

Phenotypic Variations in Low Pathogenic H1N1 Avian Influenza Viruses Resulting from Genotypic Differences Among Isolates

Jacqueline Nolting and Richard Slemmons

The Ohio State University Department of Veterinary Preventive Medicine

1920 Coffey Road, Columbus, Ohio 43210

Abstract

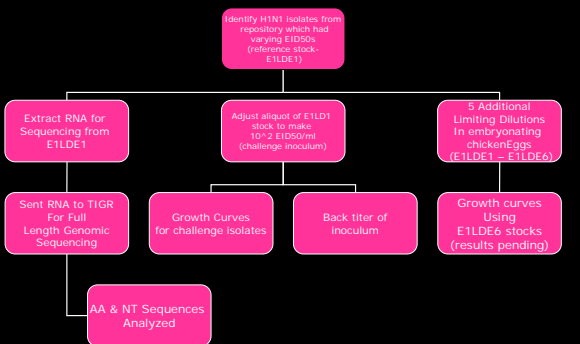
Type A influenza isolates possessing the hemagglutinin (HA) – neuraminidase (NA) combination of H1N1 have been of great interest to both public health and veterinary scientists and officials since the occurrence of the 1918 “Spanish Flu” pandemic and the emergence of classical H1N1 “swine influenza” virus. H1N1 type A influenza viruses have been recovered from wild and domestic birds, swine and humans but the isolates show relatively strong predilections for their respective hosts. However, this host specificity is not absolute and H1N1 type A influenza viruses have crossed species barriers between turkeys and swine, humans and swine, avian species and swine, and wild and domestic birds. Waterfowl-origin H1N1 type A influenza viruses in our repository appeared to demonstrate differences in infectivity and hemagglutinin titers when propagated in embryonating-chicken-eggs. Upon the completion of genomic analysis it was determined that all genomic segments were waterfowl-origin, even though determined aa sequence homology was as low as 70.5 percent between some RNA segments, thus eliminating the possibility that genetic reassortment with mammalian influenza viruses was responsible for the observed phenotypic variations. It was concluded that the phenotypic differences observed in this study were due to the nucleotide and amino acid substitutions in wild bird-origin AV/ isolates and not due to genetic reassortment among H1N1 viruses from domestic poultry, swine, or humans. Also, the amino acid differences identified were not associated with previously reported amino acid substitutions affecting replication of human-origin influenza viruses in embryonating chicken eggs.

Introduction

Waterfowl-origin H1N1 type A influenza viruses in our repository appeared to demonstrate differences in infectivity and hemagglutinin titers when propagated in embryonating chicken eggs. There are several possible explanations for these apparent differences including, but are not limited to 1) inhibitors and incomplete virus particles in original samples and first egg passage fluid which would inhibit replication of viable virus particles. 2) Varying viral concentrations in original samples could affect the final HA titer and EID50 because inoculums with higher concentrations of virus would have more particles replicating and available to attach to red blood cells in a hemagglutination test. 3) Point mutations in the genomic segments which lead to amino acid substitutions that could change the phenotypic properties of an isolate. 4) Differences in genomic constellations (all eight RNA segments in the genome) resulting from genomic reassortments with genes from other lineages of water-fowl origin type A influenza viruses and even viruses maintained in poultry, lower mammals and humans which could be influencing the biological properties. The hypotheses for this investigation are that, under the conditions of this study, the growth rates, hemagglutination titers, and maximum infectiousness of four low pathogenic waterfowl-origin (WFO) H1N1 type A influenza virus (AIV) isolates are influenced by 1.) variations in nucleotide/amino acid sequences due to prior genetic reassortment and/or 2.) point mutations.

Materials and Methods

Isolate Name	1x10 ⁶ EID50/1.0mL of E1	GMT of E1LDE1	Genbank ID
A/green-winged teal/Ohio/430/1987(H1N1)	1x10 ^{6.63} EID ₅₀ /1.0mL	256	CY11040 - CY11047
A/black duck/Ohio/95/1993(H1N1)	1x10 ^{6.33} EID ₅₀ /1.0mL	80.44	CY15443 - CY15450
A/mallard/Ohio/118/1993(H1N1)	1x10 ^{7.22} EID ₅₀ /1.0mL	8	CY018885 - CY018892
A/mallard/Maryland/350/2002(H1N1)	1x10 ^{3.67} EID ₅₀ /1.0mL	4.75	NA



