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ORIGINAL ARTICLE

In vitro comparison of acyclovir, ganciclovir and cidofovir against equid alphaherpesvirus 3 and evaluation of their efficacy against six field isolates

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Abstract Equid alphaherpesvirus 3 (EHV3) is the etiological agent of equine coital exanthema (ECE), which is a venereal, highly contagious disease, characterized by the formation of papules, vesicles, pustules and ulcers on the external genitalia of mares and stallions. EHV3 remains in a latent state after a successful infection and there are latently infected animals in which the virus is reactivated and generally re-excreted subclinically. There are no available vaccines for this condition and prevention is based on the clinical examination of mares prior to mating, which allows to segregate those showing clinical signs. As this approach does not eliminate the risk of contagion in stallions from subclinically infected mares, there is a need for a specific EHV3 treatment. Nowadays, there exist various antiviral compounds of proven effectiveness for other alphaherpesviruses affecting humans and animals. The aim of the present study was to compare the efficacy of three antiviral compounds, acyclovir, ganciclovir and cidofovir against EHV3 *in vitro*, and to assess their efficacy against six EHV3 Argentinian field isolates. To determine the efficacy of these compounds *in vitro*, three parameters were analyzed: reduction of plaque number, reduction of plaque size and reduction of viral production. Additionally, the effectiveness of the three compounds at an optimum concentration previously determined in this study was investigated for the EHV3 field isolates. Based on our results, ganciclovir was the most potent antiviral compound to reduce EHV3 replication *in vitro* and may thus be a valuable candidate for treatment and prevention of ECE in mares and stallions.

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PALABRAS CLAVE
Exantema coital equino;
Alfa-herpesvirus equino 3;
Fármacos antivirales;
Eficacia *in vitro*;
Análogos de nucleósidos

Comparación *in vitro* de aciclovir, ganciclovir y cidofovir contra alfa-herpesvirus equino 3 y evaluación de la eficacia de los mismos frente a 6 aislamientos de campo del virus

Resumen El alfa-herpesvirus equino 3 (EHV3) es el agente etiológico del exantema coital equino (ECE), enfermedad venérea, altamente contagiosa y caracterizada por la aparición de pápulas, vesículas, pústulas y úlceras en los genitales externos de yeguas y padrillos. Luego de la primo-infección, el EHV3 se mantiene en el animal en un estado de latencia a partir del cual puede reactivar y excretarse, generalmente de manera subclínica. No existen vacunas, por lo que la prevención se basa en la detección de las lesiones clínicas previo al servicio, y la segregación de estos animales. Sin embargo, este abordaje no previene la infección del padrillo por parte de yeguas que excretan el virus de manera subclínica, y por lo tanto existe la necesidad de un tratamiento específico contra el EHV3. En la actualidad, existen varios compuestos antivirales de probada eficacia contra herpesvirus humanos y veterinarios. El objetivo de este trabajo es comparar la eficacia de 3 compuestos antivirales, aciclovir, ganciclovir y cidofovir, contra EHV3 *in vitro*, y evaluar la eficacia de los mismos contra 6 cepas de campo argentinas de EHV3. Para determinar la eficacia de los compuestos *in vitro* se evaluaron 3 parámetros: reducción del número de placas de lisis, reducción del tamaño de placas de lisis y reducción de la producción de virus. Adicionalmente, la efectividad de los compuestos en una concentración óptima, previamente determinada en este estudio, fue determinada para 6 cepas de campo argentinas de EHV3. De acuerdo con los resultados obtenidos, ganciclovir fue el compuesto más potente en reducir la replicación del EHV3 *in vitro*, y por lo tanto podría considerarse un potencial candidato para el tratamiento y la prevención del ECE en yeguas y padrillos.

Introduction

Equid alphaherpesvirus 3 (EHV3), member of the subfamily *Alphaherpesvirinae*, genus *Varicellovirus*[2,10] is the etiological agent of equine coital exanthema (ECE). ECE is a highly contagious disease characterized by the formation of papules, vesicles, pustules and ulcers on the external genitalia of mares and stallions. However, signs of systemic disease (fever, anorexia and pain) are rare[1,4]. EHV3 infection is endemic in most populations of horses worldwide, and the virus is primarily transmitted by sexual intercourse but also by contact with elements contaminated with infected secretions[1,4].

As for the other alphaherpesviruses, EHV3 remains in a latent state after a successful infection[1,6]. Moreover, spontaneous reactivation of EHV3, without the typical clinical manifestation, has been also demonstrated in mares kept in isolation for 11 months[7].

Vaccines against EHV3 are not available; thus, prevention is based on the clinical examination of mares prior to the service, which allows to segregate those showing clinical signs. However, the preventive management described does not eliminate the possibility of contagion from latently infected animals that might suffer a viral reactivation, which is generally subclinical and unpredictable[4]. Despite the self-limiting nature of EHV3 infections and the fact that ECE has no direct effect on fertility or pregnancy, outbreaks of ECE have a negative impact on breeding facilities. This is due to several reasons: the need of temporarily withdrawing stallions and mares from the reproductive activity, the requirement of additional treatment for the affected animals, the extra care necessary to prevent the iatrogenic spread of EHV3, and the occurrence of outbreaks of ECE in artificial insemination and embryo transfer centers[5]. To prevent and control ECE, a specific treatment with an effective antiviral drug would be useful.

