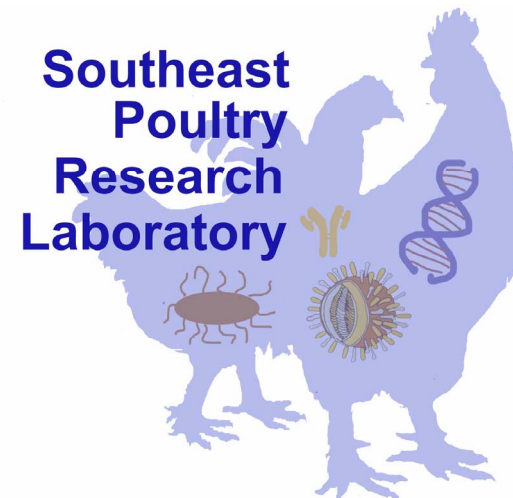


2009 Emergent H1N1 Influenza A Virus: N1 RRT-PCR differentiation test, and infectivity and transmissibility in poultry



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Introduction

- **First cases of respiratory diseases in humans in Mexico, early April, 2009**
- **17410 human cases (including 115 deaths) in 62 countries as of June 1, 2009**
- **First swine case on May 2, 2009 in Canada; human-to-pig transmission**
- **H1N1 virus reassortant:**
 - **N. American Swine: H1, NP, M & NS**
 - **N. American Avian: PA & PB2**
 - **Eurasian swine: N1 & M**
 - **Human: PB1**

Objectives

- **Develop multiplex RRT-PCR test to differentiate classic N. American H1N1 swine and 2009 emergent H1N1, from other N1 influenza A viruses**
- **Improve sensitivity of AI virus matrix gene test for detecting the emergent H1N1 virus**
- **Determine infectivity and transmissibility of the 2009 emergent H1N1 virus for chickens, turkeys (hens and poults), domestic ducks and Japanese quail**

rRT-PCR Test

- **Differential for 2009 emergent and Classic North American Swine H1N1 lineages**
- **Targets the N1 gene**
- **Optimized for:**
 - **Smart Cycler 2 (Cepheid) with OneStep Kit (Qiagen)**
 - **7500 FAST (Applied Biosystems) with AgPath ID kit (Ambion)**

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Sensitivity:

The reproducible limit of detection was determined by the lowest concentration where all replicates were positive using 1µl of template

Ct values by titer for the Classic North American Swine Lineage N1 gene rRT-PCR assay with two MDCK propagated isolates A/Swine/KY/02086/08

Virus	Titer (50% TCID/ml)	Replicate	
		1	2
A/Swine/KY/02086/08	10 ^{6.5}	25.47	25.83
	10 ^{5.5}	28.4	28.49
	10 ^{4.5}	31.15	30.59
	10 ^{3.5}	35.45	35.1
	10 ^{2.5}	38.13	38.78
	10 ^{1.5}	0.0	0.0

**Ct values by titer for the Classic North American Swine Lineage
N1 gene rRT-PCR assay with two MDCK propagated isolates:
A/Swine/NC/02023/08**

Virus	Titer (50% TCID/ml)	Replicate			
		1	2	3	4
A/Swine/NC/02023/08	10 ^{6.3}	20.22	20.22	ND ^a	ND
	10 ^{5.3}	22.6	22.97	ND	ND
	10 ^{4.3}	26.2	26.18	ND	ND
	10 ^{3.3}	29.68	29.38	ND	ND
	10 ^{2.3}	32.99	33.03	ND	ND
	10 ^{1.3}	36.43	35.53	36.53	35.82
	10 ^{0.3}	38.3	39.0	37.97	0.0
	10 ^{-1.3}	0.0	0.0	ND	ND

Ct values by titer for the 2009 Emergent Lineage N1 gene rRT-PCR assay with isolates propagated in embryonating chicken eggs: A/Mexico/4108/09.

