ELSEVIER

Antiviral Research 26 (1995) 403–413

Antiviral Research

Assays for antiviral drug resistance

David W. Kimberlin[a,*], Stephen A. Spector[b], Edgar L. Hill[c], Karen K. Biron[c], Alan J. Hay[d], Douglas L. Mayers[e], Richard J. Whitley[a,*]

[a] *Department of Pediatrics, The University of Alabama at Birmingham, 1600 Seventh Avenue South, Suite 616, Birmingham, AL 35233, USA*
[b] *University of California, San Diego, La Jolla, CA, USA*
[c] *Burroughs Wellcome, USA, Research Triangle Park, NC, USA*
[d] *National Institute for Medical Research, London, UK*
[e] *Walter Reed Army Institute of Research, Rockville, MD, USA*

1. The Herpesviruses

1.1. Herpes simplex virus (HSV)

Development of a genotypic assay to detect acyclovir (ACV)-resistant HSV isolates is problematic due to the degree of genetic heterogeneity among such mutants. However, two different in vitro assays have been utilized for the detection of antiviral resistance in HSV. In the United States, the primary assay utilized is the dye-uptake (DU) assay, while in the United Kingdom the plaque reduction assay (PRA) is used most often. Both assays measure inhibition of viral cytopathic effect (CPE) in the presence of drug.

The DU assay was developed as a high volume, rapid turn-around method for determining the in vitro susceptibilities of large numbers of clinical isolates. It utilizes a semi-automated, colorimetric method that measures a quantitative reduction in cell protection based on the uptake of neutral red dye. The assigned cutoff for identifying ACV-resistant virus is 12 μM (3 μg/ml). The DU assay has several advantages over the PRA. Its 96-well plate format allows eight replicates over nine drug dilutions. The eventual cost per assay is lower, though start-up costs are higher due to purchase of the required plate reader. With the DU assay, the final dose-

* Corresponding authors. Fax: (205) 934 8559.

SSDI 0166-3542(95)00028-3

curve is not dependent on the subjective scoring of viral plaques. In addition, the IC_{50} is generated directly from the dose-response curve. With the DU assay, small amounts (3–9%) of ACV-resistant virus in a heterogeneous population can shift the IC_{50} (Ellis et al., 1989). Plaque reduction assays, on the other hand, identify shifts in IC_{50} in a viral population only when the ACV-resistant component of a mixed population reaches 20–25%. However, small subpopulations of resistant virus can be detected at higher concentrations of drug in the PRA, provided enough plaques are analyzed. The sensitivity of the DU to the presence of small amounts of ACV-resistant virus in part is due to the fact that the DU assay does not use an inhibitory overlay (other than ACV) to limit spread of virus during the 3-day incubation period; this allows the ACV-resistant virus in a mixed population to be amplified. The sensitivity of the DU assay makes it an important tool for the detection of mixed populations of virus. The DU assay also has at least a 100-fold higher multiplicity of infection (MOI) than does the PRA. Therefore, if the same mixed viral population is inoculated into each assay, more ACV-resistant virus will be placed into the DU assay than into the PRA.

Despite these numerous advantages, the DU assay also has disadvantages when compared with the PRA. The clinical relevance of results of the DU assay is often difficult to interpret. For instance, 3% of all isolates from drug-naive, immunocompetent patients are resistant to ACV by the DU assay, and yet the problem of clinical resistance in the immunocompetent patient is virtually nonexistent. The input quantity of virus must be titered since the DU assay is virus load dependent: the DU test is considered valid only if the viral challenge dose is between 10 and 100 infectious units per well. This covers the range where the IC_{50} values are flat with respect to the input virus, with steep gradients in the dose response curve existing outside these challenge doses. The slow turn-around time of at least 2 weeks diminishes the clinical utility of the DU assay. Furthermore, the DU assay has high start-up costs due to the required initial purchase of equipment to read plates, and test results vary among cell-types utilized.

