Journal of Antimicrobial Chemotherapy (1999) **44**, 705–708

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Cytopathic effect inhibition assay for determining the in-vitro susceptibility of herpes simplex virus to antiviral agents

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We compare a rapid dilution method for the determination of antiviral susceptibility of herpes simplex virus (HSV) with the plaque reduction assay. A total of 84 HSV clinical isolates were studied by both methods to detect in-vitro resistance to acyclovir and foscarnet. The rapid method showed for the detection of HSV isolates resistant to acyclovir and foscarnet, a sensitivity of 96.8% and 100% and specificity of 100% and 100%, respectively. This method provides an easy and accurate screening procedure for the susceptibility testing of HSV to antiviral agents.

Introduction

Herpes simplex virus (HSV) produces common infections that range from self limiting localized ulcers to severe disseminated infections, especially in immunocompromised patients.[1,2] Usually, acyclovir and foscarnet are employed in the successful treatment of HSV infections;[2] but recent reports indicate treatment failures due to infections by antiviral-resistant HSV.[3–7]

In-vitro susceptibility of viruses to antiviral agents is normally measured as the inhibitory concentration 50% (IC_{50}), that is the concentration of antiviral that lowers the virus-induced cytopathic effect (CPE) and the number of plaques formed by a given inoculum, by 50%.[8] Antiviral methods for the determination of susceptibility in HSV include the plaque reduction assay (PRA), dye uptake (DU), CPE inhibition, virus yield reduction, inhibition of specific immunofluorescence and DNA hybridization. PRA has classically been considered the reference method of choice.[9] However, all these methods are tedious and time consuming.

In this study we evaluate a rapid method for the determination of HSV susceptibility to antivirals, in comparison with the PRA.

Materials and methods

HSV isolates

We studied a total of 84 HSV clinical isolates collected at the Virology Laboratory of our hospital between September 1991 and December 1995. Forty-three were HSV-1 and 41 were HSV-2.

A wild-type HSV isolate, an acyclovir-resistant laboratory mutant (presumptive thymidine kinase mutant) and an acyclovir- and foscarnet-resistant laboratory mutant (presumptive DNA polymerase mutant) obtained from the Centro Nacional de Microbiología, Virología e Inmunología Sanitarias, Instituto de Salud Carlos III were used as control strains.

Isolates were maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum (FCS) at –70°C prior to the study.

Once thawed, isolates were inoculated on to Vero cell monolayers until 75% or more of the monolayer showed a CPE. They were then centrifuged and the supernatant used as sample for the susceptibility study; an aliquot of the supernatant was stored in single use sterile tubes at –70°C.

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Antiviral agents tested

Acyclovir was provided by Glaxo–Wellcome, Madrid, Spain and foscarnet by Sigma Chemicals, Madrid, Spain.

Reference assay

The reference method was the PRA, performed according to the American Society for Microbiology guidelines,[8] with adaptation for use with 96-well flat-bottomed sterile plates and lids. Titration of the isolates was performed in order to obtain inocula concentrations of 200–400 pfu/mL. An HSV isolate was considered susceptible to acyclovir when the IC_{50} was <2 mg/L and resistant when the IC_{50} was $\geqslant 2$ mg/L. In the case of foscarnet, an HSV isolate was considered susceptible when the IC_{50} was <50 mg/L, intermediate when it was between 50 and 100 mg/L, and resistant when the IC_{50} was $\geqslant 100$ mg/L.[8]

New rapid method

The method evaluated was a qualitative method based on the PRA. Eight serial 1:10 dilutions of the viral isolates were inoculated on to Vero cell monolayers growing in 96-well plates, containing EMEM with 2% FCS as diluent, 1% glutamine and antimicrobials (penicillin, streptomycin and amphotericin B). For every HSV isolate, three sets of 1:10 serial dilutions were used, the first without any antiviral agent, the second with acyclovir (2 mg/L) and the third with foscarnet (100 mg/L). Antiviral agents were omitted in the first well of every set in order to act as growth controls. The lack of toxicity of growth medium on the Vero cells was confirmed by the inclusion of control wells.

Plates were then incubated at 37°C with 5% CO_2 for 72 h. After this incubation period, formation of a CPE was observed using an inverted light microscope.

Interpretation of results was performed by comparing the titre obtained in the sets without antiviral agent with those obtained in the sets containing each antiviral agent. The titre of an HSV isolate was the highest dilution of the isolate at which HSV growth was detected. If the titre obtained in the set without antiviral agents was at least one $\log_{10}$ greater than that obtained in the wells containing antiviral agent, then the isolate was considered susceptible to that particular antiviral agent. If titres obtained with and without antiviral were equal, meaning that the isolate was not inhibited by the antiviral, then the isolate was considered resistant to that agent (Figure). Since this method is based on serial dilutions of the initial inoculum, susceptibility results should not be affected by the viral load.

