ACICLOVIR RESISTANCE AMONG INDIAN STRAINS OF HERPES SIMPLEX VIRUS AS DETERMINED USING A DYE UPTAKE ASSAY

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Abstract

Resistance to aciclovir (ACV) among Herpes simplex virus (HSV) isolates is increasingly being reported in the literature particularly in immunocompromised patients. However, there is only limited data available from India despite widespread use of ACV in our hospital. A cross-sectional study was hence conducted to determine the aciclovir (ACV) susceptibility of HSV-1 and 2 isolates using a dye uptake (DU) assay. This study showed a 3.0% prevalence of ACV resistance among HSV-1 strains (2/66, median IC$_{50}$ 0.098 µg/mL) while in HSV-2 strains, it was 7.8% (5/64, median IC$_{50}$ 0.195 µg/mL). The IC$_{50}$ for the HSV-1 and HSV-2 strains resistant to ACV was greater than or equal to 6.25 µg/mL.

Key words: Aciclovir resistance, dye uptake assay, Herpes simplex virus

Herpes simplex virus-1 (HSV-1) and Herpes simplex virus-2 (HSV-2) are DNA viruses belonging to the Herpesviridae family that cause widespread infection in humans. Aciclovir (previously spelt acyclovir, ACV) was used from the 1980s in the treatment of HSV genital infections and infections in the immunocompromised. Since its first use, resistance to ACV has been reported from the West, particularly in immunocompromised patients in whom the prevalence of resistant virus is typically 4 to 7% (compared to a mean of 0.3% in immunocompetent hosts).

ACV has been used in India to treat HSV infections since the early 1990s. However, to our knowledge, only one study from India has been conducted that has reported ACV-resistance in vitro using a bioassay. Apart from this study, no data is available regarding the ACV susceptibility of HSV isolates despite its widespread use in our population. In addition, there has been an increase in the number of patients with human immunodeficiency virus (HIV) infection or those undergoing immunosuppressive therapy for transplants. Hence, it was considered important to determine the pattern of susceptibility of HSV to ACV in patients in south India attending a tertiary care hospital.

Materials and Methods

This study was conducted at the Department of Clinical Virology of the Christian Medical College, Vellore between September 1998 and September 2004. The minimum sample size for this study (assuming a prevalence of 5% and with a desired precision of 4%) was estimated at 115 (CI: 95%) using Epi-Info Ver. 6.03 software.

A total of 146 HSV isolates was obtained from clinical samples of 141 patients, seen in various units of the hospital (127 collected prospectively and 19 archived isolates stored from 1996). Another four isolates were obtained from Sankara Nethralaya, Chennai (of unknown ACV susceptibilities at the time of receipt). Standard and previously characterized ACV-resistant or -susceptible strains were obtained from Johns Hopkins Hospital (JHH), Baltimore, USA and Health Protection Agency (HPA), Colindale, London to verify the dye uptake (DU) assay used in the study.

All samples were collected, transported and processed as described previously, inoculated into Vero, HEp-2 or A549 cells and identified using polyclonal (Dako A/S, Denmark) immunofluorescence assays (IFA) for HSV confirmation, followed by monoclonal IFA (Imagen Herpes simplex virus direct IF test, DakoCytomation, UK) for typing. A polymerase chain reaction (PCR) based on the pol gene was performed as described previously.

In the course of the study, 20 isolates were found to be non-cultivable and hence, a total of 130 isolates was available for testing.

The titre of virus obtained from each sample was determined by a microtitre plate infectivity assay using the method of Reed and Muench. Briefly, ten-fold serial dilutions of the test viruses were made in maintenance medium (MM) consisting of minimum essential medium (MEM) with 2% fetal calf serum (FCS) held on ice. Virus dilutions (10$^{-2}$ to 10$^{-7}$) were added to the appropriate rows after which Vero cells suspended in growth medium (GM) were added to all wells with appropriate cell controls. The plates were incubated in a CO$_2$ incubator at 37°C with 5% CO$_2$ for three days, during which the cell monolayer in each well of the microtitre plate
was examined for the presence or absence of cytopathic
effect (CPE). The method of Reed and Muench was used to
determine the 50% tissue culture infectious dose (TCID₅₀) on
the third day. Subsequently, the neutral red dye uptake assay
(DUA) was performed.

