Pyrosequencing use for drug resistance detection in influenza

APHL 101 teleconference

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Pyrosequencing approach to sequence determination

- Sequencing by synthesis
- Different platforms for different applications (quasispecies analysis, full genome sequencing etc.)
- Qiagen/Biotage: a user friendly and the most suitable platform for virus surveillance purposes
- Review: *Deyde and Gubareva. (2009) Expert Reviews in Molecular Diagnostics. 9:493-509.*

Advantages:

- High throughput format
- Generates sequence data
- Single Nucleotide Polymorphism (SNP) or Sequencing Analysis (SQA) modes
- Sensitive, often used directly on clinical specimens (typically on samples with Ct values <30)
- Can be easily modified to target new markers
- Built-in data analysis for a particular target (IdentiFire software allows update of a sequence library by the user)
- Detection of minor variants (5-10%)

Disadvantages:

- High cost of equipment
- A single vendor for pyrosequencing equipment and reagents (except RNA extraction and RT-PCR steps)
- Training is necessary
- Includes several steps and is not as rapid as a real time RT-PCR assay

Detection of drug resistant viruses based on molecular markers

FDA-approved drugs for influenza: 2 classes

- **M2 blockers** (Adamantanes):
  - Amantadine
  - Rimantadine
- **NA inhibitors**:
  - Zanamivir (inhaled)
  - Oseltamivir (oral)
  - [Peramivir IV, was used in US under EUA during the 2009 pandemic]
  - Peramivir is marketed in Japan and South Korea;
  - Laninamivir (inhaled) is in clinical trials

Resistance to M2 blockers

- Amino acid change(s) in the M2 protein:
  - Variance in M2 sequences among flu A viruses:
    - (needs to be reflected in the M2-specific sequence library of IdentiFire)
  - Established markers of resistance:
    - L26F, V27A, A30V, A30T, S31N, and G34E
    - Recently added marker: S31D
    - A combination of markers (eg, V27A+S31N)
- Confer cross-resistance to amantadine and rimantadine

Cell culture assays

Cell culture-based assays (virus yield reduction) in the presence of amantadine and rimantadine are typically used to assess drug susceptibility, when

- a previously unseen change is detected in the M2 protein (25-43 aa portion) by sequencing or pyrosequencing;
- a new virus (eg, 2009 pandemic) is isolated from humans;
- a virus is resistant if its replication is not significantly affected by the M2 blocker at concentration of 1ug/ml.
- *Gubareva et al, Antivir Therapy, 2010.*

Current global situation

- H3N2 viruses collected during the two last seasons have been resistant to M2 blockers (S31N marker).
- Pandemic 2009 H1N1 viruses have been resistant (S31N marker), with the exception of 4 viruses collected in the US.
- Pre-pandemic H1N1 viruses: some sensitive and some resistant. The most recently collected (in Africa from summer 2010) pre-pandemic H1N1 viruses were sensitive to M2 blockers.
- Some swine viruses are resistant and some sensitive.
- Certain clades of H5N1 viruses are sensitive whereas the others are resistant.
- At CDC, we will continue to test a portion of viruses circulating in humans and all newly emerging viruses (eg, swine triple reassortants isolated from humans).

Pyrosequencing support: M2 Blockers

- CDC SOPs were updated to reflect the continuous virus evolution
- Updated CDC SOPs are available via APHL (Tricia Aden):
  - SOP for H3N2 viruses (also suitable for pre-pandemic H1N1 viruses)
  - SOP for pandemic 2009 H1N1 viruses
- We are working on a universal assay for all influenza A viruses, including swine and avian H5N1, but at this time we do not have an assay that covers all subtypes and clades.
- Reference viruses are available upon request (email Dr. Larisa Gubareva; lqg3@cdc.gov).

NA Inhibitors

Assessment of susceptibility to NA inhibitors

- Limited information on molecular markers associated with NAI-resistance in humans
- Cell culture-based assays are not suitable due to interference by the HA and the difference in the receptors' structure compared to the human host
- No established correlates for resistance in vitro and vivo.
- No defined cut-off values to separate sensitive viruses from resistant ones
- Current approach: testing in the NA inhibition assay(s) with reference viruses (sensitive and resistant) in combination with NA sequencing and/or NA pyrosequencing.