Results

Sixty-one isolates were susceptible and 21 resistant to acyclovir by both methods. Two were resistant to acyclovir using the rapid method but susceptible using the PRA, (IC_{50}s of 0.5 and 1 mg/L). No false-negative results were obtained (Table). These data mean that, for acyclovir, the

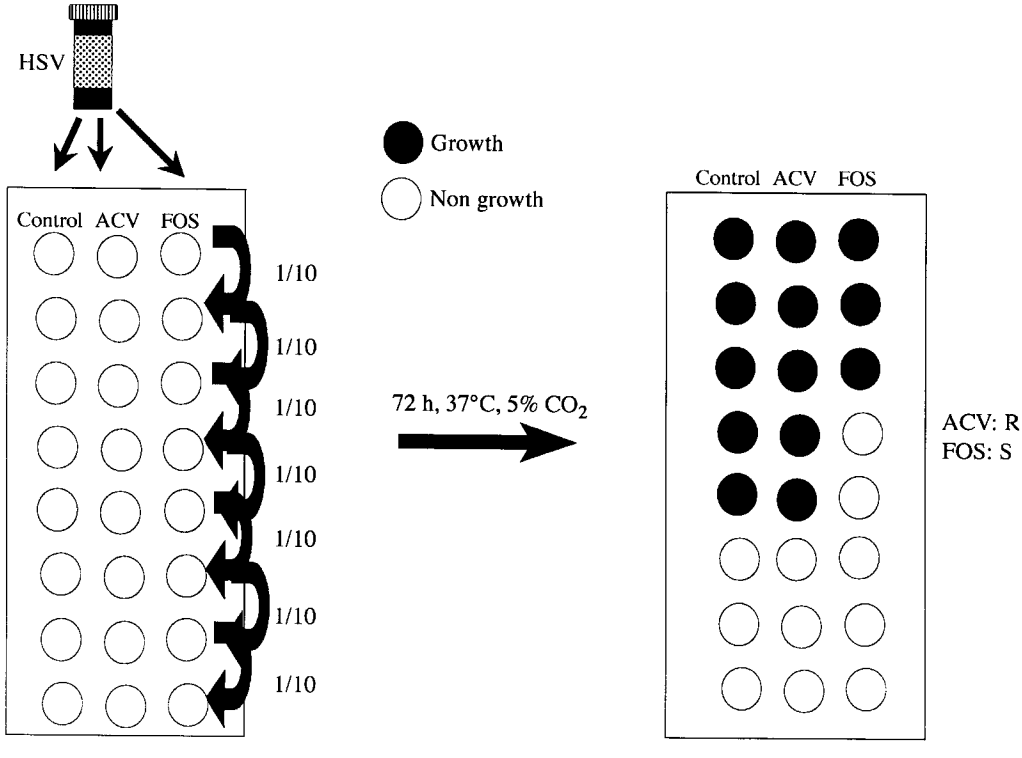

Figure. Graphical representation of the rapid method for testing HSV antiviral susceptibility and its interpretation. ACV, acyclovir; FOS, foscarnet; R, resistant; S, sensitive.

Table. Comparative susceptibility results of HSV to acyclovir and foscarnet by the rapid method and the plaque reduction assay

	Rapid method assay		Plaque reduction assay	
	susceptible[a]	resistant[a]	susceptible[a]	resistant[a]
Acyclovir	61	23	63	21
Foscarnet	81	3	81	3

[a]Number of isolates.

rapid method has 100% specificity, 96.8% sensitivity, 91.3% predictive value of the 'resistant result' and 100% predictive value of the 'susceptible result'.

Eighty-one isolates were susceptible and three were resistant to foscarnet by both methods (Table), one of these had an IC_{50} of 50 mg/L (determined using the PRA), thus being intermediate, but was considered as resistant for the purpose of this work. These data give values of specificity, sensitivity, predictive values of 'resistant and susceptible results' for foscarnet of 100%, although caution is required, as only three isolates were found to be resistant to foscarnet.

Discussion

In-vitro determination of HSV susceptibility to antiviral agents is becoming a necessary tool for the clinical management of severe HSV infections in immunocompromised hosts due to the rapid increase in resistance to antiviral agents, especially acyclovir. Antiviral resistant HSV infections are, in many cases, associated with worse prognosis and progression of the disease than susceptible HSV infections.[5,6] Rapid methods of susceptibility testing are therefore needed.

The rapid method we have described is simpler to perform than the PRA and requires no titration of inoculum. It also does not require the use of radioactive material. The rapid method takes only 72 h to perform. This is compared with the 5–7 days needed for the PRA. Another advantage of the rapid method is that since it is a qualitative method, it is more reproducible than the PRA. In addition, if results obtained using the rapid method are to be confirmed using any other method (i.e. PRA, DU or others), inocula titration will have already been performed, as the set of wells without antivirals serves this purpose.

Data on sensitivity, specificity and predictive values of 'resistant and susceptible result' obtained for our assay of acyclovir in this study might be modified when applied to other populations. The predictive value of a 'resistant result' would be reduced in populations where the prevalence of resistance was lower than in our sample. However, high predictive values of a 'sensitive result' makes our assay particularly useful in screening large series of strains.

Since acyclovir is currently the first choice antiviral agent to treat infections caused by HSV,[2] a feasible rapid method for detection of acyclovir-resistant HSV isolates such as the one we have described, would be a very important tool in clinical virology laboratories.

Acknowledgement

This work was presented in part at the Thirty-Sixth Interscience Conference on Antimicrobial Agents and Chemotherapy, September 1996, New Orleans, LA, USA

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Received 2 February 1999; returned 10 May 1999; revised 7 June 1999; accepted 27 July 1999