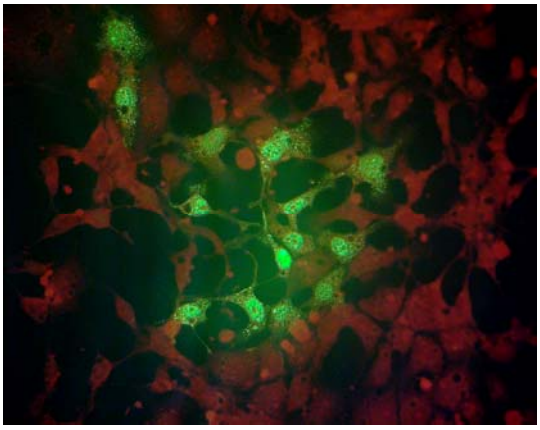
Erasmus Medical Centre, Rotterdam, The Netherlands

Influenza Resistance Information Study

PROTOCOL (CONFIDENTIAL)

Rapid algorithm for detection of influenza A(H1N1)pdm09 virus and screening for a key oseltamivir resistance (H275Y) substitution in neuraminidase.

We present a sensitive and specific approach for detection of influenza virus A(H1N1)pdm09 (A) and a rapid RT-PCR assay detecting a primary oseltamivir resistance mutation (H275Y) which can be incorporated easily into clinical virology algorithms (B).



Immuno staining of MDCK-cells infected with influenza virus A(H1N1)pdm 09

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Publication:

E. van der Vries et al. Evaluation of a rapid molecular algorithm for detection of pandemic influenza A (H1N1) 2009 virus and screening for a key oseltamivir resistance (H275Y) substitution in neuraminidase. J. Clin. Virol. 2010;47:34-7.

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Validation conditions

For validation of the RT-PCR assays, nucleic acids were extracted from respiratory specimens using 200ul. of respiratory specimen. Nucleic acids were eluted to a volume of 110ul. using the pathogen complex 200 protocol and the QiaSymphony machine (Qiagen, Venlo, The Netherlands) according to instructions of the manufacturer. The rtTH- based EZ RT-PCR kit (Applied biosystems, Nieuwerkerk a/d Ijssel, The Netherlands) was used for all RT-PCR assays and amplification and detection was performed on a LightCycler 480 instrument using the FAM (465-510nm) and Dragonfly (533-580nm) filter (Roche, Almere, The Netherlands).

EZ RT-PCR mixture/ reaction

RNAse free water	4,5 µl
5x Taqman EZ buffer	10 µl
Manganese acetate (25 mM)	6 µl
dNTP's	6 µl
primer/probe mix (1 µl/reaction)	1 µl
rTth DNA Polymerase (2,5U/µl)	2 µl
Amperase UNG (1U/µl)	0,5 µl
Total mix volume	30 µl
Input RNA	20 µl
Total volume	50 µl

Program(s)

	Time (min)	T (°C)
UNG reaction	2:00	50
cDNA reaction	30:00	60
Denaturation	5:00	95
+(45 cycles)		
Denaturation	0:20	95
Elongation	1:00	62 (A) / 60 (B)

(A) Influenza A/H1 and A/N1 RT-PCR

(B) Influenza A H275Y RT-PCR

Primers and probes

(A) Influenza A matrix, A/H1 and A/N1 RT-PCR reactions

Primers and probes	Sequence (5'-3')	Label (5'-/-3')	Position	final concentration/ reaction (pmol)
New variant influenza A/H1N1-H1 and A/H1N1-N1 2009 duplex RT-PCR				
panH1-forward ggaaagaaatgctgga	tctggta		822-844	30
panH1-reverse atgggaggctg	gtgtttatagc		904-926	30
panH1-probe	tgcaatacaacttgtcagacacccaaggg	Dragonfly /BHQ-2	874-902	10
panN1-sense	acatgtgtgtgcagggataactg		865-887	30
panN1-antisense	tccgaaaatcccactgcatat		949-969	30
panN1-probe	atcgaccgtgggtgtctttcaacca	6-FAM/BH Q-1	899-923	10
(B) Influenza A H275Y RT-PCR				
New variant influenza A/H1N1-N1 H274Y				
panN1-H275-sense	cagtcgaaatgaatgcccctaa		797-818	20
panN1-H275-antisense tgcacacacatgt	gatttcactag		854-877	20
panN1-275H-probe	ttaTCActAtgAggaatg*	6- FAM/BHQ-1	821-836	5
panN1-275Y-probe	ttaTTActAtgAggaatg*	Dragonfly/BHQ-2	821-836	5

* LNA nucleotides are denoted in upper case, DNA nucleotides are denoted in lower case and LNA nucleotide complementary to the predicted single nucleotide polymorphism (SNP) is underlined.