Virus	Titer (50% TCID/ml)	Replicate	
		1	2
A/Mexico/4108/09	$10^{6.9}$	20.72	20.83
	$10^{5.9}$	24.02	24.13
	$10^{4.9}$	27.47	27.46
	$10^{3.9}$	31.05	31.13
	$10^{2.9}$	34.43	34.73
	$10^{1.9}$	37.61	37.75
	$10^{0.9}$	0.0	0.0
	$10^{-1.9}$	0.0	0.0

Ct values by titer for the 2009 Emergent Lineage N1 gene rRT-PCR assay with isolates propagated in embryonating chicken eggs: A/CA/08/09.

Virus	Titer (50% TCID/ml)	Replicate	
		1	2
A/CA/08/09	10^{7.1}	20.81	20.83
	10^{6.1}	24.02	24.13
	10^{5.1}	27.27	27.46
	10^{4.1}	31.05	31.13
	10^{3.1}	34.43	34.73
	10^{2.1}	37.61	37.75
	10^{1.1}	0.0	0.0
	10^{0.1}	0.0	0.0

Sensitivity: Summary

- **Classic lineage assay**
 - **Two MDCK propagated North American N1 lineages viruses were each tested**
 - **A/Swine/NC/02023/08 = $10^{1.3}$ /ml TCID₅₀**
 - **A/Swine/KY/02086/08 = $10^{2.5}$ /ml TCID₅₀**
- **2009 lineage assay**
 - **Two egg propagated 2009 Emergent N1 lineages viruses were each tested**
 - **A/Mexico/4108/09 = $10^{1.9}$ /ml EID₅₀**
 - **A/CA/08/09 = $10^{2.1}$ /ml EID₅₀**

Specificity

- **RNA from a total of 49 type A influenza isolates from various lineages tested**
 - **All 9 NA subtypes included**
 - **31 N1 viruses tested of swine, human and avian origin**

Specificity with N1 isolates

Template	Subtype		Classic lineage test	2009 emergent lineage test
	HA	NA		
A/CA/04/09	H1	N1	-	+
A/CA/08/09	H1	N1	-	+
A/Mexico/4108/09	H1	N1	-	+
A/Brisbane/59/07 (Seasonal human)	H1	N1	-	-
A/Solomon Is./03/06 (Seasonal human)	H1	N1	-	-
A/Swine/IN/1726/88	H1	N1	+	-
A/Swine/NC/02023/08	H1	N1	+	-
A/Swine/OH/02026/08	H1	N1	+	-
A/Swine/MO/02060/08	H1	N1	+	-
A/Swine/IA/02096/08	H1	N1	+	-
A/Swine/KY/02086/08	H1	N1	+	-
A/Swine/MN/02053/08	H1	N1	+	-
A/Swine/MN/02093/08	H1	N1	+	-
A/Swine/NE/02013/08	H1	N1	+	-
A/Swine/NC/02084/08	H1	N1	+	-
A/Swine/MN/02011/08	H1	N1	+	-

Specificity with N1 isolates continued

Template	Subtype		Classic lineage test	2009 emergent lineage test
	HA	NA		
A/Turkey/MO/21939/87 (swine-like)	H1	N1	+	-
A/Turkey/IA/10271-3/90	H1	N1	+	-
A/Turkey/IA/21089-3/92	H1	N1	+	-
A/Turkey/NC/19762/88	H1	N1	+	-
A/Turkey/KS/4880/80	H1	N1	+	-
A/Turkey/MO/21939/87 (Classical swine-like)	H1	N1	+	-
A/Chicken/AR/30402/99 (Swine/IA/30-like)	H1	N1	+	-
A/Swine/Italy/65296/04 (European swine like)	H1	N1	-	-
A/Swine/England (European swine like)	H1	N1	-	-
A/Duck/NY/13152-13/94	H1	N1	-	-
A/Mallard/WI/428/75	H5	N1	-	-
A/Duck/Alberta/35/76 (NA Avian)	H1	N1	-	-
A/Duck/NJ/15024-21/96 (NA Avian)	H1	N1	-	-
A/Duck/NJ/7717/95 (NA Avian)	H1	N1	-	-
A/Chicken/NJ/15906-9/96	H1	N1	-	-

