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Influenza A(H9N2) Virus, Myanmar, 2014–2015

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Routine surveillance of influenza A virus was conducted in Myanmar during 2014–2015. Influenza A(H9N2) virus was isolated in Shan State, upper Myanmar. Whole-genome sequencing showed that H9N2 virus from Myanmar was closely related to H9N2 virus of clade 4.2.5 from China.

Influenza A(H9N2) virus has been found in several avian species. Despite low pathogenicity in poultry, H9N2 viruses are important to public health because of their high adaptability and frequent infection in humans. Clinical cases of H9N2 human infection have been reported in China (including Hong Kong) and Bangladesh (*1*,*2*). H9N2 viruss are constantly evolving and can reassort with other influenza A virus subtypes, resulting in novel influenza viruses. H9N2 was the likely donor of internal genes for the H5N1, H7N9, and H10N8 viruses (*3*,*4*).

During December 2014–August 2015, we conducted an influenza A surveillance program in Shan State, Myanmar. An outbreak of highly infectious avian influenza A(H5N1) in November 2007 has been the only outbreak reported in this state. For this study, we collected 648 samples from live-bird markets (LBMs) in Muse, Namkham, Laukkai, and Chinshwehaw, Shan State townships on the China–Myanmar border (online Technical Appendix 1, https://wwwnc.cdc.gov/EID/article/23/6/16-1902-Techapp1.pdf). We collected oropharygeal swab specimens from chickens (n = 273) and ducks (n = 180) as well as environmental samples (n = 195). Identification and isolation were performed at the Livestock Breeding and Veterinary Department, Yangon, Myanmar (online Technical Appendix).

Of the 648 samples subjected to virus isolation by egg inoculation, 10 were hemagglutinin (HA) positive. We further confirmed 3 samples as influenza A virus by using real-time reverse transcription PCR (RT-PCR), and we subtyped and confirmed all 3 as H9N2 (online Technical Appendix Table 1). However, the overall occurrence of H9N2 in the LBMs in this study was relatively low. The 3 H9N2 isolates, A/chicken/Myanmar/NK-2/2015(H9N2), A/chicken/Myanmar/NK-4/2015(H9N2), and A/chicken/Myanmar/NK-5/2015(H9N2), were from chickens in LBMs in Namkham Township in June 2015.

To characterize the Myanmar H9N2 virus, we performed whole-genome sequencing of these 3 isolates and submitted nucleotide sequences GenBank (accession nos. KY115364–KY115387). Although an international standard for clade nomenclature of the H9 subtype has not been well established, phylogenetic analysis showed that all 3 Myanmar H9N2 isolates were grouped into clade 4.2.5 (HA gene) and BJ94-like (neuraminidase [NA] gene) (Figure; and online Technical Appendix Figure 2) (*5*). The Myanmar H9N2 viruses clustered with avian and human H9N2 virus recovered in China in 2015. The phylogenetic analyses of internal protein genes showed similar findings; the Myanmar H9N2 viruses were also closely related to avian H9N2 viruses from China. Five internal protein genes, polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA), nucleoprotein (NP), and membrane protein (M), were grouped with the viruses of G1 lineage, and the nonstructural (NS)