Nowadays, there exist various antiviral compounds (acyclic nucleoside analogs and phosphonates) of proven effectiveness for other alphaherpesviruses affecting humans and animals[26]. Acyclovir, valaciclovir and famciclovir are the drugs currently approved for the treatment of mucocutaneous disease caused by human alphaherpesvirus 1 and 2[8,18]; and human alphaherpesvirus 3 (*Varicella-zoster virus*, VZV). Brivudin is also another choice for the treatment of VZV infections as it has demonstrated to be more potent than acyclovir against this virus[8,18]. Moreover, even if ganciclovir and cidofovir have been proven to be efficacious against human alphaherpesviruses, their use is principally restricted to betaherpesvirus infections[8,18]. With regard to antiviral application in animals, goats infected with caprine alphaherpesvirus (CpHV1) have been treated topically with cidofovir with promising results[21–23]. Regarding felid alphaherpesvirus 1 (FeHV1) infection, cidofovir has been added to the list of drugs recommended for the treatment of ocular disease[11,13,15,25]. In turn, acyclovir is used both orally and topically, in combination with human interferon (IFN)-α, for the treatment of ocular FeHV1[13,25]. With respect to equid alphaherpesvirus 1 (EHV1), several nucleoside analogs have been studied *in vitro* with encouraging results. Cidofovir was found to be the most potent antiviral agent in

reducing plaque size of EHV1 when compared with other nucleoside analogs[12]. Furthermore, ganciclovir was found to be effective against EHV1 *in vitro*[3,12,20].

Concerning EHV3, there is limited information about the efficacy of nucleoside analogs to reduce its replication *in vitro*. The effective concentration 50% (known as the amount of drug per ml of medium that reduces the formation of plaques by 50%, EC_{50}) reported for acyclovir and ganciclovir for EHV3 plaque number was 4.25 μg/ml[9] and 0.16 μg/ml[20], respectively. However, there is no information available on the efficacy of cidofovir against EHV3. The aim of the present study was to compare the efficacy of three antiviral compounds, acyclovir, ganciclovir and cidofovir against EHV3 *in vitro*, and to assess their efficacy against six field isolates.

Materials and methods

Drugs

Acyclovir (Cat. No. A4669; ≥99% purity by HPLC), ganciclovir (Cat. No. G2536; ≥99% purity by HPLC) and cidofovir (Cat. No. C5874; ≥98% purity by HPLC), purchased from Sigma–Aldrich, USA were used. The compounds were dissolved in Roswell Park Memorial Institute (RPMI)-1640 (Cat. No. 56510C, Sigma Aldrich) medium at a concentration of 1 mg/ml, and subsequently filtered through 0.45 and 0.22 μm filters and stored at 4 °C as stock solutions.

Cell culture

Equine dermis (EDerm) NBL-6 cell line (Cat. No. CCL-57, ATCC) was grown and maintained in complete medium consisting of Dulbecco Minimum Essential Medium modified (D-MEM) supplemented with 10% fetal bovine serum (FBS). To minimize variability between the experiments, EDerm cells were used only on passages 30–34, inclusive.

Viruses

The EHV3 strain, namely E/9283/07 C3A, isolated from ECE lesions of a polo mare in an embryo transfer facility[6], was used for the *in vitro* comparative assays. To assure the genetic homogeneity of this strain, the virus was cloned by three plaque purifications in EDerm cells and replicated four additional times to obtain sufficient volume for the entire study. In addition, six Argentinian field isolates of EHV3 were selected according to their phenotypic characteristics and genetic diversity in gG gene (based on one-base substitutions at positions 904, 1103 and 1264) to compose a group of viruses that best represents the strains circulating in Argentina. The six isolates of EHV3 were replicated independently in EDerm cells to obtain sufficient volume for the entire study. Viral stocks were aliquoted and stored at −70 °C. Virus infectious titers were estimated by the Reed and Muench method[19] and expressed both as the tissue culture infective dose 50% ($TCID_{50}$) per ml and plaque forming unit (PFU) per ml (Table 1).

Comparison of the efficacy of antiviral compounds by plaque number and plaque size assay

Monolayers of EDerm cells seeded in 12-wells tissue culture plates were infected with 500 μl of a dilution of the virus to obtain up to 30 PFU/well. Overlay medium (D-MEM supplemented with 5% FBS) with carboxymethyl cellulose (CMC) 0.75%, supplemented independently with the different concentrations (0–100 μg/ml) of each antiviral compound (acyclovir, ganciclovir and cidofovir), was immediately added to four wells of the inoculated EDerm cells monolayers. For the comparative analysis of the six EHV3 isolates, only one concentration of each antiviral was used, namely acyclovir at 5 μg/ml, ganciclovir at 0.05 μg/ml and cidofovir at 2 μg/ml. These concentrations have been chosen based on the results obtained previously in the comparative analysis of plaque number, plaque size and viral load on the control strain, E/9283/07 C3A. Cells not infected but treated with each antiviral compound were used as control to test the potential cytotoxicity of the compounds. After an incubation period of 72 h at 37 °C in a 5% CO_2 incubator, the cells were fixed and stained with 0.1% formalin-buffered crystal violet solution[12,16]. The number of plaques in each replicate, at each concentration of the three antiviral compounds and in untreated control wells, were macroscopically counted. The inhibitory effect of the antiviral compounds on plaque number was calculated by the formula already described[12,16].

Plaque size was determined using the IMAGE J program (Wright Cell Imaging Facility, Toronto Western Research Institute, www.uhnresearch.ca/wcif – http://rsb.info.nih.gov/ij), to measure the pictures of a minimum of 16 plaques visualized under an inverted microscope with a magnification of 100×. The size of each plaque was expressed as its area, in pixels (pi). The inhibitory effect of the antiviral compounds on plaque size was calculated by the formula already described[12,16]. The three compounds were tested simultaneously, in three independent experiments.

