Sun, Y. and Xu, Q. and Shen, Y. and Liu, L. and Wei, K. and Sun, H. and Pu, J. and Chang, K.-C. and Liu, J. (2014) Naturally occurring mutations in the PA gene are key contributors to increased virulence of pandemic H1N1/09 influenza virus in mice. Journal of Virology, 88 (8). pp. 4600-4604. ISSN 0022-538X

JVI Journals.ASM.org

Naturally Occurring Mutations in the PA Gene Are Key Contributors to Increased Virulence of Pandemic H1N1/09 Influenza Virus in Mice

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We examined the molecular basis of virulence of pandemic H1N1/09 influenza viruses by reverse genetics based on two H1N1/09 virus isolates (A/California/04/2009 [CA04] and A/swine/Shandong/731/2009 [SD731]) with contrasting pathogenicities in mice. We found that four amino acid mutations (P224S in the PA protein [PA-P224S], PB2-T588I, NA-V106I, and NS1-I123V) contributed to the lethal phenotype of SD731. In particular, the PA-P224S mutation when combined with PA-A70V in CA04 drastically reduced the virus's 50% mouse lethal dose (LD_{50}), by almost 1,000-fold.

The outbreak of H1N1/09 influenza in North America in April 2009 marked the global spread of the virus as the first pandemic of the 21st century (1, 2). Although most H1N1/09 infections were mild (1, 3), experts estimated that 284,500 people died from the virus in the first 12 months of its circulation (4). Since 2009, strains of H1N1/09 influenza with differing virulences have been observed in humans and animal models (5, 6). However, the genetic basis of virulence of H1N1/09 viruses is not well understood. Previously characterized molecular markers associated with adaptation of influenza virus to humans, such as 627K in the PB2 protein (PB2-627K), are not present in the H1N1/09 influenza virus (7). Introduction of known virulence markers into the H1N1/09 virus did not affect its pathogenicity (8, 9). A PB2-591K residue was shown to compensate for the lack of PB2-627K in the H1N1/09 virus (10). By serial passage of H1N1/09 virus in mice, Zhou et al. found that the PB2-E158G mutation increased the morbidity and mortality of H1N1/09 in mice (11). Ye et al. reported that five amino acid substitutions distributed in HA, PA, or NP protein from a mouse-adapted A/California/04/2009 (CA04) virus contributed to the lethal phenotype in mice (12). The role of the HA gene in the virulence of H1N1/09 viruses has also been recognized in other studies (13, 14). Since mouse-adapted H1N1/09 virulent strains are far removed from prevailing infections in natural hosts, in the present study, we identified natural isolates of H1N1/09 influenza viruses in China that are more virulent in mice than an early representative isolate (CA04). The virulence markers previously identified in H1N1/09 viruses are absent in these isolates, indicating that they possess hitherto-undefined molecular determinants of pathogenicity.

Six natural occurring H1N1/09 viruses, including four swine isolates and two human strains isolated in China, were identified in our laboratory. A/Beijing/7/2009 and A/Beijing/132/2010 were isolated from patients who exhibited influenza symptoms, including rhinorrhea, sneezing, coughing, and fever. Four swine H1N1/09 isolates were isolated from diseased pigs characterized by coughing, fever, abdominal respiration, and anorexia. These isolates, listed in Table 1, were passaged twice in MDCK cells before they were sequenced. The virulence of the six H1N1/09 strains in 6-week-old female BALB/c mice was examined and compared with that of the early CA04 virus. The 50% lethal dose (LD_{50}) was determined for each isolate as previously described; mice that developed ≥25% body weight loss were euthanized and classified as fatalities (15, 16). The pathogenicity of all six H1N1/09 isolates was greater than that of CA04 (Table 1). In particular, the LD_{50} of A/swine/Shandong/731/2009 (SD731) virus was more than 1,000-fold lower than that of CA04.