Results

Initial Virus Isolation

First egg passage (E1) and first egg passage followed by a limiting dilution in eggs (E1LDE1) reference stocks of the four H1N1 WFO AIV isolates demonstrated varying concentrations of infectious viral particles (Table 1 and 2) and E1 through E1LDE6 stocks demonstrated varying HA titers. (Table 3). These results indicate that the isolate with lower initial infectivity (02-350) increased infectivity to within one log of the other isolates, at passage E1LDE2. Isolate 02-350 also began with the lowest HA titers, and continued to exhibit the lowest HA titers among the four isolates through the six limiting dilution, the duration of the study. (Table 3)

Isolate Name	87-430	93-95	93-118	02-350
Passage #				
E1	5.63	6.33	7.22	3.67
E1LDE1	8.5	8	8.48	6
E1LDE2	9.33	9.33	9.66	8.5
E1LDE3	9.25	9.55	8.76	7.33
E1LDE4	8.5	9.5	8.3	8.5
E1LDE5	8.83	8.33	9.5	7.5

Isolate	87-430	93-95	93-118	02-350
Passage #				
E1LDE1	256	80.44	8	4.75
E1LDE2	32	181.01	64	11.31
E1LDE3	315.17	604.69	8	5.65
E1LDE4	256	8	512	8.81
E1LDE5	1024	512	90.5	7.33
E1LDE6	1024	256	22.6	9.51

Growth Curves

HA activity for 02-350 (standardized E1LDE1) was first detected at 32 hours post inoculation with a HA GMT of 11.31. The HA GMT at 48 hours post inoculation was 9.85. In contrast, the highest HA titer of 2048 exhibited by isolate 87-430 was first detected at 20 hours post inoculation. The remaining two isolates also demonstrated an HA titer at 20h post inoculation, and GMTs ranged from 90.51 to >4096.

Back titrations confirmed the EID50 of the 0.1mL challenge inoculums for 87-430, 93-95, 93-118, and 02-350 were 1x10^{2.17} EID50/1.0mL, 1x10^{2.76} EID50/1.0mL, 1x10^{4.67} EID50/1.0mL, and 1x10^{2.00} EID50/1.0mL, respectively.

Sequence Analysis

Nucleotide homology among isolates ranged from 93.3% with the matrix gene (M1) to 71.1% with the non-structural gene (NS). (Table 4) The matrix gene, which was the most conserved in this study, had nucleotide (nt) and amino acid (aa) identities at 93.3% and 100%, respectively. The largest identity differences among the isolates were demonstrated in the non-structural genomic segments with only 71.1% nt identity, and the NS1 protein with 70.5% aa identity, with all other proteins having identities within the range of 70.5% - 100%. (Table 4)

Using information published by Suarez, et al. a phylogenetic tree was created to determine whether the NS genomic segment of isolates within the study was non structural allele A or B. (5) It was determined based on the phylogenetic tree that all the isolates in this study were allele A.

Both nt and aa substitutions appeared to be randomly distributed throughout each segment, and did not concentrate or group near the open reading frames. (Table 4 and 5)

When compared to other previously published gene segments using BLAST (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>), all 8 segments of each of the four isolates matched multiple other wild-bird origin type A influenza gene segment with at least 99.0% identity.

An alignment of the HA segments of the four isolates showed amino acid residue glutamate (E) was present at aa position 204 and glycine (G) is present at position 239 suggesting all isolates bind to sialic acid α2-3gal, further confirming the avian origin of all four virus isolates.

Gene/protein	# NT/AA	% nucleotide identity	% amino acid identity
MP	975/349	93.3	100
NP	1259/498	90.8	99.2
PB2	2295/765	89.4	98.9
PB1	2284/757	91.42	98.9
PA	2190/716	85.6	97.4
HA	1725/566	7.76	97.9
NA	1424/469	91.57	97
PB1 F2	-/90		85.6
NS	855/351	71.1	80.5
NS2	-/230		73.5
NS1	-/121		80.5

