Virulence of pandemic (H1N1) 2009 influenza A polymerase reassortant viruses

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Infections due to the pandemic (H1N1) 2009 influenza A viruses have been considerably mild relative to previous pandemics. However, its continued circulation among human and animal populations heightened concerns for the generation of virulent variants with greater threat to public health. Thus, we explored the potential role of the influenza viral polymerases, including known molecular markers, in altering the virulence phenotype of the 2009 pandemic A/California/04/09 (CA04, H1N1) virus. By examining in vitro polymerase activities and in vivo pathogenicities in mice model, we were able to show that individual or simultaneous expression of virulence factors in PB2, PB1 and PA might not significantly elevate pathogenicity. Nevertheless, we demonstrated that PB2627K or PA97I derived from different genetic backgrounds and other unknown polymerase markers have the potential to enhance virulence of CA04. Virus rescue and replication studies identified PA as a critical factor in maintaining genetic stability of the CA04 (H1N1) virus.

Influenza A viruses are the perennial cause of recurrent human influenza epidemics and occasional pandemics. The emergence and global spread of the novel pandemic H1N1 virus in the early spring of 2009 marked the start of the most recent pandemic of the new century.1,2 Despite slight pathogenicity and high transmissibility over seasonal influenza in animal models,3,5 virulence of the pandemic (H1N1) 2009 virus among human populations in general had been relatively mild compared with its pandemic virus predecessors in 1918 (Spanish Flu), 1957 (Asian Flu), and 1968 (Hong Kong Flu), which caused millions of deaths on a worldwide basis.6 However, this relatively benign infection may potentially shift with significant genetic evolution through continued circulation.

The influenza viral genome contains single strands of eight viral RNA segments with negative orientation.7 Such segmented nature coupled with the error-prone RNA polymerase transcription and replication of the viral genome are key elements that drive genetic evolution of influenza viruses through the accumulation of mutation (antigenic drift) and/or reassortment (antigenic shift) oftentimes resulting to enhanced pathogenesis and expanded host range. Influenza virulence is a polygenic trait.8,9 The viral RNA-dependent RNA polymerase subunits (PB2, PB1 and PA) are responsible for the transcription and replication of the viral RNA genome in the nuclei of infected cells.10 In addition to the specific contributions of the surface glycoprotein hemagglutinin (HA) and the non-structural 1 (NS1) protein, each of the polymerase genes have been continuously implicated to harbor molecular determinants for host range and virulence such as the presence of lysine (K) and/or asparagine (N) residue at positions 627 and 701 in PB211,12 expression of the alternatively-spliced PB1-F2 protein,14,15 and the isoleucine (I) substitution in PA at position 97.16 The absence of these characteristic molecular factors in the genetic make-up of the pandemic (H1N1) 2009 virus may partially contribute to its low virulence among humans.

Key words: influenza A virus, pandemic, viral polymerases, genetic evolution, virulence, compatibility

Submitted: 06/23/11
Revised: 07/29/11
Accepted: 08/01/11
http://dx.doi.org/10.4161/viru.2.5.17267
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Addendum to: Song MS, Pascua PN, Lee JH, Baek YH, Park KJ, Kwon HI, et al. Virulence and genetic compatibility of polymerase reassortant viruses derived from the pandemic (H1N1) 2009 virus and circulating influenza A viruses. J Virol 2011; 85:6275–86; PMID: 21507962; http://dx.doi.org/10.1128/JVI.02125-10.
In our recent work, we examined the potential alteration of virulence of the pandemic A/California/04/2009 (CA04) (H1N1) virus focusing on the role of the viral polymerases. Therefore to address this, we generated various polymerase point mutant pandemic viruses (PB2, 627K/701N; PB1, expression of PB1-F2 protein; PA, 97I) by an established reverse genetics system. We found in our work that expression of the 627K or 701N mutation in PB2 or driving the PB1-F2 protein expression enhanced reporter gene activity in vitro compared with that of the wild-type (WT) CA04 polymerase complex. However, the individual or even the simultaneous incorporation of these virulence markers did not essentially increase replication kinetics of the pandemic virus in MDCK cells or its pathogenicity in mice which conform to the findings of other works.

