

IDENTIFICATION OF MUTATIONS IN THE INFLUENZA A GENOME ASSOCIATED WITH ADAMANTANE AND NEURAMINIDASE INHIBITOR RESISTANCE DIRECTLY FROM CLINICAL SPECIMENS



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ABSTRACT

Objective: To evaluate the ability of pyrosequencing performed directly on clinical specimens (CS) to detect specific point mutations in the influenza A genome known to confer resistance to the antivirals adamantane (amantadine and rimantadine) and oseltamivir, a neuraminidase inhibitor.
Methods: A sampling of respiratory virus surveillance specimens from 2006 to 2008 positive for influenza A by RT-PCR were tested. Viral RNA was extracted from CS using the Total Nucleic Acid kit with the MagNA Pure LC instrument (Roche Diagnostics). RT-PCR was performed to amplify products encompassing the region of the M2 gene and the NA gene known to confer resistance to adamantanes and oseltamivir, respectively. Pyrosequencing of the PCR products were performed according to the manufacturer's protocol (Biotage AB) to identify a change at the S31N in the M2 gene, and at the H274Y in the NA gene. CS tested resulting in either a "Passed" or "Check" pyrosequencing quality rating were determined acceptable. Any CS with a "Failed" pyrosequencing quality rating was determined unacceptable.
Results: Over the past two influenza seasons to date (2006-2008), 207 CS positive for influenza A virus by RT-PCR were tested for the S31N mutation by pyrosequencing. Of those, 17 (8.2%) were determined unacceptable, 11 of 17 (64.7%) had a real-time RT-PCR crossing threshold (Ct) of >26.0. Over the previous influenza season (2007-2008), 43 CS positive for influenza A(H1N1) virus were tested for the H274Y mutation in the NA gene. Of those, 4 (11.7%) were unacceptable and all had a Ct value >26. Of all influenza A detected in CS from the 2006-2007 season in Wisconsin, the majority 376 out of 443 (84.9%) had Ct values <26.0. Of the 33 A/H1 and 7 A/H3 CS tested during the 2007-2008 respiratory virus season, 1 A/H1 (3.0%) and 7 (100%) A/H3 were determined to be resistant to the adamantanes.
Conclusion: Previous studies (ASM abstract C71, 2007) indicated that direct pyrosequencing of CS using a Ct threshold of <26.0 was comparable to testing from cell culture isolates. The results of this study confirm that pyrosequencing for resistance mutations from CS, using a Ct threshold of <26.0, is applicable for the majority of CS positive for influenza A. Antiviral resistance mutation can be detected directly from CS in one day compared to up to seven days from cell culture isolates. With emergence of antiviral resistance, this rapid testing has both clinical and public health implications for determining appropriate and timely antiviral treatment, especially in outbreak settings or with emergence of novel influenza strains.

OBJECTIVE

- To evaluate the ability of pyrosequencing to detect resistance mutations associated with adamantane and neuraminidase inhibitor antivirals in the influenza A genome directly from clinical specimens (CS).
- To discuss the importance of monitoring for antiviral resistance in influenza and the impact on pandemic preparedness.

METHODS

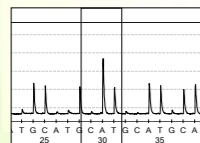
- Surveillance:** A representative sample of respiratory specimens from 2006-2008 were obtained from the virus surveillance network sites throughout Wisconsin.
- Nucleic Acid Purification:** Nucleic acid was extracted from 200ul of respiratory specimens using the Roche MagNA Pure[®] LC instrument with the Total Nucleic Acid kit and eluted in 50ul.
- Real-time PCR:** Nucleic acid was tested for influenza and subtyped utilizing the CDC influenza branch RT-PCR method on the ABI 7500 Fast.
- Pyrosequencing:** PCR was performed on positive influenza A/H1 and A/H3 samples with primers encompassing the M2 (matrix) region, and on positive influenza A/H1 samples with primers encompassing the NA (neuraminidase) region specific for A/H1N1 viruses (Figure 1). Pyrosequencing was performed on the PCR products according to the manufacturer's (Biotage) instructions.
- Data Analysis:**
 - Sequencing data resulting in "passed" or "check" ratings were determined to be acceptable for the M2 regions, and "passed" only ratings were determined to be acceptable for the NA regions tested. Sequences resulting in "failed" were unacceptable for both regions tested.
 - Specimens exhibiting a change at S31N in the M2 gene were considered 'resistant' to the adamantanes (adamantane and rimantadine); Specimens exhibiting a change at H274Y in the NA gene were considered 'resistant' to oseltamivir (Tamiflu[®]).

