Assays for monitoring susceptibility of influenza viruses to neuraminidase inhibitors

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Close monitoring of drug susceptibility among human influenza viruses was necessitated by widespread resistance to M2 inhibitors in influenza H1N1 (pre-pandemic and 2009 pandemic) and H3N2 viruses, and of oseltamivir resistance in pre-pandemic H1N1 viruses. The FDA-approved neuraminidase (NA) inhibitors (NAIs), oseltamivir and zanamivir, as well as investigational NAIs, peramivir and laninamivir, are currently the principal treatment options for managing influenza infection. However, there are challenges associated with assessing virus susceptibility to this class of drugs. Traditional cell culture–based assays are not reliable for phenotypic testing of NAI susceptibility due to complexity in interpretation. Two types of laboratory assays are currently available for monitoring NAI susceptibility, phenotypic such as the neuraminidase inhibition (NI) assay and genotypic. The NI assay's requirement for propagated virus lengthens testing turnaround; therefore, the need for timely detection of molecular markers associated with NAI resistance (e.g., H275Y in H1N1) has spurred the development of rapid, high-throughput assays, such as real-time RT-PCR and pyrosequencing. The high sensitivity of genotypic assays allows testing of clinical specimens thus eliminating the need for virus propagation in cell culture. The NI assays are especially valuable when a novel virus emerges or a new NAI becomes available. Modifications continue to be introduced into NI assays, including optimization and data analysis criteria. The optimal assay of choice for monitoring influenza drug susceptibility varies widely depending on the needs of laboratories (e.g., surveillance purposes, clinical settings). Optimally, it is desirable to combine functional and genetic analyses of virus isolates and, when possible, the respective clinical specimens.

Keywords Functional analysis, genetic analysis, influenza antiviral susceptibility, neuraminidase inhibitors.

Introduction

Neuraminidase (NA) inhibitors (NAIs) are currently the only antivirals that are effective for prophylaxis and treatment of influenza A and B infections, due to widespread resistance to M2 inhibitors among currently circulating seasonal influenza A viruses that has greatly diminished the usefulness of this class of drugs. Oseltamivir and zanamivir, administered orally and by inhalation, respectively, are currently the only FDA-approved NAIs for use against type A and type B influenza infections.[3] A newer NAI, peramivir,[4] currently investigational in the United States, has been developed as an intravenous (IV) formulation, and is licensed in Japan[5] and S. Korea, while R-125489, developed as an inhaled prodrug laninamivir (CS-8958), is licensed in Japan.[6]

The 2007–2008 influenza season experienced the dramatic emergence of oseltamivir-resistant seasonal influenza A(H1N1) viruses carrying the H275Y mutation in the NA,[7–9] which by the 2008–2009 season accounted for up to 100% of seasonal A(H1N1) viruses in many countries.[10,11] These H275Y variants were subsequently replaced by influenza A(H1N1)pdm09 viruses, which emerged in April 2009, leading to a sharp decline in circulating oseltamivir-resistant viruses. In the following 2009–2010 and 2010–2011 seasons, resistance to NAIs among the newly emergent influenza A(H1N1)pdm09 viruses remained low (<1% in the United States).[12,13] However, during the 2010–2011 season, a cluster of oseltamivir-resistant influenza A(H1N1)pdm09 viruses with the H275Y substitution, in circulation between May and September 2011, was reported in Australia.[14,15] The majority of the patients from whom these H275Y variants were recovered had no known oseltamivir exposure. Such potential for emergence and community spread of NAI-resistant variants, coupled with the limited pharmaceutical options currently available for

the control of influenza infections, emphasizes the need to monitor NAI susceptibility among influenza viruses circulating globally.

Assessment of influenza antiviral susceptibility to NAIs is primarily performed using functional phenotypic NA inhibition (NI) assays coupled with genotypic methods such as pyrosequencing[16] and Sanger dideoxy sequence analysis[17] of the NA gene to detect mutations that are associated with NAI resistance. While these methods are effective in detecting influenza viruses resistant to NAIs, there is a need for more high-throughput, affordable and low turnaround approaches for monitoring influenza antiviral susceptibility. This review highlights phenotypic and genotypic approaches that are currently available for assessing influenza virus susceptibility to NAIs.

