INVESTIGATION

Herpes simplex virus: isolation, cytopathological characterization and antiviral sensitivity

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Abstract: BACKGROUND: Herpes simplex virus (HSV) infection is an endemic disease and it is estimated that 60-95% of the adult population are infected with symptoms that are usually self-limiting, though they can be serious, extensive and prolonged in immunocompromised individuals, highlighted by the emergence of drug-resistant strains. The study of the wild-type HSV strains based on the cytopathogenic features and its antiviral sensitivity are important in the establishment of an antivirogram for controlling the infection.

OBJECTIVE: This study sought to isolate and examine the cytopathological characteristics of circulating strains of the Herpes simplex virus, from clinical specimens and their sensitivity to commercially available antiherpervirus drugs, acyclovir, phosphonophormic acid and trifluridine.

METHODS: Herpes simplex virus isolation, cytopathological features and antiviral sensitivity assays were performed in cell culture by tissue culture infectious dose or plaque forming unit assay.

RESULTS: From twenty-two clinical specimens, we isolated and adapted nine strains. Overall, the cytopathic effect was detected 24 h post-infection (p.i.) and the presence of syncytia was remarkable 48 h p.i., observed after cell staining. Out of eight isolates, four developed plaques of varying sizes. All the isolates were sensitive to acyclovir, phosphonophormic and trifluridine, with the percentage of virus inhibition (%VI) ranging from 49.7-100%.

CONCLUSIONS: The methodology for HSV isolation and characterization is a straightforward approach, but the drug sensitivity test, regarded as being of great practical importance, needs to be better understood.

Keywords: Antiviral agents; Herpesvirus 1, human; Herpesvirus 2, human

INTRODUCTION

Herpes simplex virus (HSV) or human herpesvirus (HHV) is a member of the Herpesvirales order, Herpesviridae family, Alphaherpevirinae subfamily, Simplexvirus genus. HSV is an enveloped virus with a genome consisting of a large linear molecule of 152 kb double-stranded DNA. HSV genes are classified into three groups: immediate early (IE), early (E), and late (L) genes that encode regulatory proteins of replication, replication proteins and structural proteins. The virus establishes latent infection in sensory neurons and ganglia (trigeminal and sacral) for the lifetime of the host. During latency, there is no expression of viral genes, but some transcripts, known as LAT (Latency-Associated Transcripts) can be detected and found at high levels. There are two serological types, 1 and 2, which mostly cause mucocutaneous infections, resulting in recurrent lesions. HSV-1 is associated primarily with orofacial infections, whereas HSV-2 is generally associated with anogenital infections, transmitted through sexual contact. HSV infection is endemic in all human populations and it is estimated that 60-95% of the adult population is infec-
ted by at least one of the species. The symptoms associated with both virus types are usually self-limiting in healthy individuals, but can be extensive and prolonged in immunocompromised individuals.

In this study, we isolated and studied the cytopathological features of circulating strains of HSV from clinical specimens collected from in- and outpatients with either oral or genital lesions, clinically compatible with herpes simplex infection. Moreover, we analyzed the sensitivity of these isolates against commercially available antiviral drugs, acyclovir (ACV), phosphonoformic acid (PFA) and trifluridine (F3T).

**MATERIAL AND METHODS**

### 2.1 Cells and virus

HEp-2 cell cultures (human larynx epithelial cells carcinoma - ATCC, CCL-23) were grown in Dulbecco’s Modified Eagle Medium (DMEM) (*Gibco-BRL, EUA), supplemented with 10% fetal bovine serum (*), 100 µg/ml streptomycin (**) and 2.5 µg/ml amphotericin B (Bristol Myers-Squibb, Brasil).

Cell monolayers were used for experiments after a growth period of approximately 48-72 h. Trypsin/versene were used as cell dispersing agents.

Standard HSV-1 supplied by the Departamento de Virologia/IMPG/UFRJ, Rio de Janeiro-RJ, was used as internal control.

**Antiviral drugs**

Acyclovir - ACV (Zyvir, New Farma, Brazil), phosphonoformic acid - PFA (Foscarnet, Hospira, USA) and trifluridine - F3T (Viropic, Monarch Pharmaceuticals, USA) were used. PFA and F3T were kindly donated by Trade Farma, Brazil.

**Clinical specimens**

Clinical specimens were collected between June 2009 and June 2010, of patients aged 12-53, male and female, in- and outpatients of the Hospital Universitario Regional do Norte do Parana - Londrina (HUNRPr/UEL), and a few from university staff and students. Informed consent form was signed by all participants. The project was approved by the UEL Ethics Committee under the registration n° 225/09 PF.

Twenty-two specimens were collected with swabs from skin/mucosa blisters or wounds in areas including the mouth (13), oral cavity wound (1), genitalia (2), perianus (2), conjunctiva (1), leg (1), buttocks (1) and back (1). The specimens were collected in DMEM containing penicillin (1000 IU/ml), streptomycin (1000 µg/ml) and amphotericin B (25 µg/ml), and kept at 4-8°C until laboratory manipulation. The specimens were clarified by centrifugation at 1000 xg for 10 minutes, in order to remove cell debris.

