DETECTION OF EQUINE HERPESVIRUS-1 AND EQUINE HERPESVIRUS-4 IN MULES AND DONKEYS BY REAL TIME PCR

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ABSTRACT

The equine herpesviruses (*EHVs*) are important pathogens in all members of *Equidae* family world wide. Because of the causative agents of several diseases and latency makes major challenges to equine industry. In the present study blood samples of 108 mules and donkeys from different rural areas of North-East of Iran, as a region with most equine population, were examined for presence of *Equine herpesvirus -1* and *4* using real time PCR as a rapid molecular diagnostic test that has high sensitivity and specificity. Based on results, it was conclude that *EHV-1* DNA was not detected in any blood samples whereas *EHV-4* was present in 14.8% of donkeys and mules. Which can have important role as a source of infection for other equids population.

Key words: *Equine herpesvirus*-1 and 4, donkeys and mules, respiratory disease, real time PCR, Iran

Introduction

Equine herpes virus are DNA virus with linear double-strand and belong to Herpesviridae family. The two most common types major importance are Equine herpes virus type 1 (EHV-1), which causes abortion, respiratory disease and neurologic disease; and Equine herpes virus type 4 (EHV-4), which causes only respiratory disease but also occasionally causes abortion. EHV-1 and EHV-4 are the members of varicello virus genus in Alpha herpes virinae subfamily and are endemic in equine populations worldwide (Reed and Toribio, 2004; Fortier *et al*., 2010; MacLachlan and Dubovi, 2011). These viruses have 145 to 150 kb genomic DNA and glycoproteins that are important for cell attachment, entry and cell-to-cell spreading and induction of immune responses (Reed and Toribio, 2004; Azab *et al*., 2010). The common characteristics between all herpes virus infections are latency and persistent infection with periodic or continuous shedding (Sellon, 2007; Ataseven *et al*., 2009; MacLachlan and Dubovi, 2011)

EHV-1 and EHV-4 can establish latent infections in trigeminal ganglia and T-lymphocytes. Latently infected animals are the major reservoirs of disease and frequent shedding of the virus from these asymptomatic carriers cause spread of virus to susceptible population (Slater *et al.,* 1994; Chesters *et al.,* 1997; Pusterla *et al.,* 2009; Fortier *et al.,* 2010)

Epidemiological studies, suggest that the infection occurs during the few early weeks or months after birth occurs, generally before or after weaning, from adult mares that asymptomatically shed virus (Nicola Pusterla *et al.,* 2005; Sellon, 2007).

Techniques that are used for the diagnosis of EHV infection include: virus isolation as “gold standard”, serological tests and nucleic acid detection techniques (PCR). Several PCR-based methods have been developed for detection and identification of EHV-1 and EHV-4 DNA in aborted foetuses or nasal swabs (Sellon, 2007).

qPCR technology offers a flexible and rapid method as compared to virus isolation. This is sensitive, specific and quantitative method which provides a very useful diagnostic tool for infectious diseases studies, so also a valuable aid for screening large numbers of samples (Diallo *et al.,* 2006; Perkins *et al.,* 2008; Dzieciatkowski *et al.,* 2009; Hoffmann *et al.,* 2009). The main target of this study is to survey circulation of virus and possible source of infection in equine population in North-East district of Iran that has the highest equine population with high prevalence of EHV-4.

Materials and Methods

A total 108 blood samples from donkeys and mules in different rural areas in North-East of Iran were collected during 2011 to 2012 and examined in this study. These regions are having the highest equine population in Iran. From each case, 2 ml of blood was collected from jugular vein by venipuncture into tubes containing EDTA (Vacumed® K3 EDTA. FL medical. Italy).

DNA was extracted from 180 µl of each whole blood sample using a commercial DNA extraction kit (DNA Extraction kit, MBST Inc., Iran) according to manufacturer’s instructions. Quality of extracted DNAs was confirmed by the agarose gel electrophoresis and spectrophotometerical analysis.

Among members of Alpha herpes virinae glycoprotein B is highly conserved and contains very specific sequences that can be used for differentiation between closely related equid herpes viruses (Wagner *et al.,* 1992; Diallo *et al.,* 2007). The conserved and variable regions of glycoprotein B gene of each equine herpesvirus (EHV-1 and EHV-4) were determined by alignment of sequences available in Gene Bank and the type specific primers for EHV-1 and EHV-4 were designed by Primer software, (PREMIER Biosoft International, Inc). Primer sequence specificity was checked BLASTn in the NCBI data base and the sequences have 100% homology to those of EHV-1 and EHV-4 strains deposited in the Gene Bank (http://www.ncbi.nlm.nih.gov/BLAST/).

