D/N752 CODING CHANGE IN DNA POLYMERASE GENE (ORF30) PLAYS NO ROLE IN EQUID HERPESVIRUS TYPE 1 (EHV-1) GROWTH IN VITRO

Rania Abo-Sakaya[1,4], Mohamed H. Ebeid[1], El Sayed M. Galila[1], Mohamed Nayel [2], Samy Kasem[3], Mohamed G. Abdelwahab[1], Abdel-moneim M. Moustafa[1], Faysal K. Arnaout[1], and Hideto Fukushi[4]

[1]Department of Animal Medicine, Faculty of Veterinary Medicine, Banha University. [2]Department of Animal Medicine and Infectious Diseases, Faculty of Veterinary Medicine, Sadat City University
[3]Department of Virology, Faculty of Veterinary Medicine, Kafr Elsheikh University. [4]Department of Applied Veterinary Sciences, United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193

A B S T R A C T

The growth kinetics of neuropathogenic and non-neuropathogenic equine herpesvirus type 1 (EHV-1) strains in fetal horse kidney (FHK) and mouse cerebral cortex cell line were compared. The relevance of the aspartic acid/asparagnic (D/N) amino acid change at position 752 of open reading fram (ORF) 30 was examined in Japanese isolates. Neuropathogenic strains 01c1 exhibited similar growth kinetics to nonneuropathogenic strain 90c16 in FHK and cultured neurons. Open reading fram (ORF30) (DNA polymerase) mRNA expression levels by real-time RT-PCR were the same among the FHK cells infected by both virus -strains. The amino acid encoded at 752 of ORF30 in 01c1 was aspartic acid; while asparagine was encoded in 90c16 strains isolates. The D/N752 difference in ORF30 may not be related to replication ability in FHK and neurons cell line.

KEY WORDS: DNA polymerase, EHV-1, FHK, Neuron, RT-PCR.

(BVMJ-26(2):1-9, 2014)

1. INTRODUCTION

Equine herpesvirus 1 (EHV-1) is a ubiquitous respiratory viral pathogen that causes serious economic losses in the horse industry worldwide (Allen and Bryans, 1986; Bryans and Allen, 1989; Brosnahan and Osterrieder, 2009). EHV-1 exerts its impact by causing respiratory tract disease, but it can also cause abortion, neonatal foal death and nervous system disorders (Patel and Heldens, 2005; Lunn *et al.,* 2009). The mucosa of the upper airway tract is the first line of defence against respiratory diseases (Timoney, 2004). It is also the primary replication site of EHV-1, as it is for most alphaherpesviruses (Kydd *et al.,* 1994a; Van Maanen, 2002; Van Maanen and Cullinane, 2002; Gryspeerdt *et al.,* 2010). Subsequently, the virus disseminates via a leukocyte-associated viraemia, which enables EHV-1 to reach end-vessel endothelia in the uterus and central nervous system (Allen and Bryans, 1986; Kydd *et al.,* 1994b). Allen and Breathnach (2006) reported that the mutation of ORF30 in EHV-1 may be related to the high intensity and long duration of leukocyte-associated viremia, which leads to a higher risk for development of neurological signs in horses infected with neuropathogenic EHV-1. A single nucleotide polymorphism (SNP) in the catalytic subunit of the viral DNA polymerase, encoded by open reading frame (ORF) 30, causing a substitution of asparagine (N) by aspartic acid (D) at amino acid 752, is significantly associated with the neurovirulent potential of

naturally occurring strains. They reported that 78 out of 82 (95%) nonneurological isolates investigated encoded amino acid N752 (asparagine), whereas 42 out of 49 (86%) neurological isolates investigated amino acid D752 (asparagic acid) (Allen and Breathnach, 2006). To compare neurovirulent and non-neurovirulent EHV-1 isolates the murine model of EHV-1 infection is a valuable model to study EHV-1 in vivo (Awan *et al.,* 1990; Van Woensel *et al.,* 1995; Galosi *et al.,* 2004).