The DUA is a quantitative colorimetric test based on
the principle of preferential uptake of vital dye by viable
cells over damaged cells. It was performed as described in
a microtitre plate format. Briefly, neutral red solution at pH
6.0 was added to each well after taking the visual reading.
The plates were incubated for 45 min after which the dye
was removed and the monolayer washed with pH 6.0 buffer.
An elution buffer was then added to each well and gently
rocked for 10 min to ensure even elution. Eluate in each
well was transferred to an enzyme-linked immunosorbent
assay (ELISA) plate in an exact template of the original
microtitre plate and the OD of the wells was read using a
spectrophotometer at 540 nm. Using the method of Reed and
Muench, the 50% dye uptake infectious dose (DU₅₀) endpoint
determination was determined.

The susceptibility of the isolates to ACV was determined
using a method described previously in a microtitre plate
format with Vero cells and ACV IV (Zovirax by Burroughs
Wellcome, Mumbai, India and Vir by Troikaa, Gujarat, India).
Briefly, ACV IV 250 mg was reconstituted in sterile distilled
water, aliquoted and stored at -20°C. When required, dilutions
were made to obtain an in-use concentration of 25 µg/mL.
Titrated HSV isolates were diluted in MM to give an in-use
solution of 10-100 DU₅₀/50 µL. In-use ACV solution was
delivered and diluted serially to give concentrations ranging
from 0.012 to 6.25 µg/mL (each drug concentration set up in 4
replicates). The respective virus dilutions were added followed
by a Vero cell suspension. The plates were incubated in a CO₂
incubator at 37°C with 5% CO₂ for three days and examined
for the presence or absence of CPE. After visual readings
were taken, the neutral red DUA was performed as described
previously.

The 50% inhibitory concentration (IC₅₀) for each isolate
was then determined (lowest concentration of ACV that reduced
CPE by 50%). The IC₅₀ was calculated by determining the
mean OD obtained for each drug dilution (the highest dilution
that had a mean OD less than that of cell controls was taken
to be the IC₅₀ for that isolate) with threshold IC₅₀ values for
resistance taken to be greater than 3.0 µg/mL. Each assay was
run with a standard susceptible and resistant strain respectively
that had to perform satisfactorily for the run to be deemed
valid.

Results

Of 141 patients whose samples yielded HSV, medical
records showed that 50 (35.5%) were immunosuppressed,
19 (13.5%) were immunocompetent and there was no
record of the immune status in 72 (51.1%). Fifty-five (39%)
were not treated with ACV, 19 (13.5%) were previously
treated with ACV and in 67 (47.5%) there was no record of
ACV treatment. Hence, where information was available,
the majority was immunosuppressed (from treatment for
malignancies or infection with human immunodeficiency
virus) and not previously treated with ACV.

Out of the total number of 130 viable isolates, 66 (50.8%)
were found to give the HSV-1 specific product (469 bp size by
pol PCR) while 64 (49.2%) gave the HSV-2 specific product
(391 bp size). The type-wise ACV susceptibility of the isolates
is shown in the table. Of the 130 HSV strains that were tested,
7 (5.4%) were ACV resistant, of which 5 (71.4%) were HSV-
2 isolates. The prevalence of ACV resistance among HSV-1
strains was two (3.0%) of the 66 tested. The prevalence of
resistance in HSV-2 strains was 5 (7.8%) of 64.