The PRA is considered the gold standard for detection of antiviral resistance in HSV. Because larger amounts of ACV-resistant virus are required to shift the dose-response curve in the PRA than in the DU assay, the accuracy of the PRA to predict clinical failure based on elevated in vitro susceptibilities has been reported to be as high as 95% (Safrin et al., 1994). As a result, the PRA correlates better with clinical outcome than does the DU assay. The prevalence of ACV-resistant HSV isolates by PRA is 0.25% in immunocompetent patients, regardless of prior history of ACV exposure; this is despite the fact that the cutoff for identifying ACV-resistant virus is lower with the PRA than with the DU assay (3 μM, or 0.7 mg/ml). This indicates that the results of the PRA more closely approximate the composition of the original clinical isolate than is the case with the DU assay.

The major disadvantage of the PRA is that it is more labor intensive than is the DU assay. Smaller plaques at higher drug concentrations may be missed during the

reading of the assay, introducing greater subjectivity and lowering the assay's accuracy. As with the DU assay, the PRA is virus load dependent, so the input virus must be titered. The amount of input virus is limited by the number of plaques that can be accurately counted in a tissue culture dish. For assays utilizing duplicate 35-mm wells, each well can receive 15 to 500 plaques and be counted accurately. This range is a little greater than for the DU assay. Like the DU assay, the turn-around time of at least 2 weeks also diminishes this assay's clinical utility, and test results vary among cell-types utilized.

These two assay systems have been used to evaluate the prevalence and emergence of ACV-resistant clinical isolates. Although the DU and PRA assays have different endpoints, they both have demonstrated the ability to identify ACV-resistant HSV samples recovered from human immunodeficiency virus (HIV)-infected patients who were failing therapy; 88% were identified by the DU assay (Hill et al., 1991), and 91% were identified by the PRA (Safrin et al., 1994). At the current time no consensus exists as to which of the two assays should be routinely utilized, though the PRA remains the gold standard. Analysis of clinical isolates over the last two decades has detected no increase in the prevalence of ACV-resistant isolates by either the DU assay or the PRA, despite the greatly increased use of ACV over the last ten years.

Once an isolate is identified as resistant by either the PRA or the DU assay, additional phenotypic characterization is warranted. Plaque autoradiography utilizing ^{125}I-labeled iododeoxycytidine (IDC) can distinguish between wild-type (thymidine kinase-positive, or TK^+) and thymidine kinase-negative (TK^-) isolates. Detection of TK-altered (TKa) mutants can be problematic, but a [^{14}C]thymidine plaque labelling experiment performed in a TK-deficient cell line is helpful in combination with the IDC results (Martin et al., 1985). Enzyme assays on crude extracts of HSV-infected TK-deficient cells can determine the percentage of TK phosphorylation activity present when compared to appropriate controls, and therefore can be useful in the characterization of TK mutants. DNA polymerase assays can be performed if one suspects a DNA polymerase mutant. Final phenotypic characterization of an ACV-resistant mutant is based on its high IC_{50} value, its autoradiographic findings, and the percentage of TK enzyme phosphorylating activity. Following identification of an isolate as ACV-resistant, the susceptibility profile to other antiviral agents can be determined.

1.2. Cytomegalovirus (CMV)

As with HSV, the PRA is the gold standard for detection of antiviral resistance in CMV. Due to the amount of labor and the degree of expertise involved in performing PRAs, much work has been performed toward developing alternative phenotypic assays, as well as perfecting even more rapid genotypic assays (Chou et al., 1995; Wolf et al., 1995a,b). While much work remains, significant progress has

been achieved in each of these endeavors.

Of the phenotypic assays, the commercially available Hybriwix assay is a DNA-DNA hybridization assay that can be completed within 4–6 days, making it much more rapid than the PRA (Dankner et al., 1990). Other phenotypic assays utilize specific monoclonal antibodies for the detection of CMV early or late proteins, such as pp65. By attempting to grow the cells in the presence of drug, these assays allow for direct evaluation of antiviral susceptibility, with only resistant isolates being capable of producing such protein products. A variation on this approach involves direct culture of urine or buffy-coat specimens; resistant isolates produce cytopathic effect (CPE) in the presence of drug as well as in the control culture, whereas susceptible isolates produce CPE only in the control culture. Culture of clinical specimens has the advantage of direct evaluation of antiviral susceptibility from the primary isolate.

