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Characterization of a thymidine kinase-deficient mutant of equine herpesvirus 4 and in vitro susceptibility of the virus to antiviral agents

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ABSTRACT

Equine herpesvirus 4 (EHV-4) is an important equine pathogen that causes respiratory tract disease among horses worldwide. A thymidine kinase (TK)-deletion mutant has been generated by using bacterial artificial chromosome (BAC) technology to investigate the role of TK in pathogenesis. Deletion of TK had virtually no effect on the growth characteristics of WA79ΔTK in cell culture when compared to the parent virus. Also, virus titers and plaque formation were unaffected in the absence of the TK gene. The sensitivity of EHV-4 to inhibition by acyclovir (ACV) and ganciclovir (GCV) was studied by means of a plaque reduction assay. GCV proved to be more potent and showed a superior anti-EHV-4 activity. On the other hand, ACV showed very poor ability to inhibit EHV-4 replication. As predicted, WA79ΔTK was insensitive to GCV. Although EHV-4 is normally insensitive to ACV, it showed >20-fold increase in sensitivity when the equine herpesvirus-1 (EHV-1) TK was supplied in trans. Furthermore, both ACV and GCV resulted in a significant reduction of plaque size induced by EHV-4 and 1. Taken together, these data provided direct evidence that GCV is a potent selective inhibitor of EHV-4 and that the virus-encoded TK is an important determinant of the virus susceptibility to nucleoside analogues.

1. Introduction

Equine herpesvirus 4 (EHV-4) is reported worldwide as an etiologic agent of respiratory disease, occasionally abortion and neonatal infection in horses. EHV-4 and its close relative equine herpesvirus 1 (EHV-1) are major pathogens of horses with considerable economic and veterinary importance (Allen and Bryans, 1986; Patel and Heldens, 2005; Slater et al., 2006). Both viruses have been classified into the *Alphaherpesvirinae* subfamily, genus *Varicellovirus* (Davison et al., 2009; Roizman, 1996). Although EHV-1 and EHV-4 are closely related, antigenic variations were recognized as early as 1959. More recently, molecular analysis and specific gene sequencing have revealed genetic differences between these two viruses. To date, control measures have proved inadequate, and although vaccines are available, their efficacy is controversial (Harless and Pusterla, 2006; Reed and Toribio, 2004).

EHV-4 genome manipulation with subsequent understanding of the viral gene functions has always been difficult due to the limited number of susceptible cell lines and the absence of small-animal models of the infection. Efficient generation of mutants of EHV-4 would significantly contribute to the rapid and accurate characterization of the viral genes. This problem has been solved recently by the cloning of the genome of EHV-4 as a stable and infectious bacterial artificial chromosome (BAC) without any deletions of the viral genes (Azab et al., 2009). Very low copy BAC vectors are the mainstay of present genomic research because of the high stability of inserted clones and the possibility of mutating any gene target in a relatively short time.

The study of thymidine kinase (TK)-deficient mutants of other herpesviruses has received considerable attention for many years. Deletion of this viral encoded enzyme in alphaherpesviruses, such as herpes simplex virus (HSV) types 1 and 2, bovine herpesvirus 1, and pseudorabies virus, resulted in a reduction in virulence and the rate of re-activation of latent viruses (Coen et al., 1989; Field and Wildy, 1978; Kit et al., 1985a; Mittal and Field, 1989; Slater et al., 1993; Stanberry et al., 1985). Previous reports of EHV-1, the close relative of EHV-4, TK-deficient mutants suggested that they are markedly less pathogenic than the wild-type virus, despite being able to replicate in the host (Cornick et al., 1990; Slater et al., 1993).

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However, the role of TK in EHV-4 pathogenesis has not been yet elucidated.