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Conclusions

We have evaluated a novel RT-PCR assay for sub typing the pandemic influenza A/H1N1 virus. Both RT-PCRs are sensitive and specific and can be incorporated directly into existing influenza detection and sub typing algorithms. Up to 5% for the H275Y oseltamivir resistant mutants could be detected in mixed virus populations using the H275Y discrimination assay. For diagnostic purposes, the discrimination RT-PCR assays should be performed in parallel with influenza RT-PCR assays targeting more conserved regions of the influenza viral genome. The sub typing RT-PCR assays described appear sensitive and specific in this validation pilot. Using the H275Y discrimination RT-PCR, A(H1N1)pdm09 viruses can be screened for oseltamivir susceptibility within a 2-3 hours time, which makes it useful for surveillance and valuable in clinical patient management.

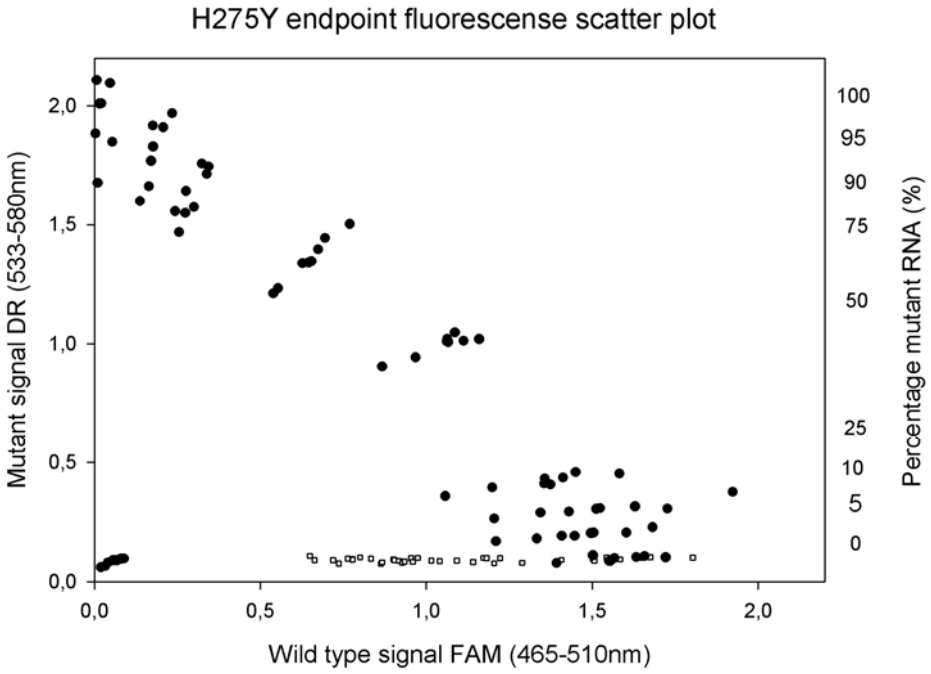

Figure. Detection of the H275Y resistance mutation. Mixtures of *in vitro* transcribed wild type and mutant RNA (total input $1.0X10^5$ vp/ml) were analyzed using the H275Y discrimination assay (black dots). Relative H275 wild type (465-533nm) and 275Y mutant (533-580nm) fluorescence emissions were plotted on the x-axis and y-axis respectively. In addition, 37 clinical isolates from naïve influenza virus A(H1N1)pdm09 infected patients were analyzed (white squares) to determine a threshold for detecting mutant genotypes in mixed virus populations. The threshold was set to be 5%.

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Interpretation of results

Influenza A Matrix PCR	275H	275Y	Interpretation*
Neg			Patient Flu negative
Ct>33		Neg	Patient low positive and resistance PCR not interpretable
Ct>33		Pos	Patient low positive and resistant
Ct<33	Pos	Neg	Patient positive not resistant
Ct<33	Neg	Pos	Patient positive and resistant
Ct<33	Pos	Pos	Patient positive and resistant
Ct<33	Neg	Neg	Discrepancy between Matrix and resistance PCR. Repeat both.
Repeated assay Ct<33	Neg	Neg	Consider virus culture followed by NA sequencing and /or phenotypic assay if there is clinical urgency or indication for resistance.

* The resistance PCR only monitors for the H275Y substitution. A “Not resistant” result actually means that the virus does not have the H275Y substitution that has been associated previously with resistance against oseltamivir. The virus can have other mutations that confer the virus less susceptible or resistant. If the patient does not respond to oseltamivir therapy and the result of this PCR is “Not resistant”, consider virus culture followed by NA sequencing and / or phenotypic assay.