

Detection of respiratory viral infections by real-time PCR

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Respiratory viruses

- Majority of these are RNA viruses
- Seasonality of respiratory viral infections
- Role in disease of some viruses is uncertain
- Most susceptible groups are young children, elderly and immune-suppressed.
- Outbreaks may occur in closed environments

Respiratory viruses

- RNA viruses
 - RSV (A & B subtypes)
 - Influenza, A (16 subtypes)
 - H3 & H1 – annual epidemics
 - H5 - a threatening pandemic
 - Influenza B
 - Parainfluenza 1,2, 3 and 4
 - Rhino – over 100 serotypes
 - Entero –over 100 serotypes

Respiratory viruses

- RNA viruses

 - Coronaviruses – 229E

 - OC43

 - SARS

 - NL63 & HKUI

 - Metapneumoviruses

- DNA viruses

 - Adenoviruses - 51 serotypes

 - Bocaviruses

 - KI & WU polyomaviruses

Detection methods

- Isolation of virus by culture
 - time consuming (4-21 days)
 - gold standard
- Rapid antigen detection (DIF)
 - shorter TAT
 - reagents not available for all viruses,
limited to RSV, influenza, parainfluenza
and adenovirus
 - expertise required

Detection methods

- Nucleic acid amplification by PCR
 - TAT is shorter
 - Gel based
 - Real time assays
 - Taqman & FRET probe based
 - short TAT, allows multiplexing
- Microarray based systems (Virochip)
 - new and costly
 - evaluation ?

Real time detection of respiratory viruses at ICPMR

Multiplex assays

LightCycler with 6 channels

Taqman (hydrolysis) probes

Reporter dyes with different excitation/emission spectrum

Combination of 3 different channels is possible with the use of colour compensation files

530 is the dominant channel (FAM), 610 (Cal Fluor red, Texas red) and 705 (Pulsar 650)

Multiplex assays (cont)

Available assays

- 1 – H5N1, influenza A (M gene) and betaglobin (house keeping gene)
- 2 – RSV, influenza A (M gene) and influenza B
- 3 – parainfluenza 1, 2 and 3
- 4 – rhinoviruses and enteroviruses (duplex)
- 5 – metapneumovirus
- 6 – adenovirus and betaglobin (duplex)
- 6 – pan coronavirus
- 7 – influenza A subtypes H1& H3 (melt curve)

Method

RNA viruses

Reverse transcription of target RNA to cDNA using Invitrogen Superscript III

Random hexamers for priming (allows us to use the cDNA for multiple tests)