TABLE 1 Virulence of pandemic H1N1 influenza viruses in mice

Virus name	LD_{50} (log_{10} PFU)	Residue at protein position				
		PB2-588	PA-70	PA-224	NA-106	NS1-123
A/California/04/2009	≥6.5	T	A	P	V	I
A/Beijing/7/2009	4.7	.	.	S	I	V
A/Beijing/132/2010	4.3	.	.	S	I	V
A/swine/Shandong/94/2010	4.5	.	V	S	I	V
A/swine/Shandong/361/2010	4.3	.	V	S	I	V
A/swine/Shandong/327/2010	4.3	.	V	S	I	V
A/swine/Shandong/731/2009	3.5	I	V	S	I	V

Subsequently, we examined the virulent SD731 and avirulent CA04 viruses for molecular changes that would account for their difference in virulence. Sequence comparison of their genomes revealed 13 amino acid differences between SD731 and CA04, located in the PB2, PA, HA, NP, NA, and NS genes (Fig. 1A). To determine the role of each substitution in the virulence phenotype, we first generated six single-gene reassortant viruses by introducing each individual gene from SD731 into the CA04 backbone by reverse genetics: CA04-SD731PB2, CA04-SD731PA, CA04-SD731HA, CA04-SD731NP, CA04-SD731NA, and CA04-SD731NS. Sequences of all reconstructed viruses were confirmed by whole-genome sequencing. Five 6-week-old female BALB/c mice were intranasally inoculated with 10^5 PFU of each virus and were monitored for 14 days. SD731 genes displayed various contributions to viral pathogenicity in mice (Fig. 1B). Introducing the PA gene from SD731 alone caused fatality for all mice by 6 days

Received 27 October 2013 **Accepted** 3 February 2014
Published ahead of print 12 February 2014
Editor: D. S. Lyles
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doi:10.1128/JVI.03158-13