Herpesvirus-encoded TKs also have an important role in the mode of action of several acyclic nucleoside analogues [e.g. acyclovir (ACV) and ganciclovir (GCV)] which are potent anti-herpetic drugs. The ultimately active metabolites of most nucleoside analogues are their triphosphates. Studies on the mode of action of ACV have shown that it is phosphorylated in infected cells by the virus-induced TK to ACV monophosphate (ACV-MP). After monophosphorylation, host cellular kinases convert ACV-MP to the di- and triphosphate (ACV-TP). ACV-TP is the active form that inhibits herpesvirus DNA polymerase (De Clercq, 1995; Field and Whitley, 2005; Morfin and Thouvenot, 2003). Although antiviral chemotherapy is a standard practice in the management of herpesvirus infection in humans, the veterinary use of antiviral drugs is relatively uncommon. Perhaps the most frequently reported use of antiviral drugs in veterinary medicine is for the treatment of feline herpesvirus 1 infections. However, there are some reports declared the efficacy of antiviral drugs against EHV-1, but not EHV-4, in vitro (De Clercq et al., 2006; Garré et al., 2007b; Hussein et al., 2008). Furthermore, the efficacy of 5-ethyldeoxyuridine and penciclovir against cercopithecine herpesvirus 1 was recently investigated (Focher et al., 2007). One of the objectives of this study was to compare the anti-EHV-4 activity of ACV and GCV by plaque reduction assay (PRA). GCV displayed a superior anti-EHV-4 activity, while ACV showed very poor ability to inhibit EHV-4 replication. Furthermore, the TK-negative mutant was resistant to the effect of the potent anti-herpetic drug GCV. These observations directed our attention toward investigating the role of TK-mediated phosphorylation in determining the susceptibility of EHV-4 to nucleoside analogues. To address this point, transfected fetal horse kidney (FHK) cells expressing EHV-1 TK gene was produced. Although EHV-1 seems to be less sensitive to ACV as compared with HSV-1, several recent reports have described the use of ACV for the treatment of horses during herpesvirus outbreaks (Garré et al., 2007a; Henninger, 2003; van der Meulen et al., 2003). The sensitivity of EHV-4 to ACV and GCV, TK-dependent drugs, in the EHV-1 TK-transformed FHK cells was investigated. We found that supplying EHV-1 TK in trans resulted in a marked increase in the sensitivity of EHV-4 to ACV.

2. Materials and methods

2.1. Viruses, cells and antiviral drugs

EHV-4 strain TH20p was plaque purified from the Japanese EHV-4 prototype strain TH20 (Kawakami et al., 1962; Maeda et al., 2004). The recombinant WA79, EHV-4 infectious BAC clone, was generated by the insertion of a *loxP*-flanked BAC vector into the intergenic region between genes 58 and 59 (Azab et al., 2009). EHV-1 strain 89c25, a virulent strain of EHV-1, was isolated from a race horse during an epizootic of EHV-1 respiratory infection in Japan in 1989 (Matsumura et al., 1992). Primary fetal horse kidney (FHK, within the fifth passage), Rabbit kidney (RK13), and human embryonic kidney (293) cells were propagated in Dulbecco's modified Eagle's medium (DMEM: Nissui) supplemented with 10% fetal calf serum (FCS) (JRH biosciences).

The antiviral compounds used were acyclovir (Sigma) and ganciclovir (LKT Laboratories, St. Paul, MN). The drugs were dissolved at 2 mg/ml in sterile distilled water and stored at −20 °C prior to dilution in cell culture medium.

2.2. Plasmids

The entire EHV-4 and 1 TK genes were polymerase chain reaction (PCR)-amplified using the primers listed in Table 1. The PCR products were digested with *Eco*RI and *Eco*RV and inserted into the expression vector pFLAG-CMV-2 (sigma), resulting in recombinant plasmids pFEH4TK and pFEH1TK, respectively. Correct amplification and insertion was confirmed by sequencing. Transfection of the pFEH4TK or the pFEH1TK plasmids into FHK cells was performed using TransIT-LT1 according to the Manufacturer's instructions (Mirus, Madison, WI).