One advantage of phenotypic assays is that they can detect all resistant viruses that can be grown in vitro. The results of such assays are frequently expressed as inhibitory concentrations, a format that often can be more relevant to clinical care than the results of genotypic assays. Phenotypic assays such as the PRA remain the gold standard and are currently being used by many laboratories. Procedural standardization of CMV phenotypic assays is currently under development within the AIDS Clinical Trial Group (ACTG).

The primary disadvantage of phenotypic assays is that they are tedious and time consuming. Results are often obtained too late to be useful to the clinician. In addition, phenotypic assays require the isolation and expansion of viral isolates prior to performing the assay, a process that can take weeks to months. Furthermore, such expansion may lead to selection of minority populations within the isolate.

Genotypic assays are designed to detect specific genotypic mutations which have been demonstrated to confer resistance to either ganciclovir (GCV) or foscarnet (PFA). One method of performing such genotypic analysis involves direct sequencing of polymerase chain reaction (PCR) amplified products. While this technique requires the most amount of time of the genotypic assays, it also provides the greatest amount of information. Such PCR amplification is necessary for identifying specific genotypic mutations that are associated with antiviral resistance. Other methods for performing genotypic assays include PCR/ligase chain reaction (LCR) amplification with probe detection of the specific mutants, as well as utilization of other nucleic acid detection systems following PCR amplification. These detection systems include the evaluation of gain or loss of specific restriction enzyme sites (restriction fragment length polymorphisms) in the mutant virus (Chou et al., 1995).

Specific mutations that confer resistance of CMV clinical strains to GCV have been identified in the UL97 region, which encodes the viral phosphotransferase, and in the UL54 region, which encodes the viral DNA polymerase. Mutations within the UL97 region appear to occur much more often than do those within the

DNA polymerase (Stanat et al., 1991; Chou et al., 1995). Mutations in the DNA polymerase gene are also known to confer resistance to PFA; however, specific mutations have yet to be confirmed by recombinant viruses.

Genotypic assays offer the advantage of being performed directly on clinical material or primary isolates. They can often be performed even when virus cannot be cultured. As such, the risks of selection of minority populations of virus are reduced. These assays are rapid and potentially applicable to guide therapeutic decisions by clinicians. The major disadvantage of genotypic assays is that only mutations known to confer phenotypic resistance can be identified with certainty. Mutations not recognized to confer resistance will not be appreciated with these assays.

1.3. Varicella-zoster virus (VZV)

The PRA remains the gold standard for detection of VZV drug resistance. Other phenotypic assays which have been applied to the evaluation of VZV susceptibilities include DNA-DNA hybridization assays and an ELISA which utilizes varicella-zoster immune globulin (VZIG). Of the DNA-DNA hybridization assays, the Hybriwix assay is more suitable for smaller laboratories that evaluate only a few isolates each month. The 96-well dot blot, on the other hand, is a relatively automated technique that can evaluate large numbers of isolates; however, this feature makes it less applicable for smaller laboratories. For the phenotypic assays, variables that affect the absolute IC_{50} values include: input multiplicity of infection (MOI); the type of cell line utilized (human diploid fibroblast versus the Mewo human melanoma cell line); medium composition; drug purity and concentration; the type of overlay utilized (semisolid or fluid); the presence of antibody; incubation time; and assay endpoints. The genetic heterogeneity among ACV-resistant VZV isolates probably will preclude the development of genotypic assays to detect such mutants (Talarico et al., 1993; Boivin et al., 1994).

Resistance to ACV has only been documented in patients with acquired immunodeficiency syndrome (AIDS) following prolonged oral therapy. Clinical isolates of VZV can consist of mixtures of drug-sensitive and drug-resistant phenotypes. The majority of ACV-resistant isolates recovered from these patients have consisted of TK-negative (TK$^-$) virus or, less frequently, TK-altered (TKa) virus. Analysis of serial isolates in several patients indicate that viral phenotypes can change within a 2-week period of time. In four cases, follow-up isolates were again wild-type (TK$^+$) following a ≥3-week interval off ACV therapy.