Table 5	87-430	93-95	93-118	02-350
PB2	V147L, I255V, K586R	None	None	R3K, V147L, I255V, K340R, T559I, K586R, S690Q, G682S, V688I
PB1	S152L, E638E, N654S	None	None	E176Q, T359V, R368K, A529I, N654S
PB1 F2	E2G, H33P, L38S, L38S, A56V, S77L, V78L	None	None	H6T, K29R, H33P, M39T, Y42C, I45T, A56V, W58L, S77L, V78L
PA	D272E, I387V, P400Q, N409S, M423I, I432V, I659V	None	None	V63I, R256K, R269K, V323I, I48L, N350S, S380S, P400Q, N409S, M423I, I432V, I659V, K626R
HA	A11T, V64I, E116G, N138S, K236R, N291K, H300Y	T11A, I64V, A413G	T11A, I64V, K419R, F454L, D518N	A11T, S53R, V64I, T169I, T212I, D123N, H530S
NP	V67I	None	None	V67I, I655X, N417S
NA	I13V, R45H, A76T, K78Q, V81A	None	None	V13I, I19V, S42N, R45H, T48I, K78Q, T78A, T89V, V81V, I258V, I264V, D311E, V388A
MP1	None	None	None	None
NS1	V6I, S7T, F14V, V18I, R21L, F22L, A23S, R24M, Q26R, E26D, L27M, Q28C, L33D, S42A, R44K, I54L, E55R, T65V, R65M, A68E, Q69R, R67D, E70K, E71S, S73T, A76N, M79I, T80A, V84S, S87P, L90I, T94S, L98I, N99I, D101E, F103Y, K108R, V111I, A112T, S114G, C116M, I117V, R118K, N127R, I129M, I137L, R140Q, I45V, L146S, E152D, E153D, G155A, L163I, L166M, D171T, V180I, V184A, R204Q, S205V, S266R, N270I, D269N, R211G, K221Y, T225R, I262V	None	I68L	I18V, R59C, S165X
NS2	V6I, S7T, M14Q, G22E, E26V, S37R, L49I, A48S, S57F, G59A, G64Q, Q68E, E81A, V83C, H82A, R86I, K88T, I89K, M100L, Q111S	None	S57F	None

Summary

This study confirmed genotypic differences existed in isolates serologically characterized as H1N1 AIV. Amino acid homology varied from 70-100% among the genomic segments but, it was determined that all genomic segments were likely waterfowl-origin. Thus the possibility that genetic reassortment with type A influenza viruses from poultry, humans and lower mammals was not responsible for the phenotypic variations, resulting in the rejection of hypothesis one. Therefore we conclude that the phenotypic differences observed in this study were a result of accumulated nucleotide point mutations and amino acid substitutions observed among the isolates, thus supporting hypothesis two. The aa changes in the HA gene identified did not match previously reported amino acid substitutions affecting replication of human-origin influenza viruses in embryonating chicken eggs and the persistent differences in HA titers could not be attributed to a specific amino acid substitution.

1. Conderbury, P. K. and Richard Slemmons. Biological Properties of Waterfowl-Origin Type A Influenza Viruses in Chickens. Avian Diseases 36(1): 23, 1992
 2. Dugan, Vivian, Ruling Chen, David Spino, Naomi Sengemeyer, Jennifer Zatorsky, Elyse Ghedin, Jacqueline Nolting, David Beayne, Jonathan Rundstedter, George Hagg, Dennis Serna, Ruisun Wang, Richard Slemmons, Edward Holmes, and Jeffrey Tashenberg. The evolutionary genomics and emergence of AIV in wild birds. PLoS Pathogens 4(5), 1-9, 2009
 3. Ghedin, Elyse, Naomi A. Sengemeyer, Martin Shumway, Jennifer Zatorsky, Tamara Felblym, Jill Davy, David Spino, Jeff Snel, Hean Koo, Pavel Bittov, Dmitry Deminoy, Taisara Takano, Yimeng Bai, Kristin B. George, Jill Taylor, David Loman, Claire M. Fraser, M. Levent Yildirim, and Jeffrey K. Tashenberg. Large-scale sequencing of human influenza reveals the dynamic nature of viral genome evolution. Nature 437(2): 1162-66, 10-20, 2005
 4. Slemmons, Richard and Beayne, D. E. Tissue Tropism and Replicative Properties of Waterfowl-Origin Influenza Viruses in Chickens. Avian Diseases 35, 521-27, 1991
 5. Suarez David L. and Michael Peiris. Multiple alignment comparison of the non-structural genes of influenza A viruses. Virus Research 54, 89-93, 1998.

Author disclosures: This paper was funded by the USDA/ACIP and The Ohio State University Veterinary Preventive Medicine. Type A Influenza Sequence Laboratory. A special thanks goes to Dr. Allison Rogers for her laboratory assistance, Dr. Naomi Sengemeyer for her work as TIGR, and Drs. Jeff Tashenberg and Vivian Dugan for their willingness to provide insight and wisdom.