The primer sets were synthesized by Bioneer (Bioneer Inc. Korea) and Macro Gene Corporation (Macro Gen Inc. South Korea). The resulting amplicons for EHV-1 and EHV-4 were 113bp and 100 bp in long, respectively. Primers are listed in

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Table 1.

To determine the efficiency of nucleic acid extraction and verify the absence of PCR inhibitors in the DNA templates, all samples were tested for the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (an equine housekeeping gene) as an internal control (IC) by specific primers (forward:52 -ATCTGACCTGCCGCCTGGAG -32 , Reverse: 52 -CGATGCCTGCTTCACCACCTTC -32) (Katia Cappelli, 2008; Hoffmann *et al.,* 2009).

Reference virus strains

Purified DNA of EHV-1 strain 89C25 and EHV-4strain TH20p, the Japanese prototype of EHV-4 (Kawakami *et al.,* 1962) (provided by Dr. Matsumura, Japan Racing Association, Tochigi, Japan) were used as controls at real-time PCR assay. The DNAs had been purified from foetal horse kidney cells infected with EHV-1 (89C25p strain) or EHV-4 (TH20p strain) using QIAamp DNA Blood Mini Kit (QIAGEN).

qPCR assay

Samples were tested for the presence of EHV-4 by a SYBR Green-based real-time PCR assay. The amplification carried out in a 25 µl reaction volume containing 12.5 µl of the Fermentas Maxima®SYBR Green/ROX qPCR master mix (2X) (Thermo Fisher Scientific Inc. USA),100 nM of each primer, 2 µl of template DNA (Fig.1). The EHV-1 real time PCR was performed in 20 µl reaction volume containing 4 µl of the Solis Bio DyneHOTFIREPol® Eva Green® qPCR Mix Plus (ROX) (Solis Bio Dyne Inc., Estonia), 5X,100 nM of each primer and 2 µl of template DNA. qPCR was performed in a Bio-Rad CFX 96 qPCR Detection System. Thermal cycler conditions for EHV-1 were : 95°C for 15min, followed by 40 cycles of 10 sec 95°C denaturation, 30 sec 64.1°C annealing, 30 sec 72°C extension and for EHV-4: 95°C for 10min, followed by 40 cycles of 10 sec 95°C denaturation, 30 sec 59.3°C annealing, 30 sec 72°C extension.

All samples were tested in triplicate and sample threshold and baseline values were calculated by the CFX manager software (Bio-Rad) (Fig. 2a and b). All reactions included no-template controls (NTC). EHV-1 and EHV-4 DNAs were used as positive and negative control reciprocally to determine the specificity of the assays. The sensitivity of the qPCR reaction was determined using standard curve that were run for both EHV-1 and EHV-4 assays and amplification efficiency of the gB gene was calculated from the coefficient of determination and the slope value of a standard curve generated on log10 dilutions as described by Hussey *et al*. (2006).

Data processing was based on standard curve method and efficiencies of each reaction was calculated as Efficiency = 10 (-1/Slope) -1[12] that amplification of the log dilution series showed linearity with slope = -3.1 and R2 =0.998 for EHV-4 (Fig. 3).

No fluorescence signal was detected in the tubes that contained negative (no template control) samples. However in the tubes with positive reference template high fluorescence signal was detected, confirming that the assay was highly specific for the detection of EHV-1 and EHV-4 in the samples. The analytical speciûcity of the assay was determined by testing cross-reaction to EHV-1 and EHV-4 reference strains. No cross-reaction was observed with any of the herpesviruses tested.

The species specificity of the PCR products was confirmed by sequencing. The purified PCR products were then sequenced using the respective sequencing primers; sequence of the EHV-4 PCR products was 100% identical to the sequence of EHV-4 strains available in the Gene bank data base (Fig.4).

Results and Discussion

Results indicated that EHV-1 DNA was not present in any samples while EHV-4 DNA in 14.8% of donkeys and mules blood samples was detectable (Fig. 4). The Internal Control (GAPDH) was detected in all samples; therefore, DNA losses did not occur during nucleic acid extraction and DNA polymerase inhibition was not observed during real-time PCR amplification.

On equine in Iran population was in Chaharmahal and Bakhtiyari province which showed the prevalence rate of EHV-1and EHV-4 to be 39.08 and 68.96%, respectively. However, these studies were based on indirect recognition of virus by ELISA (Momtaz and Hematzadeh, 2003). In another serologic investigation 3 EHV 1 and 15 EHV 4 of the 600 sera were found positive. Thus suggested that infection was associated with sex, age and respiratory disease status. EHV l and EHV 4 showed extensive antigenic cross reactivity and serological determinations of the infection caused by either of the two virus types have been difficult (Pusterla *et al*., 2009).

Pervious study in equine population of north east of Iran by real time PCR showed the high prevalence of equine herpes virus-4 (88%) in equine population of this regions (Sarani, 2013).