Figure 1. M2 and NA sequencing primers

Primer/probe ID	Sequence (5'-3')	Orientation	Reference
M2F	cag atg car cga ttc agt g	Forward	Bright et al.
NAF	aga tcg aga agg gga agg tta cta		CDC Flu Branch
MR	BIO-agt aga aac aag gta gtt ttc tac tc	Reverse	Bright et al.
NAR	BIO-gt cyc tgc ata cac aca tea ct		CDC Flu Branch
M2FS	cag atg car cga ttc agt g	Sequencing	Bright et al.
NAFS	aaa tgc acc caa t		CDC Flu Branch

Figure 2. Influenza A/H3N2 pyrogram from direct clinical specimens showing a change at S31N in the M2 protein.

Adamantane-resistant
Result: GCG**A**ATATCA



Adamantane-susceptible
Result: GCG**A**GTATCA

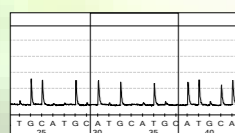
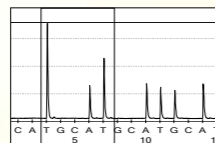


Figure 3. Influenza A/H1N1 pyrogram from direct clinical specimens showing a change at H274Y in the N1 region.

Oseltamivir-resistant
Result: TTT**A**ITATGA



Oseltamivir-susceptible
Result: TTT**C**ATATG

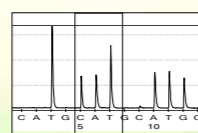


Figure 4. Upon initiation of surveillance for the 2006-2007 influenza season, a real-time PCR crossing point (Ct value) of <26.0 was determined and later verified as an acceptable testing cut-off point from 33 influenza A CS tested for adamantane resistance surveillance to ensure that sufficient RNA was present for pyrosequencing.

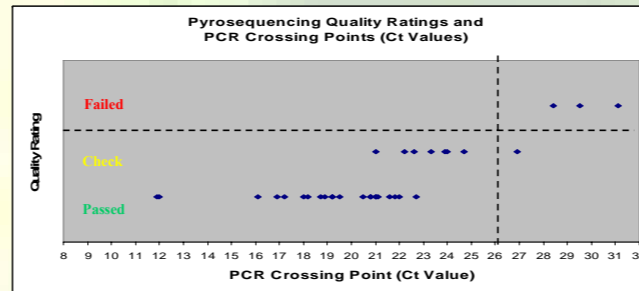
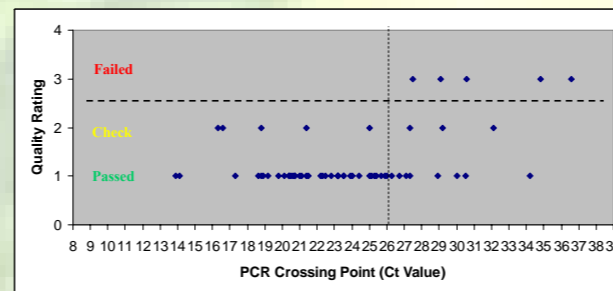


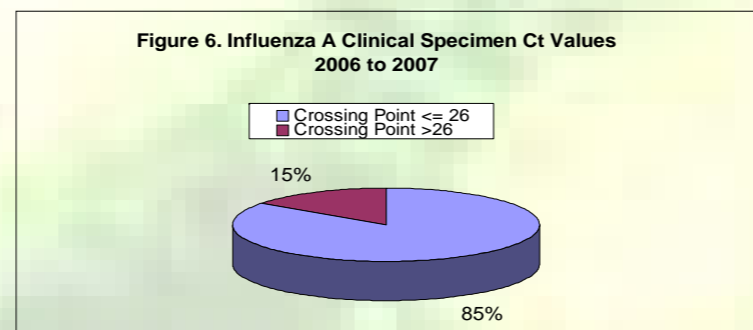
Figure 5. During the 2007-2008 influenza season, a real-time PCR crossing point (Ct value) of <26.0 was also proposed as a testing cut-off point from 59 A/H1 CS tested for oseltamivir resistance to ensure that sufficient RNA was present for pyrosequencing.