2.3. Mutagenesis and generation of recombinant viruses

The EHV-4 BAC clone pYO03 has been generated previously (Azab et al., 2009). Mini F plasmid sequences flanked by *loxP* sites were inserted into the intergenic region between genes 58 and 59. DNA of pYO03 was manipulated by red recombination (Lee et al., 2001) in *E. coli* EL250 strain (Fig. 1), a kind gift from Dr. Neal G. Copeland. PCR primers were selected such that the recombination arms of 50 nucleotides (*nt*) enabled the substitution of *nt* 1 to 1058 of the EHV-4 TK gene by the kanamycin-resistant (Kan^R) gene amplified out of plasmid pEPkan-S, a kind gift from Dr. N. Osterrieder (Tischer et al., 2006). Primers used for the amplification of the Kan^R gene are listed in Table 1. PCR product was treated with *Dpn*I, to digest the template, and electroporated into EL250 containing pYO03. Kanamycin-resistant colonies were purified and screened by PCR, restriction enzyme digestion and DNA sequencing analyses to detect *E. coli* harboring the mutant pYOΔTK. The TK deletion mutant virus WA79ΔTK was reconstituted by transfection of pYOΔTK into 293 cells as described earlier (Azab et al., 2009). Three days later, the supernatant and cells were collected and used to infect confluent FHK cells. Viral plaques were observed 2 or 3 days after infection.

Table 1
Oligonucleotide primers used in this study.

Gene	Direction	Sequence
EHV-4 TK	Forward	*ACT***gaattc**ATGGCTGCTTGCGTACCCAC[a]
	Reverse	*AAT***gatatc**TCAGACGCCCATCTCCGCGT[a]
EHV-1 TK	Forward	*ACT***gaattc**ATGGCGGCTCGCGTACCTTC[a]
	Reverse	*AAT***gatatc**TCAGACGCCCATCTCGGCGT [a]
TK-Kan	Forward	ATGGCTGCTTGCGTACCCACGGGAGAAGCTCCACGAAGCGCCAGCGGAACAGGATGACGACGATAAGTAGGG[b]
	Reverse	TCAGACGCCCATCTCCGCGTTAAAGGTGCGTGCCGCCCGCTCTAAAGCAGCAACCAATTAACCAATTCTGATTAG[b]
TK	Forward	TTAGTGGTATTTACGACACC
	Reverse	TGCTTATAAGCCCCACAGGA
Kan	Forward	AGGATGACGACGATAAGTAGGG
	Reverse	CAACCAATTAACCAATTCTGATTAG

[a] Restriction enzyme sites are given in lower case bold letters; sequences in italics indicate additional bases which are not present in the EHV-4 sequence.
[b] Underlined sequences indicate the template binding region of the primers for PCR amplification with pEBkan-S.