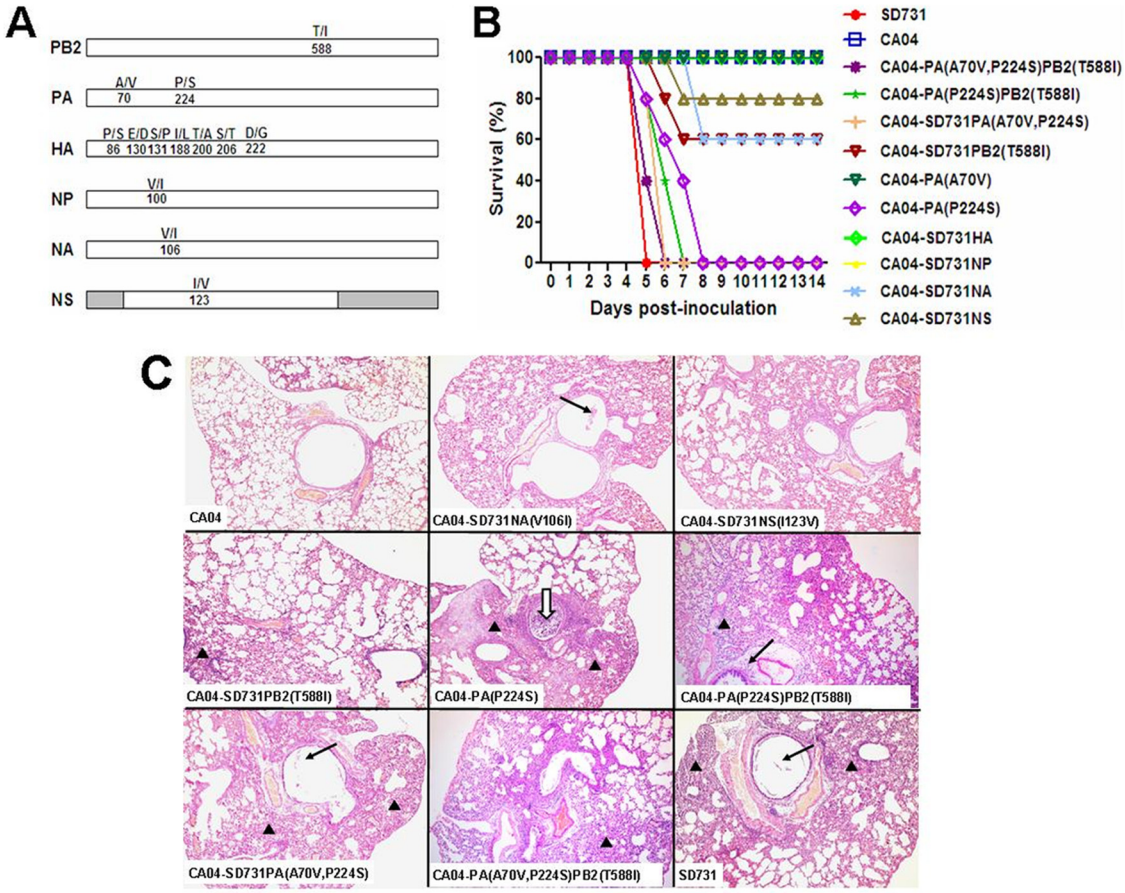

FIG 1 Relative pathogenicities of pandemic H1N1/09 influenza viruses with different amino acid mutations in mice. (A) Amino acid differences between the avirulent CA04 and pathogenic SD731 viruses. (B) Survival percentages of mice infected with 10^5 PFU of each virus. (C) Photomicrographs of hematoxylin and eosin (H&E)-stained lungs from mice infected with 10^4 PFU of virus at 6 dpi. Solid arrows, erosion of epithelial lining in the bronchioles; open arrow, extensive epithelial damage and inflammatory cell infiltration in the bronchioles; triangle, alveolar interstitial consolidation and extensive inflammatory cell infiltration. Magnification, ×100.

postinoculation (dpi). Introducing the SD731 PB2, NA, or NS gene individually resulted in some deaths, while other reassortants did not lead to mortality.

Since there was only one amino acid substitution for each reassortant carrying the SD731 PB2 (T588I), NA (V106I), or NS (I123V) protein in the CA04 backbone, we propose that these mutations are likely to be contributors to the increased virulence of SD731. There are two amino acid differences in the PA protein (PA carrying A70V [PA-A70V] and PA-P224S) between CA04 and SD731. To establish the relative contribution of each amino acid substitution in PA to the virulence of SD731, we generated separately recombinant viruses housing a PA-A70V or PA-P224S mutation in the CA04 backbone [CA04-PA(A70V) and CA04-PA(P224S), respectively]. CA04-PA(A70V) in mice had no effect even at an inoculation dose of 10^6 PFU, whereas the LD_{50} of CA04-PA(P224S) was $10^{4.3}$ PFU (Table 2). However, the pathogenicity of CA04-PA(P224S) was still lower than that of CA04-SD731PA (which contained both A70V and P224S and had an LD_{50} of $10^{3.7}$ PFU). To determine if there was synergy between the two viral polymerase subunits PA-P224S and PA-(A70V,P224S) with PB2-T588I, we generated the CA04-PA(P224S)PB2(T588I) and CA04-PA(A70V,P224S)PB2(T588I) viruses and evaluated their pathogenicity in mice. We found that the pathogenicity of CA04 virus with mutations in both the PA and PB2 genes [CA04-PA(P224S)PB2(T588I) and CA04-PA(A70V,P224S)PB2(T588I)] was higher than that of the corresponding virus with a mutation(s) in a single PA or PB2 gene and that the LD_{50} of CA04-PA(A70V,P224S)PB2(T588I) was the same as that of wild-type SD731 virus ($LD_{50} = 10^{3.5}$ PFU).

TABLE 2 Replication and pathogenicities of H1N1/09 viruses in mice

Virus	Titer of virus in lungs (mean $\log_{10}$ PFU/g ±SD)[a]		LD_{50}
	3 dpi	5 dpi	
CA04	7.1 ± 0.0	6.5 ± 0.2	≥6.5
SD731	7.8 ± 0.1**	7.5 ± 0.2**	3.5
CA04-PA(A70V,P224S)PB2(T588I)	7.9 ± 0.3**	7.5 ± 0.1**	3.5
CA04-PA(P224S)PB2(T588I)	7.6 ± 0.4*	7.3 ± 0.2**	4.3
CA04-SD731PA(A70V,P224S)	7.8 ± 0.2**	7.4 ± 0.2**	3.7
CA04-SD731PB2(T588I)	7.5 ± 0.1*	6.8 ± 0.4	5.5
CA04-PA(A70V)	7.0 ± 0.2	6.6 ± 0.2	≥6.5
CA04-PA(P224S)	7.7 ± 0.1**	7.2 ± 0.2**	4.7
CA04-SD731NA(V106I)	7.5 ± 0.1*	6.8 ± 0.3	5.3
CA04-SD731NS(I123V)	7.7 ± 0.4*	7.3 ± 0.3**	5.7

[a] *, $P < 0.05$ compared with the value for CA04 virus (analysis of variance [ANOVA]); **, $P < 0.01$ compared with the value for CA04 virus (ANOVA).