In determining drug resistance in VZV clinical isolates, close analysis of the dose response curve can suggest the presence of a small proportion of resistant virus. If an emerging fraction of resistant virus is present, changes in the IC_{90} will be seen prior to changes in the IC_{50}. Tests that evaluate the function of the viral TK can also be of benefit in evaluating clinical isolates. As with HSV, the incorporation

Table 1
Drug IC_{50} ranges for susceptible VZV isolates

Drug	Type of assay (μM)		
	Plaque-reduction assay	Hybriwix assay	DNA dot blot assay
ACV	2.0–17.0	0.5–4.0	1.0–8.0
PFA	–	70.0–200.0	not done
BV-ara-U	0.006–0.025	0.001–0.03	0.002–0.025
PCV	9.0–17.0	4.0–6.0	not done

tion of [^{125}I]iododeoxycytidine in the VZV plaque autoradiographic format correlates well with the ability of TK to phosphorylate ACV (Martin et al., 1985). The TKa phenotype can be distinguished by [^{14}C]thymidine incorporation in the TK 143 cell line. None of the TK , TKa, or DNA polymerase mutants generated in the laboratory or encountered in the clinic to date are impaired for growth in vitro when compared with wild-type strains.

Reference ranges from various phenotypic assays for several antiviral agents are presented in Table 1. Drug resistant mutants with a TK phenotype can be expected to have a 10–50-fold elevation in IC_{50} for all TK-activated drugs [ACV, penciclovir (PCV), 1-B-D-arabinofuranosyl-*E*-5-(2-bromovinyl)uracil (BV-ara-U)]. Isolates with a TKa phenotype will have a ≥10-fold elevation in IC_{50} to the antiviral agent being evaluated, but resistance to related drugs is variable, ranging from fully susceptible to fully resistant. And while DNA polymerase mutants exhibit a ≥4-fold elevation in IC_{50} to the antiviral drug being tested, the cross-resistance profiles of these isolates are variable.

2. Influenza

Detection of resistance of influenza A viruses to amantadine and rimantadine can be effectively monitored either by the loss of sensitivity to drug or the acquisition of well defined mutations in the M2 protein. The simplest and most definitive assay for inhibition of viral replication by these drugs is an ELISA of MDCK cells infected at low multiplicity, using antibodies to the hemagglutinin, nucleoprotein, or matrix protein (Belshe et al., 1988; Kendal, 1991). Results are obtained within two days and the assay is reproducible between laboratories. Although sensitive viruses differ in their drug susceptibility, M2-determined resistance generally abolishes susceptibility, giving a definitive criterion for resistance. The results, therefore, provide a clear contrast between susceptible and resistant isolates, making the ELISA assay the gold standard for detection of amantadine and rimantadine resistance (Douglas, 1990). Plaque reduction assays (PRAs), in general, are less discriminating and have in many instances proven unsatisfactory. In addition, for

human influenza A viruses, the ELISA assay is the only test which has been found to correlate reproducibly with the genotypic changes known to confer resistance to amantadine and rimantadine.

Drug resistance of influenza A mutants, selected in vivo or in vitro, has to date been shown to be due to single amino acid substitutions at one of five positions within a nine amino acid stretch (residues 26 to 34) of the transmembrane domain of M2, the particular amino acid change selected depending on the virus strain. Mutations have been monitored by: 1) direct sequencing of the appropriate short region of the M RNA segment (Belshe et al., 1988; Hayden et al., 1989); 2) sequencing of PCR-amplified products, which facilitates direct analysis of clinical samples; or 3) changes in restriction enzyme patterns of amplified fragments, which allows the screening of larger numbers of samples (N. Cox, personal communication).

Measurement of proton channel activity of the M2 protein expressed in mouse erythroleukemia (MEL) cells (Chizhmakov et al., 1995) provides a sensitive assay for detecting changes in the ion conductance properties and pH-dependent activation characteristics of mutant M2 protein channels. This assay should be helpful in studies of the consequences of amantadine/rimantadine resistance mutations, which may aid an understanding of the potential epidemiological impact of drug-resistant influenza A viruses.

Assays which evaluate cytopathic effects have also been developed for detection of influenza resistance. Along with PRAs, these alternative assays may be of benefit in evaluating resistance to newer antiviral compounds with activity against influenza. For instance, the PRA detects resistance to the topical neuraminidase inhibitor GG167, while the ELISA assay lacks sufficient sensitivity for such detection.