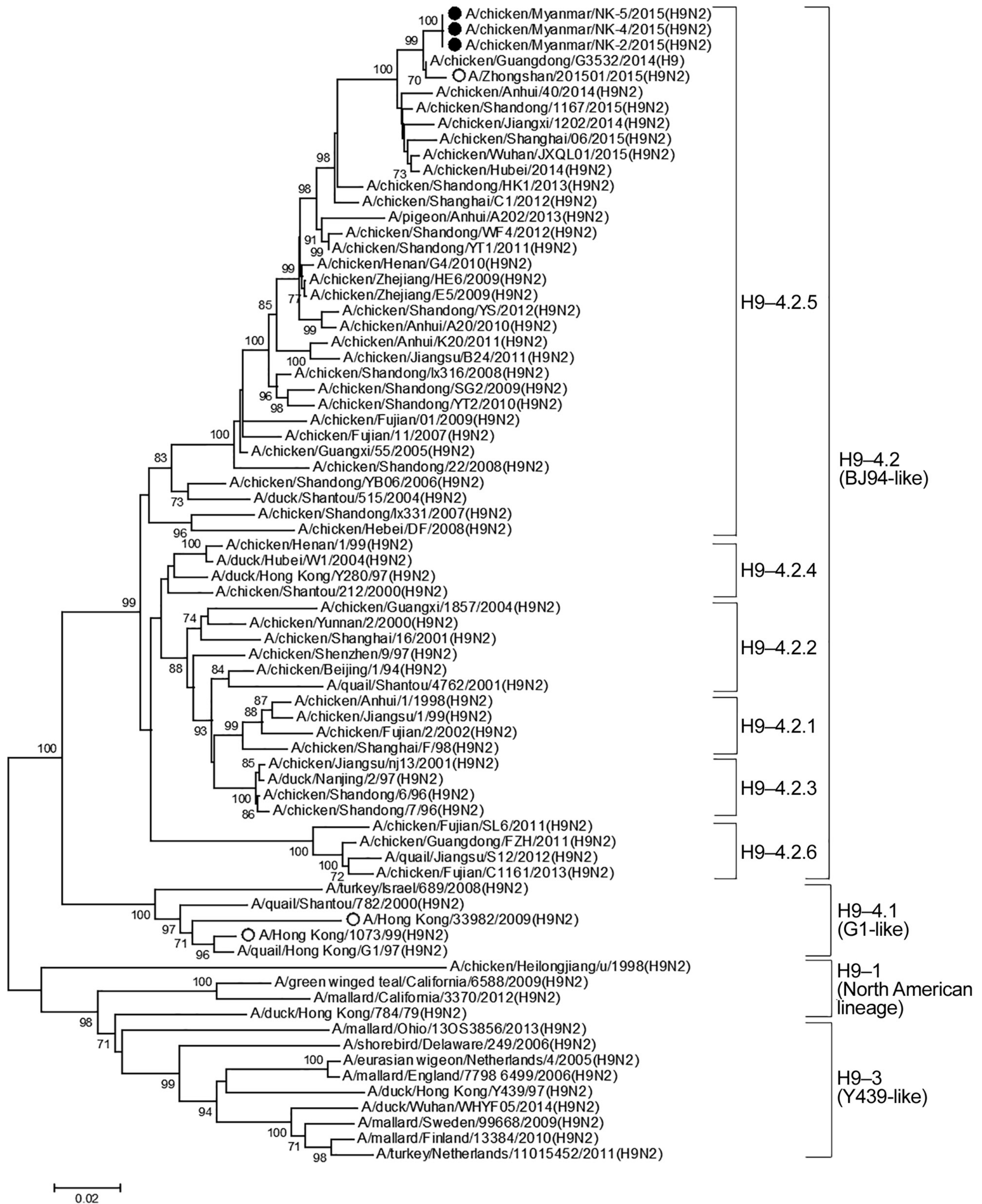

Figure. Phylogenetic tree of H9 gene of influenza A(H9N2) viruses from Myanmar and reference viruses. Phylogenetic trees were constructed by using MEGA version 6.0 (http://www.megasoftware.net/) and a neighbor-joining algorithm with the Kimura 2-parameter model and 1,000 replications of bootstrap analysis. Only bootstrap numbers >70% are shown. Black circles represent isolates from this study, and open circles represent human H9N2 isolates. Virus clades are indicated at right. Scale bar indicates nucleotide substitutions per site.

gene was grouped in BJ-94–like lineage (online Technical Appendix Figure 3). In addition, BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of the 8 gene segments of the Myanmar H9N2 viruses showed high percentages of nucleotide identities with H9N2 viruses from China (online Technical Appendix Table 2).

Genetic analysis showed that the Myanmar H9N2 viruses possessed R-S-S-R at the HA cleavage site, indicating low pathogenic characteristics (*6*). At the HA receptor binding sites, all Myanmar H9N2 isolates carried Q226L substitution, showing the affinity to human α-2,6-glycan receptors (*7*). The Myanmar H9N2 viruses possessed all 7 HA receptor binding sites, identical to human H9N2 virus A/Zhongshan/201501/2015 (online Technical Appendix Table 3) (*8*). For NA gene analysis, all Myanmar H9N2 viruses had a 3-aa deletion (positions 62 to 64) in the NA stalk region, suggesting virus adaptation from wild birds to poultry (*8*).