RESULTS

- Adamantane Resistance (2006-2008 Respiratory Virus Season)**
 - Of the 266 influenza A CS with Ct values <26.0 tested for resistance to adamantanes, 12 (4.5%) were determined to be "unacceptable".
 - Of the 42 A/H1 and 44 A/H3 strains tested during the 2007-2008 respiratory season, 1 (2.4%) A/H1 and 44 (100%) A/H3 had the S31N resistance mutation. The one resistant A/H1 was determined to have had a recent travel history in Asia.
- Neuraminidase Inhibitor Resistance (2007-2008 Respiratory Virus Season)**
 - Of the 59 A/H1 CS tested for resistance to oseltamivir, 5 (8.5%) had a quality rating of "Failed", and 8 (13.6%) had a quality rating of "Check". All 13 (22.0%) were determined to be "unacceptable".
 - Of these 13, 8 (61.5%) had PCR crossing points >26.0.
 - Of the 46 A/H1 with acceptable sequences, 8 (17.4%) had the H274Y resistance mutation.

Figure 6. Influenza A Clinical Specimen Ct Values 2006 to 2007



DISCUSSION

- Virus surveillance:** A Ct value cutoff point of <26 is useful to rapidly monitor influenza antiviral resistance patterns from season to season in "real time" on direct clinical specimens.
- Rapid results:** Pyrosequencing directly from CS and analysis can be accomplished in one day compared to up to seven days for tissue culture isolates.
- Time/Cost savings with high throughput:** Considerable time savings over conventional sequencing from virus isolates. Cost savings compared to conventional sequencing is superior when testing large numbers of samples due to the 96-well format of pyrosequencing.
- Outbreak Response:** Availability of antiviral resistance data can impact treatment in high risk populations such as nursing home resident and immunocompromised patients.
- Pandemic Preparedness:** Can be used to rapidly identify known mutations in novel strains that confer resistance to antivirals.
- Limitation:** Only small fragments (<150bp) can be analyzed efficiently using pyrosequencing.
- SPHL Network:** To support CDC influenza antiviral resistance testing, a network of state public health laboratories with pyrosequencing technology should be formed. This is especially important as new oseltamivir resistance is developing and would be useful surge capacity during an influenza pandemic.

CONCLUSIONS

- Increasing resistance to oseltamivir (neuraminidase inhibitor) among H1N1 isolates tested at CDC has recently been documented from <1% in 2006-2007 to 9.1% in late 2007 (Klimov, 2008).
- Adamantane resistance continues to remain high: 87/271 (32%) of influenza A viruses were resistant (MMWR, 2008).
 - Influenza A/H3N2 99% resistance
 - Influenza A/H1N1 7.6% resistance
- No antiviral resistance to zanamivir (Relenza[®]) has been detected in any influenza subtype (MMWR, 2008).
- Direct pyrosequencing from CS using a crossing threshold (Ct) of <26.0 was comparable to testing cell culture isolates.
- The majority of influenza positive CS tested at WSLH have a Ct <26.0 [Figure 6].

REFERENCES

- Bright RA et al. (2006) Adamantane resistance among influenza A viruses isolated early during the 2005-2006 influenza season in the United States. JAMA. Feb 22;295(8):891-4. Epub 2006 Feb 2.
- Bright RA et al. (2005) Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. Lancet. Oct 1;366(9492):1175-81. Epub 2005 Sep 22.
- Klimov A (2008) CDC Antiviral Resistance Surveillance Report. 11 March.
- Update: Influenza Activity---United States, September 30, 2007-February 9, 2008 MMWR Feb. 22, 2008: 57(07) 179-183

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