3. Human immunodeficiency virus-1 (HIV-1)

One of the first assays to be employed in the assessment of antiretroviral activity evaluated the inhibition of CPE in lymphoblastoid cell lines such as ATH8, MT-2, and MT-4. Despite the subsequent development of additional assays, this assay remains useful, especially in high-volume, automated laboratory screening of antiretroviral activity.

The syncytial focus (plaque)-forming assay involves co-cultivation of a patient's peripheral blood mononuclear cells (PBMCs) with lymphoblastoid cells (such as MT-2 cells), followed by analysis using a HeLa-CD4 drug susceptibility assay. It is faster and less expensive than other assays, and it generates a monotonic sigmoid curve that is highly reproducible (Richman et al., 1993). This allows for the calculation of quantitative values for susceptibilities, as well as detection of phenotypic mixtures. However, HIV isolation rates of 35–40% on MT-2 cells limits the clinical application of this assay system. HeLa-CD4 assays have also been performed with

Table 2
AZT susceptibility ranges as determined by the PBMC-based assay

Drug susceptibility	IC_{50} value (μM)
Sensitive	≤0.20
Partial resistance	0.21–0.99
Resistant	≥1.0

high titers of virus generated in PBMCs, but low isolation rates still hinder this technique.

The PBMC-based assay can be applied to most clinical isolates (Japour et al., 1993). Utilizing the PBMC-based assay system, isolation rates have been increased to in excess of 85%, thereby decreasing selection bias when compared with assays that require passage in cell lines. This phenotypic method involves viral culture and expansion, followed by titration of the virus to achieve a standardized inoculum. Viral growth is then evaluated in the presence of drug. The PBMC-based assay system has a run-to-run variability of 2–3-fold, and 4–6 weeks are required for final results. Isolation rates of 99% are attainable when the patient's CD4 count is less than 200; when the CD4 count is between 200 and 500, the yield is 85%. Due to such high isolation rates, the PBMC-based assay is more useful in assessing susceptibilities of clinical isolates to zidovudine (AZT) than are the previously described assay systems.

The PBMC-based assay has been used to perform drug susceptibility testing of clinical HIV isolates with nucleoside and non-nucleoside RT inhibitors (NNRTIs), protease inhibitors (PIs), PFA, methylation inhibitors, and antisense agents. This assay works well for drugs such as AZT, for which resistance is clearly demarcated by large differences in IC_{50} between susceptible and resistant isolates (Table 2). However, it is problematic for the assessment of many nucleoside RT inhibitors other than AZT. For example, PBMC-based assays of 2′,3′-dideoxyinosine (ddI), 2′,3′-dideoxycytosine (ddC), and 2′,3′-dideoxydidehydrothymidine (d4T) are significantly affected by viral replication characteristics mediated by envelope determinants. However, alternative assays for ddI, ddC, and d4T have yet to be developed.

The disadvantages of the PBMC-based assay primarily are the variability among donor cells and the expense of the assay. In addition, the 4 to 6 weeks required to complete the assay limits its application in the clinical setting.

Genotypic assays offer the potential advantage of being rapid enough to provide useful information to the clinician. Such assays detect point mutations associated with HIV drug resistance. Following nested PCR amplification of a segment of the RT gene, point mutations are detected by differential PCR, ligase reaction, differential hybridization of a probe, or addition of a labelled ddN. Sites which have been evaluated by these methods include codon 41 (AZT resistance), codon 74 (ddI

resistance), codon 181 (resistance to the NNRTIs), codon 184 (3TC resistance), codon 188 (resistance to the NNRTIs), and codon 215 (AZT resistance). Of these, detection of mutations at codon 215 has been evaluated most thoroughly (Larder et al., 1991; Richman et al., 1991; Boucher et al., 1992; Kozal et al., 1993). However, this assay is only positive when more than 10% of the viral population from the clinical specimen carries a mutation at codon 215. In addition, a PCR result that demonstrates the presence of a mutation at codon 215 must be confirmed by repeat PCR following dilution of the sample because erroneous faint bands of mutant virus can sometimes be seen with very strong bands of wild-type virus.