Comparison of the efficacy of antiviral compounds by viral load determination by quantitative real time PCR and infectious titer

Monolayers of EDerm cells seeded in 12-well tissue culture plates were infected with 500 μl of a dilution of the virus in order to obtain up to 30 PFU/well, as it was previously determined by a plaque titration method. For assessing the antiviral activity against the E/9283/07 C3A strain, overlay medium supplemented with acyclovir (5, 20 and 40 μg/ml), ganciclovir (0.05, 0.5 and 1 μg/ml) and cidofovir (2, 20 and 40 μg/ml) was added after 2 h incubation, and plates were incubated for 72 h, at 37 °C in a 5% CO_2 incubator. For assessing the antiviral activity against the six Argentinian EHV3 isolates only one concentration of each antiviral was used, namely acyclovir at 5 μg/ml, ganciclovir at 0.05 μg/ml and cidofovir at 2 μg/ml. Each antiviral concentration was tested in triplicate. Cells infected with the virus without antiviral treatment were used as reference positive control. To quantify EHV3 load with and without antiviral treatment, aliquots of the supernatant

Table 1 Control strain and field isolates of EHV3 used to evaluate *in vitro* efficacy of acyclovir, ganciclovir and cidofovir.

	Identification	Origin	Infective Titer		Phenotypic differences		Genetic differences at positions		
			Tissue culture infective dose ($TCID_{50}$)	Plaque forming unit (PFU)	Plaque size[a]	Clinical manifestation	904	1103	1264
Control	E/2983/07-3	E-Polo horse	10^6	$10^{5.53}$	Large	Typical ECE	C	A	G
1	E/3900/08-1	A-Thoroughbred	$10^{5.5}$	$10^{3.38}$	Small	Typical ECE	A	C	T
2	E/3900/08-1	A-Thoroughbred	$10^{4.66}$	$10^{4.19}$	Small	Typical ECE	A	C	T
3	E/1333/07-2	B-Thoroughbred	$10^{5.5}$	$10^{4.49}$	Large	Typical ECE	A	C	T
4	E/976/07-154	A-Thoroughbred	10^5	$10^{4.80}$	Large	Subclinical	A	C	T
5	E/1331/07-5	C-Thoroughbred	10^5	$10^{4.74}$	Large	Typical ECE	C	A	G
6	E/3767/08-11	D-Polo horse	10^5	$10^{4.77}$	Large	Typical ECE	C	A	G
Reference	Cornell University	–	–	–	Standard	–	A	A	T

[a] Plaque size (arithmetic mean of the sizes of 20 lysis plaques) compared with the plaque size obtained for the reference strain (standard): Small was 1.64 times larger than the reference strain; Large was 2.88 times larger than the reference strain.

of each concentration of the antiviral compounds were taken at serial times post infection: for strain E/9283/07 C3A 0, 24, 48 and 72 h and for the six field isolates 0, 48 and 72 h. DNA was extracted from those samples by a commercial kit (High Pure PCR Template Preparation Kit, Cat No. 11796828001, ROCHE®), following the manufacturer's instructions. A quantitative real time PCR (qPCR) assay, described by Barrandeguy et al.[6], which targets a region of the EHV3 glycoprotein G (gG) gene was conducted. Briefly, the 25 μl reaction contained 12.5 μl of TaqMan Universal Master Mix™ (Applied Biosystems®), 5 μl (1 μM) of TaqMan® specific probe (5′-FAM-TGTGTCTCCTCATCGGCCTCATTGTCT-TAMRA-3′) and 1.5 μl (10 μM) of both, the forward (5′-GGGTATCGGCTTTCTCATCTTG-3′) and the reverse 5mst Real-time PCR System (Applied Biosystems®, Life Technologies, Grand Island, NY). The cycling program included 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles at 95 °C for 5 s and 60 °C for 1 min. To assure the sensitivity of qPCR, the infectious virus titer was estimated by the Reed and Muench method[19] and expressed as $TCID_{50}$.

Each PCR run contained two negative controls and 10-fold serially diluted E/9283/07 C3A strain to generate the standard curve. These dilutions had previously been quantified in terms of $TCID_{50}$/ml and PFU/ml. Viral load was derived from Ct using the standard curve generated in parallel and expressed as the number of DNA copies (or viral equivalents) per volume unit. The percentage of inhibition was calculated by the formula already described by other authors[12,16] and properly adapted to calculate viral load inhibition:

$$\text{Percentage inhibition} = \frac{1 - (\text{DNA copies})\,\text{antiviral}}{(\text{DNA copies})\,\text{control}} \times 100\%$$

For assessing the antiviral activity against the E/9283/07 C3A strain, the three compounds (each one at three concentrations) were tested simultaneously in two independent experiments. For the analysis of the antiviral activity against the six EHV3 isolates, the three antivirals (each one at one concentration) were tested simultaneously in three independent experiments.

Statistical analysis

The EC_{50} for both plaque number and plaque size was obtained by a non-linear regression fit from a symmetrical sigmoidal dose (log inhibitory)–response curve using the Variable Slope model, which fitted the Hill Slope from the data, using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA (www.graphpad.com). The statistical analysis for plaque number and plaque size assays was based on analysis of variance (ANOVA) using the MedCalc version 12.7.0.0 for Windows XP/Vista/7/8 (www.medcalc.org).

For both the quantification by qPCR and viral infectious titer, a non-linear regression fit from a symmetrical sigmoidal dose–response curve was obtained using the Variable Slope model as described above. The statistical analysis was based on the analysis of the area under the curve (AUC) calculated using the MedCalc version 12.7.0.0 for Windows XP/Vista/7/8 (www.medcalc.org).

