QUANTITATION OF INFLUENZA A VIRUS FROM NASAL AND LUNG TISSUE OF COTTON RATS USING REAL-TIME RT-PCR AND CULTURE

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Background

The 2007/08 influenza season was the worst in three seasons in terms of overall morbidity and mortality (1), particularly due to antigenic drift in the current trivalent influenza vaccine. Additionally, an increase in the cumulative confirmed cases of high pathogenic H5N1 has raised concerns of a potential pandemic in humans. Since Jan 1, 2008 avian influenza has continued to circulate in Indonesia, Vietnam, and Egypt with 30 confirmed human cases and 23 fatalities (76% mortality) (2).

A highly sensitive and specific set of real-time RT-PCR (rRT-PCR) assays for point of care detection of influenza virus typing (influenza A or B) and subtyping (H3N2, H1N1, and H5N1) would facilitate patient care and decrease costly evaluations. In a potential pandemic, rapid detection of infected individuals would enhance intervention and patient cohorting, and could help prevent widespread dissemination of disease in the community.

The cotton rat (*Sigmodon hispidus*) is a useful small animal model for influenza pathogenesis (Figure 1) and several studies exploring influenza nasal and pulmonary infection, immunological response, and antiviral therapy have been performed (3-5). This study compared the sensitivity of 'gold standard' culture to the recently developed PrimeMix real-time RT-PCR System for the detection of influenza virus in primary infected and non-infected (sentinel) cotton rats.

Figure 1. ***Sigmodon hispidus*, a commonly used cotton rat in biomedical research.**

Objective

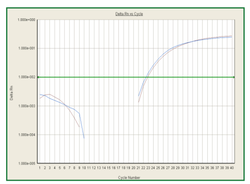
To compare quantitative sensitivities rRT-PCR and culture for detecting influenza virus in lung and nasal tissue from primary infected and sentinel (non-infected) cotton rats.

Methods

Cotton rats (primary infected) were inoculated intranasally with 10^7 $TCID_{50}$ influenza A (H3N2) virus and housed with non- infected (sentinel) rats. Nasal and lung tissue homogenates obtained on post infection day 1,4,10,21, and 28 were analyzed using quantitative culture and real-time with an exogenous control and calibrator sample.

The exogenous control is a distinct nucleic acid sequence (e.g., influenza B) of known concentration added to each sample and serves as a reference to control for variations from extraction and pipetting (6). The calibrator is an untreated control used for normalizing viral concentration (Figure 2).

RNA extractions from homogenized nose and lung samples were lysed and preserved in PrimeStore Solution. Extraction was performed using the RNaqueous Micro Kit (Ambion). Real-time RT-PCR assays for influenza A were designed, optimized, and evaluated according to Daum *et al.* (7) and adapted into PrimeMix, a ready-use simplified rRT-PCR blend.



ABI 7500 real-time Instrument

Figure 2. **Real-Time RT-PCR Assays Detect Known Amounts of Calibrator and Exogenous Template and can Quantify Viral Unknowns in Nose and Lung Samples**. The calibrator and exogenous control reaction represent 0.1 pg of template cRNA corresponding to approximately 5.8 x 10^5 influenza copies. The calibrator and exogenous controls were used in subsequent real-time RT-PCR to quantify viral copy number from cotton rat nose /lung samples inflected with influenza A virus (6).

Results

Table 1. Influenza detection in nose and lung tissue using real-time RT-PCR and culture .