Internal protein gene analysis showed that the Myanmar H9N2 isolates possessed both avian- and human-specific amino acids. For example, no mutation was observed at E627 and D701 in PB2, retaining the avian characteristics (*9*), whereas human-specific amino acids were observed at 13P in PB1 and 409N in PA (*10*). Amino acids concerning virulence of the H9N2 viruses were also indicated. For example, the PA gene carried 672L and the NS1 gene contained 149A, relating to increased virulence of the virus (*10*). All Myanmar H9N2 viruses also possessed 31N in the M2 gene, suggesting amantadine resistance. Our results, based on the whole-genome analysis, showed that the Myanmar H9N2 viruses possessed all genetic signatures and virulence determinants similar to avian and human H9N2 viruses circulating in China in 2015 (online Technical Appendix Tables 3–5).

In conclusion, the Myanmar H9N2 viruses were considered of low pathogenicity in chickens. However, public health concerns should be raised because the viruses are closely related tom and possess virulence determinants similar to, human H9N2 reported in China in 2015. The human cases likely represent a spillover of the virus to humans from poultry (*8*). The Myanmar H9N2 viruses were obtained from healthy chickens in LBMs near the China–Myanmar border, where sources of poultry are mostly from China. Because LBMs are the major sources of several subtypes of influenza viruses, this environment provides opportunities for influenza viruses to mix, be transmitted, and exchange their gene segments. Infection and transmission in humans and poultry can frequently occur in LBM settings. Therefore, public awareness, control measures, and routine disease surveillance should be implemented.

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Influenza A(H9N2) Virus, Myanmar, 2014–2015

Technical Appendix

Materials and Methods

Sample Collection from Live-Bird Markets, Myanmar

In Myanmar, information on influenza, especially low-pathogenic avian influenza, is limited. Only the outbreaks of highly pathogenic avian influenza (HPAI) H5N1 were reported in 2006 (*1*). Moreover, information on the outbreaks and genome sequences of influenza virus subtypes (IAVs) in Myanmar remains insufficient to depict the genesis of the viruses. Therefore, we carried out a routine influenza A surveillance during December 2014–August 2015 to monitor the status of IAVs in Shan State, Myanmar. Live-bird markets Muse, Namkham, Laukkai, and Chinshwehaw, townships on the China–Myanmar border, were included in this study (Technical Appendix Figure 1). In this study, we collected 648 samples, including oropharygeal swab specimens of chickens (n = 273) and ducks (n = 180) and environmental samples (n = 195), from live-bird markets in the 4 townships of Shan State (Technical Appendix Table 1). We examined all samples for influenza A virus.

Influenza A Virus Identification and Subtyping

We performed the influenza A virus identification and isolation at the Livestock Breeding and Veterinary Department, Veterinary Diagnostic Laboratory, Yangon, Myanmar. We conducted the virus isolation in accordance with World Organisation for Animal Health guidelines, using 9–11-day-old specific pathogen-free embryonated chicken eggs (*2*). We inoculated viral swab suspension into allantoic sacs of the embryonated eggs, which were then incubated at 37°C. We monitored the incubated eggs every 12 hours, and collectged allantoic fluid from infected eggs that died post-inoculation. After 72 hours of inoculation, we harvested allantoic fluid from all remaining eggs. We confirmed the presence of virus by hemagglutination (HA) test using 1% chicken erythrocytes. For influenza A virus identification, we extracted RNA

using the NucleoSpin RNA virus extraction kit (Macherey-Nagel, Düren, Germany). We performed real-time reverse transcription PCR to detect the Matrix gene of influenza A virus (*3*). We also performed subtyping of viruses by using specific primers for HA 1–16 and NA 1–9 genes (*4*,*5*).

Influenza A Virus Characterization

In this study, we characterized 3 H9N2-IAVs, A/Chicken/Myanmar/NK-2/2015 (H9N2), A/Chicken/Myanmar/NK-4/2015 (H9N2), and A/Chicken/Myanmar/NK-5/2015 (H9N2). For whole-genome sequencing of the viruses, we amplified all 8 gene segments with specific primers for each gene segment. We purified the amplified PCR products with NucleoSpin PCR cleanup and gel extraction kits (Macherey-Nagel). We then subjected the purified PCR products to nucleotide sequencing. We performed nucleotide sequence assembling and alignment with DNASTAR (DNASTAR, Madison, WI, USA). We submitted whole-genome sequences of the 3 H9N2 IAVs to the Genbank database under accession numbers KY115364–KY115387. We performed phylogenetic analysis by comparing nucleotide sequence of each gene segment of H9N2-IAVs with those of other influenza viruses in the public database. We constructed phylogenetic trees with the MEGA v.6.0 program using the neighbor-joining algorithm with the Kimura-2-parameter model and 1,000 replications for bootstrap analysis (*6*).

