpes simplex virus type 1 (HSV-1) causes labial herpes and is distributed by kiss in human. HSV-2 genome consists of a large linear double strand DNA with 125 - 229 kbp length (Chan, 2007; Fotouhi et al., 2005; Knipe et al., 2007; Lowhagen et al., 2002; Murata et al., 2002; Nishiyama, 1996).

Primary infection in women usually involves the vulva, vagina and cervix. In men, initial infection is most often associated with lesions on the glans, penis and prepuce or penis shaft. In individuals of either sex, primary disease is associated with fever, malaise, anorexia and bilateral inguinal adenopathy. Women frequently have dysuria and urinary retention due to urethral involvement. Neonatal Herpes simplex virus infection is transferred from mother to infant in birth and causes skin, eye and mouth diseases, central neurons system lesions and other organs, limitation in infant growth and death in

\*Corresponding author. E-mail: [payamgator62@yahoo.com](mailto:payamgator62@yahoo.com).  
Tel: +983812222260. Fax: +983812222260.

**Abbreviations:** HSV-2, Herpes simplex virus type 2; *gD*, Glycoprotein D; PCR, Polymerase chain reaction.



**Figure 1.** Restriction enzyme analysis of recombinant pCR 4-TOPO (Line M: Molecular weight marker 1kb DNA ladder; Line 1 - 4: digestion of plasmid with *EcoRI* enzyme).

infancy (Carter et al., 2007; Fotouhi et al., 2005; Knipe et al., 2007; Qutub et al., 2001).

Herpes simplex virus, approximately coded 11 glycoproteins in which glycoprotein D (*gD*) is one of the most important adhesions of virus to attach and import to the surface of the host epithelial cells and is essential for replication of the virus in cell culture. Mutant with deletions in *gD* gene of virus is not ably invasive and transmitted to the host cells and neutralize with antibodies against glycoprotein D to prevent the virus from entering into the cells. Both glycoprotein D and B (*gB*), are two important factors for making a vaccine against the virus (Carter et al., 2007; Knipe et al., 2007; Regge et al., 2006).

The aim of this study was to determine the sequence of *gD* gene of HSV-2 samples which were collected from Esfahan and Chaharmahal Va Bakhtiari provinces in west south of Iran and then compared with nucleotide sequences registered for this gene in NCBI, in order to make a good vaccine against this virus in the near future and colonization of *gD* gene of the virus in bacterial cell.

## MATERIALS AND METHODS

### Sampling

After agreement with private and governmental clinical and pathological laboratories and clinical centers in both Esfahan and Chaharmahal Va Bakhtiari provinces in west south of Iran, 100 serum samples were collected from the suspected patients for Herpes simplex virus, with high IgG and IgM (IgG > 12 and IgM > 1.1). With the patients permission (82 samples from Esfahan province and 18 samples from Chaharmahal Va Bakhtiari province), the samples were then transferred to the biotechnology laboratory of Shahrekord Islamic Azad university of Iran situated in the ice. Included for each sample was information such as age, sex, previous abortion in women, previous diseases such as HIV and history of genital ulcers and till the end of the experiment, samples were preserved in -20°C.

### DNA extraction

For DNA extraction from samples, viral DNA isolation kit from

serum and DNA made by Rima company were used based on the conformity instruction of the company.

### Primers design

Primers were designed according to the published sequence for *gD* gene of HSV-2 (accession number: EU445527). The forward primer for *gD* was F:5'- AAATACGCCTTAGCAGACC-3' and the reverse primer was R:5' ACCCAAACGCAATACC-3'.

### Gene amplification of *gD* (encoding the glycoprotein D of HSV-2)

PCR was performed in a 50 µl total volume containing 1 µg of template DNA, 1 µM of each primer, 2 mM MgCl<sub>2</sub>, 200 µM dNTP, 5 µM of 10X PCR buffer and 1 unit of Taq DNA polymerase (Roche applied science). The following conditions were used for amplification: Initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72° for 1 min. The program was followed by a final extension at 72°C for 6 min. The PCR product was analyzed by electrophoresis in 1% agarose gel in 1X TBE buffer and visualized by ethidium bromide staining on UV transilluminator. The PCR product was purified by high pure PCR product purification kit (Roche applied science) according to the manufacturer's recommendation.

### Cloning of *gD* gene

The PCR product's single from positive samples was cloned in *E. coli* using the pCR<sup>®</sup> 4-TOPO<sup>®</sup> (TOPO TA Cloning<sup>®</sup> kit for sequencing Invitrogen) at 37°C over night. The transformed bacteria were selected by screening the colonies on LB media containing kanamycin. To determine the replicated gene in cloning plasmid, two methods were used. These methods were endonuclease digestion and sequencing.

