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Multiple amino acid mutations in viral RNA polymerase may synergistically enhance the transmissibility and/or virulence of the 2009 pandemic influenza (H1N1) virus

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Summary. – Influenza viruses may change their transmissibility and virulence via single or multiple point mutations in the functional regions of their structural proteins. In this study, we compared sequences of all three subunits of viral RNA polymerase, i.e. PA, PB1 and PB2, of the 2009 pandemic influenza A (H1N1) virus isolates from different stages of the pandemic and found that the frequencies of three mutations, including V14I and K716Q in PA and K736G in PB1, showed a similar trend. Interestingly, the death rate of infected patients during the pandemic matched the frequency of the three mutations with a 2-months delay. These findings suggest that the combined mutations may have acted synergistically in enhancing the transmissibility and/or virulence of the 2009 pandemic virus. If definitely proved, this hypothesis could guide the rational design of antiviral therapeutics targeting the RNA polymerase of influenza viruses.

Keywords: influenza A virus; 2009 pandemic; RNA polymerase; PA subunit; PB1 subunit; PB2 subunit; mutation; death rate

Introduction

With its increased virulence and transmissibility among humans, the 2009 pandemic influenza A (H1N1) virus (2009 pandemic virus) resulted in over 18,000 deaths worldwide (http://www.who.int/csr/don/2010_07_30/en/). The World Health Organization (WHO) announced on August 10, 2010, that the H1N1 pandemic had ended, but, so far, researchers have been unable to characterize the way this virus acquired its enhanced transmissibility and the virulence required to cause the pandemic.

In viruses, mutation involves changes in DNA or RNA sequences. When challenged by the host immune system, such changes provide the candidate strains with new characteristics for natural selection. The evolutionary history of the H1N1 influenza A virus suggests that the 2009 pandemic virus had undergone a long period of evolution in animals and humans, including changes in antigenicity, long before reaching the level of transmissibility and virulence required to cause the pandemic in 2009 (York and Donis, 2012). It was reported that the avian influenza virus with E627K substitution in PB2 could infect human cells (Hatta *et al*., 2007; Subbarao *et al*., 1993). However, K627 is not present in the 2009 pandemic virus. Instead, R591 is reported to compensate for it and is responsible for infecting humans (Medina and Garcia-Sastre, 2011; Mehle and Doudna, 2009; Yamada *et al*., 2010). Interestingly, patients infected with the 2009 pandemic virus with D222G mutation in hemagglutinin exhibited severe forms of the disease,

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such as pneumonia, suggesting that this mutation may have increased viral virulence (Kilander *et al.*, 2010). However, subsequent studies revealed that the 2009 pandemic virus with only this mutation exhibited no significant increase in receptor binding, pathogenesis or transmissibility (Belser *et al.*, 2011). Strikingly, however, the virus having both D222G and K163E mutations in hemagglutinin, as well as F35L in PA, did show enhanced virulence (Seyer *et al.*, 2012). Overall, these findings suggest that a combination of mutations in one or more viral proteins may, in fact, equip the virus with enhanced virulence and/or transmissibility, leading to pandemic.

To investigate the potential effect of multiple amino acid mutations in viral proteins on the transmissibility and/or virulence of the 2009 pandemic virus, we analyzed the trends in the frequency of single and multiple mutations in RNA polymerase in the 2009 pandemic virus isolates from April to December 2009, and in the death rate of infected patients. We also conducted protein modeling analysis to investigate the potential role of the multiple mutations in the enhancement of transmissibility and/or virulence of the 2009 pandemic virus.

Materials and Methods

Sequence analysis. To analyze the mutational trends, we accessed the NCBI Influenza Database and downloaded the full-length sequences of genes of the 2009 pandemic viral isolates obtained from April 2009 and February 2011. The sequences of all genes in the influenza viruses were analyzed by multiple sequence alignments using an online program (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html).

Protein modeling. To probe the position and the potential influence of the mutations we discovered, the structural models were constructed by the PYMOL (1.97) program with templates downloaded from the Protein Data Bank, including the N-terminal domain of PA (PDBID:2W69), the C-terminal domain of PA (PDBID:3CM3) and the interaction site of PB1 and PB2 (PDBID:3A1G). The mutational analysis of the proteins was accomplished by the "Mutagenesis" function of the PYMOL program. In addition, we drew the schematic figure of the whole influenza polymerase, using the Mei Tu Xiu Xiu program, as described previously (He *et al.*, 2008).