In the present study, the infectivity and growth kinetics of neuropathogenic and nonneuropathogenic EHV-1 strains previously investigated in our lab using cells from the mouse cerebral cortex (CX (M)) as an in vitro model (Yamada *et al.,* 2008) completed using FHK cell line and the growth activities of examined strains were evaluated through estimating ORF30 (DNA polymerase) mRNA expression by real-time RT-PCR. In addition, to determine the relevance of the D/N752 coding changes to neuropathogenicity, we compared two EHV-1 isolates from Japan. The 01c1 (D752) strains isolated from a mare and was the cause of neurological disorder on a ranch with a population of horses affected by abortions and neurological disorders and a nonneuropathological strain 90c16 (N752) isolated from horses with only respiratory tract manifistation.

2. MATERIAL AND METHODS

2.1 Virus strains.

EHV-1 Ab4p strain, which was kindly provided by Dr. A. J. Davison, Glasgow University, Scotland, was used as a reference strain, The 01c1 (D752) neuropathgenic strains and a nonneuropathological strain 90c16 (N752).

2.2 cell lines

The viruses were propagated in fetal horse kidney (FHK) cells and Madin-Darby bovine kidney (MDBK) cells. All of these cells were cultivated with Eagle's minimum essential medium (MEM) (Nissui, Tokyo, Japan) supplemented with 5-10% fetal bovine serum (FBS) and 100 units/ml penicillin and 100 μg /ml streptomycin. Cultured murine neuronal cells CX (M) cells (Sumitomo Bakelite, Tokyo, Japan) derived from mouse cerebral cortexes and FHK cells were used for investigating the infectivity of EHV-1cultivated in neuron culture medium (Sumitomo Bakelite).

2.3 Sequence analysis

To confirm the D/N752 coding changes. The EHV-1 Ab4p strain (GenBank accession number AY665713), the 01c1 neuropathgenic strains (DDBJ, National Institute of Genetics, Japan; accession numbers: AB363612) and a nonneuropathological strain 90c16 (DDBJ, National Institute of Genetics, Japan; accession numbers: AB363636) sequences were used to compared a 471-bp region of ORF30 to confirm amino acid position 752 of ORF30 using CLUSTAL O (1.2.0) multiple sequence alignment softwear.

2.4 Viruses infectivity and kinetics in FHK cell line and mouse neurons

Monolayers of FHK cells prepared in 24-well plates were inoculated with 01c1 (D752) neuropathgenic strains and a nonneuropathological strain 90c16 (N752) at a multiplicity of infection (m.o.i.) of 0.1 plaque forming unit (PFU)/cell. After 1.5 h adsorption time, cells were washed three times with MEM and incubated at 37°C in a 5% CO2 atmosphere in 0.5 ml/well of MEM with 5% FBS. At 0, 12, 24, 36, 48 and 60 hrs intervals after inoculation, culture fluids with scraped cells were centrifuged to sediment the infected cells. The supernatants were used as the extracellular (cell free) samples. Following two washes with EMEM, the cell pellets were resuspended in 0.5 ml of MEM and subjected to three freeze–thaw cycles. After centrifugation, the resulting supernatants were used as the intracellular (cell associated) samples. Both

extracellular and intracellular samples were titrated for viral infectivity-by-infectivity titerations. Also the infectivity and growth kinetics of EHV-1 including neuropathogenic strains (01c1) and nonneuropathogenic strain 90c16 along with Ab4p as a refrence strain were investigated using cultured murine neuronal cells as described by Yamada *et al.,* (2008).

2.5 Evaluation of growth activity by real-time PCR

The growth activities of the viruses and β-actin gene in FHK cells which were infected with 01c1 (D752) neuropathgenic strains and a nonneuropathological strain 90c16 (N752), were evaluated by real time PCR. Total RNA was extracted from the infected and uninfected FHK cells harvested at 0, 2, 4 and 8 h post infection. The growth activities of all viruses were evaluated through estimating a copy number of ORF30 (DNA polymerase). Real-time PCR assay were carried out by using 12.5 μl of SYBR Premix Ex Taq (TAKARA), 10 μM of specific primers and 10 ng of cDNA in the Thermal Cycler Dice Real Time System (TAKARA). Primers sequences are for ORF30 (DNA polymerase) primers (ORF30A 5'- GTC AGG CCC ACA AAC TTG AT-3' and ORF30B 5'- ACT CGG TTT ACG GAT TCA CG-3'). Relative quantities were measured by the ΔΔCt Method (Livak and Schmittgen, 2001).