### Sequence analysis

The nucleotide sequences were edited using Edit View v.1.0.1 (Applied Bioscience, Australia) and the 10 sequences registered in Genbank (accession numbers: EU018093.1, EU018125.1, EU445527.1, EU029158.1, AF021342.1, AY779754.1, AY779750.1, AY517492.1, K01408.1 and K02373.1) were aligned separately using the ClustalX v1.81 in order to obtain a consensus sequence. Subsequently, the sequences were analyzed using the BioEdit package v.7.0.4.1 to compare the nucleotide sequences.

The nucleotide sequence of the HSV-2 *gD* gene was compared with the corresponding sequences from other regions of Iran and other countries in the world. An unrooted dendrogramme was constructed using the Njplot software and statistical support for it was obtained by bootstrapping, using 1000 replicates.

## RESULTS

The purified PCR product (1013bp fragment) was cloned in pCR<sup>®</sup> 4-TOPO<sup>®</sup> (TOPO TA Cloning<sup>®</sup> kit for sequencing Invitrogen) vector and digestion with *EcoRI* enzyme. Figure 1 shows recombinant plasmids after digestion. The recombinant plasmid was sequenced by specific primers and Sanger sequencing method (Macrogen, Korea). The

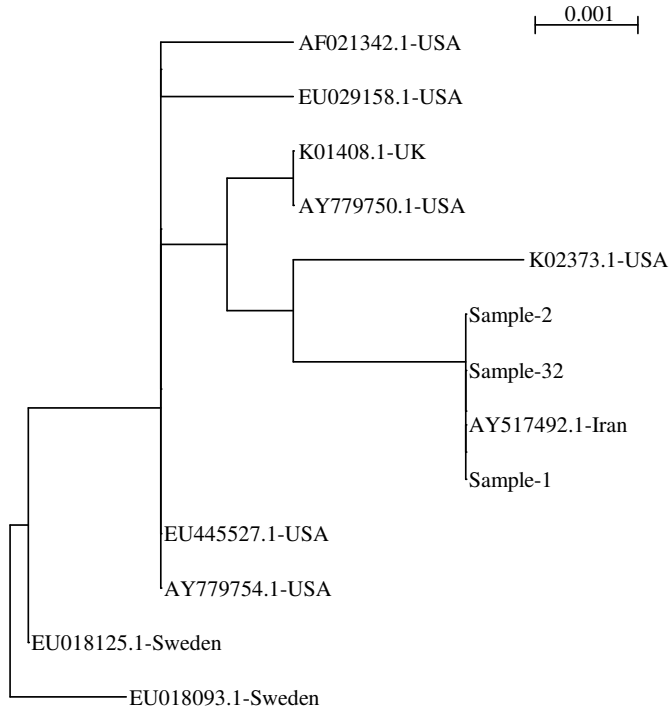
**Table 1.** The results of comparing the sequence of the HSV-2 *gD* gene in Iran (Esfahan and Chaharmahal Va Bakhtiari provinces) with other countries (Sequence Identity Matrix).

Seq- ^	EU018093.1-Sweden	EU018125.1-Sweden	EU445527.1-USA	EU029158.1-USA	AF021342.1-USA	AY779754.1-USA	Sample-1	AY779750.1-USA	AY517492.1-Iran	Sample-32	K01408. 1-UK	K02373. 1-USA	Sample-2
EU018093.1-Sweden	ID	0.997	0.996	0.997	0.957	0.994	0.91	0.992	0.906	0.901	0.899	0.858	<b>0.9</b>
EU018125.1-Sweden	0.997	ID	0.997	0.996	0.958	0.993	0.908	0.991	0.91	0.902	0.899	0.858	0.901
EU445527.1-USA	0.996	0.997	ID	0.997	0.959	0.994	0.911	0.992	0.969	0.905	0.899	0.858	0.923
EU029158.1-USA	0.997	0.996	0.997	ID	0.958	0.995	0.912	0.993	0.97	0.907	0.9	0.859	0.924
AF021342.1-USA	0.957	0.958	0.959	0.958	ID	0.955	0.914	0.955	0.963	0.916	0.896	0.857	0.92
AY779754.1-USA	0.994	0.993	0.994	0.995	0.955	ID	0.908	0.994	0.971	0.918	0.903	0.861	0.925
Sample-1	0.91	0.908	0.911	0.912	0.914	0.908	ID	0.915	0.917	0.922	0.905	<b>0.972</b>	0.918
AY779750.1-USA	0.992	0.991	0.992	0.993	0.955	0.994	0.915	ID	0.969	0.925	0.904	0.862	0.923
AY517492.1-Iran	0.906	0.91	0.969	0.97	0.963	0.971	0.917	0.969	ID	0.931	0.921	0.882	0.914
Sample-32	0.901	0.902	0.905	0.906	0.916	0.918	0.922	0.925	0.931	ID	0.932	0.969	0.936
K01408. 1-UK	0.899	0.899	0.899	0.9	0.896	0.903	0.905	0.904	0.921	0.932	ID	0.936	0.92
K02373. 1-USA	0.858	0.858	0.858	0.859	0.857	0.861	0.972	0.862	0.882	0.969	0.936	ID	0.971
Sample-2	<b>0.9</b>	0.901	0.923	0.924	0.92	0.925	0.918	0.923	0.914	<b>0.936</b>	0.92	0.917	ID