Results and Discussion

Trends in single and multiple mutations in the RNA polymerase

We compared the sequences of the 2009 pandemic virus isolates from April 1, 2009 to December 31, 2009. Three mutations in the polymerase, V14I and K716Q in PA and K736G in PB1, first appeared in July 2009, suddenly rising to more than 20% frequency in October 2009 (Table 1 and Fig. 1a). In December 2009, the mutation rates of PA V14I, K716Q, and PB1 K736G were 17.9%, 16.8%, and 16.4%, respectively, maintaining a relatively high level. Since the curves of these mutations were similar, we inferred a possible synergistic

Table 1. Trends in the frequency of V14I, K716Q and K736G mutations in the RNA polymerase in the 2009 pandemic virus isolates from April to December 2009

Mutation	Positives/tested (%)						
	Apr-Jun	Jul	Aug	Sep	Oct	Nov	Dec
PA V14I	11/1352 (0.8)	16/335 (4.8)	6/173 (3.5)	22/228 (9.6)	76/295 (25.8)	78/386 (20.2)	51/285 (17.9)
PA K716Q	3/1352 (0.2)	3/335 (1.0)	3/173 (1.7)	16/228 (7.0)	65/295 (22.0)	77/386 (20.0)	48/285 (16.8)
PB1K736G	5/1320 (0.4)	7/301 (2.3)	7/161 (4.4)	16/222 (7.2)	65/287 (22.6)	73/369 (19.8)	46/280 (16.4)

Table 2. Trends in the frequency of the multiple mutations in the RNA polymerase in the 2009 pandemic virus isolates from April to December 2009 and in the death rate of infected patients

	Apr-Jun	Jul	Aug	Sep	Oct	Nov	Dec
Mutation frequency %)	0/1290 (0.0)	0/298 (0.0)	3/156 (1.9)	11/219 (5.0)	62/283 (21.9)	71/365 (19.5)	45/279 (16.1)
Death rate (%)	(0.44)	(0.79)	(1.83)	(1.58)	(1.46)	(1.17)	–

(–) = unavailable.

relationship among them. Synergism, by definition, suggests a cooperative interaction, which culminates in a result not independently attainable. In our case, the combined mutation may have synergistic effect on the enhancement of viral transmissibility and/or virulence, leading to eventual pandemic. Consistent with our expectations, the mutation rate curve of viruses having all three mutations is almost the same as the mutation rate curve of viruses having any one of the three mutations in the polymerase (Table 2 and Fig. 1b). In October 2009, over one-fifth of the viruses detected could be classified as having undergone these three mutations, suggesting that the combined mutations in the polymerase may have synergistic effect on the activity of the polymerase and on the enhancement of the viral transmissibility and/or or virulence.

Trend in death rate

We then compared the change in frequency of the viruses carrying all three mutations with the change in mortality rate of patients infected with the 2009 pandemic virus during the pandemic period (Table 2). Surprisingly, the rate of mortality displayed a trend very similar to the rate of frequency of viruses having all three mutations in the polymerase (Fig. 1b). However, the frequency of the combined mutations peaked about two months later than the highest death rate recorded for the 2009 pandemic virus-infected patients. This could be explained by the time of collection of blood samples, which occurred well after the virus had already mutated in the patients. These results also suggest that the combined mutations in the polymerase of the 2009 pandemic virus may have synergistic effect on the enhancement of viral virulence and/or transmissibility, resulting in the increased death rate of the infected patients.

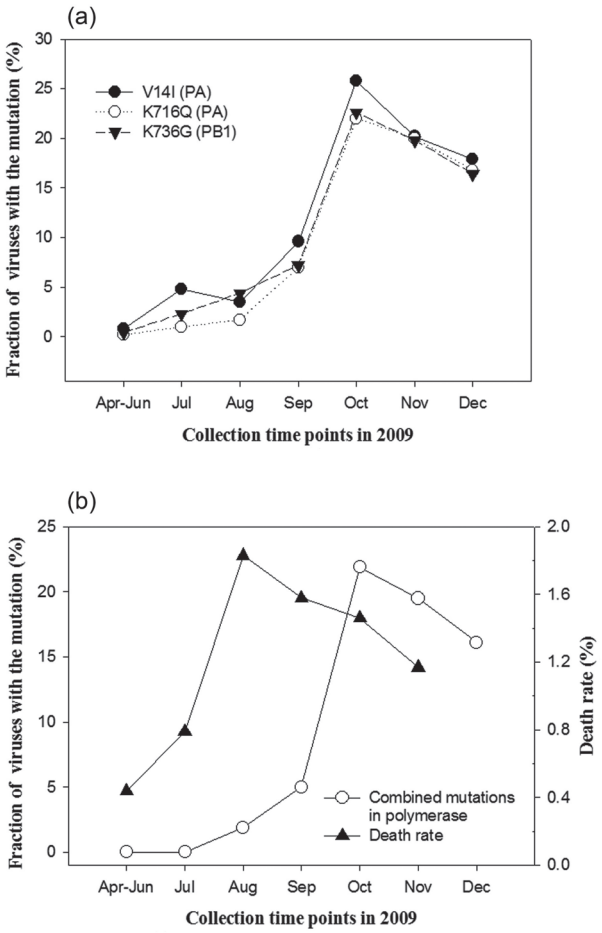

Fig. 1

Trends in single (a) and multiple mutations in the RNA polymerase in the 2009 pandemic virus isolates from April to December 2009 and in the death rate of infected patients (b)