3. RESULTS

3.1 Sequence analysis

We compared a 471-bp region of ORF30 among the 01c1 neuropathgenic strains (DDBJ, National Institute of Genetics, Japan; accession numbers: AB363612) and a nonneuropathogenic strain 90c16 (DDBJ, National Institute of Genetics, Japan; accession numbers: AB363636) in order to reveal the relationship between neuropathogenesis and the amino acid changes at amino acid position 752 of ORF30 in the two Japanese EHV-1 isolates. The genomic sequence of EHV-1 strain Ab4p (GenBank accession number AY665713) was used for comparison. The results showed the 01c1 strain (neuropathogenic strain) encoded D752, while the other strain encoded N752 (Fig. 1).

3.2 Characterization of in-vitro growth properties of 01c1 and 90c16 strains in FHK cell line and mouse neurons

The extracellular 01c1 and 90c16 strains titers in FHK cells by infectivity titerations were nearly similar in all examined post infection times (Fig. 2). The infectivity of EHV-1 in neuronal cells investigated (previously in our lab) was evaluated by inoculating CX (M) cells with EHV-1 including 01c1 neuropathogenic strains and 90c16 nonneuropathogenic strain EHV-1 antigens were detected in the neurons for all strains examined 7 days postinfection. The virus antigens were mainly recognized in the neuronal cell bodies. In vitro growth properties of neuropathogenic strain 01c1 were comparable to those of the nonneuropathogenic strain 90c16 in cultured mouse neurons.

3.3 Analysis of transcription kinetics of ORF 30 of 01c1 and 90c16 by real-time PCR

The FHK cells were infected with 01c1 and 90c16. The growth activities of two viruses were evaluated through estimating ORF30 (DNA polymerase) mRNA expression by real-time RT-PCR with using β-actin gene expression as a control. β-actin gene expression levels were the same among the FHK cells infected by each viruses. The expression of ORF30 of 01c1 and 90c16 was nearly the same at all examined times (Fig. 3).