Specificity with Non-N1 isolates

Template	Subtype		Classic Lineage Test	2009 Emergent Lineage test
	HA	NA		
<i>A/Duck/NY/484680/07</i>	H5	N2	-	-
<i>A/Chicken/CA/6643/01</i>	H6	N2	-	-
<i>A/Chicken/NY/14677-13/98</i>	H6	N2	-	-
<i>A/Duck/CA/D0406028/04</i>	H6	N2	-	-
<i>A/Turkey/PA/7975/97</i>	H7	N2	-	-
<i>A/Chicken/NJ/12220/97</i>	H9	N2	-	-
<i>A/Gull/Mongolia/1756/06</i>	H16	N3	-	-
<i>A/Chicken/NY/15086-3/94</i>	H7	N3	-	-
<i>A/Turkey/ON/6118/67</i>	H8	N4	-	-
<i>A/Duck/AB/60/76</i>	H12	N5	-	-
<i>A/Mallard/Gurjev/263/82</i>	H14	N5	-	-
<i>A/Gull/MD/704/77</i>	H13	N6	-	-
<i>A/Shearwater/Australia/2576/79</i>	H15	N6	-	-
<i>A/GreenWingedTeal/LA/169GW/88</i>	H10	N7	-	-
<i>A/Environmental/NY/19019-6/98</i>	H3	N8	-	-
<i>A/BlueWingedTeal/LA/69B/87</i>	H4	N8	-	-
<i>A/Mallard/MN/263/99</i>	H4	N9	-	-
<i>A/Turkey/WI/68</i>	H5	N9	-	-

Specificity

- **The 2009 emergent H1N1 lineage test only detected the N1 gene from emergent 2009 H1N1 viruses**
- **The classic lineage test only detected N1 genes from swine viruses with North American swine lineage N1 genes**
- **Neither test detected European swine N1 gene, N1 gene from Eurasian H5N1, N. American avian N1 or human seasonal N1 viruses**

Matrix Gene Test Update

(Spackman et al. J. Clin. Microbiol. 40(9):3256-3260, 2002)

- **Poor sensitivity for the 2009 Emergent H1N1 lineage observed with the USDA Type A rRT-PCR test (M gene test)**
- **Four mismatches identified in the reverse primer**

2009 North American 3'-cagagactggaaagtgtctttgca-5'

2002 M-124 primer 3'-cagagacttgaa**ga**tgt**t**tttgca-5'

2009 M-124 primer 3'-cagagactggaaagtgtctttgca-5'

- **Updated reverse 124 primer to be a 100% match with the 2009 Emergent H1N1 Lineage M gene sequence and optimized test with both the 2002 and 2009 reverse primers in the same reaction**
- **Initial testing showed that sensitivity was improved to the new lineage and unchanged for other lineage viruses**

Ct values of selected influenza A isolates with the M gene test with 2002 and/or 2009 reverse primers

Template	Reverse primer		
	2002 & 2009	2002	2009
CA/04/09	24.74	32.12	24.52
CA/08/09	15.38	24.03	15.17
Mex/4108/09	18.93	27.31	18.72
Duck/NY/15024/96	17.90	17.43	20.70
Brisbane/59/07	17.58	17.57	25.46
Turkey/IA/10271-3/90	18.98	18.61	28.61
Gull/Mong/1756/06	17.36	16.61	34.34
Gull/MD/704/77	18.17	18.18	28.14
Chicken/CA/6643/01	14.65	14.69	17.33
Swine/IN/1726/88	18.10	17.94	27.42
Duck/AB/35/76	15.87	15.88	18.80
Turkey/MO/21939/87	16.84	16.92	20.15
Chicken/PA/35154/	14.44	14.48	25.21
Swine/NC/3070408	18.02	17.26	28.44
Swine/IA/8548	17.70	17.31	29.18
Swine/MN/02011/08	16.27	16.03	22.79
Swine/MN/02093/08	16.76	16.60	23.83
Solomon Is./03/06	15.54	15.66	25.31