### Cell culture

Clarified specimens were inoculated into HEp-2 cell cultures established in tubes, as previously carried out. Briefly, 48 h cell cultures were inoculated with 100 µl of the clarified suspension. The cultures were observed for approximately 10 days, in order to monitor for the cytopathic effect (CPE). Up to three blind passages were performed for negative cultures by submitting inoculated cultures to three freezing and thawing cycles, and homogenates were then re-inoculated. Negative specimens after three blind passages were discarded. Following serial passages in cells, adapted strains were stored at -20°C with 10% glycerin.

**Hematoxylin-eosin (HE) staining.**

Cell cultures infected with the isolates were subjected to HE staining for better analysis of the CPE. Fifty to sixty percent of confluent HEp-2 cells grown in coverslips were infected with 200 µl of the isolates diluted to the tenth. The cultures were monitored for 24-72 h for the development of CPE. Cultures on coverslips were washed, fixed with phosphate-buffered saline (PBS)/methanol (7 vol:1 vol), (1 vol:1 vol), and finally with undiluted methanol, and stained with HE solution. Coverslips with stained cultures were mounted on slides and observed under a microscope.

**Cytotoxic assay of the antivirals**

The cytotoxicity of the commercially available antiviral drugs was determined by MTT kit (dimethylthiazol-2,5-diphenyl tetrazolium bromide) (***) in HEp-2 cells, according to the manufacturer’s instructions. HEp-2 cells grown in 96-well microplates (Nunc A/S, Denmark), at 37°C under 5% CO₂ in a confluence of 70%, were submitted to the treatment with varying concentrations of the antiviral drugs. The following concentrations were used: ACV (0.156, 0.312, 0.625, 1.25, 2.5, 5.0, 10 mg/ml), PFA (0.312, 0.625, 1.25, 2.5, 5.0, 10 mg/ml) and F3T (0.078, 0.156, 0.312, 0.625, 1.25, 2.5 mg/ml). The percentage of cell viability was calculated as follows, 100 - (A_t/A_c) x 100, where A_t and A_c refer to absorbance with the drug and control (untreated cells), respectively. The 50% cytotoxic concentration (CC₅₀) was determined as the concentration of the drugs capable of reducing absorbance by 50%, in comparison with the control by regression analysis.

**Plaque reduction assay**

HEP-2 cells grown in 24-well plates (TPP, Switzerland), with 95-100% confluence, were infected with the isolates and incubated at 37°C under 5% CO₂ for 1 h. The cell monolayer was washed and overlaid with nutrient agarose (DMEM [2x] and 1.8%...
DISCUSSION

The study demonstrated the isolation and adaptation of HSV from clinical specimens in cell culture. Further, the cytopathological features of the isolates were assessed, in addition to their sensitivity to some commercially available antiherpesvirus drugs. The development of the CPE was used to monitor the isolation and adaptation of the isolates in fresh preparation or after cell HE staining. HEP-2 cell cultures were used as the cell of choice for their susceptibility. The CPE was observed 24-48h after the inoculation of the clinical specimens, as described.
Infected cells presented increased cytoplasm refringence, cell rounding and clumping, initially in a focal pattern. Later, this effect was disseminated throughout the cell monolayer, in accordance with previous results. Characteristically, giant multinucleated cells were observed after HE staining of infected cells, as previously shown in epithelial keratinocytes. The isolation of HSV in cell culture as a diagnostic method for HSV infection has been recognized for its efficiency, rapidity and low cost, as has been demonstrated elsewhere. Furthermore, HSV plaque size has been used to distinguish HSV-1 from HSV-2. In those isolates that produced plaques of varying sizes, there was a prevalence of large plaques, suggesting the occurrence of mutants. It was shown that HSV wild and mutant strains produced plaques with diameters of 0.7-1.2 mm and 1.4-2.4 mm, respectively, and it was suggested that large plaque mutants be selected after several passages in cell cultures. Concerning the sensitivity of the isolates to commercially available antiviral drugs, we demonstrated that ACV, PFA and F3T inhibited the isolates to varying degrees, from 49.7-100%, based on the percentage of viral inhibition. The study of HSV sensitivity antiviral drugs is a valuable tool for evaluating HSV infections, particularly in immunocompromised patients, with respect to the emergence of ACV-resistant mutants. Yet today, there is no consensus between in vitro drug susceptibility and drug resistance in vivo for a given virus infection. However, clinical studies have demonstrated a significant correlation of in vitro results with the corresponding treatment response. It has been shown that both assays based on plaque reduction and infectious dose reduction are leading methods for evaluating drug sensitivity. The importance of in vitro evaluation of virus sensitivity to drugs is highlighted in understanding the mechanisms of resistant emergence, drug concentration and the limit of susceptibility and resistance, amongst other factors.

### CONCLUSION

In conclusion, we suggest that the methodology for HSV isolation and characterization is a straightforward approach, though the drug sensitivity test, considered to be of great practical importance, needs to be better understood.
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