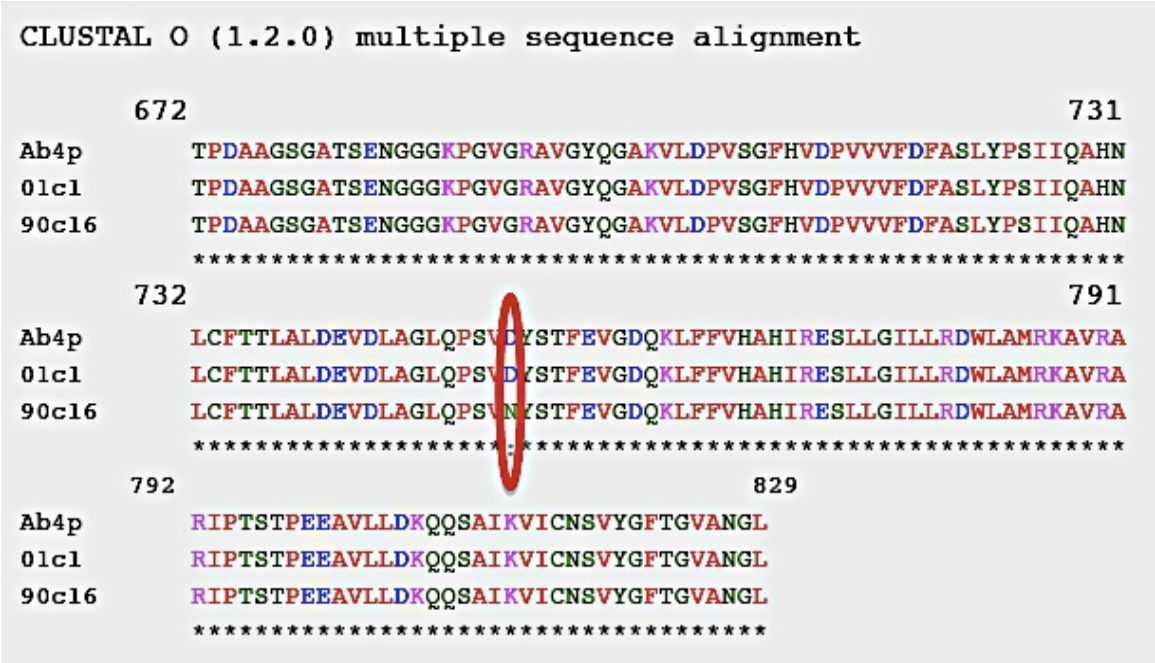

Fig. 1: Multiple alignment of predicted amino acid sequences of ORF30 in Ab4p, 01c1 and 90c16 strains. The predicted amino acid sequences from residues 672 to 829 in ORF30 are shown. The dot (*) indicates an identical amino acid residue related to that of Ab4p.

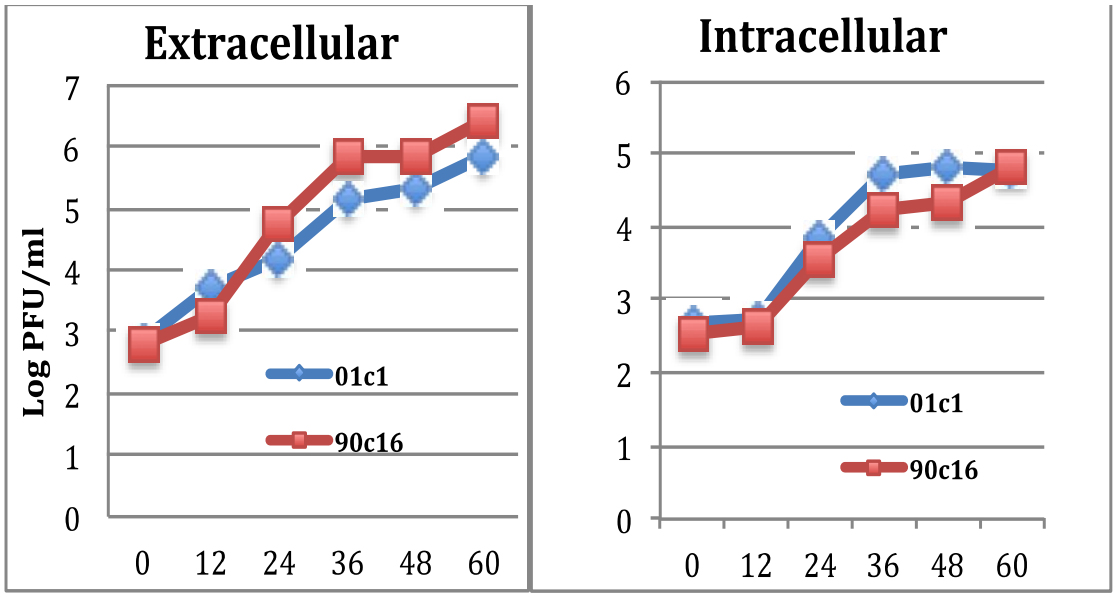

Fig. 2: Comparison of growth curves of 01c1 and 90c16 viruses on FHK Cell at MOI of 0.01. Extracellular and Intracellular viruses were titrated by plaque formation on MDBK cells. The experiments were performed in duplicate.