Phenotypic methods for assessing influenza virus susceptibility to NAIs

Traditional cell culture–based assays are desirable for initial screening in antiviral susceptibility studies, due to their ability to detect a broad range of resistant phenotypes. However, when applied to influenza, antiviral susceptibility assessed in cell culture lacks correlation with the susceptibility assessed *in vivo* in humans or animal models.[18] In this respect, the NI assay, which functionally assesses the inhibition of the enzyme by the NAI, is beneficial.

Functional methods such as the NI assay allow detection of drug-resistant viruses with established and/or novel changes in the target enzyme. Either the fluorescent[19] or chemiluminescent[20] NI assays are typically the choice for surveillance purposes. Both assays require propagation of virus prior to testing and small synthetic substrates, namely methyl umbelliferone N-acetyl neuraminic acid (MUNANA)[21] for the fluorescent assay and a 1,2-dioxetane derivative of neuraminic acid[22] for the chemiluminescent assay.

The chemiluminescent and fluorescent NI assays (Table 1) each have advantages and disadvantages associated with their use; for example, the fluorescence-based assay is less costly but requires viruses with higher titers,[23] compared to the chemiluminescence-based assay, which has been shown to provide greater linearity of signal and higher sensitivity in measuring NA activity.[24] The fluorescent assay is preferable for detecting resistance when viral sample permits, as it typically offers better discrimination between NAI susceptible and resistant viruses compared to the chemiluminescent assay.[23] Nevertheless, NAI-resistant mutants can accurately be detected by either NI assay; therefore, the choice of method to use as the primary assay depends on the objectives and requirements of individual surveillance laboratories. Sometimes, an array of assays is applied in characterizing resistance caused by a novel mutation(s).

The NI assay determines the concentration of an NAI needed to reduce enzyme activity by 50% (IC_{50}). To determine IC_{50} values, raw fluorescent NI assay data (expressed as relative fluorescence units, RFUs) or raw chemiluminescent NI assay data (expressed as relative light units, RLUs) are plotted against drug concentration (nM) using curve fitting software such as JASPR (in-house, CDC)[25] or Robosage (in-house, GlaxoSmithKline). Statistical analyses to determine IC_{50} cutoff values for outliers are previously described.[17,25,26]

The reagents used in the chemiluminescent and fluorescent NI assays are commercially available as the NA-Star® Influenza Neuraminidase Inhibitor Reagent Kit and the NA-Fluor™ Influenza Neuraminidase Assay Kit, respectively (Applied Biosystems, Foster City, CA, USA). These kits provide validated reagents (except NAIs and reference virus strains) for rapid and sensitive quantitation of influenza NA activity in 96-well microplate formats, enabling improved global assay standardization and more accurate comparison of results between laboratories. The manufacturers' protocols provided in the kits can be optimized to meet individual laboratories' needs. Alternatively, the fluorescent NI assay can be performed using reagents that are prepared in-house using standard chemicals and MUN-ANA (Sigma Aldrich, St. Louis, MO, USA), which is purchased separately. A next-generation chemiluminescence-based assay, the NA-XTD™ Influenza Neuraminidase Assay Kit (Applied Biosystems) is also commercially available. This kit provides longer signal readouts compared to the first-generation NA-Star® kit and includes detection reagents that eliminate the need for luminometers equipped with a reagent injector, thereby improving ease-in the-use.

Reference panels of NAI-sensitive and -resistant viruses, to aid in standardizing NI assays and assessing influenza virus susceptibility to NAIs, are available through the ISIRV-Antiviral Group (ISIRV-AVG),[27] the Centers for Disease Control and Prevention (CDC; email: fluantiviral@cdc.gov), or the Influenza Reagent Resource (IRR).