PCR - Single round

For/Rev Primers

Taqman probe

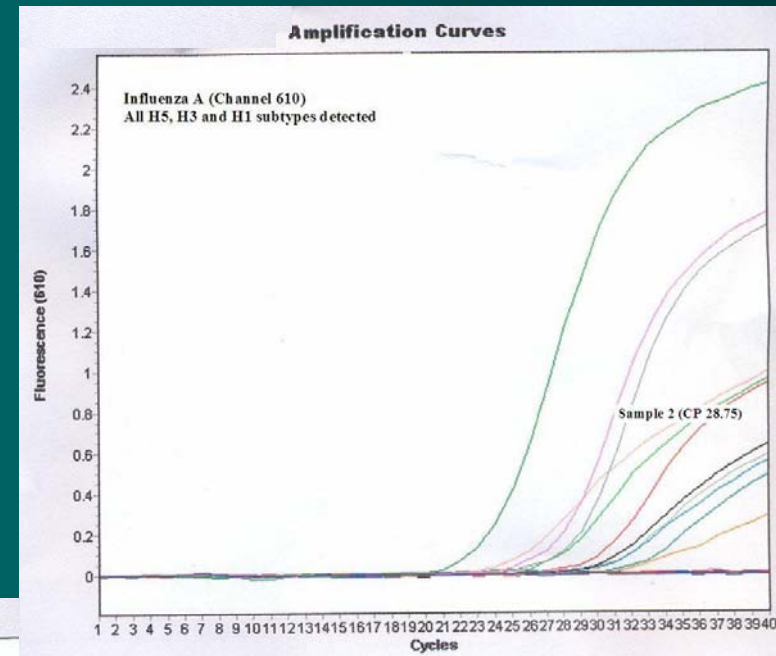
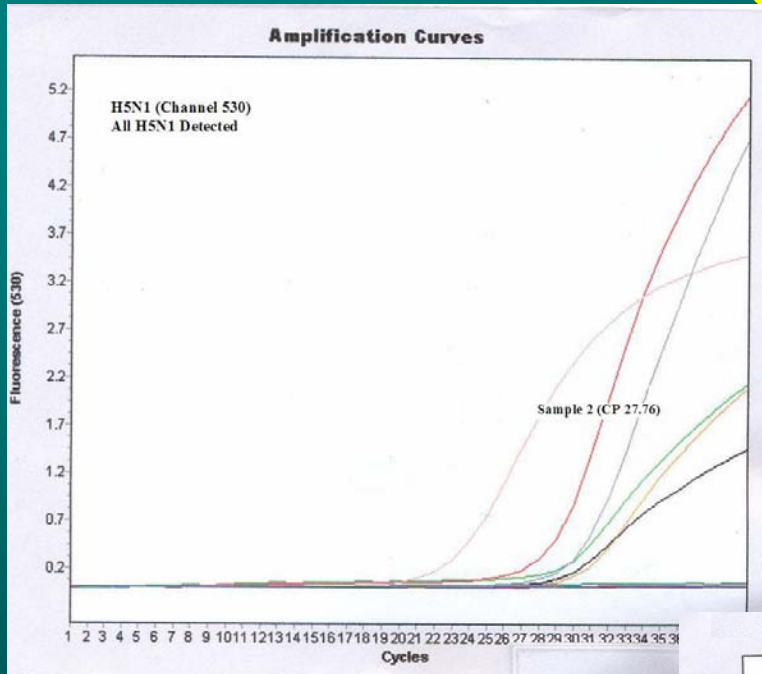
Roche Hybridization Master+

DNA/cDNA (2 to 4 μ l)

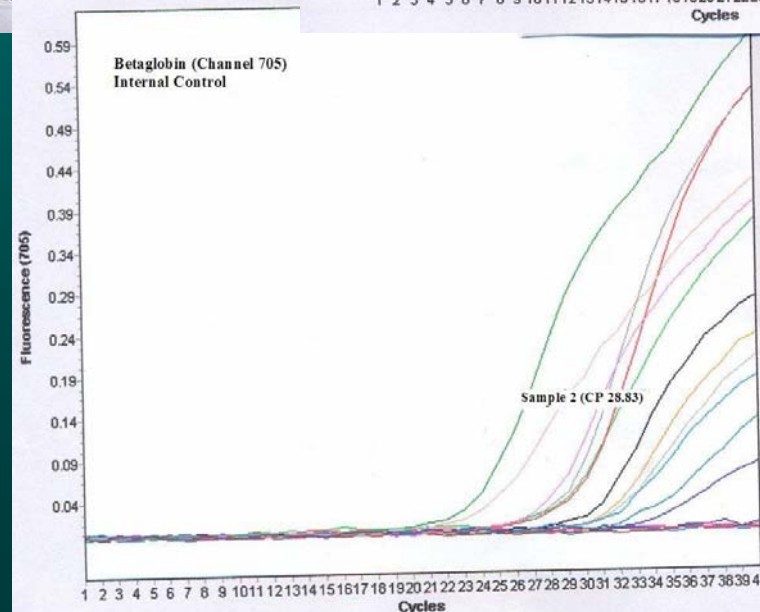
Optimization of the assays

- Primer design for each target
 - Conserved gene
 - Testing multiple primer pairs to choose the best
- Competitive inhibition of targets
 - Testing series of dilutions of each target against a constant cDNA of the other 2 targets
 - Extensive work

Amplification of H5, Influenza A and Internal control.