These types of equids are traditionally used as transport in Iran. Due to traditional housing of horses in nearby of donkeys and mules and dispatch of horses for competition and breeding in North-East of Iran, spread of EHVs between populations is easier resulting into a greater incidence of infection and subsequent latency as compared to other populations. Prevalence of this virus in donkeys and mules has also been reported in nearby countries of Iran (Tekelioglu *et al*., 2005; Ataseven *et al*., 2009). Unlike of serologic investigation in Iran by all samples were negative for EHV-

Table 1: Nucleotide sequence of primers and products used in this study

Virus	Target Gen	Genome Position	Primers sequence 5´-3´ (*Forward, Reverse)*	Amplicon Size (bp)
*EHV-4*gB* (Accession#:M26171.1)	2629 - 2610	2530 - 2549	TACCCCTGGAGGTTTACACG	100
*EHV-1*gB (Accession#:M36298)	1102 - 1084	990 - 1007	ATACTCGCTGAGGATGGA GTGAAGTTTCTCCCAAGGT	113

*Glyco protein B

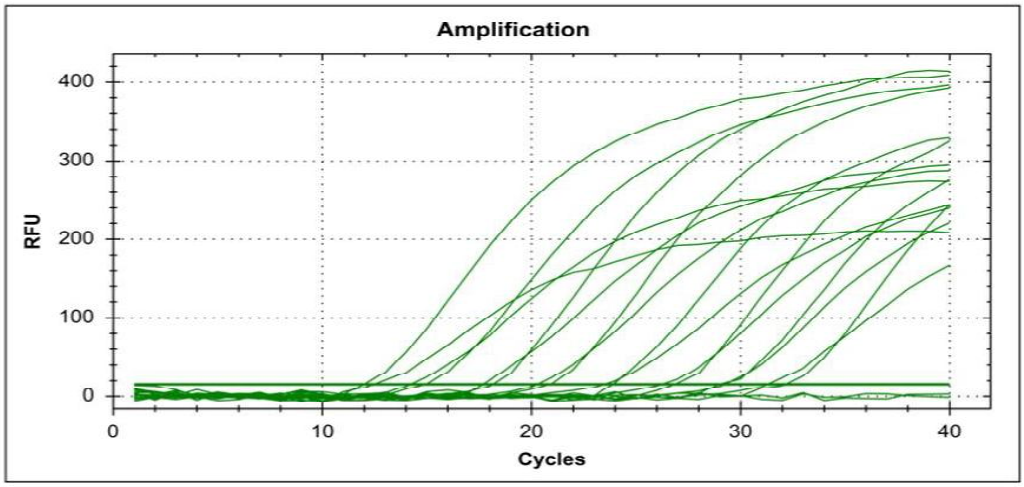

Fig. 1: Amplification plots of EHV-4 (This figures was generated automatically by the CFX manager software (Bio-Rad) version 2.1)