Molecular markers associated with resistance or reduced susceptibility in NI assay

- Markers are drug- and (sub)type specific
- A long list of NA changes associated with reduced susceptibility (resistance) to one or several NAIs. Examples of markers to watch for:

Virus	Change in NA	Oseltamivir	Zanamivir	Peramivir
H1N1 and H5N1	**H275Y** (H274Y in N2 numbering)	R	S	R
H1N1 and H5N1	I223R (I222 in N2 numbering)	R	?	?
H3N2	R292K	R	R	R
H3N2	N294S	R	?	?
H3N2	E119V	R	S	S
B	R152K (N2 numbering)	R	R	R
B	D198N (N2 numbering)	R	R	S

Pyrosequencing assay to detect most common changes associated with NAI-resistance or reduced susceptibility

- H275Y in seasonal H1N1 viruses (disappeared from circulation?)
- H275Y in 2009 pandemic H1N1 viruses
- I223 in 2009 pandemic H1N1 viruses (?)
- E119V in H3N2 viruses
- R292K and N294S in H3N2 viruses

- Changes found in virus isolates, but not in matching clinical specimens:
  - D151N/S/E… H1N1 and H3N2 viruses
  - Q136K in H1N1 viruses
  - No apparent relevance for susceptibility in humans
  - *Okomo-Adhiambo et al, (2010) Antiviral Res. 85:381-388.*

Assessment of drug susceptibility to NAIs at CDC

- All viruses submitted to CDC (by domestic and foreign partners) and tested in HI assay have also been tested in NI assay with oseltamivir and zanamivir.
- A portion of virus isolates has also been tested with the investigational NAIs peramivir and laninamivir.
- Viruses identified as potentially resistant in NI assay have been tested in the pyrosequencing assay and/or by Sanger sequencing to detect any change(s) in NA responsible for reduced susceptibility. In addition, their matching clinical specimens, when available, were tested as well.
- At times, such analysis requires comparison to NA sequences in the public databases and additional studies.
- The data generated using the NI assay have been posted on the FluView website.

Enhanced surveillance for oseltamivir resistance

- Testing of clinical specimens in the pyrosequencing assay to detect the oseltamivir resistance-conferring mutation H275Y in H1N1 viruses.
- Screening of large numbers of specimens
- Reduces delays due to virus propagation thus providing near real time surveillance data
- Pyrosequencing has been conducted at CDC and more recently by the three contractor labs (WI, CA, and NY). The generated data have been posted on the FluView website.
- Need to avoid duplication of testing and reporting when the same specimen is submitted for a routine antigenic (HI) and antiviral analysis and for enhanced surveillance (H275Y only).

Pyrosequencing: H275Y

- Clinical specimens collected from drug treated patients may contain a mix of virus variants (resistant and wild type variants)
- Limit of mutation detection: 5-10%
- SNP mode is preferable
- If the presence of 2009 pandemic virus is confirmed (Ct value <30) and the SNP test failed, submit this sample to CDC for NA sequence analysis;
- Last season, agreed to report as “resistant” if virus population contained ≥10% of the mutant H275Y variant and this percent was recorded in the report (database).
- Pyrosequencing does not tell if virus is sensitive or resistant to oseltamivir (or zanamivir) but only tells if it contains a variant with this particular mutation.

NAI-pyrosequencing testing support:

- CDC SOPs are constantly evaluated and are updated when necessary
- Updated CDC SOPs are available via APHL (Tricia Aden).
- Use of CDC SOPs for RT-PCR and pyrosequencing reactions is desirable for data consistency and for ease of troubleshooting
- When submitting a request for SOPs, please indicate clearly which markers are to be tested (H275Y in H1N1, M2 markers…)

Reagents for pyrosequencing activity

- **A virus panel** for performance evaluation (marker-specific) testing will be available upon request in the near future.
- Requests to be sent to Larisa Gubareva, lqg3@cdc.gov
- In addition, reference viruses will become available via ATCC (IRR) in the near future
- **Reagents**:
- Requests for reagents to be sent to IRR via website (not yet activated)
- Need to decide on details of this operation:
  - Performance evaluation completion
  - Need common terminology to record the assay outcomes
  - Reporting the generated data to CDC to be posted on FluView
  - Prevent duplication of testing

QUESTIONS?