Samples				
Include	Color	Pos	Name	CP
<input checked="" type="checkbox"/>	Blue	1	NEG	[21.55]
<input checked="" type="checkbox"/>	Green	2	SAMPLE 1	28.49
<input checked="" type="checkbox"/>	Red	3	SAMPLE 2	27.76
<input checked="" type="checkbox"/>	Black	4	SAMPLE 3	28.46
<input checked="" type="checkbox"/>	Pink	5	SAMPLE 4	
<input checked="" type="checkbox"/>	Light Green	6	SAMPLE 5	
<input checked="" type="checkbox"/>	Dark Blue	7	NEG EXT	
<input checked="" type="checkbox"/>	Light Grey	8	SAMPLE 6	[25.76]
<input checked="" type="checkbox"/>	Light Orange	9	SAMPLE 7	22.14
<input checked="" type="checkbox"/>	Purple	10	SAMPLE 8	
<input checked="" type="checkbox"/>	Yellow	11	SAMPLE 9	29.39
<input checked="" type="checkbox"/>	Light Blue	12	SAMPLE 10	
<input checked="" type="checkbox"/>	Brown	13	NEG	
<input checked="" type="checkbox"/>	Pink	14	H5N1 10-3	29.52
<input checked="" type="checkbox"/>	Grey	15	H3N2 10-3	
<input checked="" type="checkbox"/>	Light Green	16	H1N1 10-2	



Problems from our experience

- RNA viruses pose more problems than DNA viruses in primer/probe design and detection.
- Error rate for RNA replication is much more than for DNA viruses.
- Phenotypic variants appear due to selection pressure – may lead to false negative results.
- Antigenic drift / antigenic shift seen with influenza A viruses

Problems (cont)

➤ Picornaviruses

- Most difficult PCR to set up
- Rhinoviruses (more common)
- Enteroviruses (entero 68, Coxsackie and echo)
- Most conserved region is the 5UTR
- Shared homology between the two viruses
- Difficulty in finding a single probe to detect all rhinoviruses.
- Two probes were necessary
- New variants

Drift - sequence of NP gene of influenza A (H3N2/H1N1)

consensus	5' <i>TTATGACAAAGAAGAAATAAGGMG</i> 3'
03_MIN_H3N2A.
01_NY_H1N1C.
03_NY_H1N1
04_NY_H3N2
05_NY_H3N2
02_Yok_H1N1
02_CAN_H3N2T.....
03_CAN_H3N2T.....
02_NY_H3N2T.....
03_NAG_H3N2
02-219-1037T.....
03-257-3548T.....

Problems (cont)

➤ DNA viruses

- Adenovirus (7/51 serotypes)
- 2 probes necessary to have all the common types detected
- Used as a singleplex assay, difficulty to multiplex a DNA virus with other RNA virus
- Duplex with internal control

Problems (contd)

Multiplex vs singleplex reactions

- QAP exercises for H5N1
- Series of dilutions
- Most dilute specimen is positive on the singleplex reaction and negative on the multiplex reaction (2 out of 6)
- Low Ct values >35

Work flow for respiratory specimens at CIDMLS

- N/T swabs, NPA and BAL
- Testing process
 - DIF antigen for common viruses as a screening assay (POCT during influenza season)
 - DIF negatives set up for culture
 - DIF negatives for PCR depending on patient status (Aged care facility/ immune-compromised)

Advantages of Real time PCR testing for respiratory viruses

- In a clinical laboratory setting
 - Speed is utmost
 - Sensitive methods
 - Cost
- Dual infections (7.4% our study)
- Real time PCR allows quantitation
 - viral load after antiviral, resistance
- Differentiate viruses with common phenotypic characteristics eg entero 68 and rhinoviruses

Other issues

- TAT for PCR vs DIF/POCT
 - Timeliness for clinical treatment
 - choice of test in influenza season
- Surge capacity for pandemic influenza (PCR first)
- Rapid response to develop tests
 - PCR is best for new viruses (eg SARS, H5N1)

Is it necessary to test for new and emerging viruses?

- Do the new coronas, metapneumoviruses and bocavirus cause disease?
- Rhinovirus was the single most frequently detected virus after RSV (Gruteke et al 2004)
- RSV and metapneumovirus were each responsible for as many hospitalized cases of respiratory infections in aged care patients (Kaye et al 2006)
- Coronavirus HKUI associated with community acquired pneumonia (Woo et al 2005)

Conclusions

- PCR for respiratory viruses allows for the detection of a larger number of viruses, not possible by culture.
- Real time platforms allows multiplexing but optimization requires extensive work
- RNA viruses are under continual selection and new lineages appear and dominate. Primer and probe design need to evolve with this, particularly for influenza A.
- Probe binding is affected by 2 or more mismatches leading to false negative results.
- “PCR only” testing has the disadvantage of not having a viral isolate for influenza vaccine formulation, or other studies.