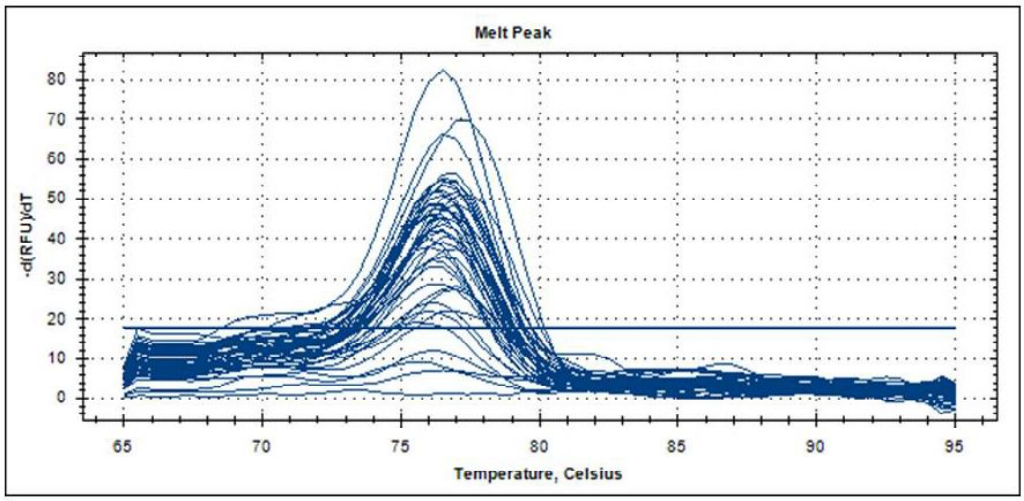

Fig. 2a: Post amplification melting pick analysis

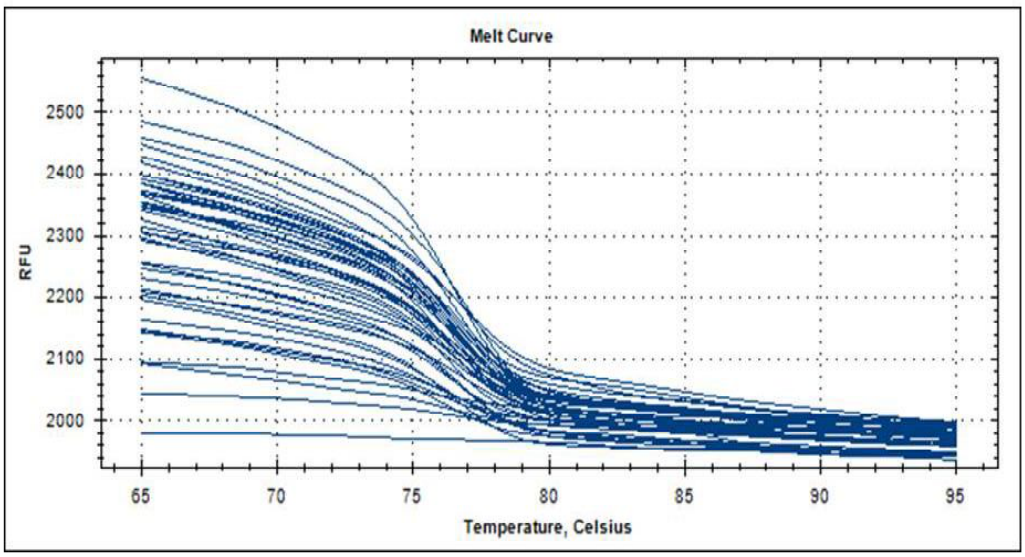

Fig. 2b: Post amplification melting curve analysis

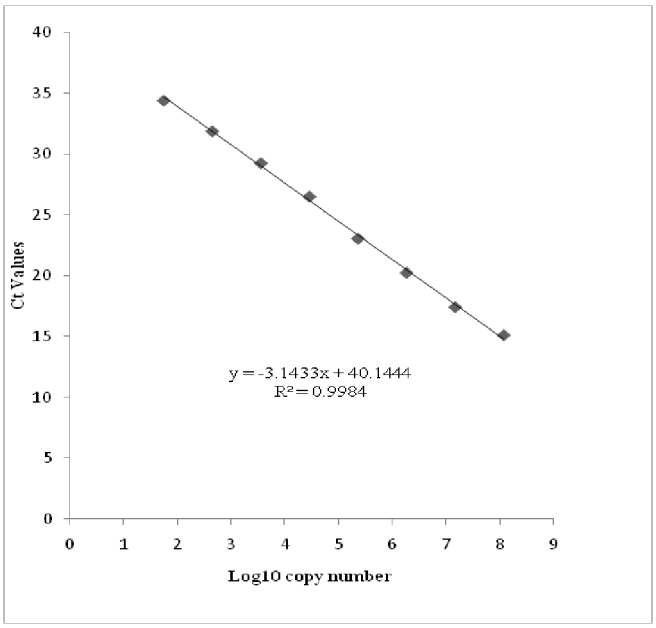

Fig. 3: Standard curve of the EHV-4 real-time PCR assay obtained from triplicates of 10-fold dilutions of standard DNA

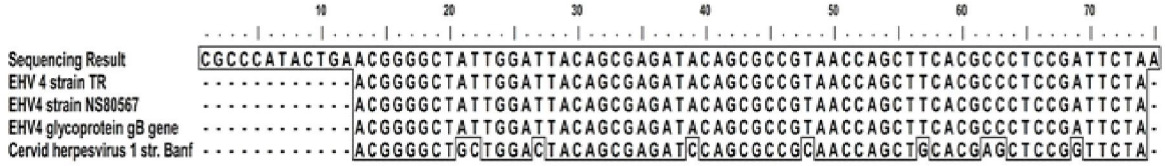

Fig. 4: Sequencing result of real time PCR products

1.The negative results are not due to absence of sensitivity in used method in this study, because the accuracy of our quantitative test was 6 copy numbers of the target gene per reaction. In clinical aspect, symptoms such as neurological signs and history of abortion did not observed in, donkeys and mules of these districts either. This finding is in according to our earlier studies in these regions (Sarani *et al*., 2013). As same as other studies although our test showed presence of EHV-4 in donkeys and mules; greater per cent of these animals did not show any clinical sign; which might be due to establishment of latency in these populations. Moreover, the qPCR can only determine number of target DNA in sample and not infective elements, this value does not show infectious potential of the viral shedding and spread in equine population (Perkins *et al*., 2008).

This study demonstrates that working mules and donkeys represent a likely reservoir of EHV-1 and EHV-4 infection and can have role in epidemic event of syndromes of EHVs, and must be included in hygienic programmes for eradication of disease.

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