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Technical Appendix Table 1. Description of poultry and environmental samples collected and tested in the study of influenza A virus, Myanmar, December 2014–August 2015

	No. samples collected			No. samples tested	
Date	Chicken	Duck	Environment	HA test (positive result)	rRT-PCR test* (positive result)
Dec 2014	42	28	30	100 (1)†	100 (0)
Jan 2015	42	28	30	100 (0)	100 (0)
Feb 2015	42	28	30	100 (2)†	100 (0)
Mar 2015	42	28	30	100 (0)	100 (0)
Apr 2015	21	12	15	48 (0)	48 (0)
May 2015	21	14	15	50 (0)	50 (0)
Jun 2015	21	14	15	50 (7)†	50 (3)‡
Jul 2015	21	14	15	50 (0)	50 (0)
Aug 2015	21	14	15	50 (0)	50 (0)
Total	273	180	195		

*rRT-PCR was used for both screening of swab samples and confirming HA positive samples.
†Samples with HA titer 2^1 HA unit (suspected and further subjected to 2nd passage egg inoculation) and HA titer ≥2^2 HA unit (positive). All 10 samples were tested positive with HA test >2^2 HA unit.
‡Three samples from chickens tested positive by rRT-PCR (C_t <40), indicating influenza A positive, and the viruses were further subtyped as influenza A virus subtype H9N2.

Technical Appendix Table 2. Nucleotide identities of H9N2-IAV (A/Myanmar/Chicken/NK-2/2015) compared with other viruses available in GenBank database using BLAST analysis

Gene*	Position	GenBank accession no.	Virus with the highest degree of nucleotide identity	Percent nucleotide identity
PB2	1–2280	KX598548	A/chicken/Sichuan/SIC36/2014 (H9N2)	99.6
PB1	1–2274	KX598569	A/chicken/Guangxi/SIC15/2013 (H9N2)	99.3
PA	1–2151	KX598632	A/chicken/Sichuan/SIC36/2014 (H9N2)	99.4
HA	1–1683	KP766779	A/chicken/Guangdong/G3532/2014 (H9)	99.2
NP	1–1497	KX598674	A/chicken/Sichuan/SIC36/2014 (H9N2)	99.7
NA	1–1401	KX598506	A/chicken/Sichuan/SIC36/2014 (H9N2)	99.4
M	1–968	KX598716	A/chicken/Sichuan/SIC36/2014 (H9N2)	100.0
NS	1–861	KT699060	A/Anser fabalis/Anhui/L139/2014 (H9N2)	99.4

*HA, hemagglutinin; M, membrane protein; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural; PA, polymerase acidic protein; PB, polymerase basic protein

Technical Appendix Table 3. Genetic analysis of nucleotide sequences of HA gene of H9N2-IAVs from Myanmar compared with other H9N2-IAVs in the public database*

			HA							
			HA binding site (H3 numbering)							HA
Viruses	Host	Lineage	158	183	189	190	226	227	228	cleavage site
A/Duck/Hong Kong/Y439/1997 (H9N2)	Duck	3	S	H	T	E	Q	Q	G	PAASNR/G
A/Quail/Hong Kong/G1/1997 (H9N2)	Quail	4.1	S	H	T	E	L	Q	G	PARSSR/G
A/Chicken/Shanghai/F/1998 (H9N2)	Chicken	4.2.1	N	N	T	A	Q	Q	G	PARSSR/G
A/Chicken/Beijing/1/1994 (H9N2)	Chicken	4.2.2	N	N	T	V	Q	Q	G	PARSSR/G
A/Duck/Hong Kong/Y280/1997 (H9N2)	Duck	4.2.4	N	N	T	T	L	Q	G	PARSSR/G
A/Chicken/Shanghai/06/2015	Chicken	4.2.5	N	N	D	T	L	M	G	PSRSSR/G
A/Chicken/Fujian/C1161/2013	Chicken	4.2.6	N	N	T	A	L	Q	G	PARSSR/G
A/Hong Kong/1073/1999	Human	4.1	S	H	T	E	L	Q	G	PARSSR/G
A/Hong Kong/33982/2009	Human	4.1	S	H	T	D	Q	Q	G	PARSNR/G