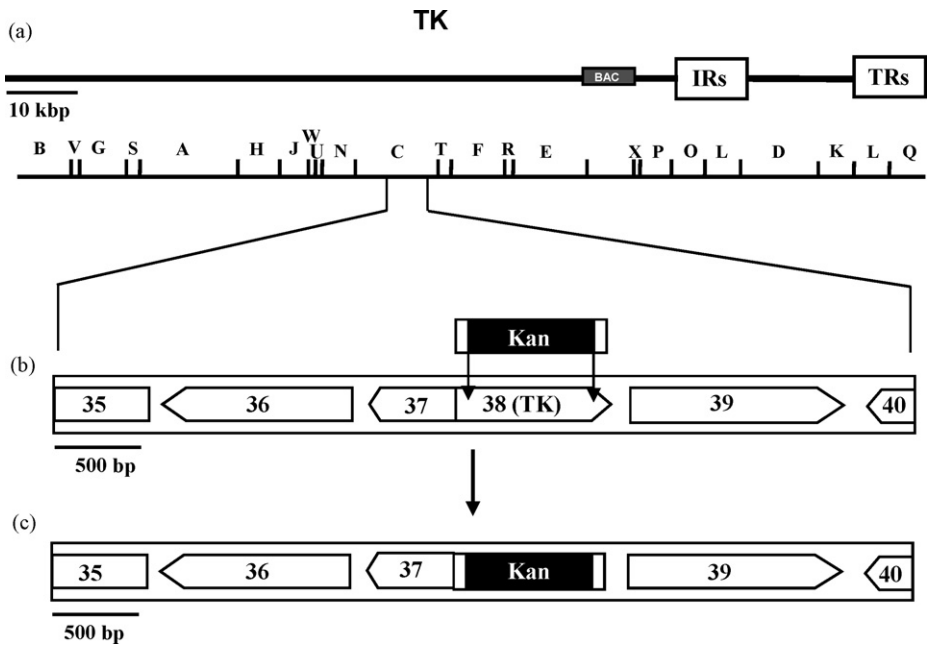

Fig. 1. Schematic diagram of the procedures used to delete the TK gene from pYO03. (a) Schematic representation of the genomic organization and the *Bam*HI restriction map of EHV-4 BAC pYO03 (Azab et al., 2009) is given. The two unique regions (U_L and U_S), the terminal and internal repeat sequences (TR_S and IR_S), and the inserted BAC cassette is shown. (b) The genomic organization of the domain that encodes the genes from 35 to 40 (Telford et al., 1998) is depicted within the *Bam*HI C restriction fragment. The PCR cassette conferring Kan^R gene was inserted into the TK locus of pYO03 using red recombination. (c) The mutant BAC DNA (pYO03ΔTK) was transfected into eukaryotic cells to reconstitute the infectious virus WA79ΔTK.

2.4. Preparation of viral DNA and Southern blot analyses

EHV-4 and WA79ΔTK viral DNAs were isolated according to the methods developed by Sinzger et al. (1999). In brief, infected FHK cells were harvested when they showed 100% cytopathic effect (CPE), pelleted by centrifugation at 250 × *g* and 4 °C for 10 min, and washed twice with cold PBS. The pellet was suspended in cell permeabilization buffer [10 mM Tris–HCl (pH 7.5), 32 mM sucrose, 5 mM $MgCl_2$, and 1% Triton X-100], incubated on ice for 10 min, and centrifuged for 15 min at 4 °C and 1300 × *g*. After micrococcal nuclease treatment, the cells were digested with 0.5% SDS and 0.1 mg/ml proteinase K (Invitrogen) at 50 °C overnight and viral DNA was extracted with phenol-chloroform and precipitated with ethanol.

Probes used in Southern blot analysis were labeled by using AlkPhos DIRECT labeling kit (GE Healthcare) according to the manufacturer's protocol as described before (Azab et al., 2009). For hybridization, DNAs were digested overnight with restriction endonucleases and separated by 0.7% agarose gels electrophoresis. Gels were stained with ethidium bromide staining, and DNA fragments were transferred to a Hybond-N^+ nylon membranes (GE Healthcare).

2.5. Virus titration and growth curve analyses

EHV-4, WA79 and WA79ΔTK viruses were titrated by plaque assay (Zhang et al., 2008). FHK cells were seeded in 6-well plates and mixed with different dilutions of infected cell suspensions. Plaques were counted by fluorescent microscopy at 3 days after inoculation. Growth curve analyses were performed to compare the growth kinetics of the parental virus with those of the recombinant and mutated viruses. Confluent FHK cells were infected with various viruses at an MOI of 0.0001 PFU per cell. After 1 h of adsorption, cells were washed and overlaid with DMEM containing 10% FCS. Infected cultures, cells and supernatants, were harvested at the indicated time post-infection (p.i.) and stored at −80 °C. The amount of infectious virus was detected by a plaque assay as described above.

2.6. Plaque size measurements

Plaque diameters on FHK cells were measured after infection of the cells cultivated in 6-well plates with various viruses and overlaid with DMEM containing 0.5% methylcellulose. Three days later, the plaques were observed for GFP expression under a fluorescent microscope. Cells were fixed with ethanol and stained with crystal violet. For each virus, 80 plaques were selected randomly and their sizes were measured by using ImageJ software vl.32j (http://rsb.info.nih.gov/ij/). Student's *t*-test was used to compare the average plaque diameter of each virus.