Of note, from a technical standpoint, the NI assay is not a true phenotypic assay and does not account for the interplay of the hemagglutinin (HA) receptor-binding and the NA receptor-destroying activities, which occurs in cell culture. Yet, virus propagation in cell culture is not without limitations, as it may select variants with changes in the virus surface glycoproteins, the HA, and/or the NA, some of which may alter the drug susceptibility profile of the virus in the NI assay.[28,29] Consequently, there is a need for sequence confirmation of both known markers of resistance and changes associated with cell culture selection in addition to functional testing. Nonetheless, virus culture remains an essential component of antiviral resistance monitoring as the NI assay requires virus.

Table 1. Phenotypic and genotypic methods for influenza antiviral susceptibility testing

Assay type	Advantages	Disadvantages/Challenges
Phenotypic (functional) methods Chemiluminescent NI assay NA-Star® Influenza Neuraminidase Inhibitor Resistance Detection Kit Fluorescent NI assay NA-XTD™ Influenza Neuraminidase Assay Kit NA-Fluor™ Influenza Neuraminidase Assay Kit Assay can be performed using in-house prepared reagents	NI assays allow accurate detection of drug-resistant viruses with established molecular markers (e.g., H275Y in N1 subtypes) and/or novel changes in the targeted NA enzyme NI assays provide valuable susceptibility profiles, which cannot be determined solely by genotypic techniques NI assays are available as commercial kits that enable antiviral susceptibility testing to be performed under standardized conditions Choice of NI assay depends on objectives and requirements of individual surveillance laboratories	NI testing cannot be carried out directly on clinical material and requires the use of cell grown isolates Elevated IC_{50} values must be combined with genotypic information to accurately define resistance There is no established cutoff IC_{50} value that is indicative of clinically relevant resistance Variations in assay conditions may affect IC_{50} values generated in the NI assay The fluorescence-based assay requires viruses with higher titers compared to the chemiluminescence-based assay
Genotypic methods Sanger dideoxy sequencing Pyrosequencing Sequence analysis (SQA) Single-nucleotide polymorphism analysis (SNP) Real-time reverse transcriptase–PCR (RT-PCR) coupled with detection methods/chemistries such as 　SYBR green agents 　MGB probes 　Single-nucleotide polymorphism (SNP) analysis 　Hybridization probes 　High-resolution melting analysis 　Rolling circle amplification Conventional end-point RT-PCR coupled with methods such as 　Single-nucleotide polymorphism (SNP) genotyping 　Restriction fragment length polymorphism (RPLP) analysis	Genotypic testing can be carried out directly on clinical material Genotypic testing allows identification of mutations on viral genome associated with amino acid substitutions conferring antiviral resistance Genotypic testing is time-saving, high throughput, relatively simple, and rapid (with exception of Sanger sequencing) Methods are not difficult to implement using existing capabilities Choice of genotypic method to use depends on laboratory testing capabilities	Genotypic assays carry a high risk for cross-contamination Some genotypic methods such as Sanger sequencing are time-consuming, labor intensive, and expensive Interpretation of mutations is difficult without phenotypic information Understanding the relationship between phenotype and genotype remains a challenge Determining the proportion of mixed virus population that is defined as resistant is a challenge

The NI assay provides valuable quantitative susceptibility data, which cannot be determined solely by sequence-based techniques. The susceptibility of viruses with intermediate IC_{50} values is usually difficult to interpret; therefore, such viruses are further investigated to determine the presence of molecular changes in the NA and to determine their frequency of detection as well as potential clinical importance.

The IC_{50} values generated in NI assays provide valuable information for detecting NAI-resistant viruses, but the lack of an established threshold IC_{50} value indicative of clinically relevant resistance does not allow IC_{50}s to be used in drawing direct correlations with drug concentrations required to inhibit virus replication in the infected human host. Nevertheless, the assessment of NAI susceptibility of influenza viruses in the NI assay, reinforced by NA sequence analysis of virus isolates with IC_{50}s above baseline values and their matching clinical specimens, provides a reliable and reasonably comprehensive approach to the identification of NAI-resistant isolates for surveillance purposes.

