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Simultaneous titration and phenotypic antiviral drug susceptibility testing for herpes simplex virus 1 and 2

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ABSTRACT

Background: Most herpes simplex virus (HSV) isolates from treatment-naïve patients are susceptible to antivirals. However, prolonged antiviral therapy can select for drug-resistant strains, especially in immunocompromised patients. Standard phenotypic methods for antiviral resistance testing are labor and time-intense and molecular resistance determinants are insufficiently understood for routine diagnostic use of genotypic resistance testing.
Objective: To enable rapid, scalable antiviral susceptibility testing and minimize viral passage, we developed a 7-day, 96-well assay for simultaneous HSV 1/2 titration and phenotypic resistance testing for acyclovir and foscarnet.
Study design: The assay was optimized and validated by testing clinical isolates and laboratory strains ($n = 39$) with known IC_{50} for acyclovir (23 resistant) and foscarnet (1 resistant) based on plaque reduction or dye-uptake assays. A chemiluminescent detection reagent is used for quantification of cytopathic effect instead of plaque counting or measuring dye-uptake. Drug concentrations inhibiting 50% of chemiluminescent signal reduction (IC_{50}) were determined concurrently at each of three virus dilutions.
Results: Results agree for 92.3% (acyclovir) and 100% (foscarnet) of isolates. For all three discordant samples, results of reference testing by plaque reduction agreed with the chemiluminescent assay. Reproducibility studies showed 100% qualitative agreement and 3–37% coefficient of variation based on IC_{50}.
Conclusions: Chemiluminescence detection as a surrogate for cellular viability with an automated plate reader provides improved throughput and workflow, as well as high accuracy and reproducibility for antiviral drug susceptibility testing.

1. Background

In the past decades, safe and effective antiviral therapies have been developed for treatment of herpes simplex virus (HSV) infections. Acyclovir is the most widely used antiviral for HSV infections. Other agents with efficacy against HSV include valacyclovir, famciclovir, foscarnet, idoxuridine, trifluridine, and vidarabine [2,14,15]. While the overwhelming majority of viral isolates from drug-naive patients are susceptible to antiviral agents, prolonged antiviral therapy can lead to the emergence of drug-resistant strains [4,16]. Due to the need for prolonged therapy or prophylaxis, drug resistance is most common in immunocompromised hosts, especially transplant recipients and AIDS patients [4]. With increasing numbers of immunocompromised patients, the development of acyclovir resistance and the morbidity and mortality associated with these resistant strains is a growing problem. Foscarnet has been used successfully as an alternative antiviral agent for treating acyclovir-resistant HSV but requires intravenous administration [12]. However, the side effect profile of foscarnet, including nephrotoxicity and metabolic disturbances, often render this a suboptimal alternative [3,7]. In addition, resistance to foscarnet has also been reported and clinical isolates resistant to both acyclovir and foscarnet have been documented [12,13]. With a growing number of immunocompromised patients, rapid advance in the development and use of additional antiviral agents, and continued emergence of drug resistance, there is a need for diagnostic laboratories to provide rapid and accurate antiviral susceptibility testing.

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The plaque reduction assay was the first test used to establish antiviral susceptibility, but this method has several limitations. In this traditional assay, HSV isolates are first titrated to determine the appropriate amount of virus to use in the second step of this assay where drug susceptibility is tested [1]. The principle of the assay is reduction of plaques formed by viral cytopathogenic effects (CPE) in the presence of antiviral drugs. Major drawbacks are that the enumeration of viral plaques is subjective and binary (i.e. plaques are either present or absent) while all stages of the assay are time consuming and labor intensive.

Because genetic mutations underlying resistance to the most commonly used antiviral drugs are still incompletely understood [8], we developed and validated a simplified phenotypic antiviral susceptibility assay for HSV-1 and HSV-2 based on chemiluminescence detection. Our assay has several advantages over the plaque reduction assay. First, it combines the viral titration and susceptibility steps of the plaque reduction assay into a single 7-day test. Second, manual counting of plaques is replaced with the use of a chemiluminescent detection reagent as a surrogate for CPE caused by drug-resistant HSV isolates. Overall, our assay provides a less labor intensive, cost-effective, less subjective, and more accurate alternative to the traditional plaque reduction assay.

2. Objectives

The goal of this study was to design, optimize, and validate an antiviral susceptibility test permitting simultaneous virus titration and HSV drug susceptibility testing for routine use in a clinical diagnostic setting.