2.7. Plaque reduction assay

Confluent monolayers of FHK cells, prepared in 24-well tissue culture plates, were infected with 50 PFU of EHV-4, WA79, EHV-1, or WA79ΔTK/well. After the virus was allowed to adsorb for 1 h at 37 °C, the inoculum was carefully aspirated, and the infected cells were rinsed twice with culture medium. The infected monolayers were overlaid with 0.5% methylcellulose medium containing an increasing concentration (0, 1.5, 3, 6, 12, 24, 50 μg/ml) of each antiviral drug; three wells were used per drug concentration. In case of EHV-1, GCV was used at concentration started from 0.01 to 50 μg/ml. the plates were incubated at 37 °C for 3 days in a 5% CO_2 incubator. The monolayers were fixed with ethanol and then stained with crystal violet. The plaques were counted for each concentration of antiviral compound microscopically at low power than previously described (Landry et al., 2000). The inhibitory effect of the antiviral drugs on plaque number was calculated according to the equation: % inhibition = (the plaque counts at positive drug doses/the plaque counts of the controls) × 100. The concentration of each compound that resulted in 50% reduction in the plaque numbers (IC_{50})

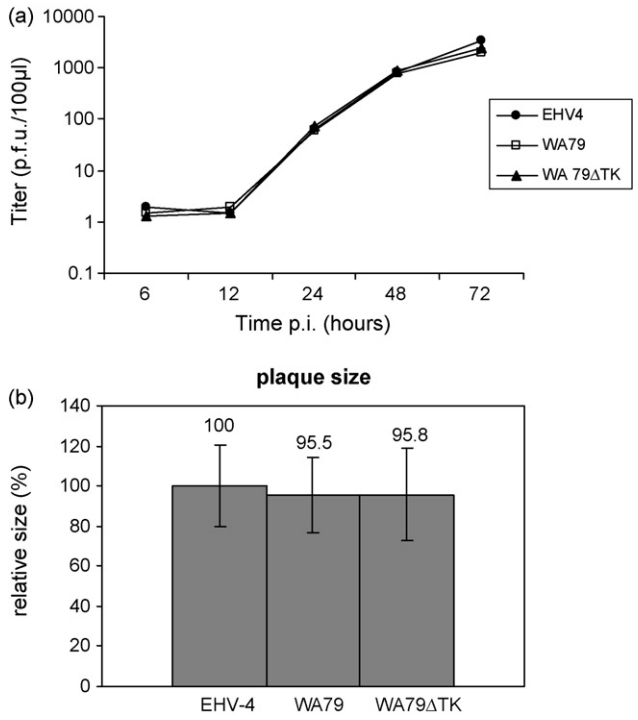

Fig. 2. Growth kinetics and plaque sizes of wild-type EHV-4, WA79 and EHV-4 mutant virus. (a) FHK cells were infected at an MOI of 0.0001. The infected cells and supernatants were collected and virus titers were determined at the indicated times p.i. The data presented are means ± SD of triplicate measurements. (b) Means ± SD of diameters of 80 plaques measured for each virus are shown. The plaque diameter of EHV-4 was set to 100%. .

was calculated from the dose-response curves generated from the data.

For EHV-4 and 1, the effect of the antiviral compounds on virus-induced plaque size was determined as described above.

3. Results

3.1. Construction of an EHV-4 TK deletion mutant

The mutant pYO03 genome lacking the TK gene was generated in order to investigate whether the UL23-homologous gene of EHV-4 (Telford et al., 1998) encodes for a functional gene product. The TK-negative virus was obtained after transfection of 293 cells as described in Materials and Methods. DNA of the recombinant virus and wild-type virus was prepared and subjected to PCR, Southern blot and DNA sequencing analyses. The expected genotype of the TK-mutant virus was confirmed and the Kan^R gene had been inserted instead of the TK gene (data not shown).

3.2. In vitro characterization of WA79ΔTK

To determine whether the TK gene is essential for EHV-4 replication in cell culture, the growth rates of the wild-type EHV-4, WA79 and WA79ΔTK were compared in FHK cells. The results showed that WA79ΔTK was capable of growing in these cells and all viruses exhibited comparable growth kinetics on FHK cells (Fig. 2a). To further explore the effect of TK deletion on direct virus cell-to-cell spread, plaque sizes of mutant viruses were determined and compared to those of parental viruses (Fig. 2b). There was no significant difference observed between plaque diameters of the parental viruses and the mutant viruses.