Genotypic methods for assessing influenza virus susceptibility to NAIs

The propensity for rapid and constant evolution of the RNA genome of the influenza virus requires flexible diagnostic tools for monitoring existing and novel drug resistance mutations.

The most commonly applied genotypic methodology for detecting NAI resistance mutations in the NA couples conventional end-point reverse transcription–polymerase chain reaction (RT-PCR) with techniques such as Sanger sequencing and pyrosequencing (Table 1). Full gene sequencing is extremely informative and accurate in detecting changes to the virus genome, but is time-consuming, laborious, and expensive, and may be indiscriminate in determining components of mixed virus populations. Nevertheless, Sanger sequencing remains the assay of choice for identifying both characterized and novel changes, which may underlie phenotypic resistance.

Pyrosequencing technology has for a number of years been applied to the detection of known molecular markers of resistance in the NA gene.[30–33] At the CDC, mutations in the NA that are most commonly screened using pyrosequencing are the oseltamivir resistance conferring H275Y substitution in A(H1N1)pdm09 viruses, the E119V, R292K, and N294S substitutions in influenza A(H3N2) viruses, as well as changes at residues H273, D197, E117, and R374 in influenza B viruses.

Initially used for sequencing target regions (SQA analysis), pyrosequencing assays have been applied in quantification of single-nucleotide polymorphisms (SNP analysis).[34,35] SNP analysis relies on the premise that most known NAI resistance markers result from single point mutations in the codons of certain critical residues in the NA. The technology has also been applied in characterizing complex mixtures in the HA.[36]

Pyrosequencing is easily scaled up for high-throughput testing and provides highly informative genetic data for known markers of drug resistance. This approach cannot, however, identify novel changes in the NA that may confer resistance or subtle differences in virus susceptibility to NAIs, and must constantly be updated to accommodate such changes. It is also associated with costly maintenance contracts, specialized equipment, and reagents.

Real-time RT-PCR is a rapid, high-throughput technology commonly used for influenza typing/subtyping in diagnostic and clinical laboratories. Recently, real-time RT-PCR has been applied to drug resistance detection (Table 1), utilizing several detection techniques, such as SYBR green intercalating agents;[37] single-nucleotide polymorphism (SNP) probes;[38–43] high-resolution melting (HRM) analysis;[44,45,46] hybridization probes;[47] minor groove binding (MGB) probes;[48] and rolling circle amplification.[49,50] The ease-of-use, accessibility to equipment, and the availability of reagents and training further contribute to the widespread application of real-time RT-PCR assays.

Additional PCR-based methods, utilizing conventional RT-PCR, have been used for detecting drug resistance mutations in influenza viruses, including SNP analysis by single-nucleotide probe extension (SNaP Shot assay)[35] and restriction fragment length polymorphism (RFLP) analysis.[51,52]

Regardless of the RT-PCR method or reaction chemistry, benefits of PCR-based characterization include lower cost, potential implementation in a high-throughput system, and generally straightforward interpretation.

The main risk of genotypic tests is cross-contamination, requiring specific procedures for its prevention. The risk of false-negative results due to insufficient extraction or procedural errors still remains. Less frequent causes of genotypic test error are mixtures (wild-type strain plus emerging mutant or multiple mutations) and silent mutations, with change of nucleotide but not amino acid.

Conclusion

The choice of assay for assessing influenza virus susceptibility to NAIs depends on factors pertaining to appropriateness to the setting, cost, sustainability, speed in obtaining valid results, reliability in terms of predictive values, and accessibility. Although slow conventional tests could still be used for epidemiological monitoring of drug resistance, rapid genotypic testing facilitates more appropriate patient management and can significantly advance large-scale epidemiological studies of drug-resistant variants. In practice, it is likely that more than one method will be needed

to ensure rapid as well as accurate detection of resistance to NAIs.

Conflicts of interest

The findings and conclusions of this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention (CDC). The authors have no potential conflicts to declare.

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