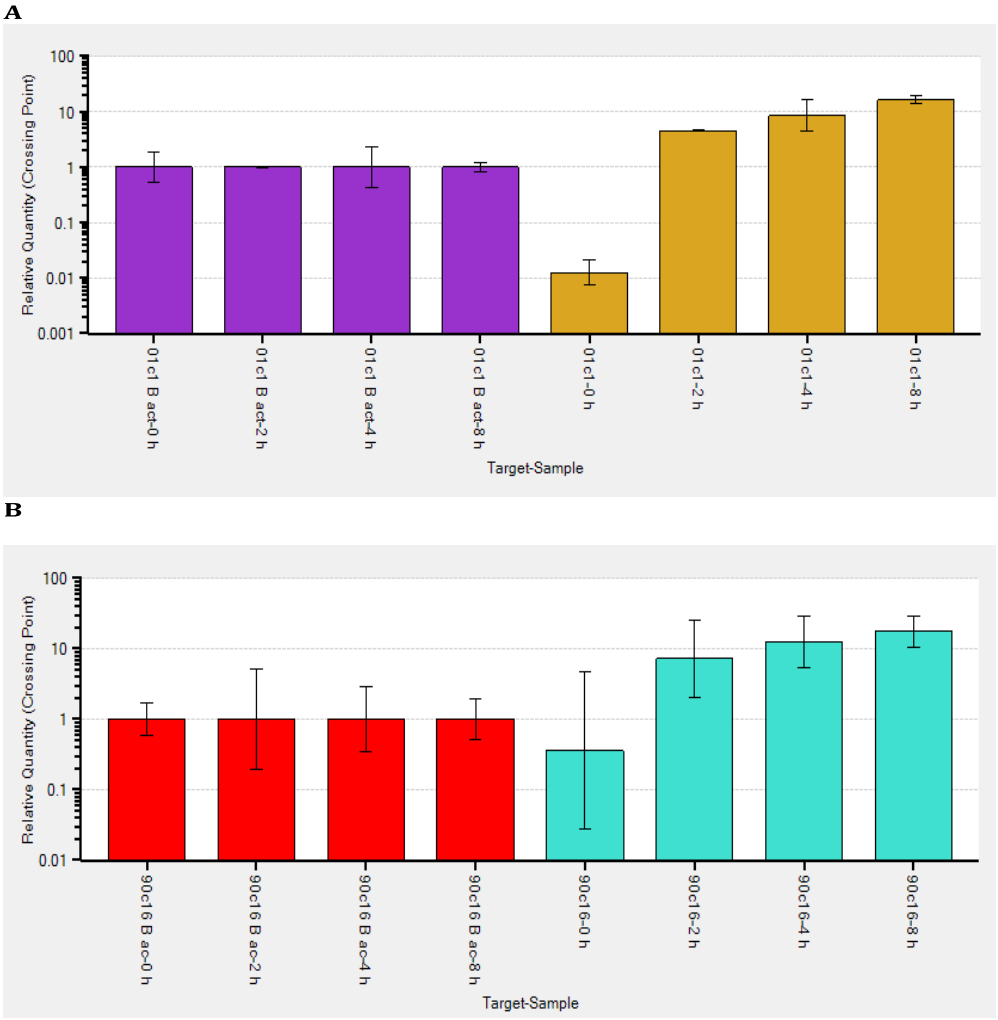

Fig. 3: Analysis of transcription activity of kinetics of ORF 30 of 01c1 (A) and 90c16 (B) by real–time RT-PCR.

4. DISCUSSION

EHV-1 harbours a 150 kb double-stranded DNA genome that is highly conserved among strains, with a nucleotide divergence of only 0.1%. However, virulence of different strains can differ significantly, especially with respect to neuropathogenicity (Nugent *et al.,* 2006). It was found that only minorities of EHV-1 strains are capable of inducing neurological disorders, although all strains can cause respiratory disease and abortion (Wilson, 1997). Recently, epidemiological as well as reverse-genetic studies have shown that a single-nucleotide polymorphism at position 2254 (G/A2254) of open reading frame 30 (ORF30), encoding viral DNA polymerase (Pol), will lead to a variation at amino acid position 752 (D/N752), which is not only necessary but also sufficient for the virus's neuropathogenic potential (Nugent *et al.,* 2006; Goodman *et al.,* 2007; Van de Walle *et al.,* 2009). The D752 genotype, in contrast to N752, can induce higher levels of viraemia in horses in vivo.

In the present study, to determine the relevance of the D/N752 coding changes in the neuropathogenicity of EHV-1, we compared a 471- bp region of ORF30 for two EHV-1 isolates from Japan with the corresponding regions in EHV-1 Ab4p strains as a reference strain. The 01c1 (D752) strains as a neuropathogenic strain and a nonneuropathological strain 90c16

(N752). Infectivity and growth kinetics of both strains were investigated using FHK cell line and cells from the mouse cerebral cortex (CX (M)) as an *in vitro* model.