sequencing result was confirmed by comparing it with databases and using basic local alignment search tool (BLAST) software. The result of sequencing of the *gD* fragment using ClustalX software was aligned with some of the registered sequences in Genbank such as the gene in USA, Sweden, UK and other regions of Iran.

After comparing the differences and similarities applying Njplot software, the phylogenetic tree was drawn which is shown in Figure 2 and the diagram is demonstrated in Table 1.

The results showed 2.8 - 10% genetic differentiation that enjoyed more affinity with nucleotide sequenced in



**Figure 2.** Phylogenetic tree of the sequence of the HSV-2 *gD* gene in Iran and in other five countries.

USA (K02373) with 97.2%, the biggest difference is in Sweden sample (EU018093) with 90% and more affinity through the research samples with 93.6 were indicated. Sample-1 is related to Chaharmahal Va Bakhtiari province and Samples-2 and 3 are related to Esfahan province.

**DISCUSSION**

Infection with Herpes simplex virus type 2 with absence of a good vaccine has caused many problems in the world. This virus causes infection in epithelial and mucosal cells, genital herpes, eye infection, abortion in women and non-infectious meningitides. Researches based on methods such as using a plasmid contained DNA that coded a HSV glycoprotein, showed an increase in the immunodeficiency in animal models (Fotouhi et al. 2005; Rekabdar et al. 2002; Zago et al. 2004).

HSV-2 approximately coded 11 glycoprotein that was necessary for primary attachment of the virus to the surface of the host cells ligand such as heparan sulfate. Others such as glycoprotein C (*gC*) and B (*gB*) via interaction with D (*gD*) are useful in attaching virus to the receptor of the mannose-6-phosphate. *gD* and *gB* and *gI* and *gL* are alone or when in combination are effective for fusion of the virus's envelope to host plasma membrane in suitable pH (Fotouhi et al., 2005; Gorander et al., 2003; Rekabdar et al., 2002; Zago et al., 2004). *gD* was necessary for entering the virus into the mammalian

cells. Also, it infects and increases their infection and creates a nerve reply. The weight of the glycoprotein D of Human herpes simplex virus type 2 was approximately 43 KD and consisted of 393 amino acids, and its high amount was effective for protecting and creating an anti-genetic virtue of HSV-1 and 2. The research showed low genetic rearrangement in *gD* and *gB* of HSV and after PCR and sequencing, most of the point mutations in this gene were silent. These characteristics due to *gD* were a suitable factor for making a vaccine (Fotouhi et al., 2005; Rekabdar et al., 2002; Smith et al. 2008; Zago et al. 2004).

This current research, for the first time, was performed for cloning and phylogenetic analysis of the *gD* gene sequence of Human herpes simplex virus type 2 in south west of Iran. Anyway, one of the main goals of this research was tracing the coded gene of *gD* protein of Human herpes simplex virus type 2 in the infected samples for the first time in Iran. Then, the presence of corresponded gene was determined by using a sequence of the fragments that multiplied. The length of the *gD* fragment that multiplied was 1013bp, and 3 samples which contain this gene were sequenced. The second goal of this study was cloning of the mentioned gene in each of the cloning pCR<sup>®</sup> 4-TOPO<sup>®</sup> (TOPO TA Cloning<sup>®</sup> kit for sequencing Invitrogen). For sequencing of *gD* gene and comparing its genetic diversity in the Iranian isolates with other available viruses in the world, a comparison of them was done with the known sequences of this gene in Genbank of NCBI.

The phylogenetic tree of compared sequences was drawn using ClustalX and Njplot softwares. As it is shown in Figure 2, the research samples and other Iranian isolates were placed in the same branch and samples of the USA which enjoyed more affinity with the researchers' own were placed in a near branch next to the Iranian isolates and the biggest difference was with the Sweden samples. The current research showed high homology in sequence of *gD* gene of HSV-2. Then, with presence of these affinities in the research samples and other species of Iran and other countries, a good vaccine can be made with high efficiency against all HSV-2 species in the world.

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