Table 2
Susceptibility of EHV-4, EHV-1 and WA79ΔTK to antiviral drugs.

Virus	Mean IC_{50} ± SD (µg/ml)	
	ACV	GCV
EHV-1	2.7 ± 0.2	0.1 ± 0.3
EHV-4	>50	2.5 ± 0.5
WA79	>50	2.1 ± 0.27
WA79ΔTK	>50	45
WA79ΔTK + pFEH4TK	>50	4 ± 0.25

Results are presented as the mean IC_{50} values of three independent experiments ± SD.

3.3. Effect of antiviral drugs on plaque number

The mean IC_{50} values ± standard deviations (SD) are shown in Table 2. In case of EHV-1, both GCV and ACV were effective in inhibiting EHV-1 replication with IC_{50} values ranging between 0.1 and 2.7 µg/ml, respectively. On the other hand, ACV was demonstrated to be inefficient in inhibiting EHV-4 or WA79 replication, while GCV proved to be more potent and inhibited EHV-4 and WA79 replication with IC_{50} of 2.5 and 2.1 µg/ml, respectively.

As a complementary parameter of assessing the antiviral drugs efficacy, the mean plaque diameter of the antiviral treated cultures was determined for both EHV-4 and -1. GCV reduced the plaque size significantly for both EHV-4 and -1 at the respective concentrations of 2.5 and 0.1 µg/ml or higher. However, ACV reduced the plaque size of EHV-1 at a concentration of 2.7 µg/ml or higher (Fig. 3).

Since the herpesvirus-encoded TK play a key role in the selective activity of acyclic nucleoside analogues, it was important to compare the activity of the tested anti-herpetic compound GCV against WA79ΔTK replication. As expected, the TK-negative viruses were resistant to the effect of GCV (Table 2). Supplying of the cells with EHV-4 TK resulted in a marked increase in the sensitivity to GCV (Table 2).

3.4. Antiviral susceptibility of EHV-4 in EHV-1 TK-transformed cells

FHK cells were transfected with pFEH1TK plasmid. The EHV-1 TK polypeptide expressed within the cells was detected by Western blotting as one band having molecular weight of approximately 38 kDa (data not shown). The antiviral activity of ACV and GCV, TK-mediated antiviral compounds, against EHV-4 was compared in pFEH1TK versus negative control FHK cells using standard PRA as described above. As shown in Table 3, supplying EHV-1 TK in trans resulted in a marked increase (IC_{50} value was 2.9 µg/ml) in the sensitivity to ACV. Also, a smaller but significant increase was observed for GCV.

4. Discussion

BAC cloning technology has opened new avenues for the manipulation of several herpesvirus genomes. The increased stability, the simplicity of DNA isolation, and the improved capability of BACs for high-throughput applications have led to their use as the preferred

Table 3
Comparative antiviral susceptibility of EHV-4 in pFEH1TK versus negative control pFLAG-CMV-2 transfected cells by PRA.

Drug	Mean IC_{50} ± SD (µg/ml)	
	pFLAG-CMV-2	pFEH1TK
ACV	>50	2.9 ± 0.26
GCV	2.7 ± 0.5	0.5 ± 0.3

Results are presented as the mean IC_{50} values of three independent experiments ± SD.