Methods to rapidly sequence stretches of the RT gene are currently being developed and validated. These sequencing technologies will be needed to assess genotypic resistance for patients on combination therapy regimens. The application of genotypic assays to the detection of antiviral resistance in HIV-1 may be complicated by several factors. Genetic variability in the mutation of interest among clinical isolates may limit the usefulness of genotypic assays. In addition, multiple mutations can be associated with a single drug resistance profile. The more complex patterns of resistance seen in combination antiretroviral regimens may be difficult to detect by PCR. For these assays to be applied on a large scale, the complex phenotypic:genotypic relationships that exist for isolates with multiple mutations must be better understood.

An alternative to the PBMC-based assay that is not used by many laboratories is the recombinant virus assay (RVA). In the RVA, a PCR fragment of the clinical isolate is mixed with an HIV molecular clone from which a portion of the DNA polymerase gene has been deleted. Alternatively, all or part of the RT gene can be deleted, or all of the protease gene can be deleted. This mixture is then transfected into T lymphocytes and, following recombination, a viable virus is produced. Susceptibility assays can then be performed on the recombinant virus. Such viruses replicate in many different cell lines and are standardized with respect to the envelope gene.

The RVA offers several advantages over the PBMC-based assay. The RVA avoids co-culture and the use of PBMCs, with recombinant virus growing well in established T-cell lines. In addition, there is no need to produce or passage viral isolates. The RVA avoids cell culture selection bias, and mixtures of viruses are faithfully reproduced. The fact that the recombinant virus is generated using PCR-amplified nucleic acids can be beneficial when viable cell samples are not available. Also, recombinant virus can be recovered from RNA samples obtained from a patient's plasma or serum.

References

Herpes simplex virus

Ellis, M.N., Waters, R., Hill, E.L., Lobe, D.E., Selleseth, D.W., Barry D.W. (1989) Orofacial infection of athymic mice with defined mixtures of acyclovir-susceptible and acyclovir-resistant herpes sim-

plex virus type 1. Antimicrob. Agents Chemother. 33, 304-10.
Hill, E.L., Hunter, G.A., Ellis, M.N. (1991) In vitro and in vivo characterization of herpes simplex clinical isolates recovered from patients infected with human immunodeficiency virus. Antimicrob. Agents Chemother. 35, 2322-28.
Martin, J.L., Ellis, M.N., Keller, P.M., Biron, K.K., Nusinoff-Lehrman, S., Barry, D.W., Furman, P.A. (1985) Plaque autoradiography assay for the detection and quantitation of thymidine kinase-deficient and thymidine kinase-altered mutants of herpes simplex virus in clinical isolates. Antimicrob. Agents Chemother. 28, 181-87.
Safrin, S., Elbeik, T., Phan, L., Robinson, D., Rush, J., Elbaggari, A., Mills, J. (1994) Correlation between response to acyclovir and foscarnet therapy and in vitro susceptibility results for isolates of herpes simplex virus from immunodeficiency virus-infected patients. Antimicrob. Agent Chemother. 38, 1246-50.

Cytomegalovirus

Chou, S., Erice, A., Jordan, M.C., Vercellotti, G.M., Michels, K.R., Talarico, C.L., Stanat, S.C., Biron, K.K. (1995) Analysis of the UL97 phosphotransferase coding sequence in clinical cytomegalovirus isolates and identification of mutations conferring ganciclovir resistance. J. Infect. Dis., in press.
Dankner, W.M., Scholl, D., Stanat, S.C., Martin, M., Sonke, R.L., Spector, S.A. (1990) Rapid antiviral DNA-DNA hybridization assay for human cytomegalovirus. J. Virology. Meth. 28, 293-98.
Stanat, S.C., Reardon, J.E., Erice, A., Jordan, C., Drew, W.L., Biron, K.K. (1991) Ganciclovir-resistant cytomegalovirus clinical isolates: mode of resistance to ganciclovir. Antimicrob. Agents Chemother. 35, 2191-97.
Wolf, D.G., Smith, I.L., Lee, D.J., Freemen, W.R., Flores-Aguilar, M., Spector, S.A. (1995a) Mutations in human cytomegalovirus UL97 gene confer clinical resistance to ganciclovir and can be detected directly in patient plasma. J. Clin. Invest. 95, 1-7.
Wolf, D.G., Lee, D.J., Spector, S.A. (1995b) Detection of human cytomegalovirus mutations associated with ganciclovir resistance in cerebrospinal fluid of AIDS patients with central nervous system disease. (submitted).