Modeling of mutiply mutated RNA polymerase

To probe the possible causal mechanisms underlying the effect of synergism on these mutations vis-à-vis their influence on the transmissibility and virulence during the 2009 pandemic virus, we used PYMOL to carry out protein modeling. The polymerase of the influenza virus consists of three different subunits: PA, PB1, and PB2. The PA subunit can be cleaved by limited tryptic digestion into two functional domains, which are in the N-terminal domain and C-terminal domain, respectively (Guu *et al.*, 2008; Hara *et al.*, 2006). The N-terminal domain is responsible for the catalysis of the endonuclease activity (Dias *et al.*, 2009; Yuan *et al.*, 2009), while the C-terminal domain is responsible for the binding to the PB1 subunit (He *et al.*, 2008; Obayashi *et al.*, 2008)]. The V14I mutation is located on the α-helix in the N-terminal domain of PA (Fig. 2a), which cleaves 10–15 nucleotides (nt) downstream of the 5′-terminal 7-methylguanosine cap of the pre-mRNA (Boivin *et al.*, 2010; Fodor *et al.*, 2002; Hara *et al.*, 2006). Since this α-helix is in the proximity of the active site of the N-terminal domain of PA, it may play a role in enhancing the stability of the active site by altering the steric hindrance.

Unlike the V14I mutation that is located in the N-terminal domain, the K716Q mutation is located in the C-terminus of the PA subunit (Fig. 2a). The K716 residue is on the α13 helix, which contributes to the formation of a hydrophobic cavity to interact with PB1. A double mutation experiment has previously proved that the W706 and F710 residues in the α13 helix are crucial residues in the interaction (He *et al.*, 2008; Obayashi *et al.*, 2008). Therefore, since the K716 residue is also located in the α13 helix, the K716Q mutation may also be involved in altering the interaction of the two polymerase subunits by changing the charge of the α13