Viruses	Host	Lineage	HA							HA cleavage site
			HA binding site (H3 numbering)							
			158	183	189	190	226	227	228	
A/Zhongshan/201501/2015	Human	4.2.5	N	N	T	T	L	M	G	PSRSSR/G
A/Chicken/Myanmar/NK-2/2015 (H9N2)	Chicken	4.2.5	N	N	T	T	L	M	G	PSRSSR/G
A/ Chicken/Myanmar/NK-4/2015 (H9N2)	Chicken	4.2.5	N	N	T	T	L	M	G	PSRSSR/G
A/ Chicken/Myanmar/NK-5/2015 (H9N2)	Chicken	4.2.5	N	N	T	T	L	M	G	PSRSSR/G

*HA, hemagglutinin

Technical Appendix Table 4. Genetic analysis of nucleotide sequences of NA, PB2, PB1 and PA genes of H9N2-IAVs from Myanmar compared with other H9N2-IAVs in the public database*

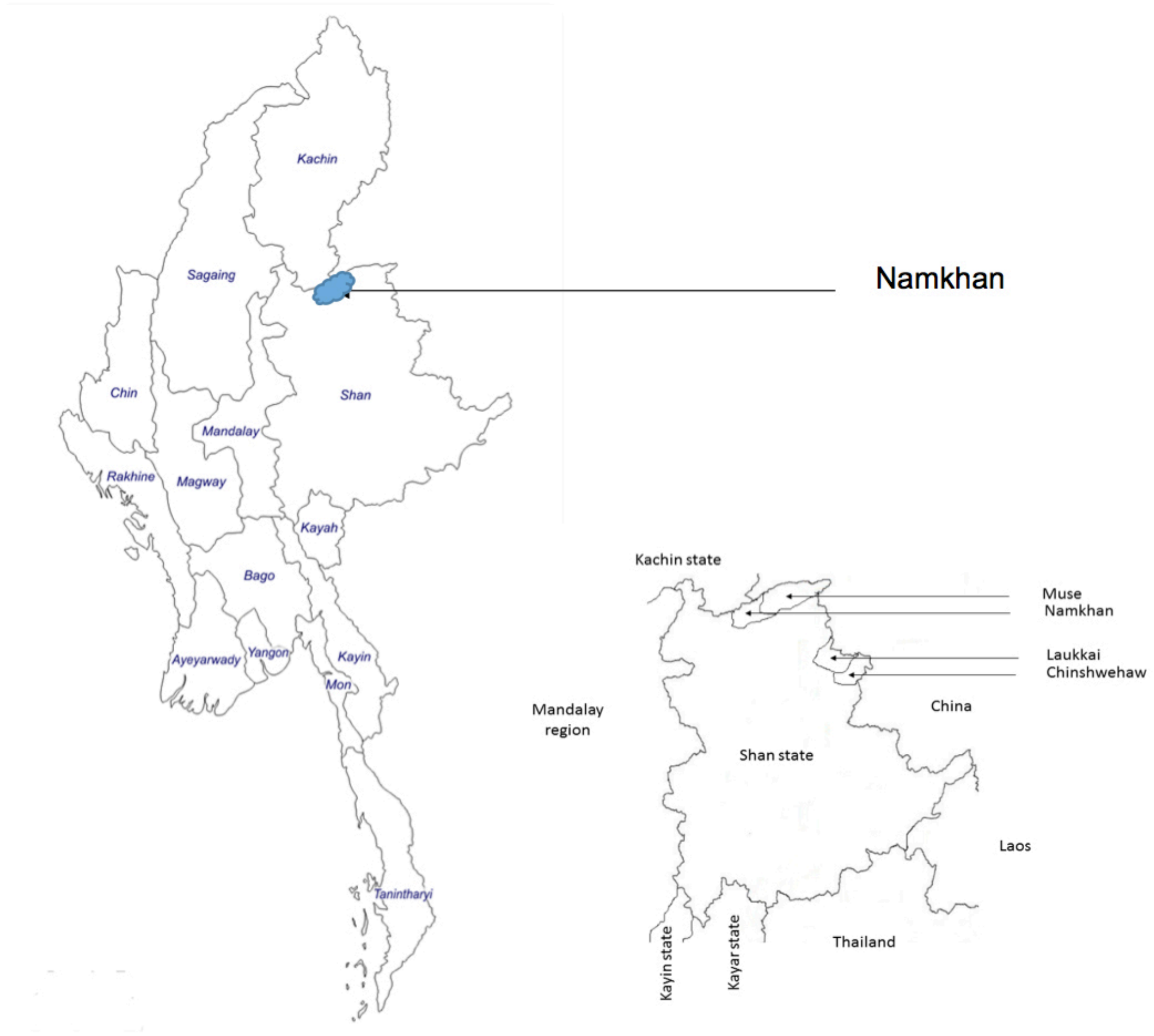
Viruses	Host	Lineage	NA			PB2			PB1	PA		
			NA stalk deletion									
			38–39	46–50	63–65	27	627	701	13	356	409	672
A/Duck/Hong Kong/Y439/1997 (H9N2)	Duck	3	No	No	No	H	E	D	P	K	S	L
A/Quail/Hong Kong/G1/1997 (H9N2)	Quail	4.1	Yes	No	No	H	E	D	P	K	S	L
A/Chicken/Shanghai/F/1998 (H9N2)	Chicken	4.2.1	No	No	Yes	H	E	D	P	K	N	L
A/Chicken/Beijing/1/1994 (H9N2)	Chicken	4.2.2	No	No	No	H	E	D	P	K	S	L
A/Duck/Hong Kong/Y280/1997 (H9N2)	Duck	4.2.4	No	No	Yes	H	E	D	P	K	N	L
A/Chicken/Shanghai/06/2015	Chicken	4.2.5	No	No	Yes	H	E	D	P	K	N	L
A/Chicken/Fujian/C1161/2013	Chicken	4.2.6	No	No	Yes	H	E	D	P	K	S	L
A/Hong Kong/1073/1999	Human	4.1	Yes	No	No	H	E	D	P	K	S	L
A/Hong Kong/33982/2009	Human	4.1	No	No	No	H	E	N	P	K	N	L
A/Zhongshan/201501/2015	Human	4.2.5	No	No	Yes	H	E	D	P	R	N	L
A/Chicken/Myanmar/NK-2/2015 (H9N2)	Chicken	4.2.5	No	No	Yes	H	E	D	P	R	N	L
A/ Chicken/Myanmar/NK-4/2015 (H9N2)	Chicken	4.2.5	No	No	Yes	H	E	D	P	R	N	L
A/ Chicken/Myanmar/NK-5/2015 (H9N2)	Chicken	4.2.5	No	No	Yes	H	E	D	P	R	N	L