Susceptibility of various animals, most notably pigs and turkeys, with the pandemic virus provides favorable opportunities for genetic reassortment with co-circulating human seasonal and animal influenza viruses (Fig. 1). Utilizing similar approach, we further evaluated reassortant viruses possessing cognate polymerase subunits from representative swine, avian and human strains. Results from our panel of assays demonstrated that the polymerase gene segments of the pandemic virus are moderately compatible with those of the different viruses tested. Furthermore, although a number of polymerase reassortants had substantially high polymerase activities or replicated well in cell culture, these were not as similarly as virulent in mice suggesting biological properties in vitro and in vivo may not always be mutually exclusive. Nevertheless, since the five potential pandemic reassortants, such as A/Aquatic bird/Korea/ma81/07 [Av/ma81 (H5N2)] PA, A/Swine/Korea/JNS06/04 [Sw/JNS06 (H3N2)] PB2, Av/ma81K (H5N2) PB2, A/Aquatic bird/Korea/ma44/07 [Av/ma44 (H7N3)] PB2 and Av/ma81K (H5N2) PA-PB2, all in the backbone of CA04, which had the capacity to surpass the virulence of the WT CA04 (H1N1) virus, had high reporter gene activities and grew well in culture,
we concluded that optimal in vitro properties might be required, although not sufficient, for high pathogenicity (Fig. 2A). Our finding that the swine-like Sw/JNS06 (H3N2) PB2 gene, devoid of any known virulent determinants but shares genetic lineage with the CA04-PB2, was able to enhance pathogenicity presented evidence on the existence of previously uncharacterized mutations in the viral polymerases that could contribute to virulence. Certainly, these potential markers merit further studies to look into. This may also imply the capacity of the pandemic virus to further adapt in humans maximizing its pathogenic potential. We have also shown that mammalian-adapted polymerase genes [Av/ma81 (H5N2), Av/ma81K (H5N2), Av/ma44 (H7N3)], bearing the PB2_{627K}, PA_{97I}, or both characteristic residues, appear to enhance pathogenesis. Surprisingly, no polymerase combinations with those from the Korean highly pathogenic avian influenza (Av/ W150) H5N1 or the seasonal human A/ Cheongju/H407/08 [H407 (H3N2)] virus had similar effect on pathogenicity. In agreement to these results, Schrauwen et al.23 also showed that pandemic (H1N1) 2009 viruses containing PB2 alone or in combination with PA of seasonal human (H1NI) virus were attenuated in ferrets. Octaviani et al. contrasting yielded polymerase reassortants, using a contemporary human HPAI H5N1 isolate, with high growth capacity in vitro. Altogether, these related findings led us to conclude that the generation of more virulent pandemic viruses through reassortment is feasible but perhaps differences in genetic background might be a key requirement.

Specific residues in PA of the pandemic (H1N1) 2009 virus have been proposed as host adaptive factors that contributed to its replication and pathogenicity in mammalian hosts, particularly humans.25 Accordingly, the results of our experiments also clearly demonstrated that PA might be crucial in maintaining genetic stability of the CA04 (H1N1) virus. Modifications on the PA gene, either by mutation or replacement, severely impaired viability during virus rescue. These data therefore suggest that changes in PA might potentially perturb overall genetic functions (i.e., genome replication and/or transcription) in the context of the pandemic virus. With respect to the mammalian-adapted Av/ma81 (H5N2) PA gene that altered virulence phenotype compared with its WT Av/W81 (H5N2) PA counterpart, three additional amino acid (aa) substitutions could be observed aside from the 97I. Therefore to verify whether the 97I mutation alone was responsible for increased virulence, we generated two reverse-mutants reflecting modifications in PA at this specific codon (Av/W81 PA_{97I} and Av/ma81 PA_{97T}) in the CA04 background and tested their pathogenicity in groups of mice intranasally inoculated with $10^{4.5}$ TCID\textsubscript{50} of each virus and monitored in 13 d for survival. Percentage values reflect average weight losses for the different pandemic polymerase reassortant viruses tested: *Av/W81 PA; †Av/ma81; ‡Av/ma81 PA\textsubscript{97I}; §Av/W81 PA\textsubscript{97I}.
polymerase gene segments. Therefore it is our interpretation that the compatibility of the polymerase subunits in the context of the CA04 (H1N1) virus background may be a restricting factor for reassortment and pathogenesis. Although only five out of the 58 successfully rescued polymerase reassortants altered virulence, emergence of more virulent variants in the future might still be possible with the inclusion of the other viral genes (HA, NP, NA, M and NS) in the course of its genetic evolution. To date, accumulating evidence on severe cases of pandemic virus infection have been attributed to amino acid substitutions from aspartic acid-to-glycine at position 222 (225 in H3 numbering) in HA and maybe the first identifiable “virulence marker” of the pandemic virus.26 Pandemic reassortants have been also reported in the field involving the NA genes27-29 indicating that the surface glycoproteins may be more active in generating more virulent variants. It should also be noted that our polymerase variants were generated in the background of the CA04 (H1N1) virus limiting our findings in the context of the pandemic virus alone. In experimental settings, several indications on the potential pathogenic impact or contribution of the viral segments derived from the pandemic virus toward contemporary viruses have been observed. Acquisition of the pandemic PA gene increased pathogenicity of an avian H9N2 (A/Chicken/Hebei/LC/2008) virus in mice30 whereas reassortants containing one or both of the PB2 and PB1 subunits from CA04 induced enhanced growth kinetics of a human HPAI H5N1 (A/Vietnam/HN31604/2009) isolate.31 Therefore, the pandemic (H1N1) 2009 virus may also become a significant genetic reservoir, a source for the generation of novel viruses with potential threat to public health. Although it may only be a time of matter when new, more virulent pandemic variants will eventually arise, the expeditious production of such viruses derived from the pandemic (H1N1) 2009 virus may be at present hampered by unknown genetic restrictions likely similar to what we have encountered in our works. This is probably the reason why pandemic viruses with altered virulence are still yet to emerge in nature. However, as such possibility is continually being underscored, it is imperative to remain vigilant and pursue surveillance particularly in key populations (i.e., humans, swine herds and avian species) where the next pandemic candidate may arise. It may as well be important to conduct complete genetic characterization of the viral gene segments of emerging viruses to discriminate previously undefined virulence markers and to rapidly identify reassortment events.

Acknowledgments
This work was supported by a National Agenda Project (NAP) grant from Korea Research Council of Fundamental Science and Technology and Korea Research Institute of Bioscience and Biotechnology (KRIBB) Initiative program (KGMO8221113) and by grant 2010-0024405 from the Korean Ministry of Science and Technology.

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