Regarding the analysis of the antiviral activity of the six field isolates, for the three parameters analyzed, a two-way ANOVA was carried out, using InfoStat version 2010, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina.

Results

Comparison of the efficacy of antiviral compounds by plaque number and plaque size assay

The three tested compounds were able to reduce plaque number and plaque size. Complete inhibition (100%) of EHV3 replication was achieved with the three compounds as seen in the dose–response curves (Fig. 1). The most remarkable effect on EHV3 for plaque number and size was observed with ganciclovir. Cidofovir displayed a lower antiviral activity and acyclovir was the least effective compound to reduce both evaluated parameters (Fig. 1). The three compounds were more effective in reducing plaque size than plaque number. No toxic effect on the EDerm cells was observed

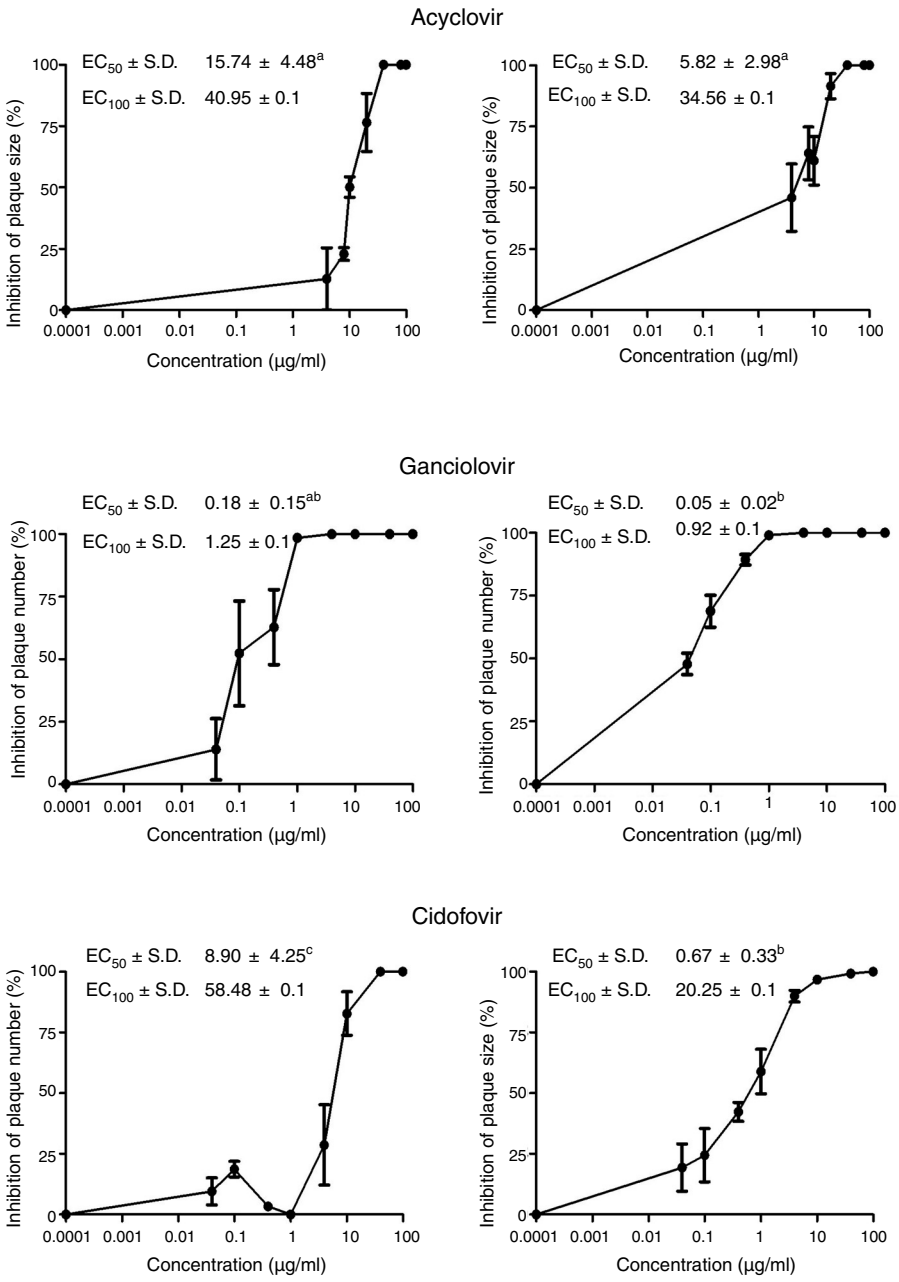

Figure 1 Dose–response curves representing the activity of acyclovir, ganciclovir and cidofovir to reduce number and size of control strain, E/79283/07 C3A, induced plaques in EDerm cells. Data are presented as the mean value of three independent assays and standard deviation. The EC_{50} and EC_{100} of antiviral compounds on plaque number and size are shown in each graph. Means with different superscript letters differ significantly for each parameter evaluated (one-way ANOVA).

with these compounds even at concentrations as high as 100 μg/ml.