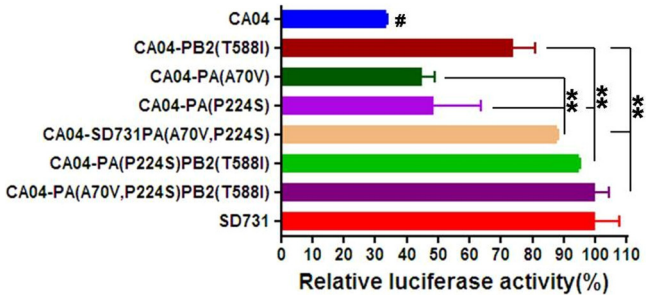

FIG 2 Polymerase activities of pandemic H1N1/09 influenza mutant viruses as determined by minigenome replication assays. Values shown are the means ± SD of data from three independent experiments and are normalized to those of SD731 (100%). #, the value for CA04 was significantly lower than those for the other mutants ($P < 0.05$ or $P < 0.01$; analysis of variance [ANOVA]); **, the values of corresponding RNPs were significantly different ($P < 0.01$, ANOVA).

The histopathology of lungs from mice, each inoculated with 10^4 PFU of H1N1/09 mutant virus, was examined at 5 dpi, as described previously (17) (Fig. 1C). Lung sections from mice inoculated with CA04 and CA04-PA(A70V) largely appeared normal. Moderate bronchopneumonitis was found in the lungs of mice infected with CA04-SD731PB2(T588I), CA04-SD731NA(V106I), and CA04-SD731NS(I123V). These changes were characterized by epithelial erosion in the bronchioles, interstitial edema, and extensive infiltration of inflammatory cells. Foci of more severe bronchopneumonitis were observed in the lungs of mice infected with CA04-PA(P224S) and CA04-PA(P224S)PB2(T588I) virus. Parent SD731, CA04-SD731PA(A70V,P224S), and CA04-PA(A70V,P224S)PB2(T588I) viruses induced the most severe bronchopneumonitis with extensive alveolar damage.

To establish if the high pathogenicity of SD731 was associated with enhanced virus replication, titers of virus in lung were determined for mice, each infected with 10^3 PFU of virus, at 3 and 5 dpi (Table 2). The titers of SD731 were significantly higher than those of CA04 at 3 and 5 dpi ($P < 0.05$). The titers of CA04-PA(A70V,P224S)PB2(T588I), CA04-PA(P224S)PB2(T588I), CA04-SD731PA(A70V,P224S), CA04-PA(P224S), and CA04-SD731NS (I123V) viruses were also significantly higher than those of CA04 virus at 3 and 5 dpi, while those of CA04-SD731PB2(T588I) and CA04-SD731NA(V106I) were significantly higher than that of CA04 at 3 dpi only ($P < 0.05$).

Previous studies showed a positive correlation between the polymerase activity of viral ribonucleoprotein (RNP) complex and viral replication and pathogenicity (18, 19). To determine the relative contributions of PA-A70V, PA-P224S, and PB2-T588I to RNP polymerase activity, minigenome replication assays were performed as previously described (17). RNP polymerase activity of CA04 was significantly lower than those of the other mutants ($P < 0.05$) (Fig. 2). However, RNP activity of CA04 virus housing the combined PA-A70V and PA-P224S mutations was significantly greater than that with the single PA-P224S or PA-A70V mutation ($P < 0.01$). The RNP polymerase activity of CA04 with mutations in both the PA and PB2 genes [CA04-PA(P224S)PB2(T588I) and CA04-PA(A70V,P224S)PB2(T588I)] was significantly higher than corresponding RNP activity with a mutation(s) in the single PA or PB2 gene ($P < 0.01$).

To trace the evolutionary frequency of mutations identified in this study, we analyzed all H1N1/09 viruses with full-length sequences available in the National Center for Biotechnology Information Influenza Virus Resource (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database). The frequency of viruses that possessed the natural mutations reported in the study had showed an upward trend over the past 4 years (Table 3). The PB2-588I and PA-70V residues were absent in early H1N1/09 strains. All reported PA-70V H1N1/09 viruses also carried the PA-224S residue. Some early H1N1/09 strains possessed proline at position PA-224; however, these viruses were rapidly replaced by those with PA-224S. Since July 2009, all H1N1/09 isolates possessed serine at the PA-224 position, except for one strain isolated in November 2009 and another isolated in December 2011. Frequencies of strains carrying NA-106I and NS1-123V, present in less than half of the H1N1/09 strains in April 2009, increased to more than 90% of the isolates in the latter half of 2009. These results suggest that viruses with these mutations could have selective advantage in replication and/or transmission in humans.