For FHK extracellular and intracellular virus titers were nearly the same titers at 6 hr, at12 hr, 24 hr, 36 hr and 48 hr of 01c1 and 90c16 viruses. In vitro growth properties of neuropathogenic strain 01c1 were comparable to those of the nonneuropathogenic strain 90c16 in cultured mouse neurons. The EHV-1 strains examined were able to propagate in FHK cell line and neurons regardless of whether they were neuropathogenic or nonneuropathogenic or whether ORF30 encoded N752 or D752. In addition the expression of ORF30 of 01c1 and 90c16 was nearly the same at all examined times when comparing the growth activities of the two viruses evaluated through estimating ORF30 (DNA polymerase) mRNA expression by real-time RT-PCR.

Allen and Breathnach, (2006) suggested a relationship between the magnitude and duration of EHV-1 cell-associated viremia and the risk for subsequent development of clinical EHV-1 myeloencephalopathy and indicated that prolonged viremia may be related to the unique viral phenotype of enhanced replicative vigor by a mutation of the DNA polymerase gene. The differences in amino acid 752 (D or N) of ORF30 may be related to the ability to replicate not only in neurons but also in other cells, such as leukocytes, endothelial cells and eoithelial cell (Allen and Breathnach, 2006; Goodman *et al.,* 2007; Van de Walle *et al.,* 2009; Allen, 2008; Annelies *et al.,* 2010). However, this mutated DNA polymerase model cannot explain the neuropathogenicity of 89c25, which exhibited similar growth properties to 01c1 in cultured neurons and possessed ORF30 encoding N752 (Yamada *et al.,* 2008).

On the other hand, Guanggang *et al.,* (2010) constructed EHV-1 strain RacL11 mutant with a deletion of ORF30 residue 752 and repaired the deletion virus again to encode D752 or N752, respectively. The D752 deleted mutant virus replicated with kinetics indistinguishable from those of D752 and N752 viruses. In equine peripheral blood mononuclear cells, no significant difference was detected between the mutants with respect to cellular tropism or virus replication. The results demonstrated that amino acid residue 752 in EHV-1 ORF30 is not required for virus growth. Annelies *et al.,* (2010) indicates that other virus characteristics beyond ORF30 are important for virus replication in the upper respiratory tract. This is in line with what has been observed in many other studies (Lunn *et al.,* 2009).

In conclusion, we have shown The single mutation of EHV-1 DNA Polymerase Gene (ORF30) alone did not lead to altered virus replication in FHK and mouse neuron cultured cells, that residue 752 in the essential DNA Polymerase of EHV-1 is not required for virus growth suggesting the involvement of other viral factors in the neuropathogenicity of EHV-1.

5. REFERENCES

Allen, G.P. 2008. Risk factors for development of neurologic disease after experimental exposure to equine herpesvirus-1 in horses. Am J Vet Res 69:1595-1600.

Allen, G.P. Breathnach, C.C. 2006. Quantification by real-time PCR of the magnitude and duration of leucocyte-associated viremia in horses infected with neuropathogenic vs non-neuropathogenic strains of EHV-1. Equine Vet J 38:252–257.

Allen, G.P. Bryans, J.T. 1986. Molecular epizootiology, pathogenesis, and prophylaxis of equine herpesvirus-1 infections. Prog Vet Microbiol Immunol 2:78-144.

Annelies, P.V., Glorieux, S., Gryspeerdt, A.C., Steukers, L., Duchateau, L., Osterrieder, N., Van de Walle, G.R., Nauwynck, H.J. 2010. Replication kinetics of neurovirulent versus non-neurovirulent equine herpesvirus type 1 strains in equine nasal mucosal explants.J Gen Virol 91:2019-2028.

Awan, A.R., Chong, Y.C., Field, H. J. 1990. The pathogenesis of equine herpesvirus type 1 in the mouse: a new model for studying host responses to the infection. J Gen Virol 71:1131-1140.

Borchers, K., Thein, R., Sterner Kock, A. 2006. Pathogenesis of equine herpesvirus-associated neurological disease: a revised explanation. Equine Vet J; 38(3): 283-287.