Comparison of the efficacy of antiviral compounds by determination of viral load by qPCR and infectious titer

All three compounds completely inhibited (100%) viral replication at 72 h post infection, at least with one of the concentrations tested. Figure 2 shows the viral load obtained at different concentrations of each antiviral compound through time, both by qPCR and $TCID_{50}$. Viral production as estimated by qPCR was inhibited 98% at 48 h and 99% at 72 h with acyclovir at 20 μg/ml, ganciclovir at 0.5 μg/ml and cidofovir at 2 μg/ml. Lower concentrations of acyclovir (5 μg/ml) and ganciclovir (0.05 μg/ml) produced 97% and 96% inhibition, respectively, at 72 h post infection. As for viral production estimated by $TCID_{50}$, an inhibition of 98% at 48 h was achieved with ganciclovir at 0.5 μg/ml and an inhibition of 100% was achieved with acyclovir at 5 μg/ml and cidofovir at 2 μg/ml. Based on these results with strain E/9283/07 C3A, we selected one concentration (optimum concentration) of each drug to assess the susceptibility of

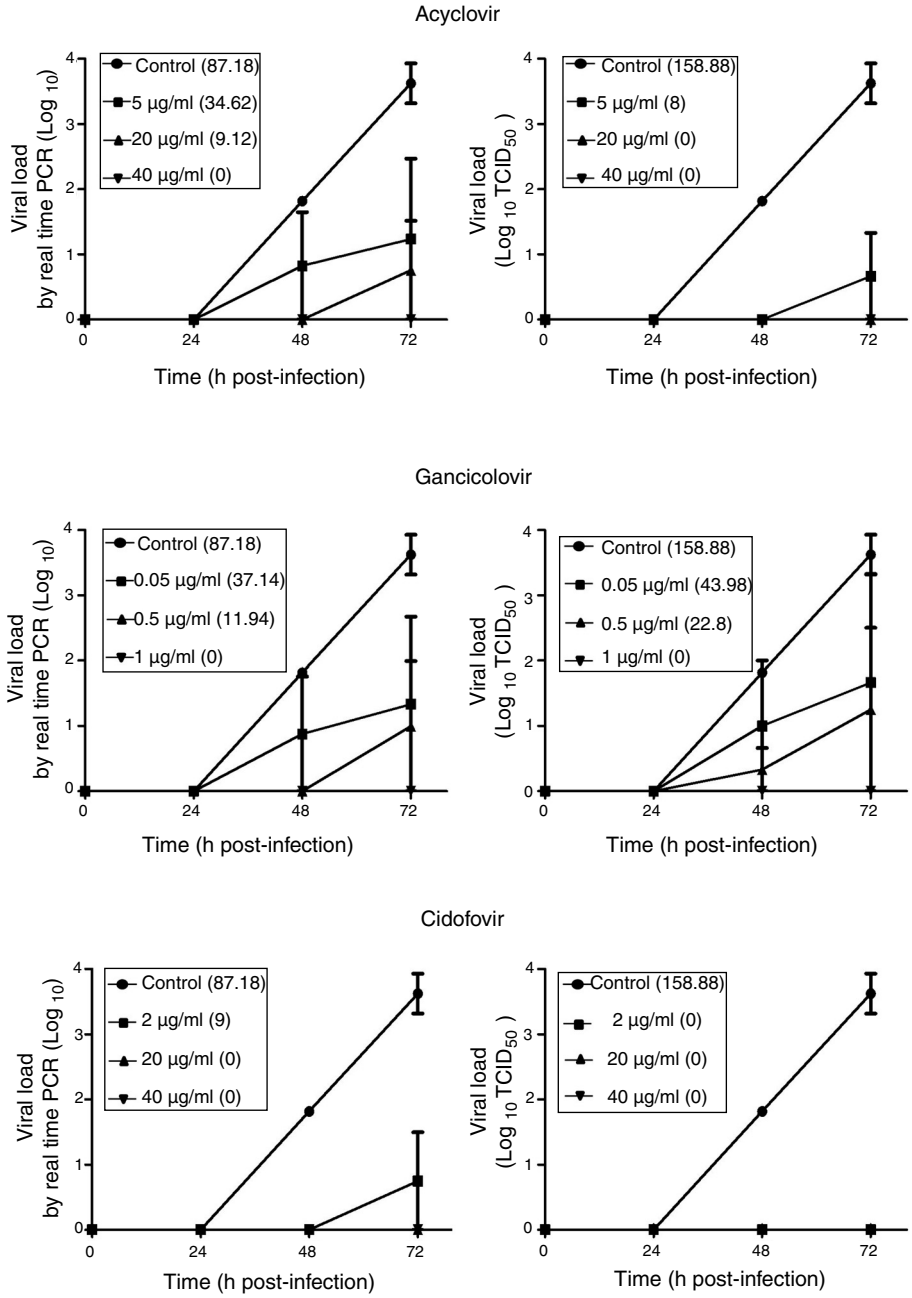

Figure 2 Viral production by qPCR and $TCID_{50}$ obtained with three concentrations of acyclovir, ganciclovir and cidofovir and without treatment (control) at different times post-infection of EDerm cells with the control strain, E/9283/07 C3A. AUC values are presented in brackets.

six different isolates of EHV3. This concentration (of each compound) was selected as the ones that produced more than 95% inhibition of viral load by qPCR in tissue cultures at 72 h. Then, we selected: 5 μg/ml of acyclovir, 0.05 μg/ml of ganciclovir and 2 μg/ml of cidofovir.