*NA, neuraminidase; PA, polymerase acidic protein; PB, polymeric basic protein

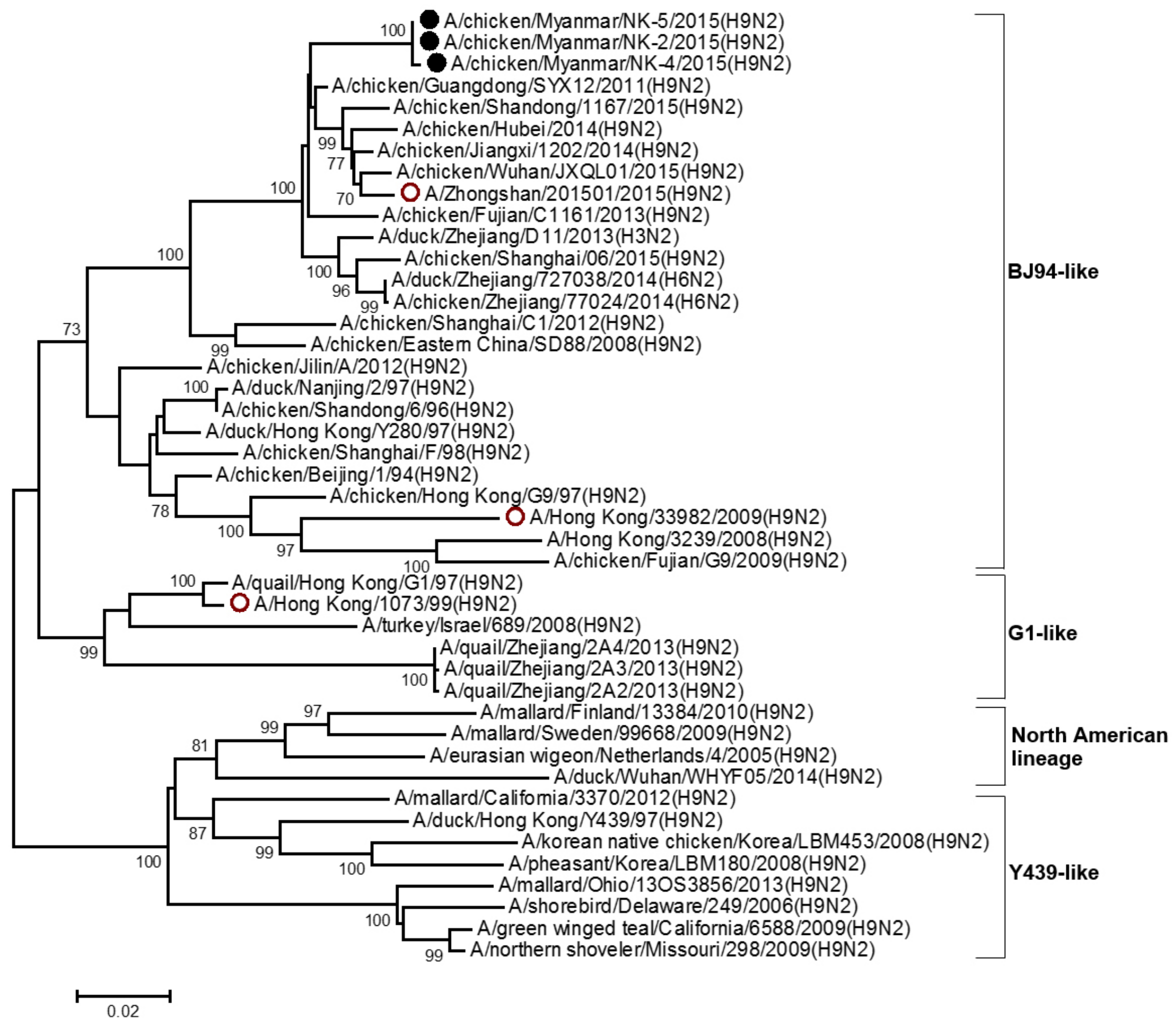
Technical Appendix Table 5. Genetic analysis of nucleotide sequences of M and NS genes of H9N2-IAVs from Myanmar compared with other H9N2-IAVs in the public database*