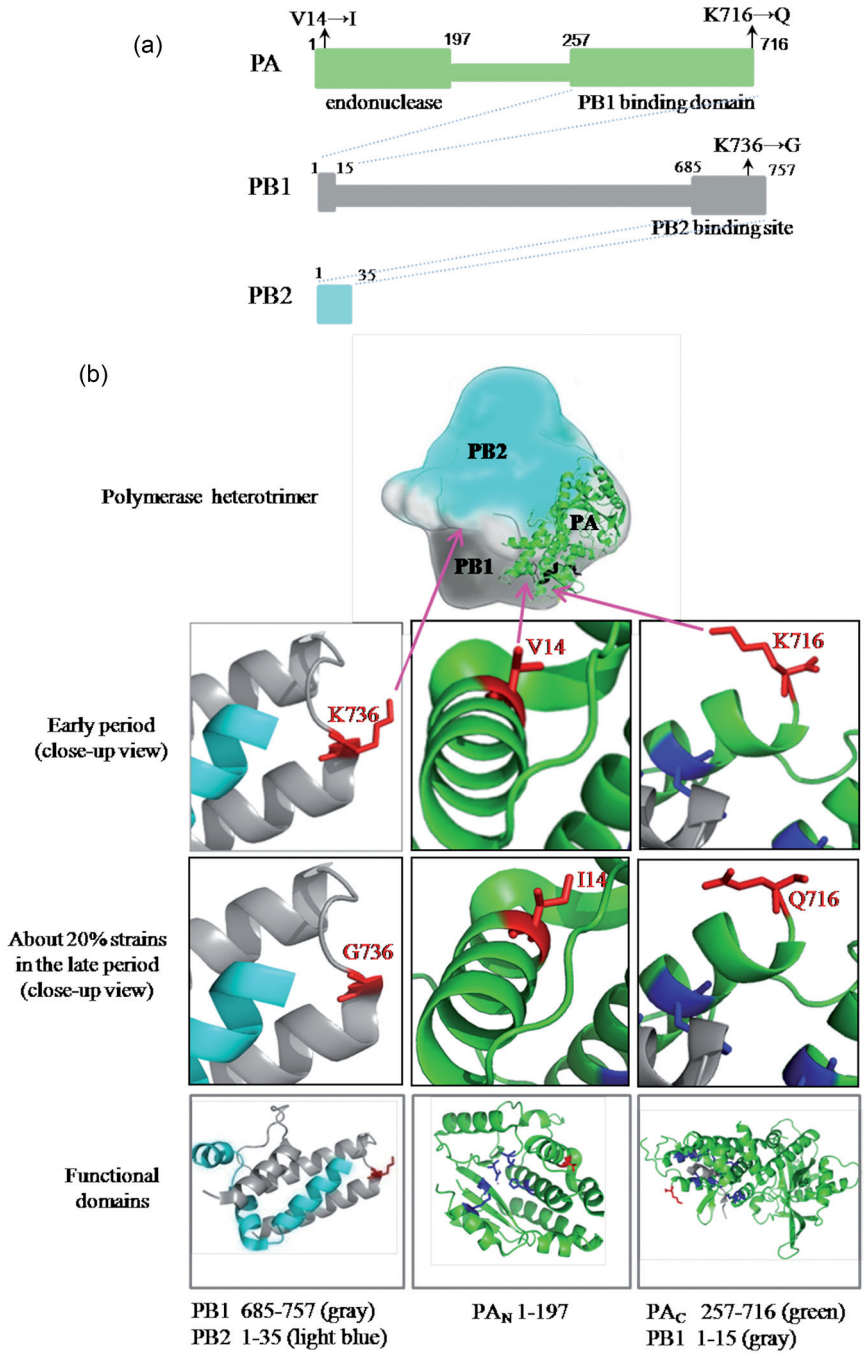

Fig. 2

Schematic presentation of the effects of the multiple mutations on functional sites in the 2009 pandemic influenza virus

(a) Linear scheme of the mutations. (b) 3D-scheme of the mutated sites in the RNA polymerase. Positions of mutated amino acids (pink arrows), all mutated amino acids (red), all functional amino acids (blue).

helix. PB1 is a polymerase subunit that interacts with both PA and PB2 subunits through its N-terminal and C-terminal residues, respectively (He *et al.*, 2008; Obayashi *et al.*, 2008; Sugiyama *et al.*, 2009). Because no direct interaction exists between PB2 and PA (Digard *et al.*, 1989; St Angelo *et al.*, 1987), the PB1 subunit serves as the bridge between PA and PB2, which integrates the whole influenza polymerase. K736 is one of the C-terminal residues of PB1, which interacts

with PB2 (Fig. 2a). Researchers have discovered that PB1 with mutations such as F699A and I750D showed increased enzyme activity, but weaker PB2 binding affinity, indicating the importance of the flexibility in the interaction between PB1 and PB2 (Sugiyama *et al.*, 2009). Residue K736 is located on the edge of the α-helix, suggesting that it may interact with other residues in different domains or subunits. We therefore inferred that the K736G mutation may enhance the flexibility of the PB1 subunit, or even of the whole polymerase.

The three polymerase subunits have different functions. PA has endonuclease activity (Dias *et al.*, 2009; Yuan *et al.*, 2009), PB1 is the active site for RNA synthesis (Argos, 1988; Biswas and Nayak, 1994; Braam *et al.*, 1983), while PB2 is responsible for cap binding and nuclear localization (Mukaigawa and Nayak, 1991). Previous studies have shown that the RNA polymerase of the influenza virus is crucial for the adaptation of the influenza virus to mammalian and human cells (Li *et al.*, 2009). Thus, it is very possible that these three mutations had synergistic effect on the 2009 influenza pandemic, resulting in the trends discussed in this work.

Since no crystal structure of the influenza virus polymerase exists, we cannot view the specific positions of each mutated residue to probe whether they form direct interactions. However, based on electron microscopic studies aimed to unravel the relative positions of PA, PB1, and PB2 subunits in the polymerase, a plausible prediction can be made (Area *et al.*, 2004; Torreira *et al.*, 2007). Accordingly, it has been predicted that the PA N-terminal domain, which is the site of the K716Q mutation (Fig. 2b), is located below the C-terminal domain (He *et al.*, 2008), indicating that these two mutations may directly interact. The locations of the attachment site of PB1 and PB2 are unknown, but it should be somewhere along the border of PB1 and PB2, which may be located in the proximity of PA, providing a prerequisite for the structural influence of the mutated residues, as shown in the polymerase model based on electron microscopy (Fig. 2b). Consequently, if these combined mutations are localized in their respective crucial functional domains, they may be strong enough to change the conformation of all subunits, an act that cannot be easily achieved by any single mutation.

Taken together, the evidence presented in the present work suggests that the combined mutations in the polymerase may have had synergistic effect on the virulence of the 2009 pandemic virus, leading to the increased death rate of infected patients. If this hypothesis can be further confirmed, novel antiviral therapeutics targeting the critical polymerase regions could be rationally designed and developed to treat patients infected with the 2009 pandemic virus.

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