Susceptibility of field isolates to each antiviral compound at a selected optimum concentration

The susceptibility of EHV3 field isolates to acyclovir (5 μg/ml), ganciclovir (0.05 μg/ml) and cidofovir (2 μg/ml) was similar among the compounds, and was in accordance with the results obtained for strain E/9283/07 C3A, referred thereafter as the control strain. With respect to plaque size, interaction was shown between the tested antiviral compounds and the different field strains ($p < 0.0048$). The effect of the three antiviral compounds on plaque size was then studied separately for each strain (Fig. 3). For field strains 1, 2 and 5, the three compounds significantly reduced plaque size ($p < 0.05$). For field strain number 3, there was a trend of reduction by the three compounds; however, it was not significant; while for field strains 4, 6 and the control strain, only acyclovir 5 μg/ml showed a

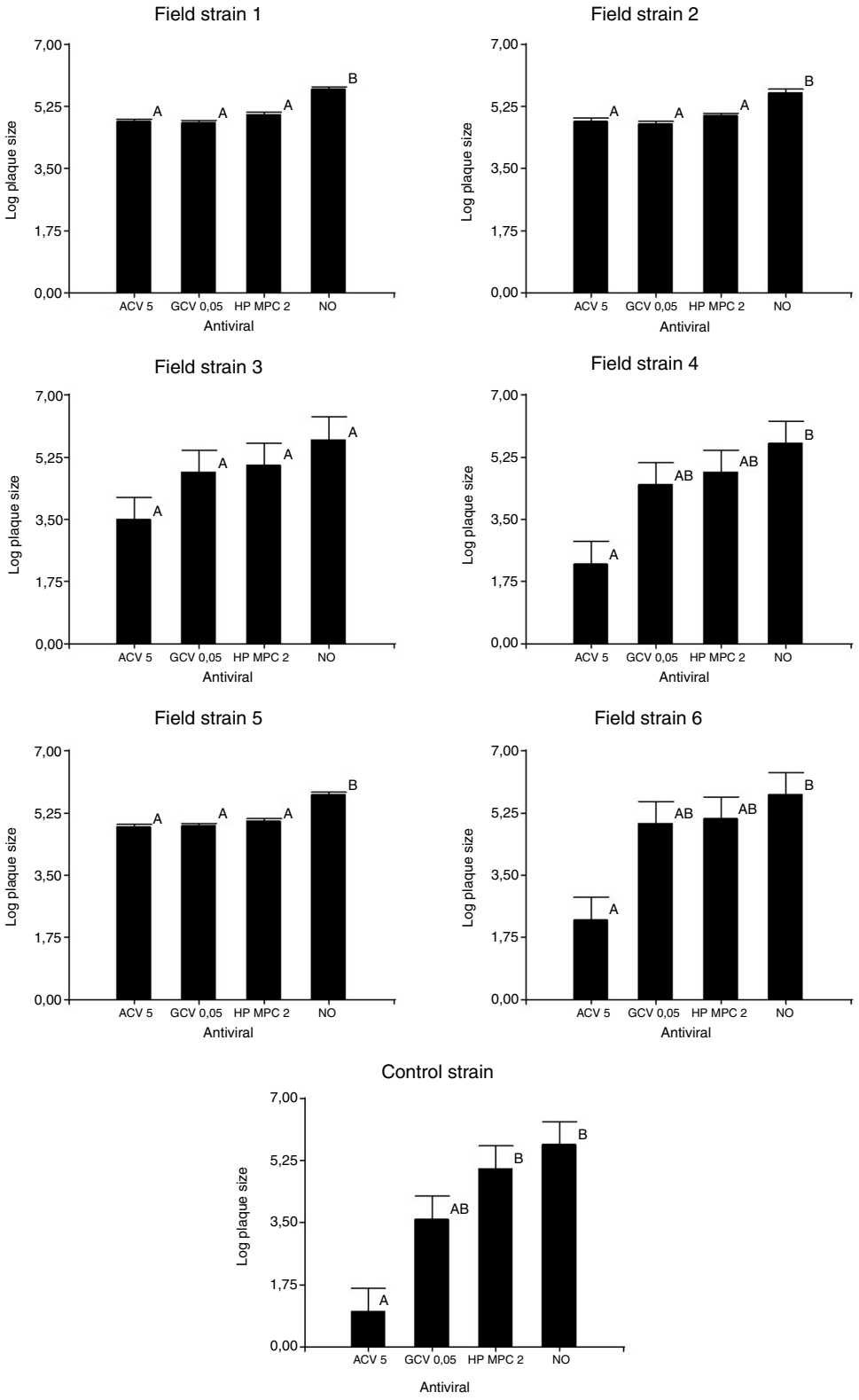

Figure 3 Effect of acyclovir at 5 μg/ml (ACV 5), ganciclovir at 0.05 μg/ml (GCV 0.05) and cidofovir at 2 μg/ml (HPMPC 2) on the size of virus-induced plaques on cells infected with different field strains (n = 6) and the control strain. Columns with different superscript letters differ significantly (two-way ANOVA; Tukey test; $p < 0.05$).