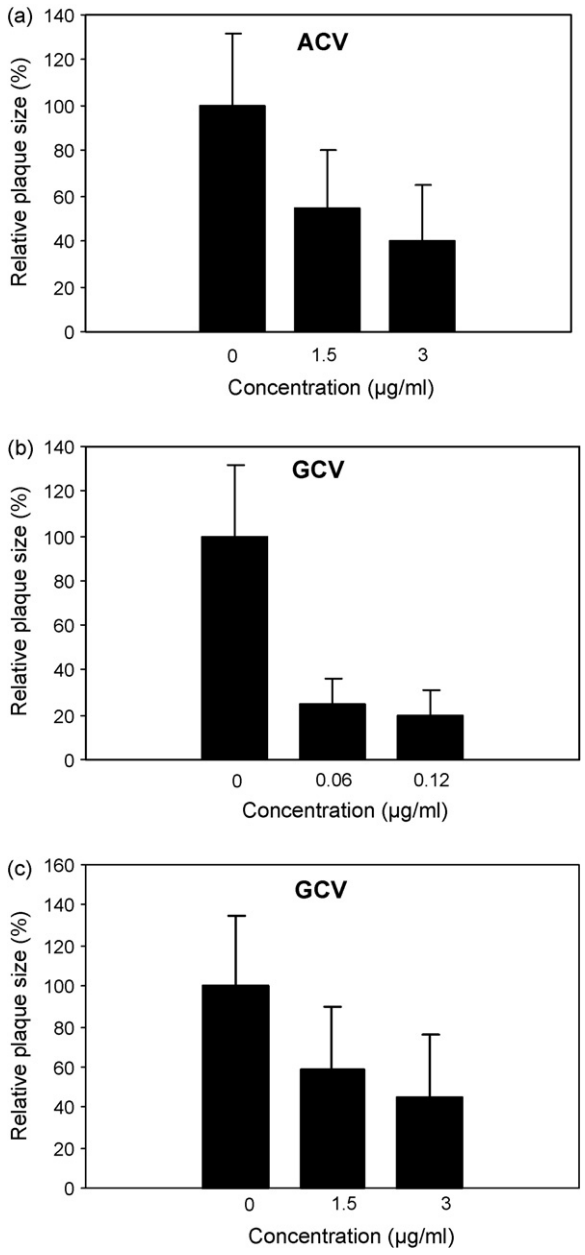

Fig. 3. The effect of antiviral drugs on the size of virus-induced plaques. ACV and GCV were able to reduce the size of plaques induced by EHV-1 (a and b) significantly. Furthermore, the size of EHV-4-induced plaques (c) was also reduced due to the effect of GCV. The data represent the mean ± SD of three independent experiments (*$P < 0.01$).

large-insert cloning system for genomic analysis and gene discovery (Heintz, 2001; Shizuya and Kouros-Mehr, 2001). Recently, we have described the cloning of the first infectious clone for EHV-4 without any deletions or destruction of the viral genes (Azab et al., 2009). Cloning of EHV-4 as infectious BAC has greatly enhanced the construction of mutants, thereby allowing assessment of the role of viral genes in host-virus interaction as well as allowing the study of the aspects of difference with EHV-1. In this study, we used the BAC technology to delete the EHV-4 TK gene and to determine the pathogenicity of TK-deficient mutants in vitro. Additionally, we compared the efficacy of two anti-herpetic drugs, ACV and GCV, against EHV-4. The role of TK-mediated phosphorylation in determining the susceptibility of EHV-4 to nucleoside analogues was also investigated.

Herpesvirus TK is an example of a viral gene product that has a specific role in pathogenesis. The deletion of TK gene of alphaherpesviruses has no effect on the propagation of viruses in cell culture. While in vivo studies revealed that TK deletion resulted in a reduction in virulence and the rate of re-activation of latent viruses (Coen et al., 1989; Cornick et al., 1990; Field and Wildy, 1978). These attributes have led to TK-negative mutants being proposed as potential agents of immunoprophylaxis. As predicted, our results showed that the virus mutant, WA79ΔTK, grows normally in cell culture and has the same plaque morphology as the parental EHV-4 virus. Previous reports of bovine herpesvirus type 1 (Kit et al., 1985b), pseudorabies virus (Kit et al., 1985a; McGregor et al., 1985), and infectious laryngotracheitis virus (Han et al., 2002) indicated that the TK-deleted recombinants were indeed less pathogenic than the wild-type viruses and they could still induce a protective immune response when inoculated in their hosts. Therefore, similarly modified EHV-4 would probably retain antigenicity and also be a candidate for a genetically engineered vaccine. However, further in vivo studies are required to evaluate the role of EHV-4 TK-negative mutants in immunoprophylaxis.