Sample	Treatment	Nose		Lung	
		Culture ($TCID_{50}$)	rRT-PCR (copy #)	Culture ($TCID_{50}$)	rRT-PCR (copy #)
71073	control	-	-	-	-
71074	control	-	-	-	-
71075	control	-	-	-	-
71076	control	-	-	-	-
71077	Primary infected	300	$5x10^5$	325	$3x10^4$
71078	Primary infected	350	$2x10^6$	375	$2x10^7$
71079	Non-infected sentinel	-	-	-	$5x10^6$
71080	Non-infected sentinel	-	-	-	$2x10^4$
71081	Primary infected	325	$6x10^5$	325	124
71082	Non-infected sentinel	-	-	-	$9x10^4$
71083	Non-infected sentinel	-	-	-	-
71084	Primary infected	300	$6x10^5$	425	$6x10^4$
71085	Primary infected	325	$6x10^5$	350	$4x10^4$
71086	Primary infected	325	$2x10^5$	375	$3x10^6$
71087	Primary infected	300	$2x10^6$	375	$3x10^4$
71088	Primary infected	300	$3x10^6$	-	9893
71089	Primary infected	275	$6x10^6$	-	$9x10^4$
71090	Non-infected sentinel	-	116	-	82
71091	Non-infected sentinel	-	-	-	-
71092	Primary infected	275	$6x10^5$	-	$5x10^4$
71093	Primary infected	300	$1x10^6$	-	$1x10^4$
71094	Primary infected	250	$6x10^5$	-	-
71095	Non-infected sentinel	-	124	-	-
71096	Non-infected sentinel	-	210	-	-
71097	Non-infected sentinel	-	53	-	-
71098	Primary infected	425	$2x10^6$	-	$5x10^5$
71099	Non-infected sentinel	-	582	-	-
71100	Primary infected	375	$1x10^9$	-	$4x10^5$
71101	Primary infected	400	$7x10^6$	-	$8x10^5$
71102	Primary infected	-	482	-	-
71103	Primary infected	-	$6x10^4$	-	193
71104	Non-infected sentinel	-	489	-	-
71105	Primary infected	-	7565	-	-
71106	Non-infected sentinel	-	-	-	-
71107	Primary infected	-	$4x10^4$	-	174
71108	Non-infected sentinel	-	114	-	-
71109	Non-infected sentinel	-	-	-	-
71110	Primary infected	-	$2x10^7$	-	-
71111	Primary infected	-	$4x10^6$	-	-
71112	Non-infected sentinel	-	-	-	-
71113	Non-infected sentinel	-	-	-	-
71114	Primary infected	-	$4x10^4$	-	17
71115	Primary infected	-	$9x10^4$	-	31
71116	Primary infected	-	-	-	-
71117	Primary infected	-	145	-	-
71118	Primary infected	-	112	-	-
71119	Non-infected sentinel	-	-	-	29
71120	Non-infected sentinel	-	-	-	-
71121	Primary infected	-	582	-	76
71122	Primary infected	-	67	-	489
71123	Primary infected	-	103	-	-
71124	Non-infected sentinel	-	33	-	-
71125	Non-infected sentinel	-	6	-	-
71126	Primary infected	-	85	-	-
71127	Non-infected sentinel	-	-	-	-
71128	Non-infected sentinel	-	-	-	-
71129	Primary infected	-	9	-	569
71130	Primary infected	-	-	-	-
71131	Primary infected	-	6	-	-
71132	Primary infected	-	-	-	-
71133	Non-infected sentinel	-	-	-	-
71134	Primary infected	-	10	-	-
71135	Non-infected sentinel	-	-	-	-
71136	Primary infected	-	-	-	-

DAY 1

DAY 4

DAY 10

DAY 21

DAY 28

Culture

❖All primary infected cotton rats (N=36) became ill and culture-positive influenza virus was detected from all nasal (6/6) and lung (6/6) samples on Day 1 post-infection.

❖On Day 4 primary infected nose samples (9/9) but no matched lung samples (0/9) were culture-positive, and thereafter (Day 10,21,28) all primary-infected lung and nasal samples were culture-negative.

❖All sentinel (non-infected) rats (Day 1-28) were culture-negative. Sentinel animals analyzed on Day 21 had increased antibody levels to influenza virus.

Real-time RT-PCR

❖rRT-PCR detected influenza in lung and nasal tissue from primary-infected and non-infected animals at times when culture was negative and at levels < 10 viral copies.

Conclusions

➢The rRT-PCR method described here is rapid (<2 hours), more sensitive than traditional culture and could be valuable for point-of-care patient influenza detection.

➢PrimeMix influenza assays could be valuable for early therapeutic intervention for high risk populations and to decrease spread of infection during a pandemic.

➢Studies are currently underway to evaluate these diagnostic assays for point of care detection in children and families.

References

1. Centers for Disease Control and Prevention. The 2007-2008 Flu Season. Available at: http://www.cdc.gov/flu/about/qa/season.htm.
2. World Health Organization. Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO. Available at: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2008_04_17/en/index.html.
3. Blanco JC, Pletneva L, Alova MB, Richardson JY, Harris KA, and Prince GA. The Cotton Rat: An underutilized animal model for human infectious diseases can now be exploited using specific reagents to cytoines, chemokines, and interferons. *Journal of Interferon and Cytokine Research* . 2004: 36 (4): 21-28.
4. Ottolini M, Blanco J, Porter D, Peterson L, Curtis S, and Prince G. Combination anti-inflammatory and antiviral therapy of influenza in a cotton rat model. Pediatric Pulmonology. 2003: (36) 290-294.
5. Straight TM, Ottolini MG, Prince GA, and Eichelberger MC. Evidence of a cross-protective immune response to influenza A in the cotton rat model. Vaccine. 2006: 24, 6264-6271.
6. Daum LT, Chambers JP, Fischer J, Fischer GW. Synthesizing Control RNAs for Real-Time RT-PCR Viral Quantification. Ambion TechNotes. 2007: Volume 14 (4); page 35.
7. Daum LT, Canas LC, Arulanandam BP, Niemeyer D, Valdes JJ, Chambers JP. Real-time RT-PCR assays for type and subtype detection of influenza A and B viruses. *Influenza and Other Respiratory Viruses*. 2007: 1(4), 167-175.

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