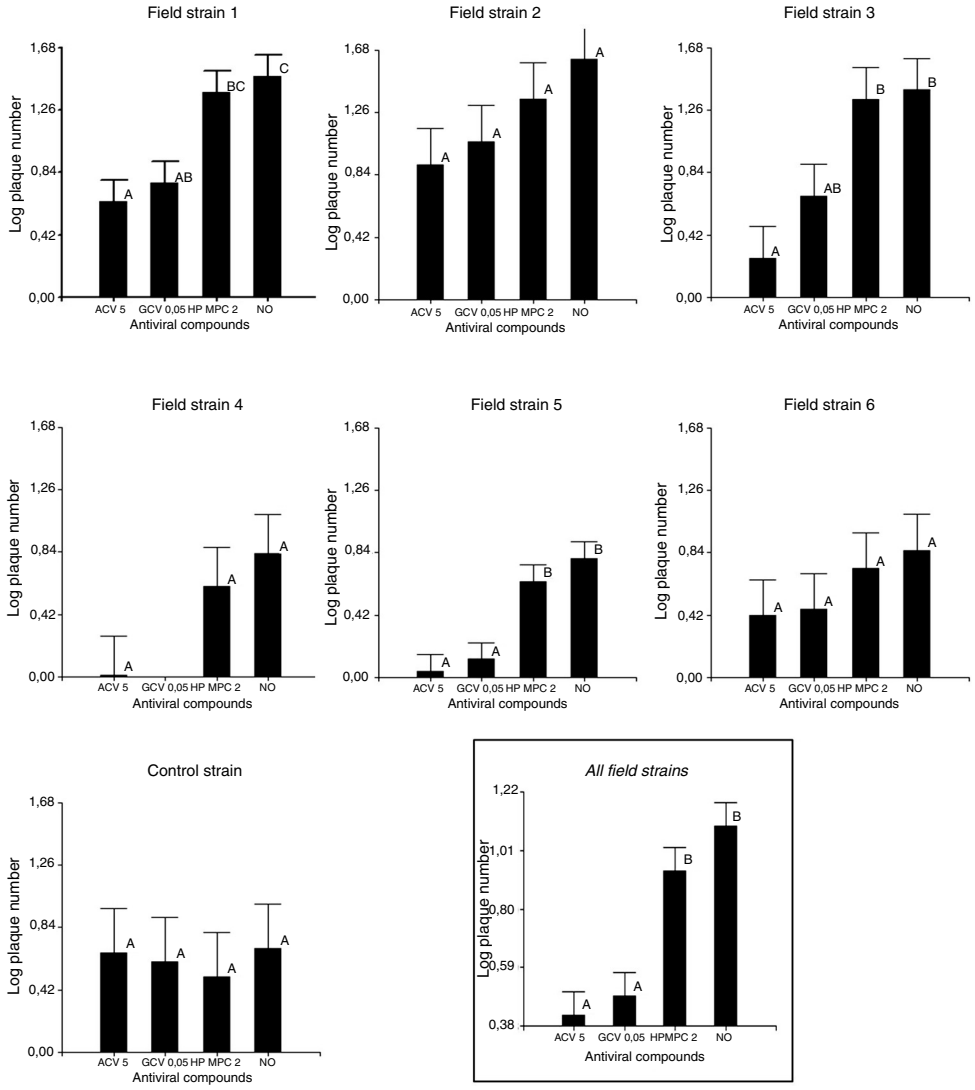

Figure 4 Effect of acyclovir 5 μg/ml (ACV 5), ganciclovir 0.05 μg/ml (GCV 0.05) and cidofovir 2 μg/ml (HPMPC 2) on the number of virus-induced plaques on cells infected with different field strains (n = 6) and the control strain. The effect of each compound is also shown on the average of all field strains and the control strain. Columns with different superscript letters differ significantly (two-way ANOVA; Tukey test; $p < 0.05$).

significant reduction in plaque size. When studying reduction of plaque number and viral load determined by qPCR at 48 h, no interaction between the two variables (field strain and antiviral compound) was observed. However, for consistency, the effects of the three antiviral compounds were presented both separately for each strain and in average for all the strains (Figs. 4 and 5). For plaque number, acyclovir and ganciclovir were the most effective compounds, both of them producing a significant reduction of the plaque number (Two way ANOVA, $p < 0.0001$). With regard to viral load at 48 h, acyclovir was the most effective compound, followed by ganciclovir and cidofovir, all of them inducing a significant reduction in viral load for all field strains (Two way ANOVA, $p = 0.0002$). Table 2 shows a summary of the efficiency of each compound against different field strains *in vitro*. Acyclovir and ganciclovir showed similar efficacy against EHV3 field strains considering the 3 evaluated parameters. Nevertheless, ganciclovir (0.05 μg/ml) was the most potent compound as it produced similar results with a lower concentration than acyclovir (5 μg/ml).

Discussion

In the present study the efficacy of acyclovir, ganciclovir and cidofovir was demonstrated against EHV3 *in vitro* by simultaneous comparison of these three compounds by plaque number, plaque size and viral load by infectious titer and qPCR. The obtained results extend those already available[9,20] for acyclovir and ganciclovir. In addition, the study reveals that cidofovir is the least efficacious among the three tested antivirals. Moreover, the efficacy of a selected optimum concentration of each antiviral compound was tested *in vitro* against a panel of six field isolates, allowing the selection of ganciclovir as the most potent antiviral to be used against EHV3.