Antiviral chemotherapy against members of the herpesvirus family is now routinely practiced in human medicine, and currently there are 11 licensed anti-herpetic drugs available (De Clercq et al., 2006). While good progress has been made in treating human infection, the veterinary use of antiviral compounds is relatively uncommon. The role of herpesvirus TK in the selective activity of these compounds has been clearly defined (Field and Whitley, 2005). ACV and GCV were previously reported to have potent anti-EHV-1 effects by PRA in vitro (Garré et al., 2007b). However, there were no previous reports regarding the effect of antiviral drugs against EHV-4. The PRA data presented here have confirmed the previous findings related to EHV-1. The IC_{50} (2.7 μg/ml and 0.1 μg/ml) of ACV and GCV, respectively, were approximately similar to the previous report conducted by Garré et al. (2007b). In case of EHV-4, our data have revealed that EHV-4 is relatively insensitive to ACV (IC_{50} value was >50 μg/ml); while GCV was found to exhibit a more potent specific anti-EHV-4 activity where the IC_{50} value was 2.5 μg/ml. Antiviral compounds not only reduce the plaque number, but also contribute in the reduction of plaque size (Garré et al., 2007b; Jenssen et al., 2004; van der Meulen et al., 2006). We found that both compounds were able to significantly reduce the plaque size. Thus, measuring reduction in plaque number and plaque size are two valuable and complementary means of assessing the antiviral efficacy.

On the other hand, WA79ΔTK was found to be resistant to GCV (22-fold change in IC_{50} compared to wild-type virus). When EHV-4 TK was supplied to FHK cells, GCV retained its potent inhibitory effect against the TK-deficient virus (Table 2). Our results are in agreement with the nucleoside analogues resistance phenotype previously reported in other studies (Boyd et al., 1993; Hussein et al., 2008; Morfin and Thouvenot, 2003; Sergerie and Boivin, 2006). GCV as well as other nucleoside analogues all rely on virus-encoded nucleoside kinases, e.g TK of HSV or EHV, or protein kinase of cytomegalovirus (CMV), for their initial phosphorylation. Therefore, TK-mutant viruses are generally insensitive to these drugs.

The results presented here showed that the IC_{50} values of ACV for EHV-1 and EHV-4 were 2.7 and >50 μg/ml, respectively. The relative insensitivity of EHV-4 to ACV could be attributed to either the inefficient phosphorylation of ACV to its 5′-monophosphate form by the EHV-4 TK or the inefficient inhibition of the viral DNA polymerase by the active form of the drug. EHV-1 TK is known to phosphorylate ACV albeit at a relatively low efficacy compared to that of HSV-TK. However, the in vitro susceptibility of EHV-1 to ACV and the anecdotal effectiveness of therapy, make it an attractive candidate for the antiviral therapy against EHV-1 infection in horses (Garré et al., 2007a; Rollinson and White, 1983; Tearle et

al., 2003). The marked reduction in the IC_{50} value of ACV (Table 3) against EHV-4 when EHV-1 TK was supplied in trans provided direct evidence that EHV-4 was sensitive to the active triphosphate form of ACV. Therefore, the inefficient phosphorylation of ACV by EHV-4 TK was responsible for the observed in vitro insensitivity of this virus to the antiviral drug action of ACV. It is noteworthy that there is a high degree of conservation, more than 90%, between both EHV-4 and 1 TK genes. On the other hand, the corresponding range of conservation between EHV-4 TK and its HSV-1 counterpart is only around 50% (Telford et al., 1998). These results clearly indicate that, despite the high sequence and structural similarity between EHV-4 TK and its EHV-1 homologue, ACV is more efficiently phosphorylated by EHV-1 TK than by EHV-4 TK. Furthermore, we can assume that EHV-4 DNA polymerase has a high affinity for the ACV triphosphates, and, hence explains the observed inhibitory effect on virus replication.

In summary, the feasibility of mutagenesis of the EHV-4 BAC clone has been studied in this paper. Our findings reported here revealed no significant difference between wild-type EHV-4 and TK-negative strain in their replication cycle in cell culture. GCV displays a potent activity against both EHV-4 and 1 infection in vitro. However, there is no direct clinical application due to its high price cost. EHV-4 appeared to be relatively insensitive to ACV; however, it acquired sensitivity in the TK-transformed cells, in which the transfected EHV-1 TK was able to phosphorylate the drug.

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