In the present study, we found several mutations, not previously recognized, that contributed to the virulence of H1N1/09 virus in mice. They include PB2-T588I, PA-P224S, PA-A70V, NA-V106I, and NS1-I123V. The PB2-588 position is located within the 627 domain and has been identified as a host-range-specific amino acid position (20, 21); it is characteristic of human influenza viruses, contributes to virus replication in mammalian cells, and partially compensates for the lack of 627K (20, 21). The PA-P224S mutation is located in the nuclear localization signal domain and may influence the transport and accumulation of PA in the nuclei of virus-infected cells (22). The PA-A70V mutation was identified in a mouse-adapted H1N1/09 virus and on its own in CA04 background did not cause lethality or a more than 10% weight loss (23), but we showed here that PA-A70V combined with PA-P224S significantly boosted the lethality and lung pathology of PA-P224S alone. The NA-V106I change was observed in A/Mexico/4482/2009, one of the first H1N1/2009 viruses iso-

TABLE 3 Frequencies of amino acids in sequenced strains of pandemic H1N1/09 influenza virus

Amino acid	Frequency, % (n^a) for strains isolated in time period (mo/yr)										
	04/2009	05/2009	06/2009	07/2009	08/2009	09/2009	10/2009	11/2009	12/2009	2010	2011–2013
PB2-588I	0 (289)	0 (495)	0 (565)	0.7 (406)	2.2 (277)	0.8 (266)	2.3 (343)	5.3 (456)	2.3 (308)	4.8 (499)	5.6 (233)
PA-224S	98.4 (247)	99.3 (451)	99.8 (609)	100 (437)	100 (293)	100 (278)	100 (336)	99.8 (460)	100 (308)	100 (496)	99.6 (234)
PA-70V,224S	0 (247)	0 (451)	0.3 (609)	0.7 (437)	0 (293)	0.4 (278)	1.5 (336)	4.3 (460)	2.0 (308)	0.8 (497)	2.6 (234)
NA-106I	47.8 (345)	64.3 (504)	80.7 (670)	91.5 (611)	91.3 (413)	95.6 (476)	97.1 (581)	96.3 (725)	98.9 (435)	98.5 (970)	97.9 (946)
NS-123V	31.2 (295)	27.5 (509)	52.0 (658)	78.1 (494)	84.3 (300)	92.4 (314)	88.2 (390)	94.7 (489)	95.1 (326)	93.2 (573)	90.3 (268)

[a] Number of analyzed sequences is in parentheses.

lated from a fatal human case (24). The NS1-I123V mutation is located in the double-stranded RNA-dependent protein kinase (PKR) binding site, which might enhance viral gene expression (25). NS1-I123 is specific for cluster I and NS1-123V for cluster II H1N1/09 viruses. Cluster I viruses appeared in early April 2009 and became extinct by the end of 2009, whereas cluster II viruses emerged in late April 2009 and are in continuing circulation (4).

The natural evolution of the current dominant virulence mutations of H1N1/09 viruses reported in this study indicates that they could not only confer a selective viral advantage in humans but also are associated with virulence in mice. Continuous surveillance and the timely evaluation of emergent virulence determinants of H1N1/09 and other influenza viruses are necessary for our preparedness to deal with future epidemics and pandemics.

Nucleotide sequence accession numbers. Sequences for the four swine isolates and two human strains identified in our laboratory were submitted to GenBank under accession numbers HQ533864, HQ533866, HQ533868, HQ533871, HQ533872, HQ533874, HQ533877, HQ533879, JF951848 to JF951855, JQ695860 to JQ695883, and KF918700 to KF918707.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (no. 31302102 and no. 31270046) and the National Basic Research Program (973 program) (no. 2011CB504702), grants from the Chang Jiang Scholars Program, Chinese Universities Scientific Fund (no. 2014XJ007 and no. 2011JS013), and a Biotechnology and Biological Sciences Research Council (UK) China Partnering Award.

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