The median IC₅₀ of the HSV-1 strains susceptible to ACV
was 0.098 µg/mL (range 0.012-0.39 µg/mL) while that for
susceptible HSV-2 was 0.195 µg/mL (range 0.012-0.78 µg/
/mL). The IC₅₀ for strains of HSV-1 and HSV-2 resistant to
ACV were greater than or equal to 6.25 µg/mL (2 and 5 strains
tested respectively).

In a previously described study, four isolates were ACV-
resistant out of seven clinically resistant HSV-1 isolates tested
by plaque reduction assay (PRA).

The in vitro method that we used for ACV susceptibility
testing was found to be robust and performed reproducibly
as assessed by the testing of known standard strains with
each batch of tests. Other methods that are described for
antiviral testing include the plaque reduction assay (PRA)
and DNA hybridization assay (DNAHA). The PRA gives
good correlation with clinical response to therapy but
requires specialized skill to perform. The DNAHA results in
mean IC₅₀ values that are significantly lower than that from
PRA, do not correlate with response to therapy and require

<table>
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<tr>
<th>Aciclovir susceptibility</th>
<th>Herpes simplex virus-1</th>
<th>Herpes simplex virus-2</th>
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<tr>
<td></td>
<td>No. (%)</td>
<td>Median IC50 µg/mL (range)</td>
</tr>
<tr>
<td>Sensitive</td>
<td>64 (97)</td>
<td>0.098 (0.012-0.39)</td>
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<tr>
<td>Resistant</td>
<td>2 (3)</td>
<td>6.25 *</td>
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<tr>
<td>Total</td>
<td>66</td>
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*All isolates had IC₅₀ ≥ 6.25 µg/mL.

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of IC50s.3 of other studies that have also shown a bimodal distribution simpler to perform and has better discriminating ability in are about two times greater than for the PRA, since it is easier to perform and has better discriminating ability in the borderline ACV category.9

Alternate breakpoints used to define resistance to ACV are 1 µg/mL for HSV-1 and 2 µg/mL for HSV-2.10 In our study, sensitive isolates had an IC50 < 1 µg/mL and resistant ones had an IC50 ≥ 6.25 µg/mL. This finding correlates well with that of other studies that have also shown a bimodal distribution of IC50.s,3

In untreated immunocompetent individuals, the mean prevalence of ACV resistant isolates was 0.3% (ranging from 0.1% to 0.7%) while the prevalence in immunosuppressed patients is between 4 and 7%.3

Of the two patients from whom ACV-resistant HSV-1 isolates were obtained in this study, one patient was immunosuppressed while the status of the other could not be determined. Of the five patients from whom isolates of ACV-resistant HSV-2 were obtained, only one patient was definitely immunosuppressed. There was no definitive record in the case of the other four patients.

Discussion
The consumption of ACV in our hospital has increased over the years that this study was conducted. Our Pharmacy records show that ACV consumption has increased from 65.5 g per month in 1999 to 101.3 g per month in 2004 for ACV suspension (I.V. preparation), from 3.00 g per month in 2000 to 5.56 g per month in 2004 for ACV eye ointment and from 1.5 g per month to 2.56 g per month in 2004 for ACV cream. However, it has been shown mathematically that HSV drug resistance is predicted to be minimal despite its widespread use (with predictions of only five in 10,000 individuals shedding drug-resistant virus despite high antiviral usage).11

In summary, the prevalence of resistance to ACV in Indian strains of HSV from this study is 5.4% (7 of 130 strains tested from immunosuppressed as well as immunocompetent individuals). The prevalence of ACV resistance in HSV-1 strains is 3% (2 of 66 tested) and 7.8% (5 of 64) in HSV-2 strains tested. The DU assay is a good screen to determine ACV susceptibility of HSV isolates as it has good reproducibility and is convenient to perform in laboratories in third world countries that have cell culture facilities and an ELISA reader. The prevalence of ACV resistance in these HSV strains is low compared to the increasing usage of ACV in our hospital. This phenomenon will have to be further investigated and seems to be comparable to that from western literature.

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References