Infectivity, Pathogenicity and Transmissibility for Poultry

Experimental design

Chickens (3 wk) and Japanese quail (yg adult)

- 5 intranasal-(IN)-inoculated (10^6 EID₅₀) and 5 contact-exposed: virus A/Mexico/4108/09
- 6 IN-inoculated sampled on 2, 4 and 7 days post-inoculation (DPI)
- Shams (IN with culture media), 2 sampled 4 DPI and 2 at termination
- Intravenous pathogenicity index (IVPI) test (chickens only)
- Turkey hens (73 weeks)
 - IN-inoculated (9), contact-exposed (3) and sham (2)
 - Sampled 2 IN and 1 contact exposed on 4 and 7 DPI
- General
 - Cloacal and oral swabs taken 2, 4, 7 and 10 DPI
 - HI serology at 15 DPI

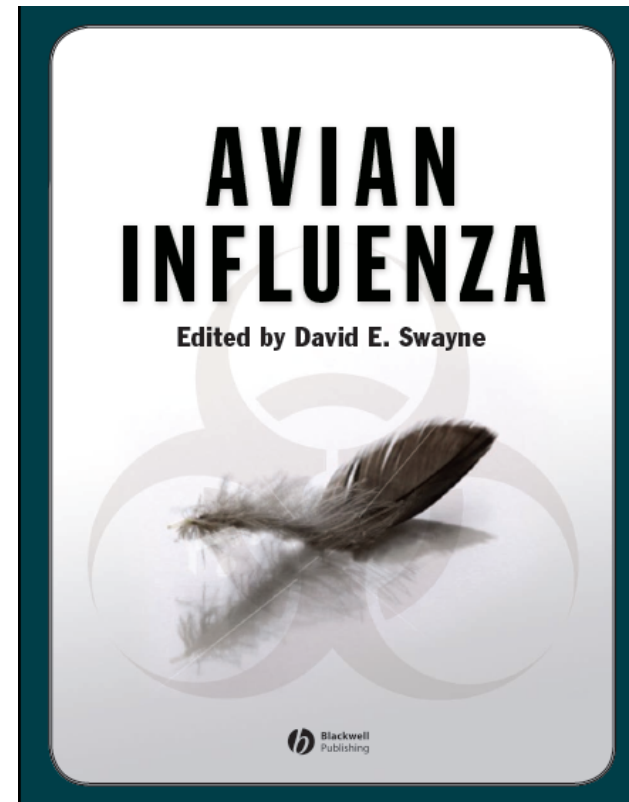
Infectivity and Pathogenicity for Poultry

- **Chickens**
 - **IVPI = 0.00**
 - **No clinical signs**
 - **No virus detected RRT-PCR or isolated from OP or CL swabs from IN-inoculated and contact-exposed birds**
 - **All HI negative at 15 DPI**
- **Quail**
 - **No clinical signs**
 - **Virus detected OP swabs at 4 DPI of IN group**
 - **IN-inoculated group = HI+, contact-exposed = HI-**
- **Turkey Hens**
 - **No clinical signs**
 - **No virus isolated from OP or Cl swabs**
 - **HI- at 15 DPI for all groups**
 - **Virus isolation for reproductive tissue pending**

Conclusions

- **N1 multiplex RRT-PCR test developed and bench validated to identify 2009 emergent H1N1 and N. American classic swine H1N1**
- **AIV matrix reverse primer RRT-PCR was modified to improve detection of 2009 emergent H1N1 viruses**
- **Poultry studies**
 - **No infections in IN-inoculated chickens and turkeys, and low level infection in quail**
 - **No contact transmission**

Thank You For Your Attention!



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