3. Study design

3.1. Clinical isolates, laboratory strains, and cells

The acyclovir and foscarnet susceptible reference strain HSV-F (ATCC VR-733) and the acyclovir and foscarnet resistant reference strain PAAr5 were cultured on Vero cells (ATCC CCL-81, maximum of 20 passages from receipt), and used to optimize the methylcellulose concentration (Fig. 2), number of Vero cells per well, and incubation time (data not shown). Twenty-two additional laboratory strains and 15 clinical isolates were used to validate the chemiluminescence assay. Laboratory strains were characterized and described in a previous study [5,6,9,17]. Aliquots of viral isolates were stored at −70 °C. In addition, 15 clinical isolates referred to ARUP Laboratories between April 2010 and March 2013 for routine patient care were also used for assay validation. All clinical isolates were initially tested with the plaque reduction or dye-uptake assays by outside laboratories (LabCorp, Burlington, NC, USA and Focus Diagnostics, Cypress, CA, USA). The same isolates were prepared for testing in the chemiluminescence assay by subculture on A549 or Mink Lung cells until at least 75% of cells showed CPE. Laboratory strains and clinical isolates with discordant results (outside laboratory vs. chemiluminescent assay) were tested with the reference method (plaque reduction assay) at the University of Alabama at Birmingham (UAB).

3.2. Chemiluminescence assay

Vero cells were quantified with an automated cell counter (Beckman Coulter Z2 Coulter Particle Count and Size Analyzer) and plated in black 96-well tissue culture plates (Greiner Bio-One) at a seeding density of 10,000 cells/well in EMEM media (Fisher Scientific) with 10% fetal bovine serum (FBS, Atlanta Biologicals) and 2 mM glutamine (Life Technologies) 24 h prior to antiviral susceptibility testing. Cells were added to all 60 interior wells of the plate to avoid evaporation in the outer wells (Fig. 1). Viral isolates grown to 3+ to 4+ CPE in shell vials with 560,000 cells for 1–3 days, were diluted 1:10, 1:40, and 1:160 in EMEM media with 2% FBS and 2 mM glutamine and 40 μL of each virus dilution was added to a 96-well plate after aspirating culture media (Fig. 1). Forty microliters of EMEM media (with 2% FBS and 2 mM glutamine) were also added to three wells for a no virus/no drug control. Cells were incubated 75 ± 15 min at 37 °C and 5% CO_2 during the infection [1]. Acyclovir (VWR Scientific) or foscarnet (Sigma–Aldrich) were diluted in 2× EMEM (Fisher Scientific) with 4% FBS and 4 mM glutamine over 9 serial dilutions (acyclovir, 64 μg/mL to 0.04 μg/mL; foscarnet, 400 μg/mL to 1.56 μg/mL). An equal volume of varying concentrations of methylcellulose (0–2%) in water was added to each drug dilution [1]. One percent methylcellulose was used for all testing after assay optimization. For each viral strain and drug combination, a separate control plate was prepared as described above using combinations of cidofovir (Caymen Chemicals) and acyclovir (for foscarnet testing) or cidofovir and foscarnet (for acyclovir testing) instead of acyclovir or foscarnet alone. This control plate was used to differentiate between viral isolates that are resistant to acyclovir or foscarnet at all tested drug concentrations and insufficiently diluted virus. In both cases, chemiluminescence is reduced at all drug concentrations when testing for acyclovir/foscarnet

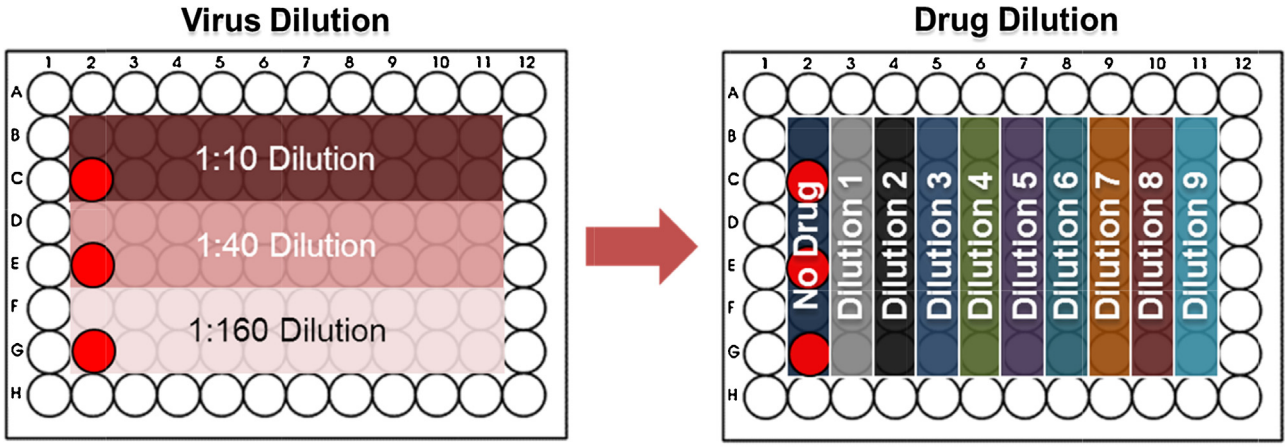

Fig. 1. Plate layout of virus dilution and drug dilution of the chemiluminescence assay. Wells highlighted in red indicate the position of the no drug and no virus control. One plate was used to test one viral strain for susceptibility to either acyclovir, foscarnet, cidofovir and acyclovir, or cidofovir and foscarnet.