Brosnahan, M.M., Osterrieder, N. 2009. Equine herpesvirus-1: a review and update. In Infectious Diseases of the Horse, pp. 41-51. Edited by T. S. Mair & R. E. Hutchinson. Fordham: Equine Veterinary Journal.

Bryans, J.T., Allen, G.P. 1989. Herpesviral diseases of the horse. In Herpesviral Diseases of Cattle, Horses and Pigs, pp. 176–229. Edited by G. Wittman. Boston: Kluwer.

Galosi, C.M., Barbeito, C.G., Vila Rosa, M.V., Cid de la Paz, V., Ayala, M.A., Corva, S.G., Etcheverrigaray, M.E., Gimeno, E.J. 2004. Argentine strain of equine herpesvirus 1 isolated from an aborted foetus shows low virulence in mouse respiratory and abortion models. Vet Microbiol 103: 1-12.

Goodman, L.B., Loregian, A., Perkins, G.A., Nugent, J., Buckles, E.L., Mercorelli, B., Kydd, J.H., Palù, G., Smith, K.C. 2007. A point mutation in a herpesvirus polymerase determines neuropathogenicity. PLoS Pathog 3, e160

Gryspeerdt, A., Vandekerckhove, A.P., Garré, B., Barbé, F., Van de Walle, G. R., Nauwynck, H. J. 2010. Differences in replication kinetics and cell tropism between neurovirulent and non-neurovirulent EHV1 strains during the acute phase of infection in horses. Vet Microbiol 142:242-253.

Guanggang, M.A., Chengping, L.U., Osterrieder, N. 2010. Residue 752 in DNA polymerase of equine herpesvirus type 1 is non-essential for virus growth. J G Viro 91:1817-1822.

Ibrahim, S.M., Pagmajav, O., Yamaguchi, T., Matsumura, T., Fukushi, H. 2004. Growth and virulence alterations of equine herpesvirus 1 by insertion of a green fluorescent protein gene in the intergenic region between ORFs 62 and 63. Microbiology and Immunology. 48(11):831-842.

Kohn, C.W., Reed, S. M., Sofaly, C.D., Henninger, R.W., Saville, W.J., Allen, G.P., Premanadan, C. 2006. Transmission of EHV-1 in horses with EHV-1 myeloencephalopathy: implications for biosecurity and review. Clin Tech Equine Pract 5:60-66.

Kydd, J.H., Smith, K.C., Hannant, D., Livesay, G.J., Mumford, J.A. 1994a. Distribution of equid herpesvirus-1 (EHV-1) in the respiratory tract associated lymphoid tissue: implications for cellular immunity. Equine Vet J 26:470-473.

Kydd, J.H., Smith, K.C., Hannant, D., Livesay, G.J., Mumford, J.A. 1994b. Distribution of equid herpesvirus-1 (EHV-1) in the respiratory tract of ponies: implications for vaccination strategies. Equine Vet J 26:466-469.

Livak, K.J., Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔ CT Method. Methods 25:402-408.

Lunn, D.P., Davis-Poynter, N., Flaminio, M.J., Horohov, D.W., Osterrieder, K., Pusterla, N., Townsend, H.G. 2009. Equine herpesvirus-1 consensus statement. J Vet Intern Med 23:450-461.

Nugent, J., Birch-Machin, I., Smith, K. C., Mumford, J.A., Swann, Z., Newton, J. R., Bowden, R.J., Allen, G.P., Davis-Poynter, N. 2006. Analysis of equid herpesvirus 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neurovirulent versus nonneurovirulent disease outbreaks. J Virol 80:4047-4060.

Patel, J.R., Heldens, J. 2005.Equine herpesvirus 1 (EHV-1) and 4 (EHV-4) epidemiology, disease and immunoprophylaxis: a brief review. Vet J 170:14-23.

Perkins, G.A., Goodman, L.B., Tsujimura, K., Van de Walle, G.R., Kim, S.G., Dubovi, E.J., Osterrieder, N. 2009.Investigation of the prevalence of neurologic equine herpes virus type 1 (EHV-1) in a 23-year retrospective analysis (1984–2007). Vet Microbiol 139:375-378.

Pusterla, N., Wilson, W.D., Madigan, J.E., Ferraro, G.L. 2009. Equine herpesvirus-1 myeloencephalopathy: a review of recent developments. Vet J 180:279-289.