Viruses	Host	Lineage	M1	M2				NS1		
			15	27	28	31	55	42	149	217
A/Duck/Hong Kong/Y439/1997 (H9N2)	Duck	3	V	V	I	S	L	S	A	K
A/Quail/Hong Kong/G1/1997 (H9N2)	Quail	4.1	I	V	V	S	F	S	A	K
A/Chicken/Shanghai/F/1998 (H9N2)	Chicken	4.2.1	I	V	V	N	F	S	A	K
A/Chicken/Beijing/1/1994 (H9N2)	Chicken	4.2.2	I	V	V	S	F	S	A	K
A/Duck/Hong Kong/Y280/1997 (H9N2)	Duck	4.2.4	I	V	V	S	F	S	A	K
A/Chicken/Shanghai/06/2015	Chicken	4.2.5	I	V	V	N	F	S	A	K
A/Chicken/Fujian/C1161/2013	Chicken	4.2.6	I	V	V	N	F	S	A	K
A/Hong Kong/1073/1999	Human	4.1	I	V	V	S	F	S	A	K
A/Hong Kong/33982/2009	Human	4.1	I	V	A	S	F	S	A	K
A/Zhongshan/201501/2015	Human	4.2.5	I	V	V	N	F	S	A	K
A/Chicken/Myanmar/NK-2/2015 (H9N2)	Chicken	4.2.5	I	V	V	N	F	S	A	K
A/ Chicken/Myanmar/NK-4/2015 (H9N2)	Chicken	4.2.5	I	V	V	N	F	S	A	K
A/ Chicken/Myanmar/NK-5/2015 (H9N2)	Chicken	4.2.5	I	V	V	N	F	S	A	K