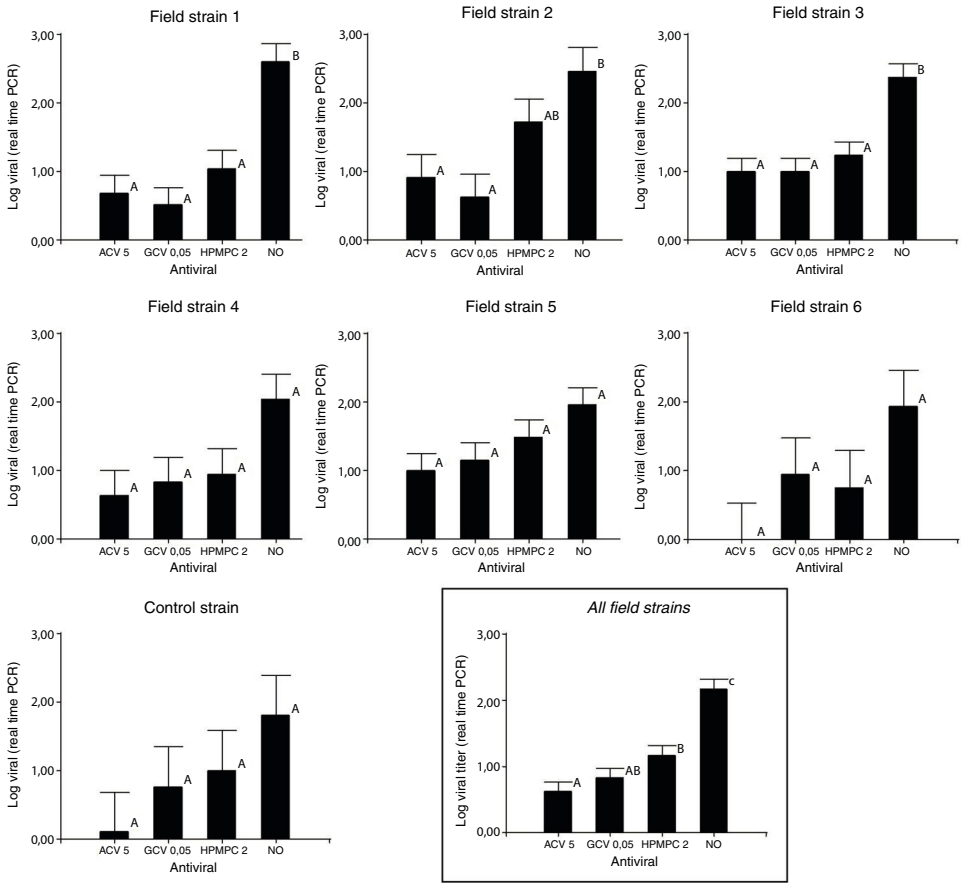

Figure 5 Effect of acyclovir at 5 μg/ml (ACV 5), ganciclovir at 0.05 μg/ml (GCV 0.05) and cidofovir at 2 μg/ml (HPMPC 2) on the viral titer determined by qPCR on cells infected with different field strains (n = 6) and the control strain. The effect of each compound is also shown on the average of all field strains and the control strain. Columns with different superscript letters differ significantly (two-way ANOVA; Tukey test; $p < 0.05$).

Table 2 Efficacy of antiviral compounds with selected concentrations on different field strains of EHV3, expressed as percentage of inhibition.

	Acyclovir 5 μg/ml	Ganciclovir 0.05 μg/ml	Cidofovir 2 μg/ml
Reduction of plaque size	84–100%	84–92%	76–85%
Reduction of plaque number	76–96%	58–93%	13–47%
Reduction of EHV3 production 48 h post-infection	88–100%	87–100%	58–100%

All three compounds exhibited an effect on viral replication, demonstrated by the three investigated parameters: plaque number, plaque size and viral load, although with variable efficacy. EDerm cells used in all assays did not evidence any change that could be associated with a cytotoxic effect of the compounds. Indeed, the toxic concentrations in equine derived cells are far above the concentrations associated with EHV3 antiviral activity[12].

The EC_{50} to reduce the plaque number of ganciclovir determined in our study was in concordance with the values previously reported[20]; however, for acyclovir, the value was three times higher than that found by Cullinane et al.[9] However, in the latter case, the EHV3 strain had not been determined in contrast to our study, where the EC_{50} values were obtained with a unique strain of EHV3, which was characterized and plaque purified to ensure homogeneity. Ganciclovir showed the lowest EC_{50}, followed by cidofovir, while the EC_{50} obtained for acyclovir was the highest one.

For the compounds evaluated in this study, the EC_{50} to reduce plaque size was lower than the EC_{50} to reduce plaque number, as also observed for another alphaherpesvirus, FeHV1[12,16]. In contrast, the three compounds behave differently between the two equid alphaherpesviruses, EHV1 and EHV3. For EHV1, the EC_{50} of acyclovir and ganciclovir for plaque size was almost the same as the one for plaque number although it was 3 times lower for EHV3. In contrast, for EHV1, cidofovir had an EC_{50} for plaque size that was 40-fold lower than for plaque number although it was only 12-fold lower for EHV3[12].

The determination of viral load at selected concentrations of antiviral compounds showed that acyclovir at 20 μg/ml, ganciclovir at 1 μg/ml and cidofovir at 2 μg/ml completely inhibit EHV3 replication *in vitro*. In addition the results showed that qPCR is more sensitive than $TCID_{50}$ to determine the efficacy of antiviral compounds as previously described for other alphaherpesviruses[14,24].

All investigated EHV3 field strains as well as the control strain exhibited similar susceptibility to selected optimum concentrations of acyclovir, ganciclovir and cidofovir. With regard to EHV1, the so-called neuropathogenic and non-neuropathogenic strains can be differentiated by one mutation in the DNA polymerase gene[17,27]. These two types of strains have comparable susceptibility to acyclovir and ganciclovir but not to cidofovir, which was found to be strain-dependent[12]. Such mutations in the DNA polymerase, which could potentially affect the susceptibility of the enzyme to the antivirals used, have not been identified in EHV3.

From our results, cidofovir was the least effective compound against different field strains of EHV3 related to the three investigated parameters. This could be attributed to the fact that the concentration used for this assay was below the EC_{50} for the plaque number previously determined in this study. However, for acyclovir and ganciclovir, the efficacy obtained for the evaluated criteria was similar at the selected concentrations. Ganciclovir was the most potent compound as it produced similar results as acyclovir but at a lower concentration.

Conclusions

From this study, it can be concluded that ganciclovir at 0.05 μg/ml displays the best overall inhibitory activity *in vitro* against EHV3. Considering the pathogenesis of the infection, especially the lesions localized in the genital area, ganciclovir in a topical formulation should be considered as a valuable candidate for the treatment and prevention of ECE in mares and stallions.

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Conflict of interest

The author declare there is not conflicts of interests

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