Varicella-zoster virus

Boivin, G., Edelman, C.K., Pedneault, L., Talarico, C.L., Biron, K.K., Balfour H.H. Jr. (1994) Phenotypic and genotypic characterization of acyclovir-resistant varicella-zoster viruses isolated from persons with AIDS. J. Infect. Dis. 170, 68-75.
Martin, J.L., Ellis, M.N., Keller, P.M., Biron, K.K., Lehrman, S.N., Barry D.W., Furman, P.A. (1985) Plaque autoradiography assay for the detection and quantitation of thymidine kinase-deficient and thymidine kinase-altered mutants of herpes simplex virus in clinical isolates. Antimicrob. Agents Chemother. 28, 181-87.
Talarico, C.L., Phelps, W.C., Biron, K.K. (1993) Analysis of the thymidine kinase genes from acyclovir-resistant mutants of varicella-zoster virus isolated from patients with AIDS. J. Virol. 67, 1024-33.

Influenza

Belshe, R.B., Hall Smith, M., Hall, C.B., Betts, R., Hay A.J. (1988) Genetic basis of resistance to rimantadine emerging during treatment of influenza virusinfection. J. Virol. 62, 1508-12.
Chizhmakov, I., Geraghty, F., Hayhurst, A., Antoniou, M., Ogend, D., Hay, A.J. (1995) Selective proton permeability and pH-dependent activation of the M2 ion channel of influenza virus. (submitted).
Douglas, R.G. Jr. (1990) Drug therapy. Prophylaxis and treatment of influenza. N. Engl. J. Med. 322, 443-50.

Hayden, F.G., Belshe, R.B., Clover, R.D., Hay, A.J., Oakes, M.G., Soo, W. (1989) Emergence and apparent transmission of rimantadine-resistant influenza A virus in families. N. Engl. J. Med. 321, 1696-1702.

Kendal, A.P. (1991) Reference viruses for use in amantadine sensitivity testing of human influenza A viruses. Antivir. Chem. Chemother. 2, 115-18.

Human immunodeficiency virus-1

Boucher, C.A., O'Sullivan, E., Mulder, J.W., Ramautarsing, C., Kellam, P., Darby, G., Lange, J.M., Goudsmit, J., Larder, B.A. (1992) Ordered appearance of zidovudine resistance mutations during treatment of 18 human immunodeficiency virus-positive subjects. J. Infect. Dis. 165, 105-10.

Japour, A.J., Mayers, D.L., Johnson, V.A., Kuritzkes, D.R., Beckett, L.A., Arduino, J.M., Lane, J., Black, R.J., Reichelderfer, P.S., D'Aquila, R.T., Crumpacker, C.S., the RV-43 Study Group, the AIDS Clinical Trials Group Virology Committee Resistance Working Group (1993) Standardized peripheral blood mononuclear cell culture assay for determination of drug susceptibilities of clinical human immunodeficiency virus type 1 isolates. Antimicrob. Agents Chemother. 37, 1095-1101.

Kozal, M.J., Kozal, M.J., Shafer, R.W., Winters, M.A., Katzenstein, D.A., Merigan, T.C. (1993) Mutation of HIV reverse transcriptase and decline of CD4 lymphocyte numbers in long term zidovudine recipients. J. Infect. Dis. 167, 526-32.

Larder, B.A., Kellam, P., Kemp, S.D. (1991) Zidovudine resistance predicted by direct detection of mutations in DNA for HIV-infected lymphocytes. AIDS 5, 137-44.

Richman, D.D., Guatelli, J.C., Grimes, J., Tsiatis, A., Gingeras, T. (1991) Detection of mutations associated with zidovudine resistance in human immunodeficiency virus by the use of the polymerase chain reaction. J. Infect. Dis. 164, 1075-81.

Richman, D.D., Johnson, V.A., Mayers, D.L., Shirasaka, T., O'Brien, M.C., Mitsuya, H. (1993) In vitro evaluation of experimental agents for anti-HIV activity. In: Coligan, J.E., Kruisbeck, A.M., Margulies, D.H., Shevach, E.M., Strober, W. (eds.), Current Protocols in Immunology, Brooklyn, NY, J. Wiley, pp. 12.9.1-21.