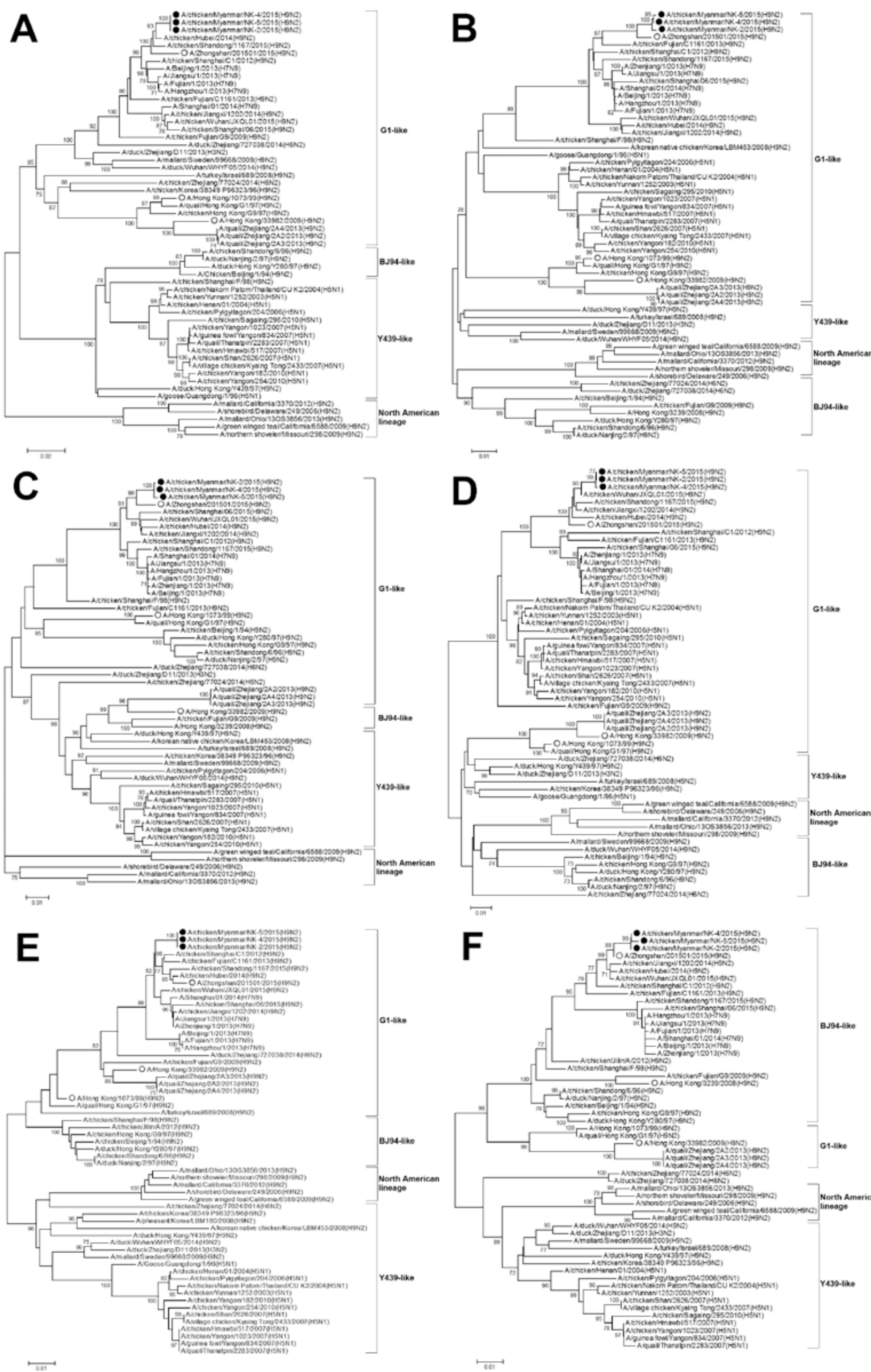
*M, membrane protein; NS, nonstructural



Technical Appendix Figure 1. Map of influenza A surveillance locations in Shan State, Myanmar, December 2014–August 2015.



Technical Appendix Figure 2. Phylogenetic tree of the N2 gene of H9N2-IAVs. We constructed phylogenetic trees by MEGA v.6.0 program (http://www.megasoftware.net/) using the neighbor-joining algorithm with Kimura-2-parameter model and 1,000 replications of bootstrap analysis. Only bootstrap numbers higher than 70% are shown. The black circles represent H9N2-IAV isolates from this study, and the open circles represent human H9N2 isolates (scale bar indicates amino acid substitutions per site).



Technical Appendix Figure 3. Phylogenetic trees of internal protein genes of Myanmar H9N2-IAVs. We generated phylogenetic trees with MEGA v.6.0 program using the neighbor-joining algorithm with Kimura-2-parameter model and 1,000 replications of bootstrap analysis. Only bootstrap numbers higher than 70%

are shown. The black circles represent H9N2-IAV isolates from this study, and the open circles represent human H9N2 isolates. A) Phylogenetic tree of polymerase basic protein (PB2) gene. B) Phylogenetic tree of polymerase basic protein (PB1) gene. C) Phylogenetic tree of polymerase acidic (PA) gene. D) Phylogenetic tree of nucleoprotein (NP) gene. E) Phylogenetic tree of membrane protein (M) gene. F) Phylogenetic tree of nonstructural (NS) gene (scale bar indicates amino acid substitutions per site).