Timoney, J.F. 2004.The pathogenic equine streptococci. Vet Res 35, 397-409.

USDA APHIS 2007. Equine herpesvirus myeloencephalopathy: a potentially emerging disease. Accessed 9 December 2008. US Department of Agriculture Animal and Plant Health Inspection Service.

Van de Walle, G.R., Goupil, R., Wishon, C., Damiani, A., Perkins, G.A., Osterrieder, N. 2009. A single-nucleotide polymorphism in a herpesvirus DNA polymerase is sufficient to cause lethal neurological disease. J Infect Dis 200:20-25.

Van Maanen, C. 2002. Equine herpesvirus 1 and 4 infections: an update. Vet Q 24:58-78.

Van Maanen, C., Cullinane, A. 2002. Equine influenza virus infections: an update. Vet Q 24:79-94.

Van Woensel, P.A.M., Goovaerts, D., Markx, D., Visser, N. 1995. A mouse model for testing the pathogenicity of equine herpes virus-1 strains. J Virol Methods 54:39-49.

Wilson, W.D. 1997. Equine herpesvirus 1 myeloencephalopathy. Vet. Clin. N. Am.: Equine Pract. 13:53-72.

Yamada, S., Matsumara, T., Tsjujimura, K., Yamaguchi, T., Ohya, K., Fukushi, H. 2008. Comparison of the growth kinetics of neurovirulent and nonneurovirulent equid herpesvirus type 1 (EHV-1) strains in cultured murine neuronal cells and the relevance of the D/N_{752} coding change in DNA polymerase gene (ORF30). J Vet Med Sci 70:505-511.

تغيير معدل ترميز D/N752 لجين بوليميريز لحامض النووى (ORF30) لا يؤثر على نمو فيروس الهيربس 1 فى الخيول معمليا

رانيا ابوسقايه [1,4]، محمد حسنين عبيد [1]، السيد مصطفي جليلة[1]، محمد ابو العز نايل [2] ، سامي قاسم [3] [1]، محمد جودة عبدالوهاب[1]، عبد المنعم محمد مصطفي[1]، فيصل خليل ارناؤط[1]، ، هيديتو فيكوشي[4]

[1]قسم طب الحيوان – كلية الطب البيطري – جامعة بنها – مصر. [2]قسم طب الحيوان والامراض المعدية -كلية الطب البيطري – جامعة مدينة السادات – مصر. [3] قسم الفيرولوجيا -كلية الطب البيطري – جامعة كفر الشيخ– مصر
[4]قسم العلوم البيطرية التطبيقية – كلية العلوم البيطرية المتحدة – جامعة جيفو، اليابان

الملخص العربي

تم مقارنة حركية النمو لعترات فيروس الهيربس 1 الممرضة وغير الممرضة عصبيا في الخيول فى كل من خلايا كلية جنين الحصان وخلايا قشرة مخ الفئران لتوضيح علاقة تغير الحامض الامينى D/N عند موضع 752 لجين ORF30 فى العزلات اليابانية. لقد أوضحت الدراسة أن العترات الممرضة عصبيا 01c1 تشابهت فى حركية النمو مع العترة الغير ممرضة عصبيا 90c16 فى خلايا كلية جنين الحصان والاعصاب المزروعة معمليا. كما تبين أن معدل تعبير الحامض النووى الرسولي الريبوسومى لجين (ORF30) بواسطة تفاعل البلمرة المتسلسل حقيقى الوقت RT–PCR كان متشابها بين خلايا كلية جنين الحصان المعدية بكلا الفيروسين. أوضحت الدراسة أن الحامض الأمينى المشفر عند موضع 752 لجين ORF30 فى عترات 01c1 هو حامض الأسبارتيك بينما الأسباراجين هو المشفر فى عزلات عترة 90c16. قد يكون إختلاف D/N752 لجين ORF30 ليس له علاقة بقدرة النسخ المتماثل أو التكاثر فى خلايا كلية جنين الحصان والخلايا العصبية.

(مجلة بنها للعلوم الطبية البيطرية: عدد 26(2):1- 9, يونيو 2014)